in vivo *20*: 431-438 (2006)

Bone Marrow from CD18–/– (MAC-1–/–) Homozygous Deletion Recombinant Negative Mice Demonstrates Increased Longevity in Long-term Bone Marrow Culture and Decreased Contribution to Irradiation Pulmonary Damage

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Abstract. *Background: Bone marrow macrophage surface expression of CD18 (MAC-1, LFA1) is involved in cellular binding to V-CAM-1 and V-CAM-2 adhesion molecules expressed on endothelial cells. We sought to determine if this interaction affected the growth of marrow in long-term bone marrow cultures (LTBMCs) and macrophage migration to the irradiated lung in pulmonary fibrosis/organizing alveolitis. Materials and Methods: Continuous bone marrow cultures from CD18–/– and CD18+/+ littermates were established. Bone marrow migration to the irradiated lung was quantitated in CD18+/+ or CD18–/– marrow chimeric mice. Anti-macrophage antibodies were administered to block monocyte/macrophage migration after lung irradiation. Results: CD18–/– LTBMCs demonstrated significantly increased longevity (over 20 weeks) of production of multilineage hematopoietic progenitor cells, total non-adherent cells and macrophage progenitors compared to those derived from CD18+/+ littermates (10 weeks). C57BL/6J female mice chimeric for male CD18–/– bone marrow showed improved (50%) survival at 120 days after pulmonary radiation compared to female mice chimeric for male CD18+/+ bone marrow (0.0%). Intraperitoneal injections (daily for 15 days) of an anti-macrophage antibody on days 80-98 after 20 Gy total lung irradiation resulted in reduction of macrophage migration to the lungs and increased survival. Conclusion: The data demonstrate a complex role of CD18 (MAC-1) in macrophage progenitor and macrophage cellular interaction involving stromal cells of the bone marrow and lung.*

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Key Words: Long-term marrow culture, hematopoiesis, CD18 (MAC-1), irradiation pulmonary fibrosis.

Monocyte/macrophage migration to sites of inflammation in tissue injury is mediated by the complex involvement of macrophage surface receptors and counter-receptors (adhesion molecules) on endothelial and other stromal cells at the site of trauma (1-9). In particular, ionizing irradiation-induced lung damage involves inflammatory reactions which occur in a biphasic acute reaction followed by a latent period, and then a secondary delayed reaction "late effect", which involves migration to the lungs of bone marrow macrophages and stromal cell progenitors (10-12). Irradiation lung damage in the mouse model and in irradiated patients is an acute response with up-regulation of TGFβ, TNFα, IL-1, and other inflammatory cytokines in the irradiated tissue (12). Infiltration of polymorphonuclear leukocytes and macrophages, alveolar fluid accumulation and endothelial cell swelling is followed by a return to normal pulmonary architecture and a latent period (10, 13). At approximately 100-120 days after irradiation, serum TGFβ levels are again detectably elevated (10), this time associated with the up-regulation of V-CAM-1 and I-CAM-1 expression on pulmonary endothelial cell surfaces (followed by pulmonary migration of bone marrow origin macrophages and stromal cells forming the lesion of organizing alveolitis/fibrosis (14, 15). Macrophage migration and homing to pulmonary endothelial cells are known to involve two classes of macrophage surface receptor, the CD18 (MAC-1) (4) and the VLA4 (3) receptors.

Here, the role of the expression of hematopoietic progenitor cell and macrophage CD18 in the development of irradiation-induced lung damage was investigated. Long-term bone marrow cultures (LTBMCs) (16) were established from CD18–/– mice and compared to those from littermate CD18+/+ mice to quantify detectable differences

in macrophage progenitor cell interaction with bone marrow stromal cells. The mouse model of pulmonary late irradiation damage in the C57BL/6J strain, the background strain for the current CD18–/– strain, was utilized to determine the effect of absence of this macrophage surface receptor on the incidence and severity of late irradiation pulmonary damage in mice chimeric for bone marrow-derived CD18–/– macrophage progenitors. The results demonstrated a clear effect of the absence of CD18 expression on the interaction of macrophage progenitor cells with stromal cells of bone marrow culture and endothelial cells in the lung.

Materials and Methods

Long-term bone marrow cultures (LTBMCs). Adult male C57BL/6J and CD18–/– (B6.12957-Itgb2^{tm1BAY}J) mice were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). The contents of a femur and tibia from adult 30-33 g mice were flushed vigorously into plastic flasks (40 cm^2, Corning plastic) in Fisher's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 25% horse serum (Flow Laboratories, Rockville, MD, USA), and 10^{-6} M hydrocortisone sodium hemisuccinate (16). For maintenance of continuous hematopoiesis, the cultures were medium changed weekly with removal of all non-adherent cells and medium, and replacement with a fresh 8-mL volume of complete medium (16). Non-adherent cells removed weekly were tested in assays for day 7 and day 14 CFU-GEMM, total cell count, and differential cell count, as previously described (16). The number of cobblestone islands of more than 50 cells, and the percent confluence of the adherent stromal cells were determined, as previously described (16).

