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*J Immunol* published online 19 September 2011
http://www.jimmunol.org/content/early/2011/09/16/jimmunol.1101141

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/09/16/jimmunol.1101141.DC1

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Selective Resistance of CD44\textsuperscript{hi} T Cells to p53-Dependent Cell Death Results in Persistence of Immunologic Memory after Total Body Irradiation

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Our previous studies showed that treatment of mice with total body irradiation (TBI) or total lymphoid tissue irradiation markedly changes the balance of residual T cell subsets to favor CD4\textsuperscript{+}CD44\textsuperscript{hi} NKT cells because of the differential resistance of the latter subset to cell death. The object of the current study was to further elucidate the changed balance and mechanisms of differential radioresistance of T cell subsets after graded doses of TBI. The experimental results showed that CD4\textsuperscript{+} T cells were markedly more resistant than CD8\textsuperscript{+} T cells, and CD44\textsuperscript{hi} T cells, including NKT cells and memory T cells, were markedly more resistant than CD44\textsuperscript{lo} (naive) T cells. The memory T cells immunized to alloantigens persisted even after myeloablative (1000 cGy) TBI and were able to prevent engraftment of bone marrow transplants. Although T cell death after 1000 cGy was prevented in p53\textsuperscript{−/−} mice, there was progressive T cell death in p53\textsuperscript{−/−} mice at higher doses. Although p53-dependent T cell death changed the balance of subsets, p53-independent T cell death did not. In conclusion, resistance of CD44\textsuperscript{hi} T cells to p53-dependent cell death results in the persistence of immunological memory after TBI and can explain the immune-mediated rejection of marrow transplants in sensitized recipients. The Journal of Immunology, 2011, 187: 000–000.
a changed balance of T cell subsets favoring the CD44<sup>hi</sup> T cells, p53-independent T cell death did not change the balance. CD44<sup>hi</sup> memory T cells immunized to alloantigens persisted even after myeloablative (1000 cGy) TBI in mice with an intact p53 gene and were able to prevent engraftment of BMTs.

**Materials and Methods**

**Animals**

Eight- to 10-wk-old male C57BL/6 WT mice and 8-wk-old male C57BL/6 mice that were homozygotic for the inactivated p53 gene (p53<sup>+/−</sup>) were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal protocols were reviewed and approved by the Stanford Administrative Panels on Laboratory Animal Care.

**Irradiation**

TBI was administered to WT C57BL/6 or p53<sup>−/−</sup> mice 24, 48, 72, or 120 h before analysis of spleen T cell subsets. TBI was performed with a Philips X-ray unit (200 kV, 10 mA; Philips Electronic Instruments, Rahway, NJ) at a rate of 84 gCy/min, with a 0.5-mm Cu filter, and delivered as a single dose.

**mAbs and chemical reagents**

Anti–CD4-FITC, anti–NK1.1-PE, anti–TCRβ-allophycocyanin, anti–CD4-Cy7-PE, anti–CD8-Cy7-allophycocyanin, anti–B220-Cy7-allophycyanin, anti–I-A<sup>d</sup>-PE, anti–Gr1-PE, anti–H-2K<sup>b</sup>-FITC, and anti–CD25-CD4-Cy7-PE, anti–CD8-Cy7-allophycyanin mAbs, as well as the FITC-conjugated anti-B-220 Ab reagent kit, BD Cytofix/Cytoperm kit, and anti-C16/12 mAb, were purchased from BD PharMingen (San Diego, CA). FITC-conjugated anti–Foxp3 mAb was purchased from eBioscience (San Diego, CA). Pan T cell isolation kit II was purchased from Miltenyi Biotec (Auburn, CA).

**Immunization to alloantigens**

Five-week-old male C57BL/6 mice were immunized by i.p. injection of 50 × 10<sup>5</sup> splenocytes from age-matched male BALB/c mice, followed by a dose of 50 × 10<sup>5</sup> splenocytes 1 wk later. Four weeks after immunization, immunized C57BL/6 mice and the splenocytes from these mice were used for further studies.

**Cell preparations**

For preparation of splenic T cells, single-cell suspensions from mice spleens were filtered through Nitex membranes. Suspensions were stained with a mixture of biotin-conjugated mAbs against CD45R, CD11c, CD19, CD3 (B220), CD49b (DX5), CD105, anti–MHC class II, and Ter-119 and monclonal anti-biotin Abs (Pan T cell Isolation Kit II; Miltenyi Biotec) and passed over two consecutive MACS LS-separation columns (Miltenyi Biotec). The purity of T cell preparation was determined by staining with anti–TCR–allophycocyanin and analyzed by flow cytometry.

