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Histone Deacetylation Critically Determines T-cell Subset Radiosensitivity₁

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Abstract

Lymphocytes are sensitive to ionizing radiation and naïve lymphocytes are more radiosensitive than their memory counterparts. Less is known about radiosensitivity of memory cell subsets. We examined the radiosensitivity of naïve (T_N), effector memory (T_{EM}), and central memory (T_{CM}) T cell subsets in C57BL/6 mice, and found T_{EM} to be more resistant to radiation-induced apoptosis than either T_N or T_{CM}. Surprisingly, we found no correlation between the extent of radiation-induced apoptosis in T cell subsets and : (i) levels of pro- and anti-apoptotic Bcl-2 family members; or (ii) the H2-AX content and maximal γH2-AX fold change. Rather, T_{EM} cell survival correlated with higher levels of immediate γH2-AX marking, immediate break binding and genome-wide open chromatin structure. T cells were able to mark DNA damage seemingly instantly (30 s), even if kept on ice. Relaxing chromatin with the histone deacetylase inhibitor valproic acid following radiation or etoposide treatment, improved the survival of T_{CM} and T_N cells up to levels seen in the resistant T_{EM} cells, but did not improve survival from caspase-mediated apoptosis. We conclude that an open genome-wide chromatin state is the key determinant of efficient immediate repair of DNA damage in T cells, explaining the observed T cell subset radiosensitivity differences.

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INTRODUCTION

Lymphocytes are highly sensitive to the lethal effects of ionizing radiation (IR), via processes commonly referred to as interphase death, with apoptosis playing a major role (1-4). However, mechanistic details of lymphocyte subset sensitivity remain incompletely understood. In general, it has been shown that mammalian cells are more sensitive to IR while undergoing mitosis, although activated, dividing T cells are slightly more resistant than their resting counterparts (2-5). Moreover, CD8 T cells were shown to be more prone to interphase death than CD4 T cells (6-8); and naïve (T_N) T cells were found to be more sensitive than their memory (T_M) counterparts (1, 2, 9). Current literature suggests that T_M cells are more radioresistant due to higher concentrations of Bcl-2 (8, 9).

Radiation-induced cell death is thought to be largely mediated by double-strand DNA breaks (DSB). H2AX is a variant of the H2A histone that is phosphorylated at Ser139 as part of the immediate DSB detection and repair, at which point this phosphorylated histone is called γ H2AX (10). Increased genomic content of the H2AX variant correlates with a survival advantage in human memory T cells (11). In addition, mouse models haploid for H2AX have shown DNA repair deficiency in lymphoid populations (12). γ H2AX detection is commonly used as a proxy for DNA damage. H2AX content, γ H2AX kinetics, and radioresistance have not been addressed in parallel in T cell subsets.

Heterochromatic DSB repair also depends on chromatin relaxation, and closed chromatin formations impair DSB repair (13, 14). Chromatin remodeling occurs during T_N to T_M cell differentiation (15). Because the relationship between DNA repair and apoptosis is a complex process (16), it remains unclear whether and how overall chromatin state contributes to radioresistance in different lymphocyte subsets.

We reexamined radioresistance of T cell subsets with a specific goal to delineate Effector Memory (T_{EM}) from Central Memory (T_{CM}) subset radiation-induced interphase death in a murine model. By excluding homeostatically dividing cells, we established interphase radiosensitivity for T cell subsets as being $T_{EM} > T_{CM} = T_N$. Radiosensitivity of T_{CM} and T_N cells could not be explained by the relative levels of pro- or anti-apoptotic Bcl-2 family members. Furthermore, an examination of γ H2AX kinetics revealed that the more resistant T_{EM} cells exhibited fast initial marking, but lower overall fold-change, relative to other subsets. Moreover, Double-Strand-Break (DSB) binding analysis by modified TUNEL and Comet assays revealed enhanced early DSB binding by T_{EM} cells. In parallel, genome-wide chromatin analysis using H3K27me3 revealed a correlation between chromatin state and radiosensitivity. This correlation was mechanistically supported by experiments showing that opening chromatin with the histone deacetylase inhibitor (HDACi) valproic acid (VPA) following radiation improved T_N and T_{CM} cell survival to the levels observed in T_{EM} cells. Our results are most consistent with the explanation that genome-wide chromatin structure is the critical determinant governing early DSB binding and survival of T cell subsets. While it is established that native DNA repair proceeds by opening chromatin at the site of repair, our results show that preexisting open chromatin can fully explain survival differences in T cell subsets, and that forcing chromatin open through HDACi is enough to radically improve survival from IR in sensitive cells.

