Radiation therapy causes loss of dermal lymphatic vessels and interferes with lymphatic function by TGF-β1 mediated tissue fibrosis.

Key words: radiation, lymphedema, lymphatic, fibrosis, endothelium, TGF-β1

Running Head: Radiation impairs lymphatic function

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Abstract

Introduction: Although radiation therapy is a major risk factor for the development of lymphedema following lymphadenectomy, the mechanisms responsible for this effect remain unknown. The purpose of this study was therefore to determine the effects of radiation on lymphatic endothelial cells (LEC) and lymphatic function.

Methods: The tails of wild-type or acid sphingomyelinase (ASM) deficient mice were treated with 0, 15 or 30 Gy of radiation and then analyzed for LEC apoptosis and lymphatic function at various time points. In order to analyze the effects of radiation fibrosis on lymphatic function, we determined the effects of TGF-B1 blockade after radiation in vivo. Finally, we determined the effects of radiation and exogenous TGF-B1 on LECs in vitro.

Results: Radiation caused mild edema that resolved after 12-24 weeks. Interestingly, despite resolution of tail edema, irradiated animals displayed persistent lymphatic dysfunction. Radiation caused loss of capillary lymphatics and was associated with a dose dependent increase in LEC apoptosis. AMS-/- mice had significantly less LEC apoptosis, however, this finding did not translate to improved lymphatic function at later time points. Short-term blockade of TGF-B1 function after radiation markedly decreased tissue fibrosis and significantly improved lymphatic function but did not alter LEC apoptosis. Conclusions: Radiation therapy decreases lymphatic reserve by causing depletion of lymphatic vessels and LECs as well as promoting soft tissue fibrosis. Short-term inhibition of TGF-β1 activity following radiation improves lymphatic function and is
associated with decreased soft tissue fibrosis. ASM deficiency confers LEC protection from radiation induced apoptosis but does not prevent lymphatic dysfunction.
Introduction

Lymphedema is a debilitating disorder that affects more than 3 million Americans (2, 48). In the United States the most common cause of lymphedema is lymph node dissection for the treatment of a variety of malignancies (44, 60). Due to the relative prevalence of breast cancer and the fact that lymphatic metastasis is the primary source of metastasis in these patients, axillary lymph node dissection (ALND) is the most commonly encountered cause of lymphedema clinically in Western Countries (23). Patients with lymphedema are easily recognizable by their dependence on tight fitting garments needed to prevent the inevitable progression of this disorder. Due to the lack of effective treatment options, these patients have significantly decreased quality of life with frequent infections, decreased function, and disfigurement (3, 60-61).

A large number of clinical studies have identified risk factors for the development of lymphedema (22, 35, 42, 59, 61). The vast majority of these studies have identified radiation therapy as an independent risk factor for the development of lymphedema (23). In fact, it is estimated that postoperative radiation therapy increases the risk of developing lymphedema by as much as 10 fold (44). In addition, it appears that the combination of surgery and radiation is necessary for the development of lymphedema since radiation alone is infrequently associated with clinically apparent lymphedema. Nevertheless, the potential mechanisms by which radiation therapy increases the risk of lymphedema remain essentially unknown.
One previous study demonstrated that intestinal lymphatic endothelial cells are relatively radioresistant as compared with microvascular endothelial cells (57). This finding was not reproduced by a more recent study demonstrating intestinal lymphatic endothelial apoptosis shortly after total body irradiation (1). The clinical relevance of studies on intestinal lymphatic endothelial cells is somewhat difficult to interpret since lymphatic dysfunction and subsequent extremity lymphedema are related to cutaneous lymphatic channels which may be structurally or functionally different than intestinal lymphatics. In fact, Jackowski, et al have shown that decreased numbers of cutaneous lymphatics after radiation therapy in breast cancer patients is associated with an increased rate of lymphedema (27). In addition, Mortimer et al have shown in limited studies that radiation therapy is associated with cutaneous lymphatic dysfunction in pigs (40). Therefore, it is possible that radiation therapy contributes to the development of lymphedema by causing depletion or dysfunction of cutaneous lymphatic channels. Radiation therapy may also increase the risk of lymphedema after ALND by promoting tissue fibrosis. Radiation therapy is well known to cause tissue fibrosis as a result of TGF-β1 dependent mechanisms (36-37). We have recently shown that fibrosis is a critical regulator of lymphatic regeneration and that this effect is largely dependent on TGF-β1 expression (8). In addition, we and others have shown that TGF-β1 is a direct inhibitor of lymphatic endothelial cell proliferation and function (8, 41). Therefore, it is feasible that at least some of the negative consequences of radiation therapy on the lymphatic system may be secondary to tissue fibrosis. This hypothesis is supported by
the fact that clinical factors that increase the risk of fibrosis (e.g. extensive surgery, infections, or obesity) also significantly increase the risk of lymphedema (34-35).

The purpose of these studies was to determine the effects of ionizing radiation therapy on cutaneous lymphatic vessels. Using a mouse tail model we show that radiation causes a dose dependent, long-term decrease in lymphatic function. Impaired lymphatic function resulting from radiation is associated with a combination of LEC apoptosis, long-term decrease in the number of cutaneous lymphatic vessels, and soft tissue fibrosis. We show that LECs in acid sphingomyelinase (ASM) deficient animals, similar to microvascular endothelial cells, are significantly protected from radiation induced apoptosis. However, this protective effect does not inhibit development of tissue fibrosis or improve lymphatic function at later time points. Short-term inhibition of TGF-B1 significantly decreased tissue fibrosis and markedly improved lymphatic function but did not provide a protective effect for LEC apoptosis or lymphatic depletion. Finally, radiated lymphatics demonstrated evidence of endothelial-mesenchymal transition (EMT) in vivo. In vitro exposure of LECs to recombinant TGF-B1 increased expression of collagen and markers of fibroblast differentiation while simultaneously decreasing the expression of lymphatic markers (LYVE-1). Taken together, these results indicate that lymphatic dysfunction resulting from radiation therapy does not cause clinically apparent lymphedema but may predispose to the development of lymphedema when combined with other insults by promoting tissue fibrosis (e.g. surgery). In addition, our results indicate that prevention of tissue fibrosis
resulting from radiation may be a means of protecting against this potentially devastating complication.

**Methods**

**Mouse Tail Irradiation**

All animal experiments were approved by the Research Animal Resource Center IACUC at Memorial Sloan-Kettering Cancer Center. C57/BL6 mice were anesthetized with Ketamine/Xylazine, then carefully shielded and their tails were then irradiated using the X-RAD 320 (Precision X-Ray, North Branford, CT) with doses of 15 or 30 Gy. We chose the mouse tail for irradiation because it is an appendage that has been previously been used extensively in studies of lymphatic function and regeneration and is also easily shielded from the rest of the animal. Control mice received anesthetic and were placed in the irradiator but received no radiation. Mice were then sacrificed for tail harvest after 4 hours, 10 hours, 1 week, 4 weeks, 12 weeks, and 24 weeks following irradiation (N=5 for each dose at each time point evaluated).

**Irradiation of acid sphingomyelinase deficient mice**

Mice deficient in the ceramide acid sphingomyelinase (ASM) have been previously shown to be protected from radiation induced apoptosis in a variety of cell types including microvascular endothelial cells (43). As such we evaluated the impact of this deficiency on LEC apoptosis following irradiation. ASM-/- mice established on the SV129/C57BL/6 back ground along with wild-type (WT) littermates underwent tail irradiation as described above. Tissues were harvested at 10 hours and 4
weeks following radiation (N=3 for each time point). The 4-week time point was chosen as the longest follow-up since this represented our longest follow-up in other groups for assessment of apoptosis.

