

Repopulation of the Irradiation Damaged Lung with Bone Marrow-derived Cells

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Abstract. Aim: The effect of lung irradiation on reduction of lung stem cells and repopulation with bone marrow-derived cells was measured. Materials and Methods: Expression of green fluorescent protein positive cells (GFP⁺) in the lungs of thoracic irradiated FVB/NHsd mice (Harlan Sprague Dawley, Indianapolis, IN, USA) was determined. This was compared to the repopulation of bone marrow-derived cells found in the lungs from naphthalene treated male FVB/NHsd mice and gangciclovir (GCV) treated FeVBN GFP⁺ male marrow chimeric HSV-TK-CCSP. The level of mRNA for lung stem cell markers clara cell (CCSP), epithelium 1 (FOXJ1) and surfactant protein C (SP-C), and sorted single cells positive for marrow origin epithelial cells (GFP⁺CD45⁻) was measured. Results: The expression of pulmonary stem cells as determined by PCR was reduced most by GCV, then naphthalene, and least by thoracic irradiation. Irradiation, like GCV, reduced mRNA expression of CCSP, CYP2F2, and FOXJ1, while naphthalene reduced that of CCSP and CYP2F2. Ultrastructural analysis showed GFP⁺ pulmonary cells of bone marrow origin, with the highest frequency being found in GCV-treated groups. Conclusion: Bone marrow progenitor cells may not participate in the repopulation of the lung following irradiation.

The bone marrow origin of epithelial cells remains a topic of intense controversy. Initial reports of the reconstitution of total-

body irradiated mice with a single hematopoietic stem cell (1) were confirmed by multiple reports of bone marrow-derived cells capable of repopulating the lung (2-11), esophagus (12-15), and other organs (16). Other studies suggested that the level of repopulation of lung epithelial organs with cells of bone marrow origin was either nonexistent or very low (17-22). Most bone marrow-derived cells in the lung were determined to be hematopoietic. While the bone marrow origin of epithelial cells in irradiation-damaged tissue remains controversial, there is clear evidence that bone marrow transplantation ameliorates the toxicity of subtotal or total-body irradiation (16, 23, 24). The magnitude of the contribution of bone marrow-derived cells in ameliorating the acute toxicity of lung irradiation damage remains controversial.

Mouse models of lung irradiation damage demonstrate genetic strain-dependent variation (25-26), as well as a histopathologic difference between acute radiation pneumonitis and late irradiation alveolitis/fibrosis (24, 27-29). To determine the role of bone marrow cells in the repair of lung irradiation damage, a positive control model for the specific removal of lung stem cells is required.

We have taken advantage of a unique model of the depletion of pulmonary stem cells in HSV-TK-CCSP transgenic mice (30-33). These mice demonstrate Herpes simplex virus (HSV) thymidine kinase (TK) expression linked to the lung stem cell (Clara-cell)-specific promoter (CCSP) (33) and when they are treated by mini-Alzet pump administration of gangciclovir (GCV) demonstrate a drug dose-dependent depletion of pulmonary stem cells (30-32). Furthermore, GCV-treated HSV-TK-CCSP mice demonstrate primitive lung stem cell depletion in addition to committed pulmonary progenitor cell depletion associated with naphthalene toxicity (30-33).

In the present studies, we utilized the FVB/NHsd (Harlan Sprague Dawley, Indianapolis, IN, USA) background strain mice, and a transgenic strain containing the green fluorescent protein expressed in all cells (FVB.Cg-Tg(ACTB-

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EGFP)B5Nagy/J). The HSV-TK-CCSP strain mice (FVB/NHsd background) were the positive control to evaluate the relative effects of total lung irradiation on pulmonary toxicity compared to the known lung toxicity induced by naphthalene or GCV treatment. We evaluated toxin-induced depletion of lung-specific mRNA levels, their elevation during repair of lung damage, and the contribution of cells of bone marrow origin in the repair and repopulation process. We utilized HSV-TK-CCSP recipient mice, chimeric for sex-mismatched GFP⁺ bone marrow to determine the bone marrow origin of both hematopoietic marked CD45⁺ cells, as well as CD45⁻ epithelial cells of bone marrow origin in the irradiated lung.

Materials and Methods

Animals. All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at University of Pittsburgh. Experiments were performed on FVB/NHsd (wild-type) and HSV-TK-CCSP transgenic littermates of 6-10 weeks of age.

Naphthalene treatment. Naphthalene administration was performed in FVB/NHsd mice as previously described to target lung cell populations for destruction (34). Briefly, naphthalene (Sigma Chemical, St. Louis, MO, USA) was dissolved in corn oil and administered at a concentration of 20 mg/ml. Each animal received 200 mg naphthalene per kilogram body weight through an intraperitoneal injection. Naphthalene was also given with or without our manganese superoxide dismutase plasma liposome (MnSOD-PL), which has been shown to reduce oxidative stress in response to irradiation (35-36), and bone marrow transplantation (BMT).

Irradiation of FVB/NHsd mice. Irradiation was administered to FVB/NHsd female or to FeVB/n female (FVB.Cg-Tg(ACTB-EGFP)B5Nagy/J male marrow chimeric) mice by a linear accelerator to a dose of 19 Gy to the thorax with head and abdomen shielded (37). Mice were followed up for one and two weeks after irradiation and then real time polymerase chain reaction (RT-PCR) studies were performed on lungs as described below. Chimeric FVB/NHsd then received a combination of granulocyte colony stimulating factor (G-CSF) (PeproTech, Rocky Hill, NJ, USA) and bone marrow transplantation (BMT) to promote homing of bone marrow including donor marrow origin GFP⁺ bone marrow progenitor cells to the lung (27).

