Histone Deacetylation Critically Determines T Cell Subset Radiosensitivity

Jason L. Pugh, Alona S. Sukhina, Thomas M. Seed, Nancy R. Manley, Gregory D. Sempowski, Marcel R. M. van den Brink, Megan J. Smithey and Janko Nikolich-Zugich

*J Immunol* published online 2 July 2014
http://www.jimmunol.org/content/early/2014/07/02/jimmunol.1400434

Supplementary Material  http://www.jimmunol.org/content/suppl/2014/07/02/jimmunol.1400434.DCSupplemental

Subscription  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Histone Deacetylation Critically Determines T Cell Subset Radiosensitivity

Jason L. Pugh,*†‡ Alona S. Sukhina,*‡ Thomas M. Seed,‖§ Nancy R. Manley,*† Gregory D. Sempowski,‖§§ Marcel R. M. van den Brink,**††† Megan J. Smithey,*‡*† and Janko Nikolich-Zugich*†‡§§

Lymphocytes are sensitive to ionizing radiation and naive lymphocytes are more radiosensitive than their memory counterparts. Less is known about radiosensitivity of memory cell subsets. We examined the radiosensitivity of naive (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 1) levels of pro- and antiapoptotic Bcl-2 family members or 2) the H2AX content and maximal γH2AX fold change. Rather, T_EM cell survival correlated with higher levels of immediate γH2AX 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. The Journal of Immunology, 2014, 193: 000–000.

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 naive (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 because of 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 γH2AX (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). γH2AX detection is commonly used as a proxy for DNA damage. H2AX content, γH2AX kinetics, and radioresistance have not been addressed in parallel in T cell subsets.

Heterochromatin DSB repair also depends on chromatin relaxation, and closed chromatin formations impair DSB repair (13, 14). Chromatin remodeling occurs during T_N to T_EM 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 antiapoptotic Bcl-2 family members. Furthermore, an examination of γH2AX kinetics revealed that the more resistant T_EM cells exhibited fast initial marking but lower overall fold-change, relative to other subsets. Moreover, 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. Although it is established that native DNA repair proceeds by opening chromatin at the site of repair, our results show that pre-existing 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 mo) male C57Bl/6 mice were acquired from The Jackson Laboratory and held under specific pathogen-free conditions in the animal facility at the University of Arizona. All experiments were conducted in accordance with the guidelines set by the University of Arizona Institutional Animal Care and Use Committee, consistent with all federal, state and local regulations. Mice were euthanized by isoflurane and spleen was collected to complete RPMI 1640 medium supplemented with 5 or 10% FBS. Blood was taken from the heart of sacrificed, or alternatively by neck bleeds from living, anesthesia-free mice. RBCs were hypotonically lysed.

Irradiation

Irradiations were performed using a on a GammaCell C135 source irradiator (Gammarcell-40 Exactor; Best Theratronics, Ottawa, ON, Canada) and calibrated using a certified ionization chamber (PTW model number N30001) by in-house physicist from the University of Arizona Health Sciences Center. The calculated radiation dose rate was 69.3 (± <1%), with total radiation dose ranging from 1 to 10 Gy. The accuracy of the radiation doses used was tested and shown to have maximal errors of <5% using both optical stimulation luminescence dosimeters (Landauer, Greenwood, IL) and thermal luminescence dosimeters (Medical Radiation Research Center, University of Wisconsin, Madison, WI) embedded milide into realistic mouse phantoms. For total body irradiation, unanesthetized mice (maximum ∼8) were placed in sterile microisolator RadiDisks (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 7 a.m.–7 p.m. In vitro irradiations were performed on ice water, and plates were kept on ice in transit. Postradiation in vitro incubations occurred in RPMI PBS. In vitro irradiations were performed on ice water, and plates were kept on ice until visualization. Samples were rehydrated with propidium iodide (PI) on the day of visualization. Microscopy was performed with a Nikon Eclipse (TE2000-U) at >×20 magnification and tail moment analysis was performed with Cometscore software (TriTek, Sumerduck, VA). One or two 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 deoxyuridine triphosphate (BD Biosciences) was applied as per the manufacturer’s instructions, with the following modifications: surface Abs were applied before radiation for 30 min and washed out before radiation, and cells were fixed directly (as per flow cytometry above) after radiation treatment in a final volume of 1 ml fixative solution per sample without RPMI 1640 medium wash and then fixed again after wash for an additional 30 min; 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 Abs as above. Ab fluorophores were chosen to account for Pt's wide emission: e450, A700, APCe780, FITC, and Pacific Orange. Samples were fixed using the BD Cytofix/Cytoperm kit (BD Biosciences) as per the manufacturer’s instructions, with following alterations: samples were fixed for 5 min rather than 20; following wash with perm solution, samples were subjected to perm solution with 10% DMSO for 20 min at 4°C, washed twice with type perm solution, and fixed again for 5 min as before (nuclear fixation). After further washing, samples were treated with a 0.6 mg/ml solution of DNASE I (Sigma-Aldrich) in BD perm solution at a final volume of 50 µl/sample at room temperature for 40 min in the dark. Samples were stained with PI + RNase (APO-DIRECT kit above) in BD perm solution (1:4), followed by three washes. A cytometer clearing regimen of Decon Condor 70 liquid detergent (Fischer Scientific, Thermo Fischer, Waltham, MA), 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 RPMI 1640 medium–10% FBS complete (see above). Staurosporine (ST) (Sigma-Aldrich, St. Louis, MO) was diluted in DMSO as stock and brought to a final concentration of 1 µg/ml. ST was washed out at 6 h, followed by a replacement of fresh medium–10% FBS complete for the remainder of the incubation. Etoside (ET) Yveside VP-16 (Sigma-Aldrich) was diluted in DMSO as stock and then to a final concentration of 5 µg/ml. ET was left in for the duration of the assay. VPA powder (Sigma-Aldrich) was diluted to a stock of 0.3M in 1X PBS and 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 min. VPA medium was applied before heat shock. Brefeldin A (eBioscience) was diluted in Methanol, and applied at a final concentration of 2 µg/ml.

