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Enhancement of T Cell Responses as a Result of Synergy between Lower Doses of Radiation and T Cell Stimulation

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As a side effect of cancer radiotherapy, immune cells receive varying doses of radiation. Whereas high doses of radiation (>10 Gy) can lead to lymphopenia, lower radiation doses (2–4 Gy) represent a valid treatment option in some hematological cancers, triggering clinically relevant immunological changes. Based on our earlier observations, we hypothesized that lower radiation doses have a direct positive effect on T cells. In this study, we show that 0.6–2.4 Gy radiation enhances proliferation and IFN-γ production of PBMC or purified T cells induced by stimulation via the TCR. Radiation with 1.2 Gy also lowered T cell activation threshold and broadened the Th1 cytokine profile. Although radiation alone did not activate T cells, when followed by TCR stimulation, ERK1/2 and Akt phosphorylation increased above that induced by stimulation alone. These changes were followed by an early increase in glucose uptake. Naive (CD45RA+) or memory (CD45RA−) T cells responses to stimulation were boosted at similar rates by radiation. Whereas increased Ag-specific cytotoxic activity of a CD8+ T cell line manifested in a 4-h assay (10–20% increase), highly significant (5- to 10-fold) differences in cytokine production were detected in 6-d Ag-stimulation assays of PBMC, probably as a net outcome of death of nonstimulated and enhanced response of Ag-stimulated T cells. T cells from patients receiving pelvic radiation (2.2–2.75 Gy) also displayed increased cytokine production when stimulated in vitro. We report in this study enhanced T cell function induced by synergistic radiation treatment, with potential physiological significance in a wide range of T cell responses. *The Journal of Immunology, 2014, 192: 3101–3110.

Radiation therapy (RT), a major form of cancer treatment, is currently undergoing a revolution mainly due to technical developments. RT has two well-known immunological links, as follows: one is the need for properly functioning host cellular immune system, especially CD8+ T cells, for maximum benefit from RT (1, 2); the second is the much discussed abscopal effect (recession of distant disease following local irradiation) that, in preclinical models (3, 4), was proved to be immune mediated. Generally, tumors are treated with a single high dose (8–20 Gy) or multiple fractions of ~2 Gy radiation, amounting to 60–70 Gy total dose. High-dose RT can provide tumor Ags from dying cells for dendritic cells that then cross-present them to CD8+ T cells. This immune mechanism contributes significantly to the control of tumor growth (5–7) and can be supported by decreasing or removing nonspecific immunosuppression in the irradiated host. Such synergy was demonstrated in a mouse glioblastoma model in which combining radiation with Ab-mediated blocking of programmed death–1 on T cells led to long-term survival (8).

In cancer therapy, 2 Gy radiation, given as a unit dose in hypofractionated localized treatment or as total body radiation, is considered a standard therapeutic dose. Total body radiation with 2 Gy or 2 × 2 Gy leads to a >80% response rate in indolent non-Hodgkin lymphoma, with local tumor control for >2 yr (9, 10). This radiation dose is expected to be less damaging for tumor cells than high doses, although treatment-related p53-mediated proliferation arrest and apoptosis of follicular lymphoma cells have been reported (11). Low-dose total body, but not localized, radiation of the tumor with 0.2 Gy induced T cell activation and decreased the incidence rate of metastasis in a preclinical tumor model, suggesting broad immune effects (12). Immune stimulation with a TLR-9 agonist, combined with 2 × 2 Gy radiation, improved clinical responses in a trial in a T cell–dependent manner in advanced lymphoma patients (13), demonstrating the role of immune involvement in low-dose radiation settings. These observations strongly suggest that the physiological effect of radiation is more complex than just DNA damage caused to tumor cells.

Whereas lymphocytes are inherently sensitive to high-dose radiation, the effect of lower doses, especially on human T cells, has received relatively little attention. Differential sensitivities, such as increased resistance to radiation by T regulatory (Treg) cells, have been reported (14, 15); however, the increased frequency was associated with compromised functionality (15). Positive effects on T cell function may be induced in a nonlinear manner by lower radiation doses, but information is scarce. Nogami et al. (16) observed enhanced TCR stimulation-induced T cell proliferation in mice receiving low-dose total body irradiation, but no mechanistic study was conducted. We have found increased IFN-γ release by human T cells to viral recall peptides in PBMC irradiated in vitro with 0.6–2.4 Gy (17), an observation that formed the basis of the hypothesis for the work presented in this study. We hypothesized that lower radiation doses directly affect T cells and influence their responses to subsequent Ag challenge. In this study, we provide detailed characterization...
of T cell functional changes to TCR-mediated stimulation following 0.6–2.4 Gy radiation and reveal some of the mechanisms behind the effect. We show that radiation causes dose-dependent death of T cells but does not activate T cells. However, when radiation is followed by stimulation, it leads to enhanced T cell responses in both the CD4+ and CD8+ subsets. The 1.2 Gy radiation and T cell activation together resulted in elevated levels of ERK1/2 and Akt phosphorylation compared with that induced by TCR stimulation alone. These signaling pathways are known to cooperate in the regulation of T cell activation and metabolism (18, 19). They facilitate activation-induced switch to glycolysis, which helps T cells to meet the increased energy and biosynthetic precursor needs of proliferation and cytokine production. Indeed, radiation was found to facilitate an earlier switch to glucose uptake by irradiated T cells following stimulation, compared with that of T cells receiving stimulation only. Our findings provide evidence that lower doses of radiation directly support increased T cell function via heightened TCR-mediated signaling and altered metabolic status of the irradiated T cells. This novel advainent effect of radiation may have clinical relevance not only in tumor treatment, but it also can affect the behavior of virus-specific or autoimmune T cells.