**Short Term TGF-β1 blockade with LY-364947**

In order to determine the effectiveness of TGF-β1 blockade in alleviating the affects of XRT on lymphatic function, we treated animals with LY-364947 (Sigma, St. Louis, Mo), a well described small molecule inhibitor. This molecule is an ATP-competitive inhibitor of TGF-β receptor I and blocks association with TGF-BRII and subsequent Smad-3 phosphorylation (52). LY-364947 was dissolved at a concentration 5mg/mL in DMSO. Experimental animals (N=5) were treated with LY-364947 (1mg/kg diluted in PBS) delivered intraperitoneally starting one day prior to tail irradiation with 15Gy. Animals were subsequently treated every other day for 3 additional doses. Control mice were irradiated in an identical manner but treated with DMSO diluted in PBS. While long term inhibition of TGF-β1 may be associated with immune dysfunction, short term inhibition as performed in our study, is well tolerated (71). No adverse side effects were noted in our animals treated with LY-364947.

Tail volume measurements

In order to evaluate the degree of acute lymphedema following irradiation, tail volumes were determined using the truncated cone formula ($V = \frac{1}{4}\pi (C_1C_2 + C_2C_3 + \ldots + C_7C_8)$) as previously described (53). Briefly, tail circumference was measured at four points at 10mm intervals starting 20mm from the tail base using a digital caliper by blinded reviewers. Tail volume changes from baseline were calculated for each animal in
order to minimize the potential effects of inter-animal baseline differences in tail volumes.

**Lymphoscintigraphy**

In order to quantify lymphatic transport following irradiation, Tc$^{99}$-sulfur colloid (100nm particle size; 400-800µCi in ~50 µl.) was injected intradermally ~20mm from the tail tip using our previously reported methods. (5) This colloid is taken up by the lymphatics and transported to the lymph nodes at the base of the tail and is a sensitive, easily quantifiable measure of lymphatic transport. Briefly, dynamic planar gamma camera images were acquired in 15- to 600-s frames for up to 130 minutes post injection using an X-SPECT™ (Gamma Medica, Northridge, CA) fitted with low-energy parallel-hole collimators. Resulting dynamic images were analyzed using ASIPro™ (CTI Molecular Imaging, Knoxville, Tennessee) and region-of-interest (ROI) analysis was performed to derive the decay adjusted activity at the lymph nodes at the base of the mouse tail. Time-activity data were then fit to a function exponentially decreasing to an asymptotic value for the injection site, or a function exponentially increasing to an asymptotic value for the individual lymph nodes. Maximal Tc$^{99}$ uptake was calculated and plotted.

**Specimen preparation, histology, and immunostaining**

Following euthanasia, mouse tails were fixed in 4% paraformaldehyde at 4°C for 24 hours and then decalcified in Immunocal (Decal Chemical Corporation, Tallman, NY). A 1 cm portion of tail was harvested 2 cm from the base of tail and sectioned.
longitudinally. Additionally, 2 mm cross-sections of tail were obtained both immediately distal and proximal to this piece. All three pieces of tail were embedded in paraffin and sectioned at 5 micrometers.

Sections were histologically stained using standard protocols and evaluated using a Leica microscope (Wetzlar, Germany). Histochemical staining for scar tissue was performed using picro-sirius red (Direct Red 80, Sigma St. Louis, MO) as previously described (16). Sirius red stained specimen were examined using polarized light microscopy (Leica TCS AOBSSP2) and the scar index was calculated as previously described using Metamorph™ Offline software (Molecular Devices Corporation, Sunnyvale, CA) in a minimum of three 70,000 micrometer sections per animal (16). A minimum of 3 animals were evaluated per group/per time point. Sirius red staining quantification of scarring and fibrosis is based on the fact that normal skin is characterized by thin, randomly oriented collagen fibers demonstrating yellow-green birefringence while scarring is associated with deposition of thick parallel collagen bundles with orange-red birefringence (15-16). The scar index is the ratio of orange-red to yellow-green pixels with higher values representing increased scarring.

Immunohistochemical and immunofluorescent staining were performed as previously described to localize lymphatic endothelial cells, growth factors, and myofibroblast markers (39). Primary antibodies used were against lymphatic vessel endothelial receptor-1 (LYVE-1, rabbit polyclonal, Abcam, Cambridge MA), Podoplanin (Syrian hamster monoclonal, Abcam, Cambridge MA), phosphorylated Smad-3 (pSmad3,
rabbit polyclonal, Abcam, Cambridge, MA), and alpha smooth muscle actin (α-SMA, Abcam, Cambridge MA). Immunofluorescent secondary antibodies used were fluorescein (R&D Systems, Minneapolis, MN) or Cy3 Zymed (Invitrogen Molecular Probes, Carlsbad, CA). For immunohistochemistry, secondary antibody was from VECTASTAIN® ABC Kit (Vector, Burlingame, CA) and developed using DAB. For all immunohistochemical or immunofluorescent studies, negative control included incubated with secondary antibody but not primary antibody.

Images were obtained using bright-field microscopy (Leica TCS) for immunohistochemistry and using a confocal microscope for immunofluorescence (Leica). Lymphatic vessel and lymphatic endothelial (LEC) cell counts were performed in 3-5 random high powered in a minimum of 3 animals/group fields by two blinded observers. There was minimal interobserver variation in the analysis. For co-localization of podoplanin and α-SMA, slides were scanned using the Mirax Slide Scanner (Zeiss). Captured bright-field and fluorescent images taken at the same location on the slide were overlaid using Adobe Photoshop (Adobe, San Jose CA).

In vivo detection of LEC apoptosis was performed on paraffin embedded tissue sections. Apoptotic cells were detected using the Apoptag Red In Situ Apoptosis Detection Kite per manufacturer’s protocol (Millipore, Billerica, MA). This assay labels apoptotic cells by using TUNEL staining. In order to localize apoptotic LECs, sections were double stained with anti LYVE-1 antibody as described above, labeled with a fluorescein secondary antibody and imaged using a confocal microscope (Leica).
quantification of LEC apoptosis the total number of LYVE-1 + cells was counted in a 3-5 random high powered fields (400x magnification) in a minimum of 3 animals/time point/group by 2 blinded reviewers. We identified cells that expressed both TUNEL and LYVE-1 and these were considered apoptotic LECs and their proportion was expressed as a percentage of the total number of LECs.

**Western Blot Analysis**

Total cellular protein was isolated from tail sections inclusive of the skin and subcutaneous tissues one week following irradiation from animals treated with LY-364947 or vehicle control. Protein was harvested using the Qiagen DNA/RNA/Protein mini kit using manufacturer’s protocol (Qiagen, Valencia, CA) and quantified using the Bradford method. Western blotting was performed as previously described (8).

Western blot analysis for TGF beta-induced (TGFBi), a protein that is upregulated by and represents a surrogate marker of TGF-β1 activity, was performed using a rabbit polyclonal antibody (Abcam, Cambridge, MA) and normalized to actin levels (24, 62).

Antibodies for N-cadherin, E-cadherin, LYVE-1, Fibroblast activating protein (FAP), and Collagen 1 were all purchased from Abcam. Immunoreactivity was determined using the ECL chemiluminescence detection system (Amersham, Arlington Heights, IL).