HSV-TK-CCSP Transgenic mice and GCV. Generation of and characterization of HSV-TK-CCSP transgenic mice has been previously described (38). Transgenic mice were treated with GCV (Roche Applied Science, Indianapolis, IN, USA) which was delivered over a 24 h period via mini osmotic pumps (ALZET, Palo Alto, CA, USA) as described previously (38). Briefly, GCV was dissolved in normal saline at concentrations of either 25 or 50 mg/ml and loaded into mini osmotic pumps. Pumps which were designed to discharge over 24 h were subcutaneously implanted and were then retrieved after 5-7 days. Control mice were implanted with mini osmotic pumps filled with normal saline. Mice were allowed *ad libitum* access to food and water and their weights were carefully monitored both prior to treatment and daily thereafter.

Tissue collection and RT-PCR analysis of lung-specific mRNA levels. After sacrificing the mouse, the heart was perfused with up to 10 ml of PBS buffer to clear the lungs of circulatory blood. The right lung was then inflated, fixed in 2% paraformaldehyde in PBS (pH 7.4) and placed into 2% paraformaldehyde in PBS solution at 4°C overnight. Fixed lungs were immersed in 30% sucrose overnight at 4°C, then frozen in liquid nitrogen-cooled 2-methylpentane. Frozen lungs were stored at -80°C until sectioned.

The left lung lobes were tied off prior to inflation and fixation of the right lung and were immediately removed and frozen on dry ice. Using a standard Trizol-based methodology (TRIzol reagent, Invitrogen, Carlsbad, CA, USA), DNA was extracted, and cDNA was generated using a reverse transcription kit (High Capacity cDNA Reverse Transcriptase Kit; Applied Biosystems, Foster City, CA, USA). mRNA expression of pulmonary stem cell markers CCSP (clara cell secretory protein) which is a pulmonary progenitor cell; CYP2F2 (cytochrome P450, family 2, subfamily f, polypeptide 2; Gene Bank: NM 007817.2), a gene responsible for the metabolism of compounds in Clara cells; FOXJ1 (Forkhead box protein J1; Gene Bank: NM 008240.3); and SPC (surfactant protein c; Gene Bank: NM 011359.2), responsible for the production of surfactant, were quantified by RT-PCR. RT-PCR was performed using a robotic automated pipetting system (39) (EPMotion 5070; Eppendorf AG, Hamburg, Germany) to ensure high precision and throughput and run on an RT-PCR machine (Realplex 2 S; Eppendorf AG). mRNA expression was compared using the $\Delta\Delta C_t$ method and a standard pooled total lung RNA preparation as the calibrator and with the GUSB gene as the housekeeping gene.

Bone marrow transplantation. Allogeneic BMT from GFP positive FVB/NHsd mice was conducted in some mice after lung toxicant administration to determine whether this would aid in repair and repopulation of depleted cells. Bone marrow was isolated from the tibia and femurs of FVB.Cg-Tg(ACTB-EGFP)B5Nagy/J mice and 1×10^6 bone marrow cells were intravenously injected into recipient mice (27).

Generation of GFP⁺ chimeric mice. Generation of HSV-TK-CCSP marrow chimeric and FVB/NHsd marrow chimeric mice has been previously described (40-41). Bone marrow was harvested from the femur of male GFP⁺ homozygous FVB.Cg-Tg(ACTB-EGFP)B5Nagy/J GFP⁺ transgenic mice. HSV-TK-CCSP or FVB/NHsd female recipient mice received 10 Gy total body irradiation using a ¹³⁷Cs irradiator (27). After irradiation, 1×10^6 GFP⁺ cells were injected into the recipient mice via the tail vein. Percentage of chimerism was then measured 60 days later using flow cytometry for GFP⁺ peripheral blood cells representing over 70% of cells.

Explant of lung and sorting for GFP⁺ cells. An experiment to determine whether BMT stimulated homing of GFP⁺ donor cells to the lungs in drug-treated or irradiated mice was carried out. Mice were sacrificed, and the circulatory system was perfused with 10 ml of PBS. Next 1 ml dispase (50U/ml; Becton Dickinson, Franklin Lakes, NJ, USA) and 1 ml 1% LMP agarose were instilled into the lungs by intratracheal cannulation and the lungs were then immediately covered in ice. Right lung lobes were then removed, minced and incubated with 2 μ g/ml collagenase/dispase (Invitrogen, Carlsbad, CA, USA) in PBS for 45 minutes in a high-humidity incubator at 37°C for digestion. Cells were then cytocentrifuged and resuspended in Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Inc., Manassas, VA, USA), drawn

through proportionately smaller gauge needles up to a 27-gauge needle and filtered twice through 40 μ m cell strainers to remove cell clumps. Cells were then resuspended in red blood cell lysis buffer for 4 minutes, washed in DMEM/10% fetal bovine serum (FBS) and resuspended in PBS/10% FBS at a density of approximately $1 \times 10^6/100 \mu$ l. Cells were then incubated in Ter119 antibody and CD45 antibody for 20 minutes to select and remove blood cells. Propidium iodide (2 μ g/ml) was added to the cells for discrimination of dead cells. The GFP+CD45+ and GFP+CD45- cells were then isolated by flow cytometry.

Immunofluorescence analysis of lungs. Lungs were sectioned at 6 μ m and affixed to charged slides (Superfrost/Plus; Fisher, Pittsburgh, PA, USA). Tissue was rinsed three times in PBS, rinsed three times in PBS containing 0.5% BSA (BSA Buffer) and blocked in 2% BSA in PBS for 30 min at room temperature. Primary antibodies diluted in BSA buffer were added to sections for 1 h at room temperature. Primary antibodies used were: rabbit anti-GFP (1:100; Abcam, Cambridge, MA, USA); rat anti-mouse CD45 (1:100; BD Pharmingen, San Diego, CA, USA) and goat anti-surfactant protein C (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sections were washed five times in BSA Buffer then fluorescently tagged secondary antibodies, diluted in BSA buffer, were added to the sections for 1 h at room temperature. Secondary antibodies used here were donkey anti-rabbit Alexa 488 (1:500; Invitrogen, Carlsbad, CA, USA), donkey anti-rat Cy3 and donkey anti-goat Cy3 (1:1000; Jackson ImmunoLabs, West Grove, PA, USA). Tissue were washed three times in PBG buffer, three times in PBS, then nuclei were stained using 0.001% Hoechst dye (bis benzimide) in double-distilled water for 30 s. Following a wash in PBS, the tissue was coverslipped using gelvatol (23 g poly(vinyl alcohol) 2000, 50 ml glycerol, 0.1% sodium azide in 100 ml PBS) and viewed on an Olympus Fluoview 1000 confocal microscope (Olympus Corporation, Center Valley, PA, USA). Collages were prepared using Photoshop CS software (Adobe Systems Incorporated, San Jose, CA, USA).