**Adoptive immunization T cell transfer and bone marrow transplantation**

In brief, immunized C57BL/6 mice were lethally irradiated (1000 cGy) from a 200 kV x-ray source. Immediately thereafter, splenic T cells were purified from these irradiated immunized mice, and 5 × 10<sup>6</sup> were injected into syngeneic C57BL/6 hosts via tail vein. Hosts were also lethally irradiated (1000 cGy) ≤2 h before injection. Control irradiated C57BL/6 host mice received the same number of purified splenic T cells from nonimmunized WT C57BL/6 mice. The next day, all C57BL/6 hosts given the adoptive T cell transfer from immunized or nonimmunized syngeneic mice were injected with 5 × 10<sup>6</sup> donor bone marrow cells from BALB/c mice via tail vein. In additional control groups, mice were given TBI 1000 cGy only, TBI 1000 cGy and BMT within 24 h (5 × 10<sup>6</sup> cells/mouse, i.v.), or TBI 1000 cGy and a BMT after two i.p. injections of BALB/c splenocytes (50 × 10<sup>6</sup> cells, i.p. on days −35 and −28 before BMT). Mice were kept on antibiotic water (25 g/ml neomycin/0.3 U/ml polymyxin B; Sigma-Aldrich) for the first 28 d. Survival was monitored daily, body weight was measured weekly, and chimerism was measured in the blood at 4 wk after transplantation.

**Immunofluorescent staining and flow cytometry analysis**

Single spleen cell suspensions lysed with ammonium chloride buffer were prepared in PBS with 1% calf serum, preincubated with anti-CD16/32 mAb to prevent nonspecific binding via FcRII/III interactions, and then incubated with the appropriate mAbs, as described in detail previously (22). Propidium iodide (Sigma, St Louis, MO) was added prior to analysis to exclude dead cells. To analyze Bcl-2 intracellular expression, all cells were first incubated with the appropriate anti-surface receptor mAbs, fixed, and permeabilized with BD Cytofix/Cytoperm kit for intracellular staining. Ethidium monoazide bromide (EMAB) (Invitrogen, Carlsbad, CA) was added prior to fixation and permeabilization to exclude dead cells. Thresholds for Bcl-2 staining were determined by using isotype-matched irrelevant mAb. All analyses were performed on a modified dual-laser LSRSkan (BD Immunocytometry Systems, San Diego, CA) in the Shared FACS Facility (Center for Molecular and Genetic Medicine, Stanford University), using FlowJo software (TreeStar, Ashland, OR) for data analysis, as described before (12).

**Statistical analysis**

Differences in the percentage and absolute number of immunophenotypic populations of cells were analyzed with the two-tailed Student t test using Prism software (GraphPad Software, San Diego, CA). For all tests, p values ≤0.05 were considered significant.

**Results**

**TBI changes the T cell subset balance in WT mice such that CD44<sup>hi</sup> memory T cells and NKT cells become predominant**

To determine the effect of in vivo irradiation on the balance of T cell subsets, groups of WT male C57BL/6 adult mice were given an approximate 10-fold range of single doses of nonmyeloablative (240 and 480 cGy) and myeloablative (1000, 2000, and 3000 cGy) TBI. Spleen cells were stained for subset surface markers 24, 48, 72, and 120 h later. Fig. 1A shows representative examples of two-color flow cytometric analyses of gated TCR<sup>β</sup> T cells in the spleens of untreated mice and irradiated (1000 cGy TBI) mice after 24 h. There was a modest increase in the percentage of total CD4<sup>+</sup> T cells from ~61% to 84% after TBI and an associated decrease in the percentage of total CD8<sup>+</sup> T cells from ~34% to 7%. This resulted in a change in the CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratio from ~2:1 to ~11:1. The mean CD4<sup>+</sup> and CD8<sup>+</sup> T cell percentages of groups of mice are shown in Fig. 1B at 24 h, and there were minimal additional changes at 48, 72, and 120 h (Fig. 1F). Changes in T cell subsets in the spleen were shown to be reflected in the bone marrow and liver (12). Yield of T cells in the lymph nodes and blood after TBI were too low to analyze accurately (data not shown).