**Cell Culture and Reagents**

Human dermal lymphatic endothelial cells (HLEC) were obtained from PromoCell (Heidelberg, Germany) and cultured in ECGM-MV containing 0.4% ECGS/H, 5% FCS, EGF (10ng/ml), hydrocortisone (1μg/ml), and Penicillin-Streptomycin (50U/ml) (Invitrogen)
and passed every 48 hours. LEC morphology was confirmed with immunofluorescent staining for LYVE-1 and prox-1 (not shown). Early passage cells (<10) were used for all experiments.

For in vitro assay of LEC apoptosis, cells were grown to near confluence in 6 well plates or chamber slides. They were then irradiated with varying doses of radiation and evaluated at various time points. Flow cytometric analysis for apoptosis by TUNEL assay was carried out using the Apo-Direct TUNEL kit per manufacturer’s protocol (Millipore, Billerica, MA). Flow cytometry was performed on the FACScaliber flow cytometer (BD Biosciences, San Jose, CA) and analyzed using Flowjo software (Tree Star, Ashland, OR). Immunofluorescent confirmation of LEC apoptosis was carried out using the Dual Apoptosis Kit per manufacturer’s protocol (Biotium, Hayward, CA). This kit allows concomitant detection of annexin V and caspase-3 as dual markers of apoptotic cell death.

In order to assess LEC senescence after radiation therapy in vitro, cells were grown to approximately 60% confluence in 6 well plates and then exposed to various doses of irradiation and grown for an additional 4 days. After 4 days, the media was removed and the cells were briefly fixed in 4% paraformaldehyde followed by staining using the Biovision Senescence Detection Kit (Biovision, Mountain View, CA). This assay is based on the fact that senescent cells stain for SA-beta-galactosidase at a pH of 6.0 using X-gal and has been used in a large number of studies to identify cellular
senescence in vitro (25). The number of stained cells as a function of total number of cells present was quantified by blinded reviewers in a minimum of 4 plates per group. In order to evaluate the direct effects of TGF-β1 on LECs, LECs were grown in vitro in fibronectin coated flasks (Sigma, St. Louis, MO). At the initiation of experiments media was changed to DMEM with 5% FCS with or without 10ng/mL rhTGF-β1 (R&D Systems, Minneapolis, MN). Cells were then evaluated at specified time points.

Statistical analysis

Multi-group comparison was performed using one-way ANOVA with the Tukey-Kramer post-hoc test. Students T-test was used for analyzing differences between 2 groups. Data are presented as mean ± standard deviation or standard error as noted with p<0.05 considered significant.

Results

Radiation is associated with severe long-term lymphatic dysfunction

In order to assess the impact of radiation therapy on tail edema, mouse tails were radiated with a single dose of 0, 15, or 30 Gy and tail volume changes as compared with baseline were evaluated using the truncated cone formula (Figure 1A). Four weeks after radiation therapy with either 15 or 30 Gy a modest, though significant and dose dependent increase in tail volumes was noted. Animals treated with a dose of 15 Gy had a more than 15% increase, while those treated with 30 Gy demonstrated an approximately 25% increase in baseline tail volume (p<0.008 and 0.002 respectively as compared with 0Gy). These acute increases in tail edema were nearly resolved by 12
weeks following radiation therapy demonstrating no significant differences among the various groups. In addition, no statistical changes in tail volumes were noted in animals followed for as long as 24 weeks after irradiation. Thus, similar to the clinical scenario, radiation therapy alone does not appear to cause chronic swelling or lymphedema in our mouse tail model.

In an effort to evaluate the effect of radiation on dermal lymphatic function, we performed lymphoscintigraphy with Tc-\(^{99}\) labeled sulfur colloid and quantified the maximal uptake of this material in the lymph node basin at the base of the tail. This analysis demonstrated that radiation therapy caused significant and dose dependent decreases in lymphatic function 4 weeks after treatment as compared with controls (\(p<0.0001\) and \(p<0.003\) for 30 Gy and 15 Gy, respectively when compared to controls; Figure 1B). Interestingly, lymphatic dysfunction persisted at the later time points examined (12 and 24 weeks) demonstrating severe lymphatic dysfunction despite resolution of measurable tail lymphedema (Figure 1B). In addition, both radiation doses appeared to cause significant lymphatic dysfunction with an apparent progression of this phenomenon in animals treated with 15 Gy such that lymph node uptake was equivalent in severity with the 30 Gy treated animals (Figure 1B). Decay adjusted lymph node uptake of radio-labeled sulfur colloid was nearly 5 fold lower in the treated animals when compared with control (\(p<0.001\)). Image analysis of lymphoscintigraphic data demonstrated gross impairment in nodal uptake of radio-labeled colloid in irradiated animals when compared with non-irradiated controls (Figure 1C).
Radiation therapy decreases the number of dermal capillary lymphatic vessels and lymphatic endothelial cells

In order to determine the impact of radiation therapy on the number of cutaneous lymphatic vessels present tail sections were stained with the lymphatic specific marker podoplanin using immunohistochemistry and positively stained luminal structures were counted (Figure 2A, B). The number of lymphatic vessels in non-irradiated skin did not vary significantly over time (not shown) and on average 8.4±1.3 lymphatic vessels were found per high powered field (HPF). In contrast, tails irradiated with 15Gy had only 3.6±1.2 lymphatic vessels/HPF four weeks after irradiation (p<0.001). There was no statistical difference in the lower and higher doses of radiation therapy used in our study (15 and 30 Gy, respectively) implying that a single 15Gy dose is sufficient to cause lymphatic vessel loss. The decrease in the number of lymphatic vessels in irradiated animals was persistent even after 12 and 24 weeks following irradiation with the number of lymphatic vessels per HPF raging from 3.4±25 to 6±1.7 (p< 0.004 for all).

As most of the dermal lymphatics are collapsed with no obvious lumen, lymphatic vessel counts may underestimate the degree of lymphatic injury and depletion resulting from radiation therapy. Therefore, in order to confirm our vessel counts we stained sections with LYVE-1, another lymphatic specific marker, and identified individually stained cells by co-localization with DAPI using confocal
microscopy (Figure 2C, D). These findings corroborated our vessel counts demonstrating that radiation therapy caused a significant and persistent decrease in the number of LECs present in the dermis and subcutaneous tissues. We noted a modest but dose dependent decrease in LEC number 1 week after radiation. This effect became more pronounced in the later time points resulting in a nearly 4-fold decrease in the number of LECs at the latest time point (24 weeks; p<0.01). In addition, similar to our findings with vessel counts other than the 1 week time point, we found no statistically significant differences between the two doses of radiation administered further implying that a dose of 15 Gy is sufficient to cause significant injury to dermal lymphatics.

Radiation is associated with LEC apoptosis and promotes cellular senescence in vitro

In light of the persistent loss of dermal LECs associated with radiation therapy and the known effects of radiation on microvascular endothelial cells we hypothesized that radiation therapy causes LECs apoptotic cell death in vivo. In order to test this hypothesis we performed immunofluorescent co-localization of the LEC marker LYVE-1 with TUNEL detection of apoptosis and the ratio of apoptotic LECs as a function of total number of LECs was calculated. Counts of TUNEL positive LECs demonstrated a dose dependent increase in LEC apoptosis that peaked at 10 hours following irradiation. We identified very few apoptotic LECs in the non-irradiated animals (1.1%). In contrast, 10 hours after irradiation, animals treated with 15 Gy demonstrated apoptosis in 8.0% of
LECs \((p<0.005\text{ as compared with 0Gy})\) while 30 Gy of radiation resulted in apoptosis in nearly 20% of LECs \((p<0.006; \text{Figure 3A, B})\).