Bone marrow chimeric mice. C57BL/6J female 30-33 g, 6 to 8-week-old mice were irradiated to 10 Gy whole body irradiation (WBI) as published (15) and were injected intravenously with $1x10^6$ bone marrow cells from either male C57BL/6J or male CD18–/– mice. Chimerism was confirmed by scoring >70% Y probe positive cells in peripheral blood on day 30 after transplant. Thirty days after marrow transplant, the mice were irradiated to 20 Gy to the pulmonary cavity, as previously described (10).

Injections of anti-macrophage monoclonal antibodies. C57BL/6J mice were irradiated to 20 Gy to the pulmonary cavity (10). Eighty days later, groups of mice were injected intraperitoneally daily with a control anti-lymphocyte (L-1) or with an anti-macrophage antibody M.170.15.11.5HL (American Type Culture Collection, Manassas, VA, USA). The mice were injected with 1 mg of antibody intraperitoneally, Monday through Friday, for 3 weeks for a total of 15 injections beginning at 80 days after irradiation. At 120 days after irradiation, or prior to time of death due to the development of irradiation-induced lung damage, subsets of mice were sacrificed, the lungs expanded in OCT, excised, frozen in OCT, sectioned and stained with an anti-macrophage F4/80 antibody (L11209, Caltag Laboratories, Burlingame, CA, USA). Immunohistochemistry was performed by fixing the cells in methanol, incubating for 2 hours at 27˚C with a 1:250 dilution of F4/80 antibody, followed by incubation for 1 hour at 27˚C with a (green) FITC-anti-mouse IgG antibody. The slides were then mounted with an anti-fade Gel/Mount (Biomedia, Foster City, CA, USA), cover-slipped and examined under a fluorescent microscope. The number of F4/80-positive macrophages (green color) were counted by examining each of 5 lobes of the lung of at least 10 mice per group; 5 slides per lobe and scoring the percent of positive cells per 10 high-powered fields, as previously described (15).

Results

LTBMCs from CD18–/– mice demonstrate increased longevity. Long-term bone marrow cultures (LTBMCs) were established from CD18–/– and CD18+/+ (C57BL/6J) mice and carried in a high humidity incubator at 33˚C according to published methods, 8 cultures from 4 mice per group (16). Non-adherent cells were harvested weekly with replacement of an equal volume of fresh medium. The non-adherent cells were counted, and then the cells plated in colony assay in semi-solid medium for assay of CFU-GEMM, in medium containing IL-3, EPO, GCSF, IL-11, 15% fetal calf serum and antibiotics, according to published methods (16).

CD18–/– and CD18+/+ mouse LTBMCs demonstrated a similar time course of establishment of the adherent layer during the first 20 weeks of culture with over 75% of the flask surface covered with adherent stromal cells by week 15 (Figure 1A). Cobblestone islands (indicative of primitive hematopoietic stem cell containing foci) have been shown to be representative of the health of the interaction between hematopoietic cells in the adherent layer and are an index of the anticipated longevity of LTBMCs. As shown in Figure 1B, cobblestone island formation was detected at a low level initially at 2-7 weeks in both groups, but progressed to a higher level in CD18–/– LTBMCs compared to those from CD18+/+ littermates. The cobblestone island numbers remained higher between weeks 7 and 20 in the CD18–/– cultures. Cumulative cobblestone island formation is shown in Figure 1C and demonstrates a plateau in the increase in number at around 20 weeks in CD18–/– LTBMCs in contrast to the CD18+/+ LTBMCs, which maintained around 3-10 cobblestone islands/flask for 20 weeks.

Non-adherent cells are produced by LTBMCs weekly depending upon the depopulation/feeding schedule and can be stably produced by flasks with few cobblestone island-containing areas (10). This production of non-adherent cells reflects self-renewal and differentiation of the primitive multilineage hematopoietic stem cells in the adherent cell layer (contained within cobblestone islands). Cyclical release into the non-adherent phase of the LTBMCs of non-adherent cells has been shown to reflect the feeding schedule and proliferative demands of the non-adherent cell compartment of marrow cultures, as reflected in the feeding schedule of the flasks (16). The cells were harvested by removal of 50% of the medium weekly and replacement with a fresh volume of new culture medium. Non-adherent cells from each flask in each group of LTBMCs were