Materials and Methods

Donors

Venous blood (20–40 ml) was collected from healthy donors and from nine prostate cancer patients, the latter participating in a local Phase II Clinical Trial of pelvic RT, as described (17). Blood was taken immediately before (RT0) and 24 h after (RT1) a single fraction of localized RT (2.75 Gy to the prostate and 2.2 Gy to the pelvis). Human CMV (HCMV) serology was carried out using ELISA (GenWay Biotech, San Diego, CA).

Tissue culture media and reagents

RPMI 1640 (Lonza, Basel, Switzerland) was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Lonza), 2 mM t-glutamine (Life Technologies), 25 mM HEPES buffer, Na-pyruvate (Sigma-Aldrich, St. Louis, MO), and 10% FBS (PAA, Pasching, Austria). Inhibitors of MEK1/2 (UO126), and ERK1/2 (FR180204) (Calbiochem, San Diego, CA) were dissolved in DMSO and added to target cells at 25 mM at the beginning of overnight incubation after irradiation (0–4.8 Gy) and CD3/CD28 cross-linking. Cell death was determined by flow cytometry after labeling cells with 35 nM 3H-labeled 2-deoxy-D-glucose (DG) (Perkin-Elmer) was added per well in a 96-well U-bottom tray. Cells were transferred into tubes (BD Biosciences); washed twice with 1 ml ice-cold serum-free and glucose-free RPMI 1640. Pellets were resuspended in 25 μM at the beginning of overnight incubation after irradiation (0–4.8 Gy) and CD3/CD28 cross-linking.

Statistics

Statistical analysis was carried out using Student t test, paired t test, one-way ANOVA with Tukey’s post hoc test, and Wilcoxon matched-pairs signed-ranks test (GraphPad InStat 3.06). Significance levels are *p < 0.05, **p < 0.01, ***p < 0.001.

T cell responses enhanced by lower doses of radiation

Cells were sorted into CD45RA+ and CD45RA− subsets using MACS separation, or HCMV immediate early-1 (IE1) peptides VLEETSVML (HLA-A2) and QIKVRDVMV (HLA-B8) (>90% purity; ProImmune, Oxford, U.K.) (22). Briefly, unsorted PBMC or separated CD3+ cells were pulsed with 0.2–20 μg/ml peptide. In the latter, CD3+ cells were added at 4:1 ratio to CD3− cells. Intracellular cytokine staining (ICCS) for IFN-γ production was carried out following overnight stimulation, or the cultures were incubated for 6 h and restimulated with peptide-pulsed or unpulsed autologous BLCL overnight for ICCS by flow cytometry, as described previously (17).

Cell counting; flow cytometry

Cells were counted with the Guava EasyCyte8 cytometer (Millipore), according to the manufacturer’s instructions, or with trypan blue exclusion. Cell death was determined by flow cytometry after labeling cells with 35 nM 3H-labeled 2-deoxy-D-glucose (DG) (Invitrogen), which accumulates in the mitochondria of cells and is released during apoptosis, and 5 μl propidium iodide to 1–2 × 105 cells/ml for 15 min. Cell proliferation was determined by labeling cells with 0.5 μM CFSE and measuring cell division–related CFSE dilution. ICCS was carried out, as described (17). Briefly, GolgiPlug and GolgiStop (BD Biosciences) (1 and 0.67 μM/ml, respectively) were added to the stimulated cells 1 h after stimulation, and the cells were cultured overnight, unless otherwise indicated. Cells were fixed, permeabilized (fixation and permeabilizing reagents; eBioscience, San Diego, CA), and labeled with fluoroconjugated anti-CD3, CD4, CD8, IFN-γ, TNF-α, and IL-2–specific Abs (eBioscience) at room temperature for 30 min. Phosphorylation of ERK1/2 (Thr202/Tyr204), Akt (T308), and mTOR (S2448) was detected by flow cytometry using phosphoprotein-specific or isotype control Abs (BD Biosciences). For phosphoflow analysis, the cells were fixed with 2% paraformaldehyde, permeabilized with ice-cold 80% methanol for 30 min at 4°C, and incubated with Ab for 1 h at room temperature. Cytokine bead array was carried out with the Th1/Th2 bead array (BD Biosciences), according to the manufacturer’s instructions. Cell surface staining was carried out on ice with CD3, CD27, and CD28 Abs (eBioscience). A FACSCanto II flow cytometer with FACSDiva software (BD Biosciences) was used for data collection and analysis.