In order to confirm our \textit{in vivo} findings, we irradiated isolated human dermal LECs \textit{in vitro} using 0, 15, or 30 Gy of gamma irradiation and identified apoptotic cells using flow cytometry and TUNEL assays 4 and 10 hours later (Figure 3C). LECs demonstrated a statistically significant increase in apoptosis compared to controls with a 30 Gy dose 10 hours after irradiation (20.4% vs 6.8%, \(p<0.001\)), but not with a 15 Gy dose. This could be a function of a higher background rate of apoptosis in this \textit{in vitro} study. Note that the baseline rate of LEC apoptosis \textit{in vivo} was 1.1%, while \textit{in vitro} it was 6.8%. This phenomena is well recognized in endothelial cells as these often have a significant rate of background apoptosis \textit{in vitro} even with meticulously maintained culture conditions (32). LEC apoptosis following exposure to XRT was confirmed by immunofluorescence for Annexin V and Caspase 3 (Figure 3D). This finding is interesting as it suggests that \textit{in vivo} tissue injury following radiation may sensitize LECs to apoptosis. Indeed, we recognize that a limitation of \textit{in vitro} irradiation studies is that these conditions fail to mimic changes in the specific niche of individual cells and may therefore underestimate the effect of irradiation on isolated cells. However, \textit{in vitro} radiation experiments have been used in multiple previous studies with the dose range described as a means of validating and supporting \textit{in vivo} observations (13, 51, 58, 63, 70).
Irradiation has been previously shown to cause cellular senescence by upregulating cell cycle regulators such as p21 and p16 (63, 74). Senescent cells are metabolically active but demonstrate significantly reduced potential for proliferation and differentiation (10). In order to determine the effects of sublethal doses of irradiation on cellular senescence in isolated dermal LECs, cells were treated with 0, 4, 8, or 12 Gy. LECs were then grown for an additional 4 days and evaluated for senescence by B-galactosidase staining at pH 6 (Figure 3E, F) (9). Interestingly, this analysis demonstrated that radiation therapy, even at low doses, causes a statistically significant and dose-dependent increase in LEC senescence as compared with controls at all doses tested. This finding implies that radiation therapy may impair lymphatic function by causing premature senescence of these cells.

Protection of LECs from radiation induced apoptosis does not prevent long-term lymphatic dysfunction

In order to study the relative impact of fibrosis and LEC apoptosis on the pathogenesis of radiation associated lymphatic dysfunction we irradiated the tails of acid sphingomyelinase deficient mice (ASM-/-) and wild-type litter mates (WT). Evaluation of LEC apoptosis 10 hours after treatment with 15Gy demonstrated that ASM-/- had significantly less apoptosis as compared with wild-type controls (4.5% vs. 8.1%, p<0.03, Figure 4 A, B). Interestingly, however, this protection from apoptosis did not translate to significantly improved lymphatic function when assessed by lymphoscintigraphy 4 weeks after radiation (Figure 4C). In addition, ASM deficiency did
not significantly decrease radiation induced fibrosis as assessed by Sirius red staining (Figure 4D, E). Radiation causes soft tissue fibrosis and increased TGF-β1 activity.

Radiation is known to cause soft tissue fibrosis (4, 68). In fact, clinically, many of the long-term wound healing and surgical complications associated radiation are directly related to soft tissue fibrosis. Previous studies have shown that post-radiation skin fibrosis is in large measure caused by activation of TGF-β1 and its down-stream mediator Smad-3 (17). Furthermore, we and others have previously demonstrated that TGF-β1 is an anti-lymphangiogenic cytokine by inhibiting LEC proliferation and function and that fibrosis is a significant inhibitor of lymphatic function during wound healing (5, 8, 41). Therefore, in order to determine the effects of fibrosis in general, and TGF-β1 function in particular, we evaluated fibrosis mouse tails treated with 0, 15, or 30 Gy of irradiation.

As expected, radiation therapy resulted in loss of hair follicles, epidermal thinning, dermal thickening, loss of fat, fibrosis, and increased inflammation at all times evaluated following radiation (not shown). Using Sirius red staining and birefringence microscopy to calculate the scar index, we found that radiation caused progressive fibrosis with a scar index that is significantly higher than control starting at 4 weeks irradiation (p<0.03 for all doses at time points when compared with control) (Figure 5A, B). In addition, immunohistochemical localization of phosphorylated Smad3 (pSmad3), an important downstream mediator of TGF-β1 signaling demonstrated a significant increase in the number of pSmad3 positive cells as early as 4 weeks following radiation
therapy. Similar to previous reports, this increase TGF-β1 activity persisted even 24 weeks after radiation therapy in animals treated with 30 Gy (p<0.03) (Figure 5C, D).

Treatment with LY-364947, a small molecule inhibitor of TGF-BRI, effectively blocks TGF-β1 signaling

Roberts and colleagues have previously shown treatment of mice with small molecule inhibitors of TGF-β1 for 3-5 weeks can decrease the fibrosis associated with radiation therapy (69). However, the effects of short-term TGF-β1 blockade on tissue fibrosis and lymphatic function remain unknown. This is important since long-term inhibition of TGF-β1 function can cause significant morbidity due to the protean effects of this growth factor. Therefore, we inhibited TGF-β1 function using a small molecule inhibitor of TGF-BRI (LY-364947) for just one week after radiation and evaluated the effects of this intervention on fibrosis, lymphatic function, and lymphatic vessel depletion.

Western blot analysis of tissues harvested 1 week after radiation (15 Gy) from animals treated with or without LY-364947 demonstrated a nearly 5 fold reduction in the expression of TGFB-induced protein (TGF-Bi) in the LY-364947 treated animals (Figure 6A, B). TGF-Bi protein expression is directly related to TGF-β1 activity therefore, reductions in TGF-Bi in LY-364947 treated animals confirm reduction in TGF-β1 signaling (24). This conclusion is further supported by immunohistochemical localization of phosphorylated Smad-3, the down-stream mediator of the TGF-β receptor, demonstrating a significant reduction in the number of pSmad3 positive
cells/high power field in LY-364947 treated animals both 4 and 12 weeks after radiation (Figure 6C, D; \(p<0.03\)). This finding is consistent with previous reports demonstrated that TGF-\(\beta\)1 expression is regulated, at least in part, by positive feedback mechanisms (50).

Short-term inhibition of TGF-\(\beta\)1 signaling reduces radiation induced soft tissue fibrosis and lymphatic dysfunction

Interestingly, even very short-term blockade of TGF-\(\beta\)1 function using LY-364947 after irradiation with 15Gy significantly decreased soft tissue fibrosis as compared to control animals. The scar index of irradiated/LY-364947 treated animals was more than 2 fold less than vehicle treated/irradiated animals even as long as 12 weeks after radiation (Figure 7A, B; \(p<0.01\)). Short-term blockade of TGF-\(\beta\)1 function was associated with decreased tail volume 4 and 12 weeks following radiation therapy although this effect was not statistically different than vehicle control treated animals (Figure 7C). This finding is not unexpected since in our earlier experiments we demonstrated that radiation only caused mild, acute increases in tail volume measurements that resolve between 4 and 12 weeks after treatment. Interestingly, however, we found that lymphatic function as assessed by lymphoscintigraphy was significantly improved in animals treated with LY-364947 as compared to controls at both time points evaluated (Figure 7D). On average, nodal uptake was nearly 4-fold and 2.5-fold higher in the LY-364947 treated animals 4 and 12 weeks after treatment respectively, as compared with controls (\(p<0.0001\) and \(p<0.028\), respectively). Image analysis of lymphoscintigraphic
data demonstrated grossly increased uptake of radio-labeled colloid in the lymph nodes of animals treated with LY-364947 when compared to vehicle control treated animals (Figure 7E).