The increase in the percentage of CD4<sup>+</sup> T cells is explained by the greater decrease in the absolute number of CD8<sup>+</sup> T cells than CD4<sup>+</sup> T cells (Fig. 1C). Although the absolute number of total CD4<sup>+</sup> T cells decreased by ~100-fold, from ~15 × 10<sup>6</sup> spleen to ~0.15 × 10<sup>6</sup> (p < 0.0001), the absolute number of CD8<sup>+</sup> T cells decreased by ~400-fold, from ~7 × 10<sup>6</sup> to ~0.02 × 10<sup>6</sup> (p < 0.0001). The greater resistance of CD4<sup>+</sup> T cells to radiation-induced cell death was observed, even when CD4<sup>+</sup> NK1.1<sup>+</sup> T cells (CD4<sup>+</sup> non-NKT cells) were compared with CD8<sup>+</sup> T cells (Fig. 1C). Previous studies showed that almost all CD4<sup>+</sup> NK1.1<sup>+</sup> T cells constitutively express high levels of CD44 and are highly resistant to radiation-induced cell death (9, 11, 12). The percentage of CD4<sup>+</sup> CD8<sup>−</sup> (double negative [DN]) T cells increased after irradiation from ~4 to 8% (Fig. 1A, 1B), and the percentage of CD8<sup>+</sup> T cells was not significantly different from DN T cells (p > 0.05) in irradiated mice. Again, the change in the balance of DN and CD8<sup>+</sup> T cells is explained by the greater decrease in the absolute number of CD8<sup>+</sup> T cells (Fig. 1C).

After irradiation, there was a nearly 7-fold increase in the percentage of NKT cells (NK1.1<sup>+</sup>TCR<sup>β</sup>+) among all T cells from ~4 to 27% (p < 0.0001). (Fig. 1A, 1B). The CD4<sup>+</sup>CD25<sup>+</sup> T cell subset (Treg) increased from ~7% of CD4<sup>+</sup> T cells in untreated mice to ~14% in irradiated mice (p < 0.001), because the absolute number of Tregs decreased to a lesser extent than did total persistence of memory T cells after irradiation.
CD4+ T cells (Fig. 1A–C). More than 80% of the CD4+CD25+ T cells were Tregs that expressed Foxp3*, as judged by intracellular staining in untreated and irradiated mice (Supplemental Fig. 1). The NKT cells and Tregs regulate allografting and can prevent rejection of allografts and graft-versus-host disease (13, 23–27). The relative resistance of CD44hi memory T cells to radiation-induced cell death compared with that of CD44lo naive T cells resulted in an increase in the percentage of memory T cells from ∼19% to ∼66% (p < 0.0001) after irradiation (Fig. 1A, 1B). Although the CD44hi T cells decreased ∼25-fold, from ∼5 × 106 to ∼0.2 × 106 cells/spleen after irradiation, the naive CD44lo T cells decreased ∼500-fold, from ∼20 × 106 to ∼0.04 × 106 (Fig. 1C).

Supplemental Fig. 2 compares representative T cell subset staining from untreated mice with those given 240, 480, 1000, 2000, and 3000 cGy. There was a gradual change in the balance going from 240 and 480 to that observed at 1000 cGy. The shift became more extreme at 2000 and 3000 cGy and continued to change in the direction established at the lower doses. The most dramatic changes occurred in the NKT cell subset, which gradually increased from ∼3% of all T cells in untreated mice to 67% at 3000 cGy, and in the CD44lo naive T cell subset, which gradually decreased from ∼80% to 3% at 3000 cGy.

Among the CD4+CD44hi T cells, there was greater resistance of the CD4+CD25+ subset to radiation-induced loss compared with the CD4+CD25 subset (p < 0.01; Fig. 1C). However, the CD4+CD25+CD44hi cells increased from 33% to ∼80% of total CD4+CD25+ T cells after 1000 cGy as a result of their greater resistance to loss than CD4+CD25+CD44lo cells (Supplemental Fig. 3).

The changes in the balance of splenic Treg and naive T cell subsets are apparent by comparing their ratios in untreated versus irradiated mice (Fig. 1D, 1E). Although the ratio of CD4+ Tregs to naive CD4+CD25+CD44lo T cells was ∼1:13 in untreated mice, the ratio after irradiation was ∼1:2 (p < 0.0001). Similarly, the ratio of NKT/naive non-NKT cells increased from ∼1:25 to ∼1:1. Because both CD4+CD25+ T cells and NKT cells are regulatory T cells that can suppress the immune function of conventional naive T cells (17, 23–27), it is clear that changes in immune function after irradiation, especially in the context of allogeneic bone marrow transplantation, must take into account depletion of absolute numbers, as well as changes in subset balance. The overall ratio of CD44hi/CD44lo T cells in untreated mice changed from ∼1:4 in untreated mice to ∼4:1 in irradiated mice (Fig. 1E). The changed ratio was similar, even when CD44hi and CD44lo non-NKT cells were compared (data not shown). In summary, the combination of NKT cells and memory T cells (both expressing high levels of CD44) accounted for ∼80% of splenic T cells after irradiation and ∼20% before irradiation as a result of their relative radioresistance.