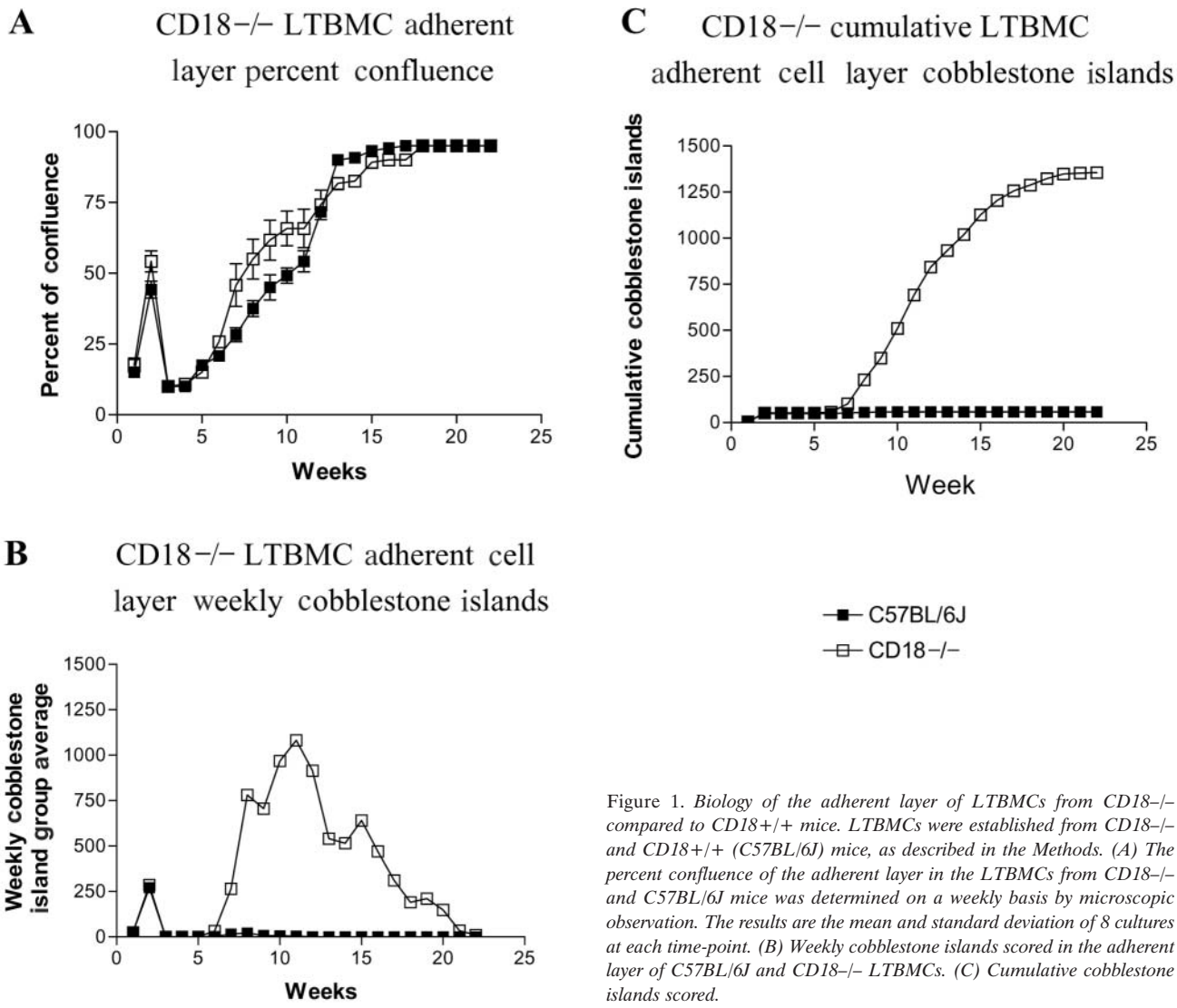

Figure 1. *Biology of the adherent layer of LTBMCs from CD18–/– compared to CD18+/+ mice. LTBMCs were established from CD18–/– and CD18+/+ (C57BL/6J) mice, as described in the Methods. (A) The percent confluence of the adherent layer in the LTBMCs from CD18–/– and C57BL/6J mice was determined on a weekly basis by microscopic observation. The results are the mean and standard deviation of 8 cultures at each time-point. (B) Weekly cobblestone islands scored in the adherent layer of C57BL/6J and CD18–/– LTBMCs. (C) Cumulative cobblestone islands scored.*

cytocentrifuged and counted. As shown in Figure 2A, the weekly production of non-adherent cells decreased over 5 weeks in both CD18–/– and CD18+/+ LTBMCs. As shown in Figure 2A, CD18–/– LTBMCs maintained a greater longevity of cycling out past 13 weeks of non-adherent cells compared to CD18+/+ cultures. The cumulative production of non-adherent cells is shown in Figure 2B and reflects the continued increase in production of non-adherent cells by the CD18–/– cultures, beginning at week 4 and continuing until week 19, while the CD18+/+ cultures produced fewer non-adherent cells after week 7.