Improved lymphatic function in LY-364947 treated animals was not due to a protective effect on LECs since quantification of dermal LECs did not show significant differences in the LY-364947 or vehicle treated animals (Figure 7F). In addition, TGF-β1 blockade did not significantly alter LEC apoptosis when evaluated 10 hours after treatment with 15Gy (Figure 7G, H). Similarly, treatment of isolated LECs with recombinant TGF-B1 (10ng/ml) did not result in increased rates of apoptosis as compared with controls.

Short-term inhibition of TGF-β1 signaling may reduce radiation induced LEC EMT and lymphatic fibrosis

TGF-β1 is a known inducer of endothelial-mesenchymal cell trans-differentiation (EMT) resulting in vascular fibrosis (45-47). In addition, we have more recently shown that LECs in animals treated with TGF-β1 or in an environment containing high endogenous expression of TGF-β1 co-express lymphatic and smooth muscle cell markers (LYVE-1 and α-SMA, respectively) in dermal lymphatic capillaries (8). This is an abnormal phenotype as dermal lymphatic capillaries are not associated with pericytes. In addition, lymphatic vessel fibrosis is a known clinical hallmark of lymphedema resulting in lymphatic vessel obliteration and dysfunction (56). Therefore, in order to determine if radiation therapy caused expression of fibroblast markers by lymphatic capillaries, we
co-localized podoplanin and α-SMA in non-irradiated animals, and in animals irradiated with 15 Gy and treated with or without LY-364947 (Figure 8A). As expected we found no LECS that expressed α-SMA in non-irradiated animals. Similarly, we found no lymphatic vessels that expressed α-SMA in irradiated animals treated with LY-364947. In contrast, we noted that in animals treated with vehicle and 15Gy of irradiation, lymphatic capillaries that co-expressed α-SMA could be seen in some sections, albeit these represented a minority of lymphatic capillaries present.

In order to more directly study the effects of TGF-B1 on LEC de-differentiation and EMT, we treated isolated LECs with recombinant TGF-B1 (10ng/ml) and analyzed the expression of a panel of fibroblast and LEC markers at various time points (Figure 8B). Interestingly, stimulation of LECs with recombinant TGF-B1 markedly increased the expression of putative EMT markers type I collagen, N-cadherin, and fibroblast activating protein (FAP) (73). Conversely, expression of the LEC marker LYVE-1 and the endothelial marker E-cadherin was markedly reduced by rhTGF-β1 treatment, providing further evidence for de-differentiation and cellular transformation.

Discussion

Although radiation therapy following lymphadenectomy is a significant risk factor for the development of chronic lymphedema, the mechanisms that contribute to this risk remain unknown (23, 49, 54). This gap in our knowledge is an important barrier to the development of targeted therapeutic or preventative strategies for lymphedema. In the current study we demonstrate that radiation therapy causes lymphatic
dysfunction due to depletion of lymphatic endothelial cells and soft tissue fibrosis.

Similar to the clinical scenario in which lymphedema is rare following radiation therapy without surgery, we found that radiation in our model resulted in only subtle increases in tail volume that resolved over time. Interestingly, however, we found that lymphatic function was markedly decreased after radiation therapy implying that radiation decreases the baseline clearance of lymphatic fluid without overwhelming the transport capacity of existing vessels. This finding is supported by the findings of Mortimer and colleagues demonstrating impaired dermal lymphatic function as measured by lymphoscintigraphy in a porcine model following 18 Gy of radiation (40). Our study expands the findings of Mortimer et al by demonstrating that lymphatic transport capacity is decreased for prolonged periods of time (even 6 months after radiation).

These findings support the hypothesis that the long-term reduction in lymphatic function resulting from radiation therapy is not usually sufficient to overwhelm the transport capacity of extremity but when combined with additional injury from surgery go on to cause chronic lymphedema.

We hypothesized that one mechanism by which radiation therapy may decrease lymphatic function is by directly injuring or depleting lymphatic endothelial cells. This hypothesis is supported by the fact that microvascular endothelial cells are highly sensitive to radiation injury and undergo apoptosis (43). In fact, depletion of these cells is thought to be the underlying pathogenic mechanism for gastrointestinal toxicity resulting from radiation therapy (43). In the present study we show that dermal
lymphatic vessels are also susceptible to apoptosis after radiation injury and that this response is, at least in part due to LEC apoptosis in a process that is mediated, at least in part, by acid sphingomyelinase. Interestingly, however, we found that prevention of LEC apoptosis did not translate to improved lymphatic function as assessed by lymphoscintigraphy suggesting that other processes such as tissue fibrosis play an overall larger role in the regulation of lymphatic function after radiation injury.

This pro-apoptotic effect of radiation was maximal 10 hours after radiation but was apparent even at the earliest time-point we selected (4 hours). Our findings are also supported by the work of Abtahian et al who demonstrated intestinal lymphatic endothelial cell apoptosis 4 hours after 12Gy of total body irradiation (1). In contrast, however Sung at al have previously shown that that intestinal and peritumoral lymphatics are resistant to radiation induced apoptosis (57). This difference from our findings may be related to a number of factors. Most importantly, Sung and colleagues used a dose of 15Gy and evaluated intestinal lymphatic endothelial cells. In the present study while we found a modest increase in LEC apoptosis after 10 hours with 15Gy (approximately 7% of LECs), 30 Gy caused a more significant increase in the number of apoptotic cells. The relative doses of radiation to the skin are also likely to be higher since they are superficial as compared to the intestinal villi exposed to total body irradiation. It is also possible that dermal lymphatics are phenotypically different than intestinal lymphatics and therefore more susceptible to apoptosis after ionizing radiation injury. This idea is supported by the fact that Intestinal lymphatics, for
example, are important in lipid transport, whereas dermal lymphatics are not. Furthermore, cellular responses to radiation injury are known to be tissue dependent and influenced by surrounding cellular niche. For example, previous studies have shown that growth factors such as heparanase that are found in the extracellular matrix may provide a radioprotective effect for local endothelial cells (19). Thus, dermal LECs may be exposed to a micro-environment that is more inductive of apoptosis than that of intestinal LECs.