Transmission electron microscopy of GFP+-sorted lung cells. Cell suspensions were fixed in 2.5% glutaraldehyde in PBS then immediately pelleted in a 1.5 ml microfuge tube at $300 \times g$. After 1 h fixation, the supernatant was removed and the cell pellets were washed three times in PBS then post-fixed in 1% OsO₄, 1% K₃Fe(CN)₆ for 1 h. Following three additional PBS washes, the pellet was dehydrated through a graded series of 30-100% ethanol, then placed in 100% propylene oxide, then infiltrated in a 1:1 mixture of propylene oxide Polybed 812 epoxy resin (Polysciences, Warrington, PA, USA) for 1 h. After several changes of 100% resin over 24 h, the pellet was embedded in a final change of resin, cured at 37°C overnight, followed by additional hardening at 65°C for two more days. Ultrathin (60 nm) sections were collected on 200 mesh copper grids, stained with 2% uranyl acetate in 50% methanol for 10 min, followed by 1% lead citrate for 7 min. Sections were imaged at 80 kV using a JEOL JEM 1011 transmission electron microscope (Peabody, MA, USA) fitted with a side mount AMT 2k digital camera (Advanced Microscopy Techniques, Danvers, MA, USA).

Results

Thoracic irradiation causes less depletion of primitive lung stem cells than does naphthalene treatment of FVB/NHsd mice. The changes in pulmonary mRNA levels of CCSP, CYP2F2, FOXJ1, and SPC were quantitated by robotic semi-automated

Table I. Effect of irradiation compared to naphthalene on lung-associated markers in FVB/NHsd mice.

Group (n=3)	CCSP	CYP2F2	FOXJ1	SPC
Naphthalene+	19.6%±06.5	37.0±21.8	53.7%±20.5	32.6%±09.9
MnSOD-PL	(p=0.0008)*	(p=0.0105)*	(p=0.0949)*	(p=0.0042)*
	(p=0.9601) ^κ	(p=0.9722) ^κ	(p=0.2412) ^κ	(p=0.1332) ^κ
Naphthalene+	19.6%±0.11	42%±08.1	80.8%±31.7	49.5%±0.7
MnSOD-PL+	(p=0.0002)*	(p=0.0043)*	(p=0.5836)*	(p=0.0010)*
BMT	(p=0.9316) ^κ	(p=0.7216) ^κ	(p=0.8336) ^κ	(p=0.305) ^κ
Naphthalene+	7.2%±1.0	18.9%±3.1	40.5%±5.3	30.9%±4
BMT	(p<0.0001)*	(p=0.0002)*	(p=0.0016)*	(p=0.0006)*
	(p=0.0367) ^κ	(p=0.1028) ^κ	(p=0.0401) ^κ	(p=0.089) ^κ
Naphthalene	20%±4.0	37.6%±8.3	88.7%±15.1	69.2%±16.7
	(p=0.0003)*	(p=0.0035)*	(p=0.5232)*	(p=0.1558)*
15 Gy day 7	62.8%±9.4	45.2%±6.0	69.3%±8.6	96.6%±2.3
	(p=0.0262)*	(p=0.0662)*	(p=0.1154)*	(p=0.6113)*
	(p=0.0277) ^Φ	(p=0.0028) ^Φ	(p=0.0415) ^Φ	(p=0.9221) ^Φ
15 Gy day 14	30%±0.0177	65.1%±0.053	49.6%±0.047	123.3%±0.363
	(p=0.0003) ^Φ	(p=0.0110) ^Φ	(p=0.0025) ^Φ	(p=0.5599) ^Φ
Control	100%	100%	100%	100%

FVB/NHsd mice were treated with naphthalene or a combination of naphthalene along with MnSOD-PL and BMT. Mice were sacrificed 3 days later and the left lobes excised, then mRNA extracted and tested for levels of CCSP, CYP2F2, FOXJ1, and SPC. Other FVB/NHsd mice were irradiated to 15 Gy to the thorax then 7 or 14 days later the left lobes excised, tested for mRNA concentrations of CCSP, CYP2F2, FOXJ1, and SPC as described in the Materials and Methods. *signifies *p*-value compared to control, ^κ signifies naphthalene alone, and ^Φ signifies 15 Gy day 7 or day 14.

RT-PCR in FVB/NHsd mice treated with lung irradiation to 15 Gy or with naphthalene (Table I and Figure 1). Naphthalene reduced expression of CCSP and CYP2F2 compared to the control untreated group after 3 days. Injection of bone marrow following naphthalene treatment may be able to prevent the decrease in primitive lung stem cells by providing a source of hematopoietic pluripotent stem cells, however naphthalene plus BMT resulted in reduction of CCSP, CYP2F2, FOXJ1, and SPC. Pretreatment with MnSOD-PL which has been demonstrated to protect the lung from the development of organizing alveolitis or fibrosis following irradiation (37) alone or with BMT did not protect the lung stem cells as seen by reduced expression of CCSP, CYP2F2, and SPC.

Thoracic irradiation reduced the mRNA expression of CCSP, CYP2F2, and FOXJ1 at seven and fourteen days after irradiation when compared to the controls (Table I and Figure 1). There was no significant change in SPC mRNA expression at day 7 or 14 after irradiation. Thoracic irradiation caused less depletion of early pulmonary cell markers by RT-PCR assay than did naphthalene treatment.