Production of colony-forming multilineage hematopoietic progenitor cells persists in CD18–/– LTBMCs. Non-adherent cells harvested from mouse LTBMCs have been demonstrated to contain multilineage hematopoietic stem cells capable of reconstituting all lineages in irradiated recipient mice (16). The longevity of production of totipotential cells is reflected by the production of more differentiated *in vitro* colony-forming cells in semi-solid medium (16). As shown in Figure 3A, CD18–/– and CD18+/+ LTBMC non-adherent cells formed hematopoietic colonies in semi-solid medium, on a weekly basis, at a similar frequency for the first 9 weeks. Beginning at week 10, the CD18–/– LTBMCs produced more day 7 colonies per $5x10^4$ cells plated. Colonies scored on day 7 reflect committed granulocyte macrophage progenitors (16). The number of these colony-forming cells decreased in frequency after week 10 and week 20 for the CD18+/+ and CD18–/– LTBMCs, respectively (Figure 3B).

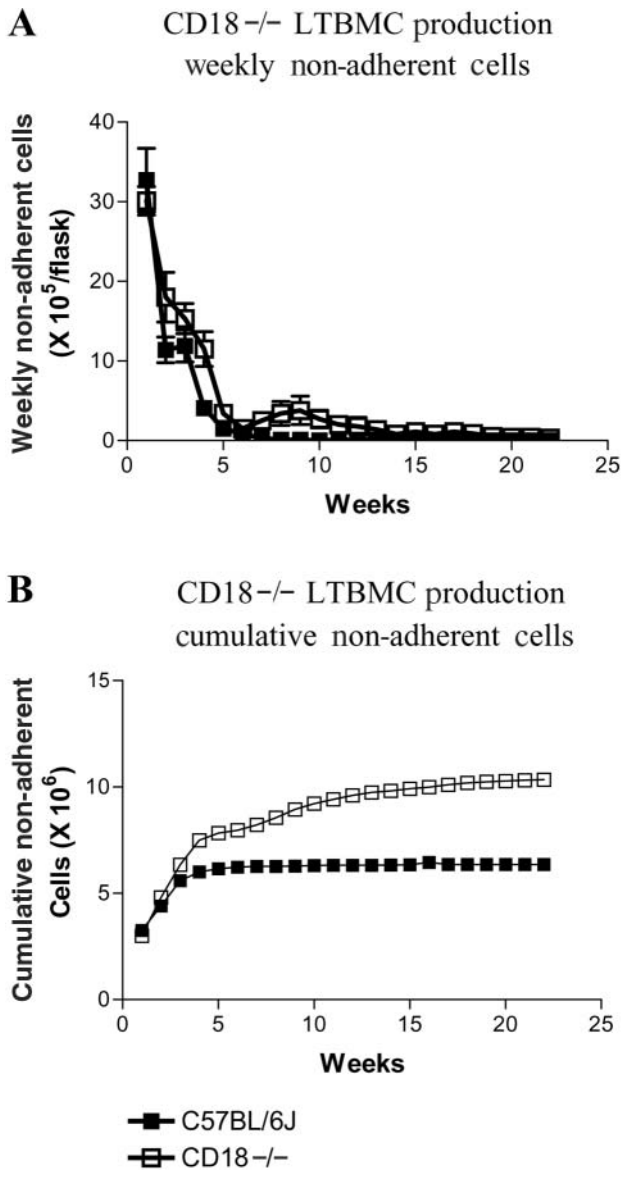

Figure 2. *Non-adherent cell production by CD18–/– compared to C57BL/6J LTBMCs. Non-adherent cells were quantified weekly from the LTBMCs described in Figure 1 by removing 50% of the media and counting the number of non-adherent cells removed (A). Mean and standard deviation of cells produced weekly from at least 8 flasks per time-point per group. (B) Cumulative cell production.*