Interestingly, we demonstrated that LECs in culture are relatively radioresistant and undergo apoptosis only when exposed to high doses such as 30Gy. The observed differences in \textit{in vivo} and \textit{in vitro} radiation susceptibility may be due to differences in the microenvironment of the cell \textit{in vivo}. This hypothesis is supported by the fact that impairment of lymphatic vessel function in response to ultra violet B radiation \textit{in vivo} is mediated by the release of VEGF-A implying that radiation therapy not only affects individual cells but also changes the cellular microenvironment (28). Furthermore, \textit{in vivo} radiation may cause generation of free oxygen radicals or other toxic substances that may increase the susceptibility to injury. Kajiya and colleagues have also demonstrated that ultraviolet B radiation therapy markedly down-regulates the expression of VEGF-C leading to lymphatic dysfunction (29). Similar changes may contribute to decreased LEC and lymphatic vessel numbers in our study since activation of VEGF-R3 is required for LEC survival and proliferation (18).
Our *in vitro* studies also demonstrated that human dermal LECs are sensitive to radiation therapy in terms of cellular senescence. We found that even relatively low doses of radiation caused an increase in the number of senescent cells. This is an interesting finding as it suggests that chronic reduction in LEC and lymphatic vessel numbers may be related to premature senescence of lymphatic endothelial cells. Similar findings have been demonstrated *in vitro* in a variety of cell types including fibroblasts, hematopoietic stem cells, and mesenchymal stem cells (7, 14, 26, 64). An important finding in our study that supports the concept of LECs are susceptible to radiation injury is the fact that radiation therapy caused a significant depletion of LECs and lymphatic vessels in tissues even up to 6 months after exposure. This is important as it demonstrates a direct mechanism by which radiation therapy decreases the transport capacity of the lymphatic system. Jackowski and colleagues have evaluated the effects of radiation therapy on lymphatic vessels using biopsies obtained at various times after treatment obtained from women treated for breast cancer (27). Similar to our study the authors demonstrated that the number of small lymphatic vessels (<10μm) was decreased in the early periods following radiation (0-5 months) although this difference was not statistically significant. However, in the patients evaluated between 11-14 months the authors found a significant increase in the number of small lymphatic vessels in the skin that they termed radiogenic lymphangiogenesis. These differences were no longer apparent 17+ months after radiation therapy. These findings imply that the timing of evaluation after radiation...
therapy is important and may be a reason for the differences observed in our study.

Furthermore, the differences in the number of lymphatic vessels after radiation between our study and that of Jackowski et al may be related to the radiation dose or fractionation protocols. Clinically patients were treated with a total of 40Gy of radiation delivered in a fractionated manner whereas animals treated in our study were treated with a single dose. Thus, fractionation may have a protective effect for lymphatic vessels. This may be reflected in the finding that the subset of patients that developed lymphedema failed to mount a lymphangiogenic effect in response to radiation implying that these patients had a more severe injury. Therefore, similar to our findings, patients with a reduction in the number of cutaneous lymphatic vessels appeared to have decreased lymphatic function and subsequent lymphedema.

The differences in the observations of Jackowski et al from our study may also stem from significant variability in the number of lymphatic vessels between patients. In fact, these differences forced the authors to use a treated:control ratio for their analysis which may have altered the findings in some way. This idea is further supported by studies demonstrating that genetic polymorphisms play a role in an individual’s tissue response to radiation (6, 31). Interestingly, polymorphisms in the gene coding for TGF-β1 have been directly implicated as modulators of tissue response to radiation (72). The use of genetically inbred animals for the study of lymphatic function obviates these differences and may simplify analysis. The inherent variability in baseline lymphatic vessel numbers observed in clinical studies is interesting as it suggest
that genetic or other differences may influence the baseline lymphatic transport capacity and therefore contribute to the risk of developing lymphedema. This concept is supported by the fact that lymphatic imaging studies have demonstrated that the rate of lymphatic transport in patients is highly variable both after surgery and at baseline, and that patients with low base-line and postsurgical clearance rates have a higher risk of developing lymphedema (55).

Another potential mechanism by which radiation therapy can decrease lymphatic function is soft tissue fibrosis. Fibrosed soft tissues lose compliance and are characterized by loss of normal structures which are replaced by scar tissue. In fact, the clinical hallmarks of lymphedema are soft tissue and lymphatic vessel fibrosis (56). Anatomical and cadaver studies have shown that lymphatic vessels undergo fibrosis after lymphadenectomy losing compliance and in many instances becoming completely stenosed and non-functional (56). We have previously shown that lymphatic fluid stasis is associated with soft tissue fibrosis and lymphatic dysfunction and that this effect is due, at least in part, to increased expression of TGF-β1 (5, 8). In addition, we and others have shown that TGF-β1 has anti-lymphangiogenic effects by inhibiting LEC proliferation and tubule formation (8, 41). These findings are important since radiation therapy is known to cause tissue fibrosis as a result of chronically increased TGF-β1 expression and may therefore be additive to the effects of lymphatic stasis in promoting soft tissue fibrosis and lymphatic dysfunction. This hypothesis is supported by our findings in ASM-
- mice in which LEC apoptosis was significant decreased but lymphatic dysfunction and
tissue fibrosis persisted at later time points.

Several studies have shown that inhibition of TGF-β1 function either in knockout
animals or using small molecule inhibitors of TGF-β1 can decrease skin fibrosis resulting
from radiation (17, 36, 69). These findings have been difficult to translate clinically,
however, since long-term inhibition of TGF-β1 function was required. In fact, the
shortest effective time for treatment reported was 3-5 weeks using the small molecule
inhibitor Halofuginone (69). Prolonged TGF-β1 inhibition can have significant negative
side effects due to immunosuppression and the protean effects of TGF-β1 in a variety of
organ systems (33, 68). To circumvent these potential difficulties, we evaluated the
effects of short-term (1 week) TGF-β1 inhibition on radiation fibrosis and lymphatic
function. We hypothesized that if the autoregulatory upregulation of TGF-β1 is avoided
then long-term sequela of radiation can be minimized. Indeed, we found that short-
term blockade of TGF-β1 with LY-364947 resulted in marked reduction of the
progressive fibrosis that is associated with radiation injury. This finding is consistent
with the study by Wormstone and colleagues, who showed that even a two day
exposure to TGF beta results in long term signaling changes in matrix contraction,
transdifferentiation, and fibrosis (66). In our study, TGF-β1 activity as measured by
Smad3 phosphorylation was attenuated far beyond the duration of therapy, suggesting
that we had at least partially succeeded in breaking the positive feedback loop of TGF-
β1 activity that is seen with progressive fibrosis (68). These findings directly correlated
with better lymphatic function in the LY-364947 treated animals, and consistent with the hypothesis that fibrosis is a mediator of lymphatic dysfunction. Interestingly, treatment with LY-364947 did not appear to confer any direct radioprotection to LECs in the present study, as evidenced by quantification of LEC depletion. While TGF-β1 has been shown to inhibit LEC tubule formation and proliferation, there is no data implicating this growth factor in LEC death. Thus the present study indicates that radiation induced LEC apoptosis is likely independent of TGF-β1.

Previous studies have shown that TGF-β1 can cause endothelial-mesenchymal transdifferentiation and microvascular vessel fibrosis (45-46, 68). In addition, we have previously shown that increased TGF-β1 expression is associated with co-expression of lymphatic and fibroblast markers in capillary lymphatics (8). In the current study we found that radiation therapy also induced the fibroblast marker α-SMA by capillary lymphatic endothelial cells and that this effect could be abrogated by short-term blockade of TGF-β1 function. These findings, together with our in vitro experiments demonstrating that TGF-B1 increases expression of EMT associated markers and simultaneous downregulation of lymphatic and endothelial markers support the hypothesis that radiation therapy may directly contribute to lymphatic dysfunction by promoting lymphatic vessel fibrosis as a result of increased TGF-B1 expression.