Statistics

Statistical calculations were performed in Prism 4.0 (Graphpad Software). Generally, paired one- and two-way ANOVA tests were used 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 subsets to radiation

For the purposes of this study, we phenotypically defined T cell subsets within both the CD8 and CD4 lineage as T N, T EM, and T CM.

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, 20), apoptosis of resting cells because of growth factor or cytokine starvation (21), and differential

...
requirements for homeostatic cytokines between subsets (22) all apply to lymphocytes and can obfuscate results. For example, T_N 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, 4, or 0 Gy (no irradiation) and then rested them for 3, 12, 17, or 24 h. 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 (Supplemental Fig. 1A). 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 d (20, 23). A slight caveat in this paper is that Ki-67 staining may miss those cells immediately primed to re-enter G1 (23, 24). However, because we used unstimulated cells and mice from a stringently specific pathogen-free colony, a short observation period post-IR, low levels of steady-state homeostatic division (18), and an in vitro environment devoid of growth factors (21), 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 0-Gy counterparts per animal per time point and per cell population. At the lowest dose tested (1 Gy), 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 and 4 Gy; Fig. 1A, 1B). 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 PBMC produced identical radiosensitivity patterns (Supplemental Fig. 1B) to those observed in spleen-derived samples.

We next tested survival trends between T cell subsets in vivo. C57BL/6 mice were exposed to 1, 2, 4 Gy, or no irradiation, then rested for 72 h. These doses were nonlethal, as expected for this strain of mice (C57BL/6) and its established LD_{50/30} (25). 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

**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 4 Gy of in vitro radiation. Samples were rested for 3, 12, 17, or 24 h, stained, and analyzed by flow cytometry as described in Materials and Methods (Supplemental Fig. 1). Counts for (A–C) are shown relative to 1, where 1 is cell numbers of the 0-Gy sample count per subset per mouse per time point. (A) Two-Gray–irradiated CD8 T cells. (B) Four-Gray–irradiated CD8 T cells. (C) Two-Gray–irradiated CD4 T cells. (D) Four-Gray–irradiated CD4 T cells. Paired two-way ANOVA results of (A, B): time ***, subtype *; (C, D): time ***, subtype ns. Bonferroni posttests show between naive and T_EM subtypes. (E and F) In vivo dose response curves. Groups of six adult male C57BL/6 mice were subjected to whole-body irradiation at 0, 1, 2, or 4 Gy and then rested for 72 h. 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 naive versus EM from linear regression: (E, F) ***. All figures representative of at least two independent experiments. ***p < 0.001; **p < 0.01.
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 with other cell cycle phases (2), prompting us to examine the relative radiosensitivity of mitotic (Ki-67<sup>HI</sup>) counterparts in each subset. T<sub>N</sub> cells undergoing homeostatic division were more resistant than their interphase counterparts, while mitotically active T<sub>EM</sub> cells, by contrast, were less resistant than their interphase counterparts (Supplemental Fig. 2A–D). No differences were found between interphase and mitotic T<sub>CM</sub> cells (Supplemental Fig. 2E, 2F).