MATERIALS AND METHODS

Mice

Adult (<8 Month) Male C57BL/6 mice were acquired from Jackson Laboratories and held under specific pathogen-free conditions in the animal facility at the University of Arizona (UA). All experiments were conducted in accordance with the guidelines set by the UA Institutional Animal Care and Use Committee, consistent with all federal, state and local regulations. Mice were euthanized by isoflurane and spleen was collected into complete RPMI supplemented with 5 or 10% Fetal Bovine Serum (FBS). Blood was taken from the heart of sacrificed, or alternatively by retro-orbital bleed from living, anesthesia-free mice. Red blood cells were hypotonically lysed.

Irradiation

Irradiations were performed using a on a Gammacell Cs¹³⁷ source irradiator (Gammacell-40 Exactor, Best Theratronics Inc., Ottawa, Canada) and calibrated using a certified ionization chamber (PTW model N30001) by in-house physicist from the UA Health Sciences Center. The calculated radiation dose rate was 69.3 ($\pm$ <1%), with total radiation doses ranging from 1 to 10 Gray (Gy). The accuracy of the radiation doses employed was tested and shown to have maximal errors of 5% or less using both optical stimulated luminescence (OSL) dosimeters (Landauer Inc., Greenwood, IL, USA) and thermal luminescence dosimeters (TLD) (Medical Radiation Research Center, University of Wisconsin, WI, USA) embedded midline into realistic mouse phantoms. For total body irradiation, unanesthetized mice (max=8) were placed in sterile microisolator RadDisks (Braintree Scientific, Braintree, MA) without dividers, and exposed uniformly at ambient conditions to deeply penetrating 662 keV gamma rays at a set dose-rate and total doses listed above. Irradiations were carried out before noon on a light-dark cycle 7am-7pm. In vitro irradiations were performed in 96-well 300ul capacity round-bottom plates with lids in the center of the irradiation field, in wells at the center of the plate, in 270ul of RPMI 10% supplemented as described (17). All remaining wells on the plate were filled with 270ul 1x PBS. In vitro irradiations were performed on ice water, and plates were kept on ice in transit. Post-radiation in vitro incubations occurred in RPMI complete with 10% FCS (as above) at 37°C with 5% CO₂.

Flow Cytometry

LIVE / DEAD yellow stain was purchased from Life Technologies (Grand Island, NY). Fluorescent conjugated α -Mouse antibodies against CD4 (S3.5) and CD19 (6D5) were acquired from Life Technologies. CD8a (S3-6.7), CD62L (MEL-14), CD44 (IM7), CD3 (17A2), NK1.1 (PK136), Bcl-XL (7B2.5), and CCR7 (4B12) were acquired from eBioscience (Dan Diego, CA). α -Ki-67 (B56), α -Active Caspase-3 (CPP32), Bcl-2 (BCL/10C4) were acquired from BD Pharmingen (San Jose, CA). CD8b (YTS156.7.7) CD49b (DX5), and γ H2AX (2F-3) were acquired from Biolegend (San Diego, CA). Monoclonal rabbit antibody against Mouse Mcl-1 (Y37) was visualized with donkey α -rabbit IgG antibody conjugated to PE or FITC. Antibodies against Bim (H-191, Santa Cruz Biotechnology, Santa Cruz, CA), Bax (B-9, Santa Cruz Biotechnology), H3K27ac and H3K27me3 (Active Motif, Carlsbad, CA), and p53 phospho-Serine 15 (D4S1H, Cell Signaling, Beverly, MA) were visualized similarly, and samples were blocked with donkey

serum to avoid nonspecific binding as needed. Staining was performed at 4°C followed by fixation and permeabilization (FoxP3 kit, eBioscience) except for CCR7, which was incubated with cells for 30 minutes at 37°C.

For *in vivo* experiments, blood and spleen counts were determined on a Hemavet cell counter (Drew Scientific, Dallas, TX). For *in vitro* experiments, equal amounts of Count Bright beads (Life Technologies) were used in the final volume of FACS buffer to count cells. Beads were employed per manufacturer's instructions, at a 1:7 dilution of beads, using appropriate calculation adjustments and a minimum of 1000 beads per any sample. Samples were acquired on a Fortessa Flow Cytometer equipped with 4 lasers and the DiVa software (BD Biosciences, Mountain View, CA). Compensation and analysis was performed using FlowJo software version 9.6.4 (Tree Star, Ashland, OR).