GCV treatment of FVB/NHsd background HSV-TK-CCSP mice causes the greatest depletion of primitive stem cell markers measured by RT-PCR. Gene expression by

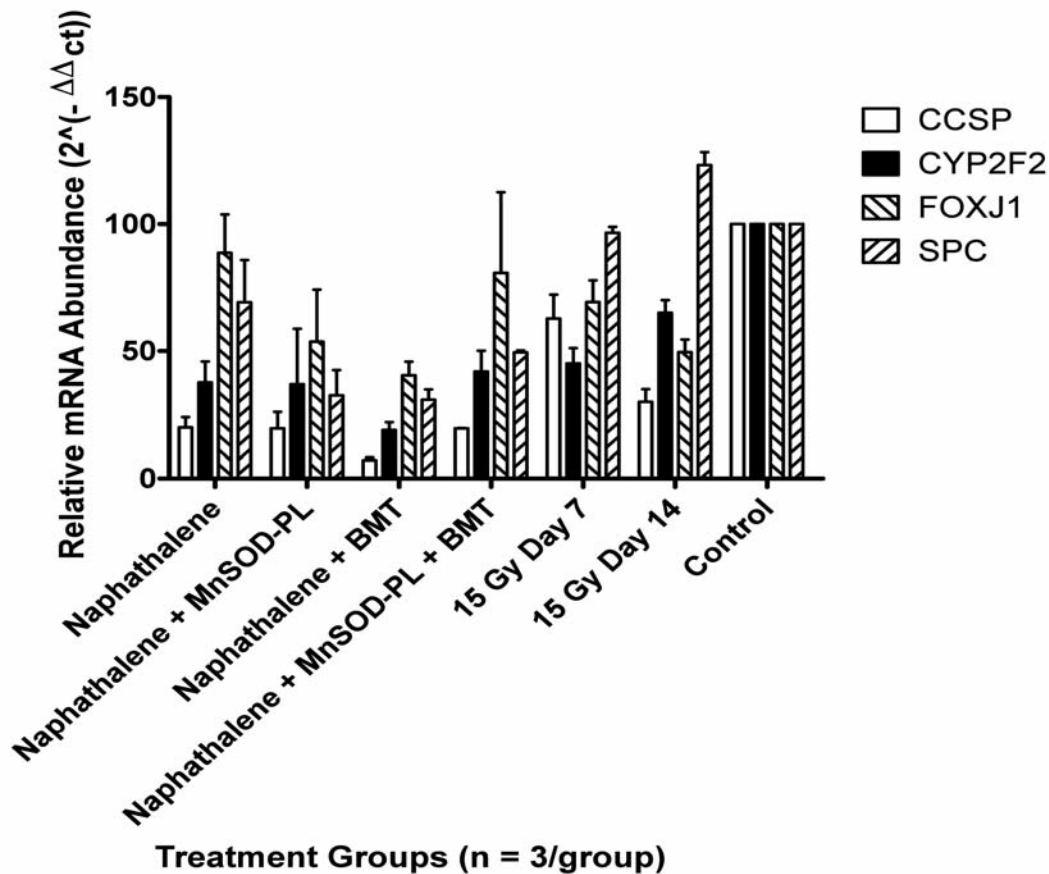


Figure 1. Levels of mRNA expression for CCSP, CYP2F2, FOXJ1, and SPC in naphthalene treated or thoracic irradiated FVB/NHsd mice. Lungs were isolated from FVB/NHsd mice which had received naphthalene, naphthalene plus MnSOD-PL, naphthalene plus bone marrow transplant (BMT) or naphthalene plus MnSOD-PL and BMT 3 days previously. The lungs from other FVB/NHsd mice which had been irradiated to 15 Gy were isolated 7 days and 14 days after irradiation with mRNA extracted and RT-PCR performed to measure gene expression for CCSP, CYP2F2, FOXJ1 and SPC.

measuring mRNA levels for CCSP, CYP2F2, FOXJ1, and SPC in HSV-TK-CCSP mice was tested after mice received either 25 mg/ml of GCV, 50 mg/ml of GCV, or saline compared to untreated control mice (Table II and Figure 2). As shown in Table II, 25 mg/ml or 50 mg/ml of GCV reduced mRNA expression for CCSP, CYP2F2, and FOXJ1 compared to the untreated control group. There was no detectable change in SPC expression. Saline treatment reduced mRNA expression of FOXJ1 and SPC, but did not change levels of CCSP or CYP2F2.

Cell sorting reveals that the greatest removal of primitive lung stem cells follows GCV treatment in GFP⁺ chimeric HSV-TK-CCSP mice. The number of GFP⁺CD45⁻ and GFP⁺CD45⁺ cells collected from the lungs of GFP⁺ chimeric transgenic CCTK mice treated with thoracic irradiation (Table III) or GCV for 7 days (Table IV) was determined by flow cytometry of excised lungs. There were low numbers of

GFP⁺CD45⁻ cells detected in both irradiated and GCV-treated HSV-TK-CCSP GFP⁺ chimeric mice.

Immunohistochemical staining and ultrastructural examination of GFP⁺ CD45⁻ and GFP⁺ CD45⁺ cells in lungs of chimeric HSV-TK-CCSP (FVB/NHsd) mice reveals rare epithelial cells of bone marrow origin. Staining and cell characterization of lung samples is shown in Figures 3-5. The chimeric FVB/NHsd mice had few GFP⁺ cells which were positive for the pulmonary marker SPC, characteristic of type II pneumocytes. Sorted cells from chimeric FVB/NHsd female mice receiving different treatments were examined by transmission electron microscopically to determine whether the GFP⁺ cells were differentiated lung cells displaying microvilli and intracellular multilamellar bodies. This would demonstrate that bone marrow cells can differentiate into mature alveolar type 2 cells. GFP⁺CD45⁻ cells were found (Figure 5); however, GFP⁺CD45⁺ cells were noted predominantly.