Radio-protective strategies are employed in a variety of clinical scenarios to decrease the morbidity of radiation (11, 20-21). For instance, these approaches have been used to decrease the early morbidity of head and neck cancers such as intraoral
blistering and swelling. In addition, radioprotection has been effectively used to
decrease the incidence of late radiation induced sequela such as osteoradionecrosis and
growth disturbances in children (12). These strategies are not commonly used for
breast cancer treatment, however since radiation in this setting is thought to be
relatively well tolerated causing little more than skin blistering and swelling. Our
findings, however, suggest that radioprotective approaches may have a role in the in
prevention of lymphedema by preventing lymphatic dysfunction. This is critical since
lymphedema remains the most dreaded complication of breast cancer treatment and is
a major source of morbidity for more than 500,000 breast cancer survivors (22, 38).
Future studies from our laboratory will explore these approaches in an effort to identify
optimal methods of limiting radiation induced injury to lymphatic channels.
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Figure 1. Radiation therapy causes severe, long-term lymphatic dysfunction. A. Tail volume changes from baseline after irradiation with 0, 15, or 30 Gy. Although there was a significant, dose dependent increase in tail volume 4 weeks after radiation therapy ($p<0.008$ and $0.002$, respectively), these differences resolved in long-term follow up (12, 24 weeks). B. Analysis of lymphatic function with lymphoscintigraphy 4, 12, and 24 weeks following radiation with 0, 15, or 30 Gy. Decay adjusted maximal uptake of Tc$^{99}$ labeled sulfur colloid in the lymph nodes at the base of the tail after injection at the tip of the tail is presented. Note significant, dose dependent impairment in lymph node uptake at the four week time point (*$p<0.001$ for both doses compared with non-irradiated control, # $p<0.05$ for 15 Gy compared to 30 Gy at 4 weeks following XRT). Also note sustained, and progressive lymphatic dysfunction in animals treated with 15 or 30 Gy and analyzed after 12 and 24 (*$p<0.001$ for both doses) weeks. C. Representative heat maps of Tc$^{99}$ radio-isotope uptake in a non-irradiated animal (upper figure) and an animal 24 weeks following exposure to 30 Gy (middle figure). Red color indicates areas of most intense radioactivity and yellow, green, and blue representing progressively lower activities. Arrow head represents the site of radio-labeled colloid near the tip of the tail, and arrow represents uptake in the lymph node basin at the base of the tail. Note far greater uptake in the lymph nodes of the non-irradiated mouse at the conclusion of the study, indicating more efficient lymphatic transport. A picture of a mouse tail is presented for orientation (bottom figure).
**Figure 2. Radiation therapy decreases number of dermal capillary lymphatic vessels and lymphatic endothelial cells.**

**A.** Analysis of capillary lymphatic vessels in the skin following irradiation with 0, 15, or 30 Gy. Tissues were stained using podoplanin antibody and lymphatic vessel counts were performed in 3-5 high powered sections in a minimum of 3 animals per group by 2 blinded reviewers. Vessel counts are presented as mean ± SD. Note significant and sustained decrease in the number of podoplanin positive luminal structures in irradiated tissue sections as compared with non-irradiated control sections (*p<0.05* compared to non-irradiated control).

**B.** Representative 200x micrographs of tissue sections from a non-irradiated animal (0 Gy; top figure) and an animal 24 weeks following a 30 Gy dose of radiation (30 Gy; bottom figure), stained immunohistochemically for podoplanin (arrow demonstrates positively stained lymphatic). **C.** Analysis of the number of LECs/HPF using immunofluorescent staining for LYVE-1. Cell counts were performed in 3-5 HPF sections/animal in a minimum of 3 animals per time point/group by blinded reviewers and are presented as mean ± SD. Again, note significant decrease in LEC counts in irradiated animals beginning 4 weeks after irradiation (*p<0.05* for all doses and time points compared to baseline; *p<0.001* at 24 weeks)

**D.** Representative 100x micrographs of sections stained for LYVE-1 (red) and DAPI nuclear counter stain (blue) in animal exposed to 0 Gy (upper figure) or 30 Gy (lower figure). Note grossly observed decreased number of LECs (red cells).
Figure 3. Radiation is associated with LEC apoptosis and promotes cellular senescence in vitro.

A. Identification of apoptotic LECs by co-localization of LYVE-1 and TUNEL. Individual LECs were identified by LYVE-1 staining and counted in 3-5 HPF sections in a minimum of 3 animals per time point/group by blinded reviewers. Percentage of apoptotic LECs was calculated by similarly counting the number LYVE-1+, TUNEL+ cells and expressing this number as a function of total LEC number. Data are presented as mean ± SD. Increased LEC apoptosis was noted as early as 4 hours after radiation and peaked at the 10 hour time point in a dose dependent manner (* p<0.01). B. Representative immunofluorescent micrographs (200x) of co-localization of the LEC marker (red) with TUNEL (green) and DAPI nuclear stain (blue) 10 hours following irradiation with 0 Gy, 15 Gy, or 30 Gy. Note the dose dependent increase in the presence of TUNEL positive nuclei. C. Flow cytometric TUNEL analysis of isolated LECs irradiated in vitro demonstrated that LECs in this setting are relatively radio-resistant requiring a 30 Gy dose of radiation to induce significant apoptosis (* p<0.0001). Data are presented as a mean of triplicate experiments ± SD. D. This finding was confirmed by immunofluorescence for Annexin V and Caspase-3. In the presented representative images Annexin V is shown by red immunofluorescence, Caspase 3 with green immunofluorescence, and DAPI nuclear counter stain in blue. Note greater number of cells staining for both Annexin V and Caspase-3 in the 30 Gy treated cells. (lower figure) E. Radiation therapy induces LEC senescence 4 days following treatment, at doses that
are much lower than those required to induce apoptosis. Cells were radiated with 0, 4, 8, or 12 Gy of ionizing radiation and senescence was analyzed with B-galactosidase staining at pH 6. Positively stained (blue) cells were identified with light microscopy and quantified in 3-5 HPF/treatment group. All experiments were performed in triplicate and data are presented as mean ± SD. Note a significant, dose dependent increase in LEC senescence (*p<0.001 for all compared to non-irradiated control). F. Representative photomicrographs (100X) of B-galactosidase staining of non-irradiated (0Gy; top figure) and irradiated (30 Gy; bottom figure) LECs.

**Figure 4. Protection of LECs from radiation induced apoptosis does not prevent induction of lymphatic dysfunction**

A. Identification of apoptotic LECs by co-localization of LYVE-1 (red) and TUNEL (green) in ASM-/- and wild-type mice (mean ± SD). Note significant decrease in LEC apoptosis 10 hours following radiation in ASM-/- mice (*p<0.034)

B. Representative immunofluorescent micrographs (200x) of co-localization of the LEC marker (red) with TUNEL (green) and DAPI nuclear stain (blue) 10 hours following irradiation with 15 Gy.

C. Lymphoscintigraphy of ASM -/- and WT mice 4 weeks after 15 Gy of irradiation. Data represent mean maximal decay adjusted uptake of a minimum of 3 animals per time point/group (not significant).

D. Scar index of tissue sections from ASM-/- and WT animals that received 15 Gy of irradiation 4 after radiation (not significant).
Representative 200X micrographs of tissue sections from ASM-/- mice (top figure) or WT (bottom figure) 4 weeks following radiation with 15 Gy. No differences are observed.

Figure 5. Radiation causes soft tissue fibrosis and increased TGF-$\beta$1 activity.