Comet Assay

Spleen or blood-derived mononuclear cells were sorted into CD8⁺CD4⁻ subsets using CD44 and CD62L to identify CD8 N (CD44^{lo}CD62L^{hi}) CM (CD44^{hi}CD62L^{hi}) and EM (CD44^{hi}CD62L^{lo}) populations. Cells were sorted on a FACS Aria cell sorter within the UA Flow Cytometry Core Facility. Directly following 5Gy irradiation on ice, cell samples were mixed with 1% agarose in TBE (37°C) and immediately pipetted onto slides and then placed back on ice. Slides were otherwise kept at 4°C in the dark. Cells were lysed with Comet lyse buffer (2.5M NaCl, 100mM EDTA, 100mM Tris, 1% DMSO, 1% Triton-X; pH 8.0) for 1.5 hours at 4°C, washed twice, and subjected to electrophoresis at 25V for 20 minutes in the TBE buffer. Samples were dried with EtOH and kept at 4°C until visualization. Samples were re-hydrated with Propidium Iodide (PI) on the day of visualization. Microscopy was performed with a Nikon Eclipse (TE2000-U) at 20x and Tail moment analysis was performed with Cometscore software (The TriTek Corporation, Sumerduck, VA). 1 or 2 slides per cell subset per mouse were processed, depending on experiment, and 10 photographs of randomly selected cells were attempted per slide.

TUNEL Assay

The APO-DIRECT TUNEL assay kit with FITC dUTP (BD Biosciences) was applied as per manufacturer's instructions, with the following modifications: surface antibodies were applied before radiation for 30 minutes and washed out before radiation; cells were fixed directly (as per flow cytometry above) after radiation treatment in a final volume of 1mL fixative solution per sample without RPMI wash, then fixed again after wash for an additional 30 minutes; the TdT reaction lasted 135 min in a 96-well plate at 37°C in 5% CO₂.

Flow-DNAse I Hypersensitivity

Splenocytes were stained with surface marker-specific antibodies as above. Antibody fluorophores were chosen to account for PI's wide emission: e450, A700, APCe780, FITC, and Pacific Orange. Samples were fixed using the BD Cytofix/Cytoperm kit (BD Biosciences) as per manufacturer's instructions, with following alterations: samples were fixed for 5 minutes rather than 20; following wash with Perm solution, samples were subjected to Perm solution with 10% DMSO for 20 minutes at 4°C, washed twice with

typical Perm solution and fixed again for 5 minutes as before (nuclear fixation). After further washing, samples were treated with a 0.6 mg/mL solution of DNase I (Sigma) in BD Perm solution at a final volume of 50ul per sample, at RT for 40 min in the dark. Samples were stained with Propidium Iodide (PI) + RNase (APO-DIRECT kit above) in BD perm solution (1:4), followed by three washes. A cytometer cleaning regimen of contrad, bleach and water, was applied between each sample to eliminate residual PI signal.

Apoptosis

All chemicals were applied to cells at their final concentration in RMPI-10% FBS complete (see above). Staurosporine (ST) (Sigma, St. Louis, MO) was diluted in DMSO as stock and brought to a final concentration of 1µg/mL. ST was washed out at 6 hours followed by a re-application of VPA or regular media for the remainder of the incubation. Etoposide (ET) (Sigma) VP-16 was diluted in DMSO as stock, then to a final concentration of 5µg/mL. ET was left in for the duration of the assay. Valproic Acid powder (Sigma) was diluted to a stock of 0.3M in 1xPBS, then further diluted to final concentration(s). All VPA stocks were made fresh from powder before application. Heat shock was accomplished in 96-well plates in a 45°C water bath for 10 minutes. VPA media was applied before heat shock. Brefeldin A (eBioscience) was diluted in Methanol, and applied at a final concentration of 2ug/mL.

Statistics

Statistical calculations were performed in Prism 4.0 (Graphpad Software). Generally, paired 1 and 2-way ANOVA tests were employed between cell types recovered from the same individual, because of the variability of overall starting counts. All error bars shown are SEM.

RESULTS

Differential sensitivity of T_{EM} and T_{CM} cell subsets to radiation

For the purposes of this study, we phenotypically defined T cell subsets within both the CD8 and CD4 lineage as T_{lo} N – Naïve T cells (CD44^{hi} CD62L^{hi}); T_{CM} – central memory T cells (CD44^{hi} CD62L^{hi}); and T_{EM} – effector memory T cells (CD44^{hi} CD62L^{lo}).

Dose-response curves, traditionally used to establish the radiosensitivity of tumors or cultured cells, cannot be readily applied to *ex vivo* immune cells. The relative rate of homeostatic division (18), the heterogeneity of memory populations (19)(23), apoptosis of resting cells due to growth factor or cytokine starvation (20) and differential requirements for homeostatic cytokines between subsets (21) all apply to lymphocytes and can obfuscate results. For example, T_N cells have different rates of homeostatic division than their memory counterparts (18), which may translate into a false display of radioresistance.