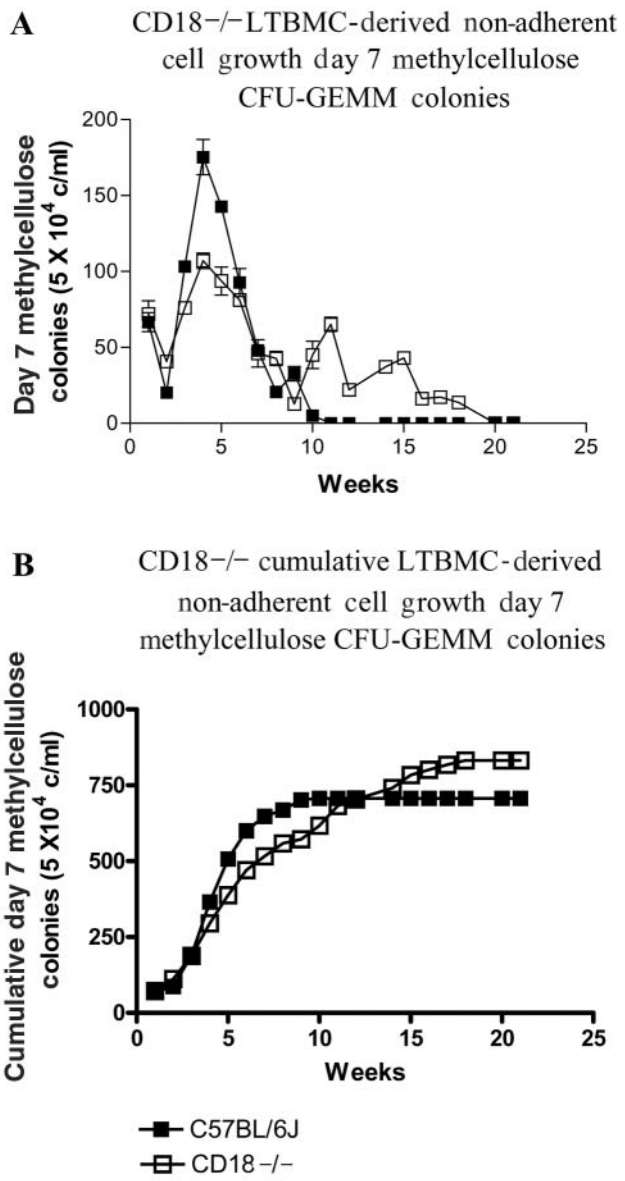

Figure 3. *Production of day 7 CFU-GEMM by non-adherent cells removed from CD18–/– compared to CD18+/+ C57BL/6J LTBMCs. Non-adherent cells were removed from each flask, cytocentrifuged, counted as described in the legend to Figure 2 and plated at a density of 5x10⁴ cells/well in 0.3% methylcellulose-containing medium supplemented with hematopoietic growth factors. The cells were incubated at 37°C for 7 days at which time colonies of more than 50 cells were counted. The results represent the mean and the standard deviation of at least 8 cultures per time-point per group. (A) Weekly colony-forming cell production by cells scored at 7 days. (B) Cumulative day 7 colony-forming production.*

Hematopoietic cells forming colonies of more than 50 cells, scored later on day 14, have been demonstrated to reflect a more primitive hematopoietic progenitor containing more diverse cell lineages, including granulocyte, macrophage, megakarocyte and erythroid cells (16). As shown in Figure 4A, non-adherent cells harvested from CD18–/– cultures produced fewer day 14 colony-forming cells during the first 7 weeks, but a greater number of day 14 colony-forming progenitors compared to non-adherent cells harvested from CD18+/+ cells after week 7. The cumulative production of day 14 colony-forming progenitors was greater in CD18–/– cultures and is shown in Figure 4B.

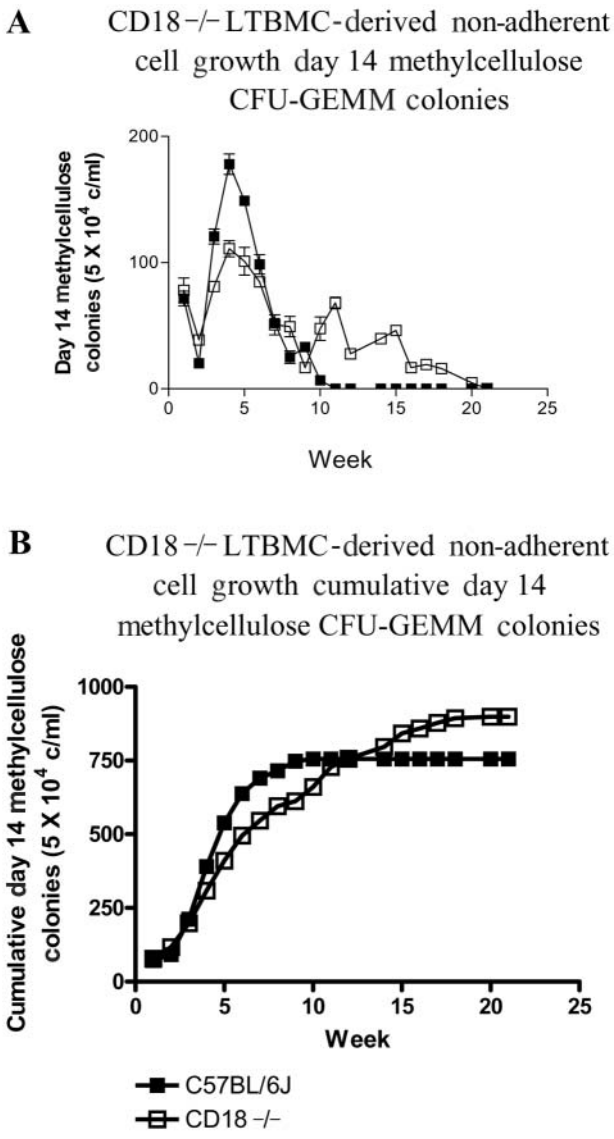

Figure 4. *Production of day 14 CFU-GEMM by nonadherent cells removed from CD18–/– compared to CD18+/+ C57BL/6J LTBMCs. The assay plates from the data in Figure 3 were scored after an additional 7 days in culture. (A) Day 14 CFU-GEMM colonies counted. (B) Cumulative day 14 CFU-GEMM.*

These data establish that, by several parameters of longevity hematopoiesis, CD18–/– LTBMCs were more robust in cell production than those from CD18+/+ mice.