Memory T cells that persist after TBI can reject BMTs

Myeloablative and nonmyeloablative TBI has been used in humans to condition recipients of hematopoietic cell transplants for the treatment of hematologic malignancies and hemoglobinopathies (1–6). In the case of the hemoglobinopathies and aplastic anemia, it is well known that multiple transfusions given prior to transplantation increase the risk for transplant rejection (7, 8). The
rejection is thought to be mediated by radioreistant memory T cells to alloantigens. To determine whether memory T cells that persisted after myeloablative TBI (1000 cGy) in the current study were functional, we developed a BMT model in which the C57BL/6 (H-2Kb) recipients conditioned with TBI were rescued with BMTs from BALB/c (H-2Kd) donor mice. Within 24 h after TBI, recipients were given an i.v. injection of 5 × 10⁶ bone marrow cells from the donors, and chimerism of peripheral blood cells and survival were monitored.

In the first series of experiments, conditioned recipients were given no bone marrow cells, bone marrow cells without recipient immunization, or bone marrow cells after recipient immunization to BALB/c alloantigens by two i.p. injections of 50 × 10⁶ BALB/c spleen cells (Fig. 2B). As expected, the recipients given TBI alone all died with marrow aplasia by 12 d, and the unimmunized recipients given TBI and BMT all survived for 50 d. The latter recipients had >90% donor type cells among blood mononuclear cells, as judged by staining for expression of the donor type, H-2Kd receptors at day 28, and subsequent analysis by flow cytometry (data not shown). The immunized recipients failed to engraft, and all died by 14 d (Fig. 2B).

To determine whether C57BL/6 radioreistant memory T cells can prevent engraftment of donor BALB/c marrow transplants, the experimental scheme shown in Fig. 2A was used. In this series of experiments, the C57BL/6 recipients were given BMTs from BALB/c donors on day 0, as well as an i.v. injection of purified splenic T cells (5 × 10⁶) on day −1 from C57BL/6 donor mice that were unimmunized or immunized to BALB/c alloantigens by two injections of BALB/c spleen cells. Just before the harvest of the spleen cells, the C57BL/6 donor mice were given TBI (1000 cGy), such that only radioreistant T cells transferred to the marrow recipients would persist. As shown in Fig. 2C, the marrow transplant recipients injected with the unimmunized T cells accepted the transplants and survived for ≥50 d. The latter recipients developed high levels of chimerism (>85% donor type cells) among TCR+ T cells, B220+ B cells, and Mac-1+/Gr-1+ monocytes and granulocytes in the blood, as judged by immunofluorescent staining on day 28 (Fig. 2C). In contrast, the marrow transplant recipients injected with the immunized T cells failed to engraft, and all died by day 16.

**TBI (1000 cGy) fails to change the balance of T cell subsets in p53−/− mice**

Because p53 is a key molecular controller of the p53/Bcl-2 apoptotic pathway (19–21), the effect of p53 gene inactivation on the changes in the balance of T cell subsets after in vivo irradiation was studied using p53−/− C57BL/6 mice. Fig. 3A and 3B show that, in striking contract to the changes observed 24 h after irradiation of 1000 cGy TBI in WT mice, the same dose of irradiation failed to induce significant differences in the mean percentages of CD4+ versus CD8+ T cells, NKT cells versus all T cells, CD4+ CD25+ T cells versus all CD4+ T cells, and CD44hi T cells among recipients at 4 wk after transplantation in the group given nonimmunized T cells. The mean percentage of donor-type cells among cells in the blood is shown. Horizontal lines represent means and brackets represent SEs.