Table II. Impact of ganciclovir (GCV) treatment on expression of mRNA of lung-associated markers in HSV-TK-CCSP mice.

Group (n=3)	CCSP	CYP2F2	FOXJ1	SPC
25 mg/ml GCV	4.3%±1.6 (<i>p</i> <0.0001)	15.0%±2.2 (<i>p</i> <0.0001)	30.5%±6.0 (<i>p</i> =0.0004)	268.3%±133.0 (<i>p</i> =0.2827)
50 mg/ml GCV	2.3%±2.1 (<i>p</i> <0.0001)	11%±10.2 (<i>p</i> =0.0010)	9.3%±5.7 (<i>p</i> =0.0001)	184.6%±46.9 (<i>p</i> =0.1589)
Saline	102.5%±12.2 (<i>p</i> =0.9499)	86.0%±11.3 (<i>p</i> =0.2144)	49.6%±4.9 (<i>p</i> =0.0008)	31.8%±12.8 (<i>p</i> =0.0056)
Control	100%	100%	100%	100%

HSV-TK-CCSP mice were treated with either 25 mg/ml of GCV, 50 mg/ml of GCV, or saline (n=3/group). Mice were sacrificed 7 days later and the left lobes excised and mRNA extracted, then concentrations of CCSP, CYP2F2, FOXJ1, and SPC were measured as described in the Materials and Methods. *P*-values compare treated mice to controls.

Table III. Quantitation of migration of GFP+ cells from bone marrow transplant to the irradiated lung.

Treatment group	GFP+ CD45 ⁻ lung cells		GFP+ CD45 ⁺ lung cells	
	n	% per 100,000 cells	N	% per 100,000 cells
0 Gy	450±95 (<i>p</i> =0.0341*)	0.4±0.1 (<i>p</i> =0.0242*)	9298±713	9.2±0.8
19 Gy	310±16 (<i>p</i> =0.0187*)	0.3±0.1 (<i>p</i> =0.0072*)	10023±1546	10.1±1.5
19 Gy+G-CSF	189±37	0.2±0.1	12539±1773 (<i>p</i> =0.0699 ^Φ)	12.5±1.8 (<i>p</i> =0.0693 ^Φ)
19 Gy+GFP+ bone marrow	299±36 (<i>p</i> =0.0766*)	0.3±0.1 (<i>p</i> =0.1053*)	8118±555	8.1±0.6
19 Gy+GFP+ bone marrow+ G-CSF	206±21 (<i>p</i> =0.0372*)	0.3±0.1 (<i>p</i> =0.0252*)	11475±198 (<i>p</i> =0.0004 ^Φ)	11.5±0.2 (<i>p</i> =0.0004 ^Φ)

FVB/NHsd female mice were irradiated to 10 Gy total body irradiation followed by intravenous injection of 1×10^6 bone marrow cells from GFP+ male mice. Sixty days later mice were divided into 5 treatment groups: 1) control nonirradiated mice; 2) irradiation to 19 Gy to the thoracic cavity (head and abdomen shielded); 3) 19 Gy followed by G-CSF injection; 4) 19 Gy followed by GFP+ intravenous injection with bone marrow 5 days later, and 5) 19 Gy plus bone marrow and G-CSF. Thirty days later, mice were sacrificed and lungs excised. Left lungs were fixed, sectioned, and examined for GFP+ cells and surfactant. Right lungs were prepared as single cell suspensions and sorted for GFP+CD45⁻ and GFP+CD45⁺ cells by flow cytometry. Irradiation resulted in few GFP+CD45⁻ lung cells. G-CSF treatment resulted in no greater numbers of GFP+CD45⁻ cells but increased the detectable number of GFP+CD45⁺ cells. *Versus 19 Gy plus G-CSF; ^Φ versus 19 Gy plus bone marrow plus G-CSF.

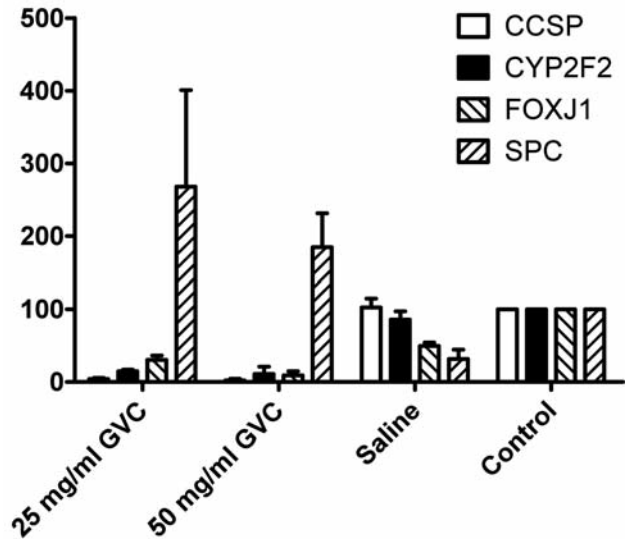


Figure 2. Levels of mRNA expression for CCSP, CYP2F2, FOXJ1, and SPC in GCV-treated HSV-TK-CCSP mice. Lungs were isolated from mice that had received either 25 or 50 mg/ml of GCV, saline or control mice 7 days after the pumps were implanted. RT-PCR was performed on mRNA extracted from the lungs to determine gene expression.

Table IV. Quantitation of migration of GFP+ cells from bone marrow transplant to the ganciclovir-treated lungs of HSV-TK-CCSP mice.

Treatment group	GFP+ CD45 ⁻ lung cells		GFP+ CD45 ⁺ lung cells	
	n	% per 100,000 lung cells	n	% per 100,000 lung cells
Control	81±39	0.1±0.1	309±102	0.3±0.1
GCV	126±8.3 (<i>p</i> =0.979)	0.1±0.1 (<i>p</i> =0.4542)	10035±1226 (<i>p</i> =0.0011)	10.0±1.2 (<i>p</i> =0.0012)

HSV-TK-CCSP female mice were total-body irradiated to 10 Gy followed by injection of 1×10^6 bone marrow cells from male GFP+ mice. Sixty days later mice were implanted with mini-Alzet pumps containing either saline (control) or 25 mg/ml ganciclovir (GCV) and treated for 14 days. Mice were sacrificed 30 days later. The left lungs were removed and examined for GFP+ cells and surfactant. The right lungs were prepared as single cell suspensions and sorted for GFP+CD45⁻ and GFP+CD45⁺ cells. GCV treatment resulted in a significant increase of GFP+CD45⁺ cells but not of GFP+CD45⁻ cells.