Improved survival of pulmonary irradiated CD18–/– marrow chimeric mice. Macrophage and macrophage progenitor cells, derived from multilineage hematopoietic progenitors produced in LTBMCs, are known to accumulate in the lungs of irradiated C57BL/6J mice at around 100 days after 20 Gy irradiation (15). The accumulation of the macrophages is known to arise from a migration of bone marrow-derived cells to the lung, and is associated with subsequent migration of bone marrow stromal cells (15). This secondary migration of stromal cells contributes significantly to irradiation-induced organizing alveolitis/fibrosis in this mouse model of late irradiation lung damage (15).

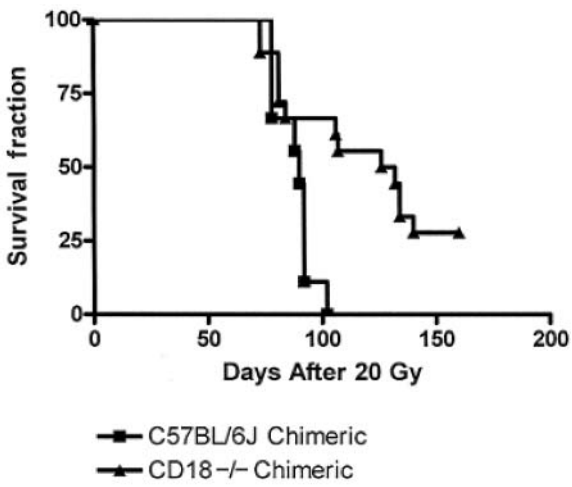

Figure 5. *Decreased ionizing irradiation-induced pulmonary toxicity in C57BL/6J female mice chimeric for male CD18–/– bone marrow. Groups of 50 female C57BL/6J mice chimeric for male CD18–/– or C57BL/6J marrow were irradiated to 20 Gy to both lungs in 2 separate experiments. The results demonstrated improved survival of CD18–/– marrow chimeric mice followed for 160 days after 20 Gy irradiation compared to control mice ($p=0.0053$). (Mice had previously received 10 Gy total body irradiation for marrow transplant to produce chimerism at day 30).*

The effect of CD18–/– or CD18+/+ macrophages on the survival of pulmonary irradiated male mice was quantified by using a chimeric marrow model. This model provided a uniform microenvironment of C57BL/6J female mice. To determine whether the absence of CD18 on the surface of macrophages and macrophage progenitor cells in CD18–/– marrow chimeric mice conferred a survival advantage compared to that of CD18+/+ marrow chimeric mice, CD18–/– chimeric mice were established by irradiating C57BL/6J female mice to 10 Gy whole body followed by intravenous injection of bone marrow from CD18–/– or C57BL/6J male mice. The mice in each group were then irradiated 30 days later to 20 Gy to the pulmonary cavity, as previously described (15). CD18–/– bone marrow chimeric mice had an increased survival compared to CD18+/+ bone marrow chimeric mice (Figure 5, $p=0.0053$). Examination of the lungs of dying mice showed fewer macrophages in CD18–/– marrow chimeric animals. The data indicate that the expression of CD18 had a role in the migration of macrophages from the marrow to the irradiated lungs and its absence decreased irradiation lung damage.