To examine the radiosensitivity of CD4 and CD8 T_{EM} and T_{CM} cell subsets to interphase death, we irradiated replicate splenocyte samples with 2 Gray (Gy), 4Gy or 0Gy (no irradiation), then rested them for 3, 12, 17, or 24 hours. We counted viable cells remaining in each sample, excluding cells that had committed or initiated apoptosis with a LIVE/DEAD stain (Life Technologies) that detects compromised cell membranes, and Anti-

Active-Caspase-3 Ab respectively (Fig. S1A). To measure interphase death, we also excluded cells undergoing recent mitosis using Ki-67 expression, which increases in lymphocytes that have crossed the S1 phase of cell cycle in the past 2-3 days (22, 23). A slight caveat here is that Ki-67 staining may miss those cells immediately primed to reenter G₁ (22, 24). However, as we used unstimulated cells and mice from a stringently specific pathogen-free (SPF) colony, a short observation period post-IR, low levels of steady-state homeostatic division (18), and an *in vitro* environment devoid of growth factors (20), we feel that cytometric distinction of Ki-67^{HI} and Ki-67^{LO} cells allowed us to focus our analysis of primary cells that were in interphase (Ki-67^{lo}), during irradiation.

Throughout this work we have normalized cell counts obtained from irradiated samples by dividing them by their 0Gy counterparts per animal per time point and per cell population. At the lowest dose tested (1Gy), we have failed to observe reproducible differences between the various T cell subset radiosensitivity in terms of rates of interphase cell death (data not shown). By contrast, we reproducibly found that CD8 T_{EM} cells showed less interphase death than their T_{CM} or T_N counterparts at higher irradiation doses (2 & 4 Gy; Fig. 1A,B). Surprisingly, CD8 T_{CM} and T_N cells were almost identical in their intrinsic interphase death sensitivity and kinetics. CD4 T_{CM} and T_N cells showed less interphase death than their CD8 counterparts (Fig. 1C-1D), but still exhibited lower resistance than CD4 T_{EM} cells. T cell subsets derived from mouse peripheral blood mononuclear cells (PBMC) produced identical radiosensitivity patterns (Fig. S1B) to those observed in spleen-derived samples.

We next tested survival trends between T cell subsets *in vivo*. C57BL/6 mice were exposed to 1Gy, 2Gy, 4Gy or no irradiation, then rested for 72 hours. These doses were non-lethal, as expected for this strain of mice (C57BL/6) and its established LD50/30 (34). Similar to the *in vitro* resistance results, CD8 T_{EM} cells were resistant and T_N cells sensitive following *in vivo* irradiation (Fig. 1E). By contrast, CD8 T_{CM} cells showed a dramatic survival improvement in an *in vivo* context, the basis of which is under examination, but which could be linked to bone marrow, or other niche, protective effects (11).

Mammalian cells in the S-phase of mitosis exhibit a slight resistance to radiation-induced death when compared to other cell cycle phases (2), prompting us to examine the relative radiosensitivity of mitotic (Ki-67^{HI}) counterparts in each subset. T_N cells undergoing homeostatic division were more resistant than their interphase counterparts, while mitotically active T_{EM} cells, by contrast, were less resistant than their interphase counterparts (Fig. S2A-S2D). No differences were found between interphase and mitotic T_{CM} cells (Fig. S2E-S2F).

Expression levels of Bcl-2 family members do not correlate with radiosensitivity

Because CD8 T_{CM} cells were relatively resistant *in vivo* but not *in vitro*, we questioned the correlation between radiosensitivity and Bcl-2. We stained T cell subsets with antibodies against Bcl-2, Mcl-1, and Bcl-XL. Standing levels of anti-apoptotic Bcl-2 family members did not correlate with survival trends between CD4 and CD8 T cells, nor between subsets. For example, T_{CM} cells expressed more Bcl-2 compared to T_{EM} cells (Fig 2A – 2B), whereas enhanced radioresistance was limited to T_{EM} cells (Fig. 1). Mcl-1 and Bcl-XL expression also failed to correlate with survival (Fig. 2B, S3A-B). When cells were followed

in time after irradiation, Bcl-2 tended to increase while Mcl-1 tended to decrease, but no clear correlation with radiosensitivity was observed (Fig. S2G – S2J). Pro-apoptotic proteins Bim and Bax also failed to correlate with CD4 and CD8 subset radiosensitivity (Fig. 2C-2D), although increased levels of these proteins were observed following radiation (Fig. S3C-S3F).