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**FIGURE 2.** Memory T cells that persist after TBI are functional and can reject the graft after allogenic bone marrow transplantation. A, Experimental scheme: C57BL/6 mice were immunized with 50 × 10⁶ BALB/c splenocytes i.p. on days −35 and −28 before i.v. transfer to C57BL/6 BMT recipients on day −1. Splenic T cells were harvested and purified from immunized mice 2 h after a single dose of 1000 cGy TBI. Purified T cells were injected into recipient mice immediately after they were given TBI 1000 cGy. On day 0, recipient mice (○, n = 10) received a BMT (5 × 10⁶ cells/mouse, i.v.) from allogenic BALB/c mice. Some C57BL/6 hosts (●, n = 10) were given the same number of splenic T cells (5 × 10⁶ cells/mouse, i.v.) from unimmunized C57BL/6 mice before bone marrow transplantation. Control groups were given TBI 1000 cGy only (▲, n = 5), TBI 1000 cGy and BMT (5 × 10⁶/mouse, i.v.) after two i.p. injections of BALB/c splenocytes (○, n = 5) (50 × 10⁶ cells/mouse, i.p. on days −35 and −28 before bone marrow transplantation). B, Survival of C57BL/6 recipients after TBI 1000 cGy and bone marrow transplantation, with or without immunization of recipients or with adoptive transferred T cells from immunized or nonimmunized syngeneic mice. C, Peripheral blood chimerism in the recipients at 4 wk after transplantation in the group given nonimmunized T cells. The mean percentage of donor-type cells among cells in the blood is shown. Horizontal lines represent means and brackets represent SEs.
all T cells ($p > 0.05$ for all comparisons). The lack of significant change in the balance of T cell subsets is explained by the lack of significant differences ($<2$-fold) between the absolute numbers of each T cell subset in untreated p53$^{−/−}$ mice versus irradiated p53$^{−/−}$ mice ($p > 0.05$) examined at 24 h after 1000 cGy TBI (Fig. 3C). As expected, the lack of significant changes in the absolute numbers of the T cell subsets after 1000 cGy TBI in p53$^{−/−}$ mice resulted in no significant difference ($p > 0.05$) in the ratios of CD4$^{+}$CD25$^{+}$ T cells versus CD4$^{+}$CD25$^{−}$ T cells (CD4$^{+}$ naive) and NKT versus total naive CD44$^{+}$ T cells before and after TBI. $p > 0.05$ for all groups. E, Mean ratios of absolute numbers of CD44$^{+}$ T versus CD44$^{−}$ T cells before and after TBI ($p > 0.05$). F, Comparison of mean (± SD) absolute numbers of NKT and NK1.1$^{+}$ T cells at different time points. $p > 0.05$, untreated versus irradiated mice at 24, 48, and 72 h; $p < 0.05$, NKT and NK1.1$^{+}$ T cells of untreated versus 120 h. Data are representative of three independent experiments (n = 6–10 mice/group).

Supralethal TBI induces T cell death but does not change the balance of T cell subsets in p53$^{−/−}$ mice

In further experiments, p53$^{−/−}$ mice were given 2000 and 3000 cGy TBI. The effects on the absolute numbers of T cells and T cell subset balance were studied. The mean absolute numbers of NKT cells and non-NKT cells in the spleen were not significantly different ($p > 0.05$) between untreated mice and those irradiated with 1000 or 2000 cGy TBI (Fig. 4). However, there was ∼10-fold reduction in both types of T cells after 3000 cGy compared with untreated p53$^{−/−}$ mice ($p < 0.05$ for both NKT and non-NKT cells) (Fig. 4B). Because this p53-independent cell loss induced by radiation was similar in both subsets of T cells, their percentages among total T cells did not change after TBI compared with untreated mice.

The percentage of total T cells among the spleen cells from the untreated p53$^{−/−}$ mice was ∼30%, and the percentage after 1000–3000 cGy was in the range of ∼25–32% (mean 31 ± 4%; $p > 0.05$). Similarly, the percentage of NKT cells among all T cells was ∼3% in untreated mice and was in the range of 2–3% after 3000 cGy (mean 3% ± 0.4; $p > 0.05$). The lack of change was in sharp contrast to the results in WT mice, because 3000 cGy induced a decrease in the percentage of total T cells from ∼30 to ∼5% and an increase in the percentage of NKT cells among all T cells to ∼59% (Fig. 4A). Fig. 4B shows that the absolute number of non-NKT cells decreased ∼50 times more than did the NKT cells in the WT mice given 3000 cGy TBI (mean fold reduction 52 ± 9; $p < 0.001$) and resulted in the major change in the balance of subsets. The results indicated that the p53-independent killing that is likely due to breaks in the dsDNA (28) causes little or no change in the percentage of total T cells in the spleen or in the balance of the T cell subsets, whereas the p53-dependent killing causes marked changes in the percentage of total T cells and the balance.

**FIGURE 3.** Effect of irradiation on different T cell subsets in p53$^{−/−}$ C57BL/6 mice. A, Gated TCR$^{+}$ cells in untreated p53$^{−/−}$ mice (upper row) or in p53$^{−/−}$ mice 24 h after 1000 cGy of TBI (lower row) were analyzed for CD4 versus CD8, NK1.1 versus TCR$^{+}$, and CD4 versus TCR$^{+}$. B, The mean percentages of CD4$^{+}$, CD8$^{+}$, NK1.1$^{+}$, CD4$^{+}$/CD25$^{+}$, CD44$^{+}$, and CD44$^{+}$/TCR$^{+}$ cells are shown. $p > 0.05$ for all groups, untreated and irradiated mice. C, Mean fold change in ratio of absolute numbers of different T cell subsets before and after TBI. All changes were <2-fold ($p > 0.05$). D, Mean ratios of absolute numbers of CD4$^{+}$/CD25$^{+}$ T cells versus CD4$^{+}$/CD25$^{−}$ T cells (CD4$^{+}$ naive) and NKT versus total naive CD44$^{+}$ T cells before and after TBI ($p > 0.05$ for all groups). E, Mean ratios of absolute numbers of CD44$^{+}$ T versus CD44$^{−}$ T cells before and after TBI ($p > 0.05$). F, Comparison of mean (± SD) absolute numbers of NKT and NK1.1$^{+}$ T cells at different time points. $p > 0.05$; untreated versus irradiated mice at 24, 48, and 72 h; $p < 0.05$, NKT and NK1.1$^{+}$ T cells of untreated versus 120 h. Data are representative of three independent experiments (n = 6–10 mice/group).
Increased expression of Bcl-2 in NKT and non-NKT cells after irradiation is p53 dependent