A. Calculation of the scar index using polarized light microscopy of Sirius red stained sections. Mean scar index was calculated from analysis of 3-5 sections/animal/group and data are presented as mean ± standard deviation. Note significant and progressive increase in scar index after radiation with 15 or 30 Gy (* $p<0.04$ for all compared to baseline except for the 15 Gy dose at 12 weeks, which approached but did not achieve significance $p<0.08$). B. Representative photomicrographs (200X) of non-irradiated (0 Gy; top figure) and irradiated (30 Gy; bottom figure) sections 24 weeks after radiation. Note increased fibrosis in irradiated section as demonstrated by more red and orange birefringence. In contrast, note yellow-green birefringence of non-irradiated skin. C. Radiation therapy increases the number of phosphorylated Smad-3 (pSmad) positive cells in tissue sections beginning as early as 4 hours after radiation and persisting as long as 24 weeks later (* $p<0.03$ for all compared to baseline). Sections were stained with phosphorylated Smad-3 antibodies and the number of positively stained cells was assessed in 3-5 HPF per section in a minimum of 3 animals per time point/group (mean ± SD). D. Representative 200x micrographs of pSmad3 immunohistochemical staining in non-irradiated (0 Gy; top figure) and irradiated (30 Gy figure) sections 4 hours after radiation. Note that non-
irradiated sections demonstrate virtually no positive dermal cells, while large numbers of positively stained cells are noted in the irradiated tissue section.

Figure 6. Treatment with LY-364947, a small molecule inhibitor of TGF-BRI, effectively blocks TGF-β1 signaling. A, B. Western blot (A) and quantification (B) for TGFβi in vehicle (2 left lanes) and LY-364947 (2 right lanes) treated animals 1 week after irradiation with 15 Gy. Each lane represents protein isolated from an individual animal. Note marked reduction (nearly 5 fold decrease) in TGFβi protein expression in LY-364947 treated animals indicating inhibition of TGF-β1 signaling (* p<0.02). C. Calculation of the number of pSmad-3 stained cells in 3-5 sections/animals in a minimum of 3 animals per group 4 and 12 weeks after irradiation with 15Gy (mean ± SD). Note significant and persistent decrease in the number of pSmad-3 positive cells in animals treated with LY-364947 for 1 week (* p<0.03). D. Representative 200X micrographs of tissue sections from animals treated with vehicle (top figure) or LY-364947 (bottom figure) for 1 week after irradiation with 15Gy. Tissue sections were harvested 12 weeks after radiation therapy. Note marked decrease in the number of pSmad-3 stained cells in the LY-364947 treated animals.

Figure 7. Short-term inhibition of TGF-β1 signaling reduces radiation induced soft tissue fibrosis and lymphatic dysfunction. A. Scar index of tissue sections from animals that received 15 Gy of irradiation and treated with vehicle or LY-364947 for 1
week after irradiation (mean scar index ± SD). Tissues were harvested 4 or 12 weeks after radiation. Note marked decrease in scar index in LY-364947 treated animals as compared with controls at both time points evaluated (* p<0.002 and # p<0.02 ). B. Representative 200X micrographs of tissue sections from animals treated with vehicle (top figure) or LY-364947 (bottom figure) 12 weeks following radiation with 15 Gy. Note decreased scarring as represented by decreased red-orange and increased yellow-green birefringence in the LY-364947 treated section. C. Tail volume measurements in animals treated with vehicle or LY-364947 four or 12 weeks after irradiation with 15 Gy (mean ± SD). Treatment with LY-364947 resulted in mild but non-significant reduction tail edema following irradiation. D. Lymphoscintigraphy of animals treated with vehicle or LY-364947 4 (D) or 12 (E) weeks after 15 Gy of irradiation. Data represent mean maximal decay adjusted uptake of a minimum of 3 animals per time point/group. Note statistically significant increased lymph node uptake in LY-364947 treated animals particularly at the 4 week time point (* p<0.0001) Statistically significant improved lymphatic function was also noted at the 12 week time point, however, this difference was less marked than the 4 week analysis (# p<0.03). E. Representative heat maps of Tc-99 radio-isotope uptake in mice irradiated with 15 Gy and treated with either vehicle (left panels) or LY-364947 (right panels), at 4 and 12 weeks following irradiation. Red color indicates areas of most intense radioactivity and yellow, green, and blue representing progressively lower activities. Arrow head represents the site of radio-labeled colloid near the tip of the tail, and arrow represents uptake in the lymph node.
basin at the base of the tail. Note far greater uptake in the lymph nodes of animals treated with LY364947, indicating more efficient lymphatic transport. 

F. Quantification of the number of LECs in tissue sections from animals treated with vehicle or LY-364496 four or twelve weeks following 15 Gy of irradiation (mean \( \pm \) SD). No significant differences were noted. 

G. Identification of apoptotic LECs by co-localization of LYVE-1 and TUNEL (mean \( \pm \) SD; not significant). 

H. Representative immunofluorescent micrographs (200x) of co-localization of the LEC marker (red) with TUNEL (green) and DAPI nuclear stain (blue) 10 hours following irradiation with 15 Gy. 

I. Flow cytometric TUNEL analysis of isolated LECs demonstrating no induction of apoptosis in response to treatment with rhTGF-B1 (10ng/ml). Data are presented as a mean of triplicate experiments \( \pm \) SD.

Figure 8. Radiation results in co-expression of lymphatic and fibroblast markers in capillary lymphatics and this effect is inhibited by TGF-\( \beta \)1 blockade. 

A. Representative 100x (left panels) and 250x (right panels) overlay images of tissue sections stained with podoplanin (brown) and \( \alpha \)-SMA (fluorescent green) obtained from animals radiated with 0 Gy (top panels), 15 Gy and treated with vehicle (middle panels), or 15 Gy and treated with LY-364947. Note no co-localization of podoplanin and \( \alpha \)-SMA in non-irradiated or irradiated/LY-364947 treated tissues. In contrast, note lymphatic vessel stained for both podoplanin and \( \alpha \)-SMA in sections obtained from an animal treated with 15 Gy of irradiation and vehicle control. 

B. Western blot analysis of LECs
grown in vitro and stimulated with 10 ng/mL rhTGF-β1 4 hours, 24 hours, and 72 hours after initiation of stimulation. Fibroblast and EMT markers Collagen I, N-cadherin, and FAP are upregulated by stimulation. Conversely, LEC marker LYVE-1 and endothelial cell marker E-cadherin are greatly down regulated.
References


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Figure 1 A
Figure 1 B, C
Figure 2 A, B
Figure 2 C, D
Figure 3 A, B
Figure 3 C, D
Figure 3 E, F
Figure 4 A, B
Figure 4 C
Figure 4 D, E
Figure 5 A, B
Figure 5 C, D
Figure 6 A, B
Figure 6 C, D
Figure 7 A, B
Figure 7 C
Figure 7 D

Vehicle LY-364947

Maximal nodal uptake (%)

Vehicle
LY-364947

4 Wks
12 Wks

* 

#
Figure 7 E
Figure 7 F
Figure 7 G, H
Figure 7 I

The bar graph shows the percentage of apoptotic cells in +TGF and -TGF conditions. The TUNEL FITC and FL3-A scatter plots illustrate the distribution of apoptotic cells. The graph indicates a higher percentage of apoptotic cells in the -TGF condition compared to the +TGF condition.
Figure 8A
Figure 8 B