Radioresistance correlates to early γ H2AX marking

To evaluate whether DNA damage sensing is comparable between T cell subsets, we examined the increase in DSB marking by γ H2AX in CD8 T cell subsets following irradiation. Maximal up-regulation of γ H2AX following irradiation over time was the highest in T_N and lowest in CD8 T_{EM} cells, which directly (and not inversely, as one might expect), correlated with radiosensitivity (Fig. 3A). We examined whether differing γ H2AX expression after irradiation could be due in part to disparate native H2AX levels, and found that CD8 T_{CM} cells indeed contain more native H2AX histone available for phosphorylation (Fig. 3B). Surprisingly, we found that radiation dose-dependent γ H2AX marking can take place even when cells were continuously maintained at $\times 4^0$ C (on ice) both during and following irradiation, up to the time of sample fixation. This early γ H2AX marking correlated with radioresistance of T_{EM} (Fig. 3D). Moreover, T_{EM} DSB marking was more robust in the first 5 minutes of 37°C incubation following irradiation (Fig. 3E), suggesting that early and rapid DSB marking may determine early protective responses following potentially lethal genomic injury.

CD8 T_{EM} cells bind DSB faster than T_{CM}

Given that T cells can sense DSB nearly instantaneously at 4°C in our hands, and given that γ H2AX marking takes place at or following DSB end binding by either PAR or Ku in many systems (25-27), we reasoned the this early γ H2AX advantage may reflect more immediate DSB binding. In order to test if T_{EM} were indeed able to hold together DSBs earlier in the repair process, we employed the TUNEL assay (28). The TUNEL assay detects DNA fragmentation due to apoptosis by enzymatic marking of DNA ends, and therefore should also detect different levels of DSBs between subsets after irradiation if DNA at the either end of DSB were unbound by repair factors, and thus available to TdT enzyme. By fixing and applying the TUNEL assay directly after *in vitro* irradiation at 4°C , we were able to detect breaks in T cells *ex vivo*. (Fig. S3G). Note that the dose of 5 Gy (rather than doses of 2 & 4 Gy) was necessary to detect reproducible damage. By this assay, CD8 T_{CM} cells showed more TUNEL activity from an identical amount of radiation compared to their counterparts (Fig. 3F), while T_{EM} cells showed the least, implying that DSB were bound more efficiently in T_{EM} than in T_{CM} . Therefore, perhaps surprisingly, T_{EM} cells were able to bind DSB ends even in the few seconds between irradiation and fixation, similar to T_N but significantly better than T_{CM} .

We also examined DSB formation by the comet assay, which has an established threshold for detecting radiation-induced DSB (29) (Fig. S3H). We reasoned that DSB ends bound *together* by Ku or PAR would produce lower tail moments under neutral conditions. Splenocytes were sorted into CD8 T_{EM} , T_{CM} and T_N cell groups. Sorted cells were kept on ice throughout radiation, transit, and after being embedded in agarose on slides. Tail

Moments of 5Gy-irradiated sorted CD8 T_{CM} cells again showed a greater proportion of unbound DSB compared to other subsets (Fig. 3G, see also Fig. S3H-S3K). Therefore, the comet assay confirmed that survival advantage of T_{EM} over T_{CM} correlated with immediate DSB binding.

Radiosensitivity correlates with closed chromatin content, and with relative chromatin shifts following radiation across T cell subsets

We noted that H2AX genomic content corresponded with enhanced radiosensitivity. Enhanced genomic H2AX content has been shown to correspond to regions of closed, non-transcribing genomic structure (30). Moreover, chromatin compaction may limit the DNA damage response (14), and it has been shown that T_N cells have a much more closed chromatin conformation than memory T cells on the whole (31). Together, these factors suggest that overall chromatin state might be limiting for early DNA damage response in T_N and T_{CM} cells.

Histone H3 that is tri-methylated on Lysine 27 (H3K27me3), is known to correspond with areas of closed chromatin architecture (32), while Histone H3 acetylated on Lysine 27 (H3K27ac) conversely correlates with areas of relaxed chromatin architecture (33). We observed that genome-wide H3K27me3 content negatively correlated with survival across T cell subsets (Fig. 4A). In order to independently validate chromatin configuration patterns observed with H3K27me3, we subjected splenocytes to a DNase I hypersensitivity assay modified for flow cytometry. We used Propidium Iodide (PI) to quantify DNA content before and after DNase treatment. As expected, PI signal in T_{EM} was more strongly reduced following DNase I treatment, compared to that from T_N and T_{CM} cells. This indicated that DNase I had access to larger segments of T_{EM} chromatin, replicating our observations using the H3K27me3 assay (Fig. 4D-4E). While CD8 T_N and T_{CM} began to open chromatin immediately following radiation, T_{EM} did not attempt significant genome reorganization in the first hour (Fig. 4B – 4C). These findings may suggest that T_{EM} cells already contain a chromatin configuration that is optimally suited for repair.