The intracellular expression of Bcl-2 in NKT and non-NKT cells was compared in untreated WT and p53−/− mice and in both types of mice given graded doses of TBI. Fig. 5 (bottom two rows) shows representative one-color staining for Bcl-2 in gated NK1.1+ and NK1.1− T cells. In the untreated WT mice, two peaks of staining were observed in the NKT cells: a large dull peak and a small bright peak that accounted for ~18% (mean 18 ± 3%, n = 10) of cells. Both peaks were clearly brighter than the isotype control staining. After administration of 3000 cGy TBI, there was a dramatic shift to brighter staining, and ~92% (mean 85 ± 8%, n = 10) of cells were contained in a single bright peak. The pattern observed for non-NKT cells in untreated mice failed to show a distinct bright peak, and ~3% (mean 3 ± 1%, n = 10) of cells stained brightly using the threshold used to distinguish the bright and dull NKT cells. After 3000 cGy, the non-NKT cells showed a marked shift in bright staining for Bcl-2, and a clear bright peak containing ~69% (mean 54 ± 18%, n = 10) of cells was observed. When the experiments were repeated using untreated p53−/− mice, the staining pattern again showed two peaks, with ~14% (mean 13 ± 1%, n = 6) of cells in the bright peak. However, after 3000 cGy, there was little shift in the Bcl-2 staining, and the bright peak contained ~19% (mean 18 ± 2%, n = 10) of cells. Similarly, there was little shift in the staining of Bcl-2 in the non-NKT cells after 3000 cGy TBI compared with the untreated mice.
Additional p53−/− mice were given graded doses of 1000, 2000, and 3000 cGy TBI, and the gated NKT cells and non-NKT cells were analyzed for bright Bcl-2 staining versus forward scatter to better delineate shifts in a discrete population of cells. As shown in Fig. 5 (top four rows), the range of bright Bcl-2 staining was ~1–19% for NKT cells and ~2–3% for non-NKT cells in the untreated p53−/− mice and all groups of irradiated p53−/− mice using the threshold developed from the one-color-staining analysis. The NKT cells and non-NKT cells from WT mice developed a marked shift in staining that reached a maximum after 3000 cGy and resulted in two clearly distinct populations of Bcl-2hi and Bcl-2lo cells with concentric contours (Fig. 5, third row from bottom). The mean percentages of Bcl-2hi non-NKT cells (2 ± 1%) and NKT cells (17 ± 0.6%) in p53−/− mice given 3000 cGy were significantly different (p < 0.0001) from that of WT mice (53 ± 18% and 85 ± 8%, respectively).

The marked increase in Bcl-2 staining intensity in the WT mice after irradiation is accounted for by the selective survival of Bcl-2hi cells compared with Bcl-2lo cells and/or the upregulation of Bcl-2 expression after exposure to irradiation. Our previous study showed that upregulation of Bcl-2 after irradiation contributed to increased Bcl-2 staining in CD4+ NKT cells but did not contribute to increased staining in non-NKT cells (12).

**Comparison of survival of Bcl-2hi and Bcl-2lo T cell subsets after irradiation**

The changes in the absolute numbers of splenic Bcl-2hi and Bcl-2lo T cell subsets were determined after 1000 cGy TBI. Fig. 6A shows the changes in CD44hi (naive) T cells and CD44lo (memory) T cells among CD4+ and CD8+ T cells. Fig. 6B shows the fold decrease in the T cells after irradiation. Among the CD4+ naive T cells, the Bcl-2hi cells decreased ~10-fold, whereas the Bcl-2lo cells decreased ~400-fold (p < 0.001). Unexpectedly, the Bcl-2hi CD8+ naive T cells decreased ~200-fold, and the Bcl-2lo CD8+ naive T cells decreased dramatically, by ~3500-fold (p < 0.001). Thus, the high levels of expression of Bcl-2 were associated with considerably greater radioresistance within the CD4+ and CD8+ naive T cells. However, there were marked differences in the radiosensitivity of Bcl-2hi CD4+ versus CD8+ naive T cells or Bcl-2hi CD4+ versus CD8+ naive T cells that were independent of the level of expression of intracellular Bcl-2.