Discussion

Thoracic irradiation causes acute and chronic (late) toxicity (23-24). Acute radiation pneumonitis in patients is dependent upon total irradiation dose, fraction size, and the duration of radiation treatment (42-48). In models of single fraction irradiation damage, a decrease in inflammatory cytokines

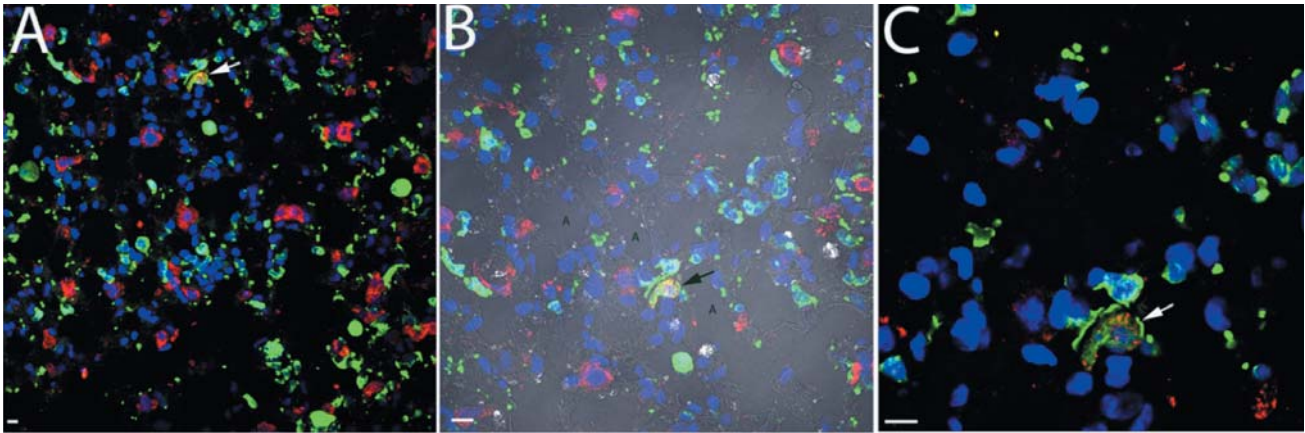


Figure 3. Immunofluorescent staining for GFP⁺ cells in the lung of thoracic-irradiated female FVB/NHsd mouse chimeric for male GFP⁺ bone marrow. Mice received 15 Gy thoracic irradiation, bone marrow transplant (BMT), and/or G-CSF as described in the Materials and Methods. Sixty days after treatment, mice were sacrificed, the left lung lobes removed, sectioned and stained for GFP (green), pulmonary surfactant protein c (red), and DNA (blue). A: Image shows a surfactant⁺ GFP⁺ cell (arrow) within the lung parenchyma ($\times 600$). B: Surfactant⁺ GFP⁺ cell (arrow) shown in relation to alveolar spaces shown in A in a differential interference microscopy overlay ($\times 600$). C: Higher magnification image ($\times 1200$) with arrow indicating GFP⁺ cell with cytoplasmic GFP and intracellular vacuolar-surfactant protein c staining (red). Bar represents 10 μ m in each panel.

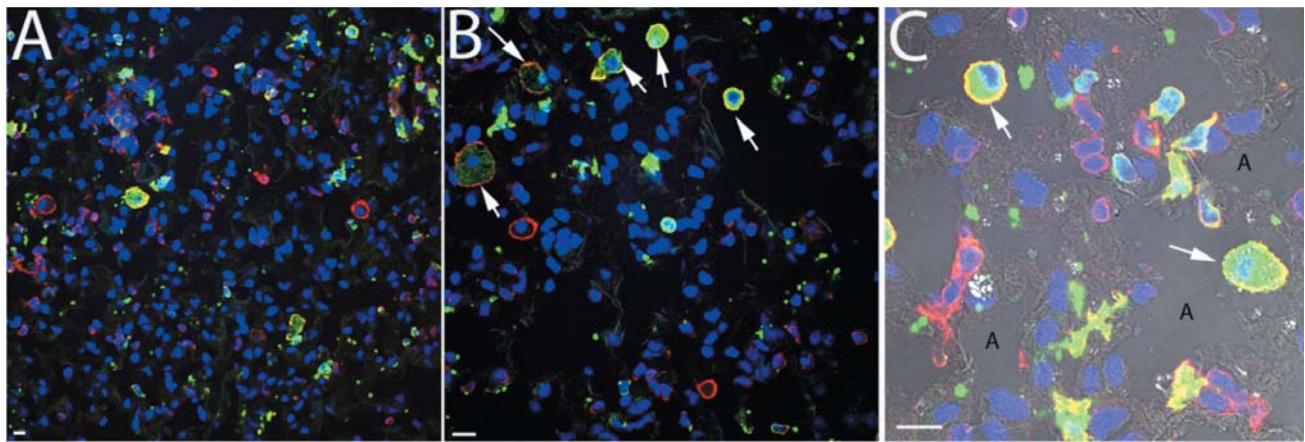


Figure 4. Immunofluorescent staining for GFP⁺ cells in lung of GCV treated female HSV-TK-CCSP mouse chimeric for GFP⁺ male bone marrow. Mice received 25 mg/ml of GCV by mini Alzet pumps as described in the Methods. Sixty days after treatment, mice were sacrificed, the left lung lobes excised, sectioned, and stained for GFP (green), CD45 (red), and DNA (blue). A: Image shows GFP⁺CD45⁺ cells ($\times 400$) which represent a large number of GFP⁺ cells. B and C: Arrows indicate macrophages ($\times 600$ and $\times 1200$, respectively). GFP⁺CD45⁺ cells were also abundant, with very few GFP⁺CD45⁻ cells observed in the sections. Bar represents 10 μ m in each panel.