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

Because CD8 T<sub>CM</sub> 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 Abs against Bcl-2, Mcl-1, and Bcl-XL. Standing levels of antiapoptotic Bcl-2 family members did not correlate with survival trends between CD4 and CD8 T cells nor between subsets. For example, T<sub>CM</sub> cells expressed more Bcl-2 compared with T<sub>EM</sub> cells (Fig. 2A, 2B), whereas enhanced radioresistance was limited to T<sub>EM</sub> cells (Fig. 1). Mcl-1 and Bcl-XL expression also failed to correlate with survival (Fig. 2B, Supplemental Fig. 3A, 3B). 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 (Supplemental Fig. 2G–J). Proapoptotic 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 (Supplemental Fig. 3C–F).

Radioresistance correlates to early γH2AX marking

To evaluate whether DNA damage sensing is comparable between T cell subsets, we examined the increase in DSB marking by γH2AX in CD8 T cell subsets following irradiation. Maximal upregulation of γH2AX following irradiation over time was the highest in T<sub>N</sub> and lowest in CD8 T<sub>EM</sub> cells, which directly (and not inversely, as one might expect), correlated with radiosensitivity (Fig. 3A). We examined whether differing γH2AX expression after irradiation could be due in part to disparate native H2AX levels and found that CD8 T<sub>CM</sub> cells indeed contain more native H2AX histone available for phosphorylation (Fig. 3B). Surprisingly, we found that radiation dose-dependent γH2AX marking can take place even when cells were continuously maintained at ~4°C (on ice) both during and following irradiation, up to the time of sample fixation. This early γH2AX marking correlated with radiosensitivity of T<sub>EM</sub> (Fig. 3D). Moreover, T<sub>EM</sub> DSB marking was more robust in the first 5 min 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<sub>EM</sub> cells bind DSB faster than T<sub>CM</sub> cells

Given that T cells can sense DSB nearly instantaneously at 4°C in our hands and given that γH2AX marking takes place at or following DSB end binding by either PAR or Ku in many systems (26–28), we reasoned that the early γH2AX advantage may reflect more immediate DSB binding. To test whether T<sub>EM</sub> cells were indeed able to hold together DSBs earlier in the repair process, we used the TUNEL assay (29). The TUNEL assay detects DNA fragmentation because of 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 (Supplemental Fig. 3G). Note that the dose of 5 Gy (rather than doses of 2 and 4 Gy) was necessary to detect reproducible damage. By this assay, CD8 T<sub>CM</sub> cells showed more TUNEL activity from an identical amount of radiation compared with their counterparts (Fig. 3F), whereas T<sub>EM</sub> cells showed the least, implying that DSB were bound more efficiently in T<sub>EM</sub> than in T<sub>CM</sub> cells. Therefore, perhaps surprisingly, T<sub>EM</sub> cells were able to bind DSB ends even in the few seconds between irradiation and fixation, similar to T<sub>N</sub> but significantly better than T<sub>CM</sub>.

We also examined DSB formation by the comet assay, which has an established threshold for detecting radiation-induced DSB (Supplemental Fig. 3H) (30). 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<sub>EM</sub>, T<sub>CM</sub>-,
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 5-Gy–irradiated sorted CD8 T_CM cells again showed a greater proportion of unbound DSB compared with other subsets (Fig. 3G, see also Supplemental Fig. 3H–K). 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 DNA (34). We observed that genome-wide H3K27me3 content negatively correlated with survival across T cell subsets (Fig. 4A). To independently validate chromatin configuration patterns observed with H3K27me3, we subjected splenocytes to a DNase I hypersensitivity assay modified for flow cytometry. We used 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 with that from T_N and T_CM cells. This indicated that genome reorganization was an important factor in T cell subset radiosensitivity differences, we experimentally forced the genome open following irradiation and asked whether global genomic architecture is the dominant factor in 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. VPA is one of a number of HDACis that allow the genome to effectively assume an open configuration that is optimally suited for repair.

Treatment with VPA 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. VPA is one of a number of HDACis that allow the genome to effectively assume an open chromatin state (35). We confirmed that VPA treatment improved T_CM and T_N cell survival across all radiation doses, whereas TEM cell survival remained unchanged (Fig. 5A, Supplemental Fig. 4C–G). The enhanced survival with VPA treatment was reflected in T cell counts (Fig. 5B).
CD8EM, CD4CM, CD4N, and CD8N. The retention in TCM and T N p53 was significantly but only slightly increased by VPA treatment (Fig. 5D, 5E), suggesting that decreased p53 retention is not the sole mechanism explaining the lowered survival. We treated cells with 6 mM VPA and subjected them to heat shock, ST (Fig. 5C), or brefeldin A (Supplemental Fig. 4F), but VPA treatment did not affect survival of cells exposed to heat shock, brefeldin A, or ST treatment to induce apoptosis. As a positive control, we treated cells with ET to induce apoptosis in a radiation-independent, but DSB-dependent manner.