A similar analysis of change in the absolute numbers of Bcl-2hi and Bcl-2lo/CD44hi (memory T cells) among CD4+ and CD8+ T cells is shown in Fig. 6. The Bcl-2lo/CD44+ memory T cells were more radioresistant than were the Bcl-2hi/CD44+ naive T cells (p < 0.001). Bcl-2lo/CD44+ naive T cells decreased 400-fold, but Bcl-2hi/CD44+ memory T cells (either CD4+CD44hi or CD4+NK1.1−CD44hi) decreased only 30-fold (Fig. 6B). Differences in the CD8+ naive and memory T cells with low levels of Bcl-2 were even more pronounced (p < 0.001). The naive cells decreased ~3500-fold compared with ~100-fold among the memory cells. Interestingly, there was no significant difference between the Bcl-2lo and Bcl-2hi/CD8+ memory T cell decreases after irradiation (p > 0.05) (Fig. 6B). In summary, the differences in reduction of memory versus naive CD4+ or CD8+ T cells could not be accounted for by differences in levels of Bcl-2 expression.

**FIGURE 6.** The effect of radiation on the absolute numbers of Bcl-2hi and Bcl-2lo cells in naive and memory CD4+ and CD8+ T cell subsets in WT mice. A. Mean (± SD) absolute numbers of Bcl-2hi and Bcl-2lo cells in CD4+ and CD8+ naive and memory T cell subsets in the spleen of WT mice 24 h after 1000 cGy TBI versus untreated (UNT) mice. Vertical line on left separates naive (CD44hi) and memory (CD44lo) cells without gating on NK1.1− cells. Vertical line on right separates memory (CD44lo) cells with or without gating for NK1.1− cells, because NKT cells are all CD44hi. B. Fold change in absolute numbers of Bcl-2hi and Bcl-2lo cells in CD4+ and CD8+ T cell subsets in A comparing untreated versus irradiated mice. The data are representative of three independent experiments (n = 6–10 mice/group). *p < 0.01, **p < 0.001.

**FIGURE 7.** Intracellular expression of Bcl-2 in naive or memory CD4+ and CD8+ T cell subsets in untreated mice or 24 h after irradiation in WT and p53−/− C57BL/6 mice. A. Splenocytes were harvested from untreated WT mice or 24 h after 1000 cGy TBI, stained for CD44lo (naive) or CD44hi (memory) CD4+ and CD8+ T cell subsets, fixed and permeabilized, and stained for intracellular Bcl-2. The mean percentages of Bcl-2hi cells among gated CD44lo or CD44hi in CD4+ and CD8+ T cell subsets are shown. A. Analysis as in A for p53−/− mice. The data are representative of three independent experiments (n = 5–10 mice/group). *p < 0.01, **p < 0.001, ***p < 0.0001.
Changes in levels of Bcl-2 expression in T cell subsets after TBI

Because levels of expression of Bcl-2 can account for the radiosensitivity of some T cell subsets and not others, it was of interest to determine changes in Bcl-2 levels in the subsets after TBI. We expected that the levels of Bcl-2 would increase markedly among surviving T cells in only those subsets in which high levels of Bcl-2 would provide a survival advantage. Fig. 7A shows the mean percentage of Bcl-2hi cells among the different T cell subsets in the spleen of untreated WT mice and after a single dose of 1000-cGy TBI. There was a 5- to 10-fold increase in the percentage of Bcl-2hi cells among naive CD4+ (p < 0.001) and naive CD8+ T cells (p < 0.001), respectively, after irradiation. The percentage of Bcl-2hi cells among naive CD4+ cells was significantly lower than that of naive CD8+ cells (p < 0.01) after irradiation. The mean percentage of Bcl-2hi cells also increased ∼10-fold (p < 0.001) among memory CD4+ T cells after irradiation (Fig. 7A). However, there was no statistical difference (p > 0.05) between the percentage of Bcl-2hi cells before and after irradiation in the memory CD8+ T cell subset. In part, this was due to the high percentage of Bcl-2hi cells among memory CD8+ T cells before irradiation, which was ∼30-fold higher than that of memory CD4+ T cells (p < 0.001). The high percentage of Bcl-2hi cells in nonirradiated memory CD8+ T cells was not influenced by the expression of p53, because the high level was also observed in p53−/− mice (Fig. 7B). The percentage of Bcl-2hi cells also increased significantly in the Treg and NK cell subsets in WT mice after TBI (data not shown). As expected, there were no significant changes (p > 0.05) in the percentages of Bcl-2hi cells in naive and memory CD4+ and CD8+ T cell subsets after irradiation in p53−/− mice (Fig. 7B). Thus, all increases in the percentage of Bcl-2hi cells among the CD4+ and CD8+ T cell subsets were dependent on p53-induced apoptosis.