and migration into the lungs of lymphocytes, polymorphonuclear leukocytes, and macrophages has been demonstrated (36-37). Irradiation damage is associated with depletion of intrinsic lung macrophages, endothelial cells in the microvasculature, and swelling of both vascular and epithelial compartments in the lung (23-24). Following recovery from pneumonitis, there is a latent period during which histopathologic evidence of lung damage is absent. A late phase of organizing alveolitis/fibrosis is then detected at around 100 to 150 days in the C57BL/6J model (27, 36). In

other mouse strains, radiation pneumonitis may be more severe and lung fibrosis less dramatic (25-26). Mouse strains both sensitive and relatively resistant to lung irradiation have been described (25). Pulmonary toxicity in humans follows the same patterns dependent upon total dose, fraction size, and volume of lung treated (49-51). Acute radiation pneumonitis remains a clear radiation dose-limiting toxicity in thoracic radiotherapy and can be fatal, particularly in a setting of opportunistic bacterial or viral infection, and toxicity of concomitant or sequential chemotherapy (52-53).

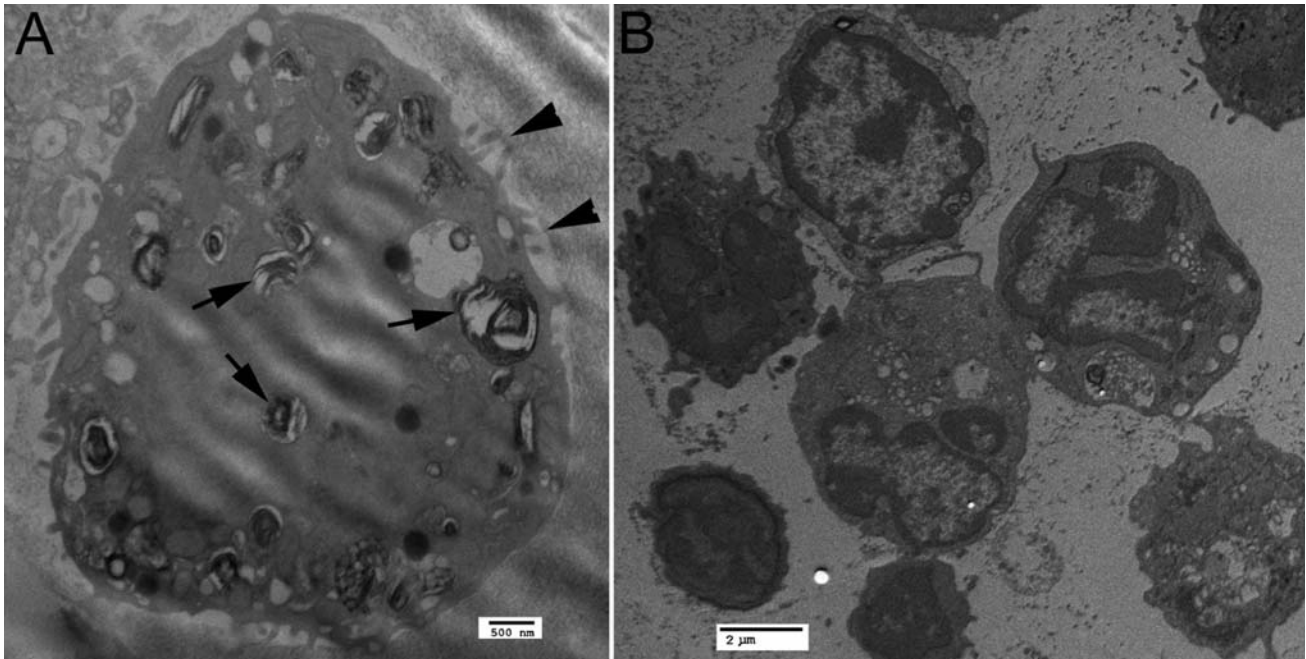


Figure 5. Transmission electron micrographs of isolated lung cells from thoracic irradiated female HSV-TK-CCSP female mouse chimeric for GFP⁺ male bone marrow treated with 25 mg/ml of GCV. Chimeric HSV-TK-CCSP female mice were sacrificed 60 days after GCV treatment, the right lung lobes excised, and single cell suspensions sorted for GFP⁺CD45⁻ and GFP⁺CD45⁺ cells using flow cytometry. Sorted cells were then processed for transmission electron microscopy as described. A: Type II alveolar pneumocyte isolated from the GFP⁺CD45⁻ fraction. Characteristic intracellular multilamellar bodies (arrows) and membrane-associated microvilli (arrowheads) are present. B: Morphology of hematopoietic cells found in the GFP⁺CD45⁺ fraction, which include neutrophils, macrophages, and lymphocytes.

There has been excitement in recent years regarding the reported plasticity of bone marrow stem cells with respect to their capacity to differentiate to cells of epithelial origin (1, 54-56). Since the initial reports of bone marrow origin of pulmonary epithelial cells, there has been much controversy and publications both confirm (2-11) or fail to confirm (17-22) the capacity of bone marrow stem cells to differentiate into pulmonary epithelial cells. A major obstacle in resolving this controversy has been the unavailability of an appropriate animal model for selected removal of lung stem cells in preparation of the 'niche' or homing cell-site for circulating stem cell progenitors of bone marrow origin. We took advantage of an appropriate positive control model that selectively eliminates lung stem cells. HSV-TK-CCSP mice demonstrate GCV-mediated effective removal of CCSP-sensitive lung stem cells in a dose-dependent manner. Primitive lung stem cells are removed in a quantifiable fashion, measurable by both drop in levels of mRNA for lung stem cell-specific markers, and through histopathologic evidence of depletion of cells at the bronchial/alveolar margin (30-33, 38). During recovery, repopulation of the lung is associated with return to normal levels of mRNA for lung cell-specific markers (38). In the present studies, we utilized the HSV-TK-CCSP mouse strain to measure

migration of bone marrow-derived cells to the lung in sex-mismatched FVB.Cg-Tg(ACTB-EGFP)B5Nagy/J chimeric mice treated with GCV compared to thoracic irradiation, and the pulmonary toxin naphthalene.