Treatment with valproic acid following irradiation abrogates T cell subset radiosensitivity differences by improving the survival of N and CM subsets

To test whether global genomic architecture is the dominant factor in T cell subset radiosensitivity differences, we experimentally forced the genome open following irradiation and asked whether T_N and T_{CM} survival improved. Valproic Acid (VPA) is one of a number of histone deacetylase inhibitors that allow the genome to effectively assume an open chromatin state (34). We confirmed that VPA treatment opened the chromatin of all CD8 T cell subsets in our hands, as evidenced by increased H3K27ac levels following exposure (Fig. S4A-4B). We irradiated cells (0, 2, 4 or 5Gy) and incubated them with or without VPA for 12 or 17 hours. Survival of VPA+ or VPA– irradiated samples was then divided by their respective VPA+ or VPA– 0Gy controls. VPA treatment improved T_{CM} and T_N cell survival across multiple doses of radiation, while T_{EM} cell survival remained unchanged (Fig 5A, Fig. S4C-S4G). The enhanced survival with VPA treatment was reflected in T cell counts (Fig. 5B).

Because HDAC inhibition potentially opens genomic areas for enhanced transcription, improved survival could be an artifact of increased transcription of one or more anti-apoptotic factors. To verify that VPA selectively improves survival from DSB-induced death, we treated cells with 6mM VPA and subjected them to heat shock (HS), Brefeldin A (BFA) or Staurosporine (ST) treatment to induce apoptosis. As a positive control, we treated cells with Etoposide (ET) to induce apoptosis in a radiation-independent, but DSB-dependent manner. As predicted for a model of enhanced DNA repair through chromatin relaxation, VPA treatment did not affect survival of cells exposed to HS, ST (Fig. 5C) or BFA (Fig. S4F), but did affect survival following Etoposide treatment in manner identical to that following radiation damage, by specifically improving CD8 T_{CM} and T_N cell subset survival (Fig 5C, Fig. S4G). Because apoptosis following IR in lymphocytes is dependent on p53 (35), we examined p53 retention in these subsets. p53 is protected from degradation following genomic damage via phosphorylation of Serine 15 by ATM (36). Fold change in p53-phospho-S15 was lowest in T_{EM} following IR, correlating with increased survival. However, p53 retention in T_{CM} and T_N p53 was significantly but only slightly lowered by VPA treatment (Fig. 5D-5E), suggesting that decreased p53 retention is not the sole mechanism explaining the efficacy of VPA-mediated increase in T_{CM} and T_N survival.

DISCUSSION

A fundamental understanding of peripheral immune subset radiation sensitivity is crucial for better understanding of the clinical impact of radiotherapy (37, 38), bone marrow transplantation (39), and immune reconstitution (40). Moreover, the discovery of factors responsible for differential lymphocyte radiation sensitivity has implications for diverse areas of immunology and biology: DNA damage response (41), natural and virus-induced hematopoietic cancers (42, 43), V(D)J recombination (44), cytotoxic killing (45), and possibly age-induced immune senescence (46), inasmuch as the latter may intersect with DNA damage repair over lifespan.

Despite the breadth and depth of the field of radiobiology, the differential response of lymphocyte subsets to radiation in different compartments remains incompletely understood (47). Previous studies have demonstrated that radiation-induced T cell apoptosis requires p53 (35). The constitutive Bcl-2 transgenic mouse model has established that anti-apoptotic machinery can be protective, but the relevance of these findings for physiological radiosensitivity remains unclear (48). Our work demonstrates that the T_{EM} cell populations are intrinsically radioresistant relative to other T cell subpopulations, and that this resistance is linked to repair advantages fostered by open chromatin. Unlike other subsets, survival of T_{EM} following DSB is not improved by forcing the chromatin open. Given that subset sensitivity and genome-wide heterochromatin content are highly correlated, we propose that chromatin state is the central arbiter of differential T cell subset radiosensitivity.

Aside from bone marrow transplant (BMT), there is no truly effective treatment for hematopoietic radiation syndrome following lethal irradiation. The therapeutic use of recombinant cytokines and growth factors can be effective, but only against exposures on the lower end of the lethal range (49). Treating large swaths of the population with BMT would be impossible in the case of mass exposure. When applied *in vivo* after potentially

lethal radiation exposure, VPA is able to abrogate T cell death in C57BL/6 mice (50). Our work suggests that this may in part operate via a mechanism whereby lymphocyte attrition is lowered throughout the animal due to enhanced DSB binding and repair, improving defense against opportunistic and other infection. This indicates that VPA-mediated HDACi provides a temporary enhancement of native repair abilities, and thus may be able to limit the damage to the immune system, although that remains to be tested *in vivo*.