Discussion

To more clearly understand the effect of radiation on T cell-mediated immunity, we studied changes in the balance of T cell subsets after graded doses of TBI. An important goal of the study was to determine whether the radiotherapy-induced depletion of T cell subsets was uniform or selective. We found that, at doses up to ∼3000 cGy in WT mice, there was a selective loss of T cell subsets that changed their balance, with CD8+ T cells being more sensitive than CD4+ T cells and CD44lo naive T cells being more sensitive than CD44hi memory T cells and NK cells. The CD4+ NK cell subsets were the least sensitive, as reported previously (12). Memory T cells that were immunized to alloantigens persisted after myeloablative (1000 cGy) TBI and were able to prevent engraftment of BMTs after adoptive transfer to transplant recipients. In the clinical setting of bone marrow transplantation, in which almost all donors are HLA matched with recipients, multiple blood transfusions of recipients can result in an increased risk for graft rejection, especially in patients with hemoglobinopathies and chronic anemia (7, 8). This is due to WBCs in the transfusions that express minor histocompatibility Ags and immunize recipients to cross-reactive minor Ags in the donor graft. In the current study, recipients were immunized to both major and minor histocompatibility Ags in the donor grafts, based on the strains under investigation. The conclusion that memory T cells directed to alloantigens are radiosensitive and can contribute to rejection is likely to be applicable to minor Ags in humans who receive more exposure to Ags over a longer period of time than in the mouse model. Because p53 is a major controller of apoptosis after irradiation (19–21), we compared the changes in the balance of T cell subsets in WT mice with those in p53−/− mice in the current study. When doses of irradiation up to 1000 cGy TBI were given to p53−/− mice, there were no significant reductions in the absolute number of any T cell subsets compared with nonirradiated p53−/− mice after 24 h. Thus, inactivation of the p53 gene prevented the early change in the balance of T cell subsets observed in the WT mice due to the prevention of cell death from apoptosis. Interestingly, when the dose of TBI was increased to 3000 cGy in p53−/− mice, there was a marked (10-fold) reduction in the absolute number of T cells compared with nonirradiated p53−/− mice. This p53-independent T cell death did not alter the balance of T cell subsets or change the level of expression of intracellular Bcl-2 within the subsets. Thus, p53-dependent T cell death resulted in a marked change in the balance of T cell subsets, whereas p53-independent T cell death did not.

Naive CD8+Bcl-2hiCD44lo T cells were the most sensitive to cell loss in WT mice and decreased ∼3500-fold after 1000 cGy TBI, whereas memory CD4+Bcl-2hiCD44hi T cells decreased <10-fold. However, different levels of Bcl-2 could not account for differences in radioresistance of CD4+ versus CD8+ T cells or naive versus memory T cells. The results suggested that antiapoptotic mechanisms other than Bcl-2 determine radioresistance in the latter subset comparisons and/or there were marked differences in the upregulation of proapoptotic proteins, such as Bax, Bim, Puma, and Noxa, after irradiation in these subsets (19, 29, 30). It is of interest that high levels of Bcl-2 or both Bcl-2 and Bim have been reported in long-lived memory CD8+ T cells in untreated mice (31, 32). Our findings that CD44hiCD8+ T cells in untreated mice have 30-fold more Bcl-2hi cells than do CD44hiCD8+ T cells is consistent with these reports. Nevertheless, the Bcl-2 levels did not determine the radioresistance among CD44hiCD8+ T cells, because Bcl-2hi and Bcl-2lo cell loss after irradiation was similar. Antiapoptotic proteins other than Bcl-2 that may affect survival after irradiation include Bcl-xL, NAIP, and MyD118 (15, 16, 33), and their impact on T cell subset balance is the subject of continuing investigations.

In conclusion, the current study showed that there is a selective p53-dependent loss of T cell subsets after a dose of TBI (1000 cGy) that is used for conditioning of BMT recipients. The selective loss changes the balance of subsets to favor CD44hi T cells, including memory T cells and NKT cells. The radiosensitive memory T cells can persist after TBI and can prevent engraftment of allogeneic BMTs.

Acknowledgments

We thank Glenna Letsinger for help in preparing the manuscript and Yinping Liu for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References