GCV treatment of HSV-TK-CCSP chimeric mice resulted in detectable clearing of stem cells that may have allowed migration to the lung of chimeric bone marrow-derived GFP⁺CD45⁻ cells shown by both histochemistry *in situ*, and by transmission electron microscopy of removed sorted cells, to be epithelial in origin. However, the majority of bone marrow-derived cells migrating into the lungs were of hematopoietic origin and GFP⁺CD45⁺. Both thoracic-irradiated and naphthalene-treated mice demonstrated a lower level of bone marrow-derived progenitor cell migration into the lungs. These studies confirm and extend previous publications demonstrating that GCV treatment in HSV-TK-CCSP mice specifically removes primitive hematopoietic stem cells compared to naphthalene treatment. Furthermore, data support the notion that the stem cell niche in the lung must be cleared by GCV treatment to allow a suitable homing site for bone marrow-derived cells (38). In an attempt to enhance homing to niche-cleared sites, we treated mice with the bone marrow mobilization drug, G-CSF. Other mice were treated by intratracheal injection of MnSOD-PL (37), shown

to facilitate improved tolerance to thoracic irradiation by removal of oxidative stress. In the present studies, BMT alone led to the highest degree of measurable GFP⁺CD45⁻ cells of bone marrow origin in the lung. Further studies will be required to determine whether an optimized schedule of G-CSF administration and/or MnSOD-PL intrapulmonary delivery can facilitate a greater degree of homing of bone marrow-derived cells to the lungs in GCV-treated mice. In contrast, naphthalene treatment resulted in non-specific toxicity of multiple lung cell phenotypes and did not lead to increased bone marrow-derived cell migration into the lungs. Thoracic irradiation did not significantly remove lung stem cells nor did it facilitate migration into the lungs of bone marrow-derived stem cells, detectable neither by mRNA level measurements, nor by bone marrow-derived cells found in single-cell suspensions of sorted lung cells after BMT. Thus, the present studies establish a relatively low level of lung stem cell depletion by single fraction thoracic irradiation. Whether fractionated irradiation depletes stem cells to a greater degree is the subject of current investigation.

In contrast to the paucity of evidence for bone marrow-derived progenitors of lung epithelium in the acute phase of irradiation damage, there is evidence that bone marrow-derived cells play a role in the late injury of organizing alveolitis/radiation fibrosis (27-29). This late injury is associated with migration into the lungs of a separate population of cells known as bone marrow stromal cells (mesenchymal stem cells, mesenchymal stromal cells). Further studies are required to determine whether effective clearance of the bone marrow stem cell niche in a setting of less oxidative stress and less toxicity can result in improved migration into the lungs of bone marrow-derived stem cells of epithelial progenitors facilitating irradiation repair (27). The mechanism by which late radiation fibrosis/organizing alveolitis is associated with robust support to the lungs of bone marrow-derived cells, although of a different cell phenotype, may be attributable to lung production of chemotactic marrow mobilizing cytokines (27-29). Further studies will be required to confirm this proposed mechanism.

The present results showed that thoracic irradiation minimally eliminates stem cells, specifically Clara cells, in lungs and this decrease is modestly present for up to 14 days after treatment. This time coincides with irradiation having a toxic effect upon rapidly dividing cells which is seen clinically in total-body irradiation and is part of the regimen for BMT in order to eliminate the bone marrow's progenitor population (57-58).

Naphthalene treatment reduced pulmonary stem cells and treatment with antioxidant MnSOD-PL did help to attenuate the depletion of pulmonary Clara cells which was correlated with MnSOD-PL protection of esophageal and lung cells from ionizing irradiation (13, 61). Both swallowed administration of MnSOD-PL and inhaled administration of

MnSOD-PL has also shown to increase homing of GFP⁺ bone marrow cells to irradiated esophagus and lung, respectively (13, 27, 62-63). Therefore, it is possible that MnSOD-PL increases homing of GFP⁺ bone marrow progenitors into the lung without altering the initial decrease in lung progenitor cells caused by the toxin exposure.

We also showed that G-CSF did not increase homing of GFP⁺ bone marrow progenitor cells into the lung. While G-CSF is responsible for increase mobilization of the bone marrow progenitor cells (20), the amount of inflammation associated with thoracic irradiation (64-66) could have prevented the mobilized progenitor cells from homing to the lung.

Clearing stem cells by GCV treatment of HSV-TK-CCSP mice was most effective. Both 25 mg/ml and 50 mg/ml GCV caused a dramatic decrease in CCSP expression. Since 50 mg/ml had a toxic effect, we used 25 mg/ml of GCV for the experiments to determine whether these cleared stem cell niches can be repopulated with bone marrow progenitor cells. GFP⁺CD45⁻ cells were present in the lungs of GCV-treated chimeric transgenic CCTK mice. The CD45⁺ antigen is a marker for hematopoietic cells (59-60). Since GFP⁺CD45⁻ cells of bone marrow origin were also present, these were likely cells that differentiated into lung epithelium. After sorting, transmission electron microscopy showed few cells to be epithelial. Immunohistochemical staining was positive for GFP⁺, and SPC, showing a bone marrow origin. Lower numbers of cells of bone marrow origin were detected in the lungs of chimeric FVB/NHsd mice treated with 19 Gy thoracic irradiation.

Further studies will be required to determine if bone marrow progenitor cells contribute significantly to damage repair of the irradiated lung or rather represent rare and insignificant events.

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