An association was found between miR-24, the expression of H2AX, and the survival of human T cells in the face of DNA damage (11). In addition, mouse models haploid for H2AX have shown repair deficiency in lymphoid populations (12). However, our study shows that greater H2AX presence is not sufficient for protection. CD8 T_{CM} cells exhibited lower survival rates following irradiation compared to their T_{EM} counterparts, while containing approximately twice as much H2AX. Maximal γ H2AX signal over time also did not correlate with survival. Rather, the initial γ H2AX marking was the strongest correlate of survival. It is likely that these changes were missed by other authors because most studies typically do not examine repair initializing at 4°C and within seconds of the insult. This work indicates that very early γ H2AX fold change, and not maximal γ H2AX response, more accurately predicts survival in T cell subsets. It also emphasizes the need for normalization of γ H2AX to total amounts of H2AX when interpreting γ H2AX signal.

We noted that active p53 retention via the DNA damage pathway negatively correlated with survival between T cell subsets, as would be expected by increased repair efficiency. However, depression of p53 retention following the VPA treatment in T_N and T_{CM} subsets did not reach the levels seen in T_{EM} cells in the absence (or the presence) of VPA treatment. Also, γ H2AX response was not altered by VPA (Fig. S4H). This implies that these DNA damage responses are not directly tuned by chromatin state in T cells. As cells were gated on Ki-67 low (non-dividing), this also suggests that competing factors in the NHEJ or alt-NHEJ pathway are able to abrogate apoptosis downstream of these key events in T cells.

While the most likely explanation for differential comet and TUNEL signals across T cell subsets is that break binding occurs at differing efficiency following radiation, at the present we cannot rule out formation of different quantities of DSB from the same radiation dose. When comet samples were treated with proteinase K following radiation, we observed extremely elongated Tail moments, and no differences between subsets (Fig S3K), indicating that damage might be equal. However, given the ability of T cells to initiate repair at 4°C, and given the amount of time needed to deliver physiologically relevant doses of radiation, analysis of possible differences in DSB formation between subsets will likely require further DSB formation analysis *in situ*.

In accordance with previous studies, CD4 T cells displayed a greater radioresistance than CD8 T cells in our hands. This pattern was extended to both CD4 T_N and T_{CM} subsets. A recent study has shown increased radioresistance in CD4 T regulatory cells (Tregs) (51). While these data could suggest that overall increased radioresistance of CD4 T cells could be due to the presence of Tregs, further characterization will be necessary to test whether the enhanced radioresistance of Tregs can completely account for the enhanced radioresistance of any specific CD4 subset, or the entire CD4 T cell population.

In the context of functional distribution of T cell subsets, the patterns of their sensitivity to DNA damage appears to correspond to their role in the immune response. T_{CM} and T_N are both responsible for clonal bursts of new effector T cells, and are thus essentially keepers of genomic integrity for a given clone. T_{EM} meanwhile reside in a variety of tertiary peripheral sites (organs), and thus must endure a hostile environment, with widely differing oxygen tensions, temperatures and other conditions. A lower threshold for apoptosis therefore seems physiologically justified for T_{CM} and T_N, while more efficient repair befits the physiological function of T_{EM}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IR	ionizing radiation
T_{CM}	central memory T cells (CD44 ^{hi} CD62L ^{hi})
T_{EM}	effector memory T cells (CD44 ^{hi} CD62L ^{lo})
T_N	Naïve T cells (CD44 ^{lo} CD62L ^{hi})

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Figure 1. Differential radiosensitivity of T cell subsets

Splenocytes from n=8 adult male C57BL/6 mice were split evenly and subjected to 0, 2, or 4Gy of *in vitro* radiation. Samples were rested for 3, 12, 17 or 24 hours, stained and analyzed by flow cytometry as described in Methods (Fig. S1). Counts for A-C are shown relative to 1, where 1 is cell numbers of the 0Gy sample count per subset per mouse per time point. (A) 2Gy irradiated CD8 T cells. (B) 4Gy irradiated CD8 T cells. (C) 2Gy irradiated CD4 T cells. (D) 4Gy irradiated CD4 T cells. Paired 2-way ANOVA results of A-B: time ***, subtype *. Bonferroni post-tests shown between Naïve and T_{EM} subtypes. (E-F): *In vivo* dose response curves. Groups of 6 adult male C57BL/6 mice were subjected to whole body irradiation at 0, 1, 2, or 4Gy, and then rested for 72 hours. Splenocytes were harvested and gated through Ki-67^{LO}. Natural Log of the surviving fraction identified by flow cytometry for phenotype and numbers for each subset is shown on the Y axis. Test of slope difference Naïve vs. EM from linear regression: (E-F) = ***. All figures representative of at least 2 independent experiments. *** = p<0.001, ** = p<0.01.