Because apoptosis following IR in lymphocytes is dependent on p53 (36), we examined p53 retention in these subsets. p53 is protected from degradation following genomic damage via phosphorylation of serine 15 by ataxia-telangiectasia mutated kinase (37). Fold change in p53–phospho-S15 was lowest in T EM following IR, correlating with increased survival. However, p53 retention in TCM and TN 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 TCM and TN survival.

**Discussion**

A fundamental understanding of peripheral immune subset radiation sensitivity is crucial for better understanding of the clinical impact of radiotherapy (38, 39), bone marrow transplantation (40), and immune reconstitution (41). Moreover, the discovery of factors responsible for differential lymphocyte radiation sensitivity has implications for diverse areas of immunology and biology: DNA damage response (42), natural and virus-induced hematopoietic cancers (43, 44), V(D)J recombination (45), cytotoxic killing (46), and possibly age-induced immune senescence (47), 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 (48). Previous studies have demonstrated that radiation-induced T cell apoptosis requires p53 (36). The constitutive Bcl-2 transgenic mouse model has established that antiapoptotic machinery can be protective, but the relevance of these findings for physiological radiosensitivity remains unclear (49). 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 transplantation, 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 (50). Treating large swaths of the population with from bone marrow transplantation 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 (51). Our work suggests that this may in part operate via a mechanism whereby lymphocyte attrition is lowered throughout the animal because of 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.

**FIGURE 4.** Standing chromatin content, and H3K27me3 downregulation following radiation, correlate with T cell subset radiosensitivity. (A) Standing H3K27me3 in T cell subsets versus relative survival of those same subsets post-4 Gy and 21 h incubation, averages from n = 5. From highest survival: CD4EM, CD8EM, CD4CM, CD4N, and CD8N. (B) CD8 H3K27me3 alterations following 4 Gy IR and 5 min incubation at 37°C. Two-way ANOVA: cell type: ****, radiation ***, (n = 4). (C) Samples as in (B), incubated for 1 h post-IR. Two-way ANOVA: Time: *** Cell subsets: *. Shown are results of Tukey’s posttests between subsets. (D) Representative histogram of CD8 H3K27me3 in TEm (solid line), TCM (dashed line), or TEM (gray). (E) DNase I hypersensitivity assay was performed in CD8 spleen samples by flow cytometry (n = 4), and results were expressed as percent decrease in PI MFI. Significance between the subsets was assessed using the Tukey posttests. (F) Representative histogram of PI staining in Ki-67 negative TEM cells either treated (solid line, left histogram) or untreated (dashed line, right histogram) with DNase I. All figures representative of at least two independent experiments. ***p < 0.001; **p < 0.01; *p < 0.05.
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 TCM cells exhibited lower survival rates following irradiation compared with their TEM counterparts while containing approximately twice as much H2AX. Maximal γH2AX signal over time also did not correlate with survival. Rather, the initial γH2AX 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 initiating at 4°C and within seconds of the insult. This work indicates that very early γH2AX fold change, and not maximal γH2AX response, more accurately predicts survival in T cell subsets. It also emphasizes the need for normalization of γH2AX to total amounts of H2AX when interpreting γH2AX 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 TCM and TEM subsets did not reach the levels seen in TEM cells in the absence (or the presence) of VPA treatment. Also, γH2AX response was not altered by VPA (Supplemental Fig. 4H). This implies that these DNA damage responses are not directly tuned by chromatin state in T cells. Because cells were gated on Ki-67 low (nondividing), this also suggests that competing factors in the nonhomologous end joining or alternative nonhomologous end joining pathway are able to abrogate apoptosis downstream of these key events in T cells.

Although 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 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 (Supplemental Fig. 3K), 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 Tn and TCM subsets. A recent study has shown increased radioresistance in CD4 T regulatory cells (Tregs) (52). Although 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. TCM and Tn are both responsible for clonal bursts of new effector T cells and are thus essentially keepers of genomic integrity for a given clone. TEM 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 TCM and Tn, whereas more efficient repair befits the physiological function of TEM.
HISTONE DEACETYLATION AND T CELL RADIOSENSITIVITY

Acknowledgments

We thank Drs. Giovanni Bosco (Dartmouth College), Ted Weinert, Kirsten Limesand, Felicia Goodrum (all from the University of Arizona), and the Goodrum laboratory. We also thank Paula Campbell at the University of Arizona Flow Core Facility and Doug Cromey at the University of Arizona Microscopy Core Facility. We give special thanks to Dr. Wendell Lutz (University of Arizona Cancer Center) for radiation dosage at the University of Arizona Microscopy Core Facility. We give special thanks to Dr. Wendell Lutz (University of Arizona) for help and advice.

Disclosures

The authors have no financial conflicts of interest.

References


Downloaded from http://www.jimmunol.org/ by guest on April 28, 2017