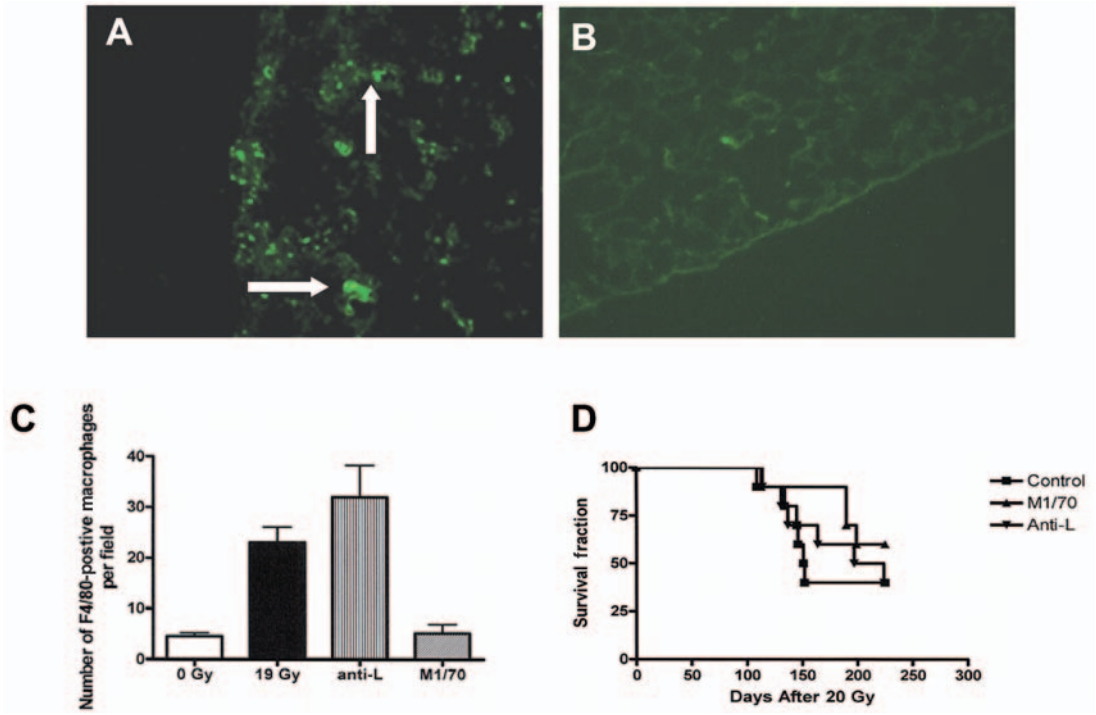

Figure 6. *Effect of injection of macrophage apoptosis-inducing M.170.15.11.5HL antibody or control anti-lymphocyte L-1 antibody on lung irradiation-induced macrophage migration and development of pulmonary fibrosis. (Each antibody was administered intraperitoneally for 15 days from day 80-98 after 20 Gy total lung irradiation, as described in Methods.) Panel B shows reduced numbers of F4/80+ macrophages in a representative high-power field (x500) in mouse treated with anti-macrophage antibody, compared to the anti-lymphoctye control antibody (A) (x500). Panel C demonstrates quantitation of F4/80+ macrophage number in 100 high-powered microscopic fields in the areas of organizing alveolitis at 120 days in 20 Gy irradiated mice compared to unirradiated mice, or mice treated after 20 Gy lung irradiation with either anti-macrophage or anti-L antibody. Mice treated with M1/70 antibody had fewer F4/80-positive macrophages than the control, irradiated or anti-L-treated mice ($p=0.0024$ or 0.0100, respectively). Panel D shows improved survival of anti-M.170-treated mice after 20 Gy irradiation compared to anti-L-treated mice ($p=0.0289$).*

Treatment of pulmonary-irradiated C57BL/6J mice at the time of initiation of pulmonary fibrosis with anti-monocyte/macrophage antibody improves survival. To confirm that the results with CD18–/– marrow chimeric mice reflected reduced pulmonary monocyte/macrophage numbers, groups of 20 Gy lung-irradiated female C57BL/6J mice were treated with anti-macrophage M antibody or with control anti-L-1 antibody at the time after 20 Gy irradiation when macrophage migration is known to be initiated in an established model (15). Beginning on day 80 after 20 Gy irradiation to the lungs with 15 injections of 1 mg intraperitoneally to each mouse for 5 days a week, the mice were treated for 3 weeks. Subsets of each group were sacrificed on day 120 or prior to the death of control irradiated mice on day 150. Control irradiated mice or mice injected with the control anti-L-1 antibody had significantly more F4/80-positive macrophages in the lungs on day 120 (Figure 6A) than did mice injected with the anti-monocyte M1/70 (Figure 6B) (17). Quantitation of pulmonary macrophages using *in situ* anti-F4/80 antibody stain demonstrated increased numbers in control irradiated or anti-L-1-treated irradiated mouse lung compared to the lungs of M1/70-treated irradiated mice (Figure 6C, $p=0.0024$ or 0.0100, respectively). M1/70-treated irradiated mice also showed improved survival compared to the control irradiated or anti-L-cell treated then irradiated mice (Figure 5D, $p=0.0289$).

Discussion

Pulmonary toxicity remains a dose-limiting complication of total body irradiation (TBI) for bone marrow transplantation and radiation therapy in the treatment of thoracic malignancies (18-22). While acute radiation pneumonitis remains a complication of TBI and some thoracic radiotherapy patients, the late lesion of pulmonary fibrosis is a significant complication in long-term survivors. Furthermore, the radiosensitizing effect of many effective chemotherapeutic drugs has been associated with an increased incidence of irradiation pulmonary fibrosis in both experimental animal models and in the clinic (18-22). There

is a need to identify new targets for intervention in preventing late irradiation side-effects in the lungs, and the identification of such targets requires analysis of the cellular and molecular events involved in pulmonary radiation damage (20).