Figure 2. Standing levels of pro- and anti-apoptotic Bcl-2 family members do not correlate with intrinsic T cell subset radiosensitivity

Splenocytes (n=6) from adult male C57BL/6 mice were subjected to flow cytometry and analyzed for levels of expression, as judged by the mean fluorescent intensity (MFI) of (A) Bcl-2, (B) Mcl-1, (C) Bim and (D) Bax. Statistics are the result of paired 1-way ANOVAs between subsets of either CD4 or CD8 T cells. All ANOVA results = ****. All figures representative of at least 2 independent experiments. *** = $p < 0.001$, ** = $p < 0.01$.

Figure 3. Early γ H2AX marking and DSB binding correlate to post-radiation survival

All samples from adult male C57BL/6 mice. (A) γ H2AX fold change in CD8+ T cell splenocyte subsets at indicated times post 4Gy irradiation (n=4) and incubation at 37°C. 2-way ANOVA for time: ****, cell type: ****. Bonferroni post-tests shown for EM vs CM. (B) Standing H2AX histone (protein) amongst the CD8 + T cell subsets from the blood of 6 mice. 1-way ANOVA = ***. (C) Representative flow plot of γ H2AX signal from isotype control (dotted line), CD8 EM T cells mock irradiated (grey filled), or CD8 EM T cells irradiated at 10Gy on ice and kept on ice until fixation. (D) γ H2AX fold change on ice as in (C) for CD8 subsets across radiation doses. 2-way ANOVA and Bonferroni post-tests shown. (E) Kinetic of γ H2AX fold change during the first five minutes post irradiation and incubation at 37°C. Bonferroni post-tests is shown for EM vs. CM. (F) TUNEL assay applied to PBMC after 5Gy irradiation and immediate fixation (n=6). Each cell type from each mouse is subtracted from its 0Gy control. 1-way paired ANOVA = *. Tukey post-test shown. (G) Splenocytes (n=8) sorted into CD8 CM, EM and Naive subsets, irradiated (5Gy) and subjected to Comet assay. 1-way ANOVA = ***, ns = not significant, **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

Figure 4. Standing chromatin content, and H3K27me3 down-regulation following radiation, correlate with T cell subset radiosensitivity

(A) Standing H3K27me3 in T cell subsets vs. relative survival of those same subsets post 4Gy and 21 hour incubation, averages from $n=5$. From highest survival: CD4_{EM}, CD8_{EM}, CD4_{CM}, CD4_N, CD8_{CM}, CD8_N. (B) H3K27me3 alterations following 4Gy IR and 5 minute incubation at 37°C. 2-way ANOVA: cell type: ****, radiation ***. ($n=4$). (C) Samples as in (B), incubated for 1 hour post-IR. 2-way ANOVA: Time: ** Cell subsets:*. Shown are results of Tukey post-tests between subsets. (D) Representative histogram of H3K27me3 in T_N (solid line), T_{CM} (dashed line), or T_{EM} (grey). (E) DNase I hypersensitivity assay was performed in spleen samples by flow cytometry ($n=4$) and results expressed as the reduction of PI signal (a measure of open, DNase-accessible chromatin) following DNase treatment. Significance between the subsets was assessed using the Tukey post-tests. (F) Representative histogram of PI staining in Ki-67 negative T_{EM} cells either treated (solid line, left histogram) or untreated (dashed line, right histogram) with DNase I. All figures representative of at least 2 independent experiments. ns = not significant, *** = $p < 0.001$. ** = $p < 0.01$, * = $p < 0.05$.

Figure 5. VPA treatment rescues CD8 T_{CM} and T_N from interphase death

(A) Following 4Gy of irradiation or 0Gy, 6 splenocyte samples were treated or not with 6mM VPA and rested for 12 hours post-radiation. Counts shown normalized to 0Gy sample for each mouse and subset. (B) Actual counts of Naïve CD8 T cell splenocytes (n=6) irradiated with 4Gy and rested for 12 hours with or without VPA. Results of paired T test shown. (C) Splenocytes (n=6) treated with heat shock (45°C for 10 minutes), Staurosporine (STE) at 1 ug/mL for 6 hours, Etoposide at 5ug/mL for 12 hours, or 4Gy irradiation. CD8 Naïve T cell subset shown. (A-C) Samples rested for a total of 12 hours post-treatment. (D) Representative histogram of p53 phospho- Serine 15 signal from T_{CM} splenocytes irradiated with 4Gy (solid line, open histogram) or 0Gy (grey closed histogram), 3 hours post-irradiation. (E) Percent change in p53 phospho- Serine 15 between n=4 splenocyte samples irradiated at 4Gy vs. 0Gy, 3 hours post-irradiation. Significance was determined using Tukey post-tests. All figures representative of at least 2 independent experiments. ns = not significant, **** = p<0.0001, *** = p<0.001, ** = p<0.01, * = p<0.05.