An effective model of human radiation fibrosis is organizing alveolitis/fibrosis in the C57BL/6J mouse (10-15). Organizing alveolitis in this model has been shown to be lung volume-, radiation dose- and radiation fraction size-dependent and, as such, follows similar parameters of radiobiological toxicity of pulmonary irradiation in humans. Furthermore, the time course and detection of late irradiation damage follows a latent period after recovery from an acute reaction, again similar to human clinical radiation fibrosis. Initiation of organizing alveolitis/fibrosis in C57BL/6J mice occurs at around 100 days after 19 Gy irradiation to both lungs and is associated with an increase in pulmonary levels of mRNA for TGFβ, up-regulation of endothelial cellular V-CAM-1 and I-CAM-1 (13), accumulation of bone marrow-derived monocyte/ macrophages beginning at day 80 (14, 15) and migration into the lungs of bone marrow-derived fibroblast progenitors (mesenchymal stem cells, marrow stromal cells) (15). The signals involved in initiation of the late lesion are unknown.

The present studies were designed to determine whether monocyte/macrophage migration from the bone marrow into the lungs was a critical step in the etiology of marrow origin fibroblast migration (15). Cell surface adhesion receptors known to be involved in bone marrow origin macrophage migration to the lungs include the CD18/MAC-1/LFA1 system and the VLA4 receptor (1-9). Since VLA4–/– mice do not survive after birth, the present studies focused on the CD18–/– mouse strain which remains healthy for the 150-200 days required to carry out studies of late irradiation toxicity.

Because bone marrow-derived macrophages arise from hematopoietic progenitor cells, specifically those forming CFU-GEMM (14 day colony-forming cells in culture) and CFU-GM/CFUM (day 7 colony-forming progenitor), we first determined whether the absence of CD18 altered the proliferation capacity of monocyte/macrophage progenitors *in vitro* in the LTBMC system. LTBMCs have been demonstrated to be a valuable resource for analyzing the effects of over production or absence of hematopoietic stimulatory or inhibitory molecules. The results demonstrated increased longevity of CD18–/– LTBMCs, increased production of non-adherent cells, and both day 7 and day 14 colony forming progenitors compared to LTBMCs from CD18+/+ mice. This data may reflect more rapid release from the adherent to non-adherent layer of marrow culture macrophage progenitors due to the absence of CD18. Alternatively, an increased production of hematopoietic growth factors may have resulted from less inhibitory factors released by adherent monocyte/macrophages, or to other factors. The adherent cell layer of CD18–/– LTBMCs were not analyzed for the frequency, relative to CD18+/+ cultures, of endothelial cells/reticular adventitial cells, marrow stromal cells, or adherent macrophages. The increased production of macrophage progenitors by CD18–/– LTBMCs over 25 weeks in culture suggested that the studies of migration of marrow-derived monocyte/macrophages to the lungs in irradiated CD18–/– mice would not be compromised by reduced numbers or decreased production of these progenitor cells.

CD18–/– male bone marrow chimeric C57BL/6J female mice which had been irradiated to 20 Gy to both lungs demonstrated a decreased migration of macrophages and monocytes (identified by anti- F4/80 antibody) to the lungs, increased longevity, and decreased accumulation of bone marrow stromal cells in the lungs at days 120 to 130 after irradiation. The persistence of migration to the lungs of some monocyte/ macrophages in irradiated CD18–/– marrow chimeric mice suggests that other cell surface molecules on macrophages may be acting in a redundant fashion to support the accumulation of a smaller cell population in the lungs at a reduced frequency. Further studies will be required to determine whether those F4/80+ monocyte/macrophages in the lungs of CD18–/– marrow chimeric mice reflect a sub-population with reduced capacity to either produce TGFβ1, or interact with pulmonary parenchymal cells to recruit bone marrow stromal cells.

We also eliminated pulmonary monocyte/macrophages by a second experimental technique using monoclonal antibodies against monocyte/macrophages. Two monoclonal antibodies were used, M.170.15.11.5HL inducing monocyte/macrophage apoptosis (2) and an anti-lymphocyte antibody which does not bind to monocytes (3). Mice treated with M.170.15.11.5HL showed reduced monocyte/macrophage migration to the lungs and improved survival.

In other studies, C57BL/6J mice chimeric for Smad3–/– bone marrow stromal cells (with bone marrow stromal cells incapable of responding to TGFβ elevation in irradiated tissues) showed comparable migration of macrophages into the lungs compared to mice chimeric for Smad3+/+ marrow (23). The Smad3–/– chimeric mice had a significant decrease in secondary bone marrow stromal cell migration to the lungs and showed improved survival after pulmonary irradiation. Thus, the present and previous (23) data suggest that both monocyte/ macrophages and bone marrow stromal cells are required to facilitate the fatal lesion of organizing alveolitis/fibrosis in the C57BL/6J mouse model.

In future studies, it will be important to determine whether elimination of both macrophage and stromal cell populations results in yet further improvement in survival of lung-irradiated mice or whether other toxic effects of

ionizing irradiation on pulmonary endothelial or alveolar cells elicit other components of the irradiation damage response and continue to cause toxicity.

Acknowledgements

This research was supported by a grant from the National Institutes of Health (#R01-HL60132), USA.

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Received April 28, 2006
Accepted May 12, 2006