T cell cytotoxicity assay

Autologous BLCL served as targets; they were labeled with 51Cr for 1 h and pulsed with 50 μg/ml peptide or 5 μl/ml DMSO for 1 h. A total of 3 × 104 BLCL was mixed with 3 × 105 T cells (10:1 E:T ratio) in 200 μl/well in triplicates. Medium or 5% Triton X-100 added to target cells without T cells served as background and maximum lysis, respectively. Four hours later, 50 μl supernatant was removed and liquid scintillation was carried out on a Perkin-Elmer Microbeta scintillation counter. Specific lysis was calculated as described (21).

Phosphokinase array

Phosphokinase activation was measured in 5 × 106 T cells per group 60 min following radiation and/or stimulation using the Human Phosphokinase Ab Array (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions. T cell groups studied were as follows: 1) untreated; 2) irradiated with 1.2 Gy; 3) stimulated with CD3/CD28 Ab-coated beads; or 4) treated with 2 and 3 together. Pixel densities of the scanned blots were quantitated using GENE-E software (The Broad Institute, Cambridge, MA).

Glucose uptake and its inhibition

Glucose uptake was measured by a modified method of Kin et al. (23). A total of 2 × 105 purified T cells was stimulated in complete RPMI 1640 with CD3/CD28 beads (2:1 ratio) or left unstimulated for 24 or 48 h, respectively, in 200 μl/well in a 96-well U-bottom tray. Cells were transferred into tubes (BD Biosciences); washed twice with 1 ml serum-free, glucose-free RPMI 1640 (Life Technologies); and incubated in this media for 30 min at 37°C. A total of 1 μCi 3H-labeled 2-deoxy-D-glucose (DG) (Perkin-Elmer) was added per tube, followed by incubation for 30 min at 37°C. Supernatants were removed, and the cell pellets were washed in 1 ml ice-cold serum-free and glucose-free RPMI 1640. Pellets were resuspended in 200 μl permeabilization reagent (eBiosciences). Radioactivity was measured by liquid scintillation.

For inhibition of glucose uptake, DG (Sigma-Aldrich) was dissolved in water and added to T cells at 25 μM at the beginning of overnight incubation after irradiation (0–4.8 Gy) and CD3/CD28 cross-linking.
Results

Lower radiation doses affect T cells directly, resulting in enhanced T cell responses

We have previously reported that stimulation of PBMC with recall viral peptides (CEF) yielded elevated frequencies of responding T cells following 2 Gy radiation of PBMC (17). To assess whether radiation affected T cells directly or via APCs in the PBMC, CD3+ and CD3− cells were isolated from healthy donor blood and the two groups of cells were treated with increasing doses of radiation separately. CD3+ cells were loaded with CEF peptides and mixed with CD3+ cells. We observed a radiation-induced increase in the frequencies of T cells responding to CEF peptides when only the CD3+ cells were irradiated with 0.6–2.4 Gy (Fig. 1A, black bars). On the contrary, T cell responses were not enhanced when only CD3− cells, serving as APCs, were irradiated (Fig. 1A, first group of bars, black versus shaded bars). In fact, at certain radiation doses, when both T cells and CD3+ cells were irradiated, T cell responses were lower compared with that following T cell irradiation alone. The increased frequencies of IFN-γ-producing T cells cannot be explained by selective elimination of nonresponding cells from the cultures, as the absolute number of responding T cells also significantly increased in the irradiated groups (Fig. 1B). Furthermore, both CD8+ and CD4+ T cell subsets followed the same pattern of functional enhancement upon radiation (data not shown). The likely explanation behind the increase in the absolute numbers of IFN-γ-producing T cell is the fact that T cell proliferation is also enhanced by radiation. This has been confirmed by stimulating irradiated T cells with CD3/CD28 Ab-coated beads that cross-link the TCR and also provide a costimulatory signal. Irradiated T cells displayed significantly increased cumulative proliferation profile over 4 d as measured by CFSE dilution (Fig. 1C). Furthermore, enhancement of cytotoxic function of the RLAR CD8+ T cell line, representing differentiated effector T cells, was also enhanced by 0.6–2.4 Gy radiation in an Ag-specific manner in a standard 4-h 51Cr assay (Fig. 1D). These experiments confirm that lower doses of radiation affect T cells directly, whereas they also demonstrate that the impact of radiation can be detected in long- and short-term T cell assays alike.

Net gain in T cell numbers following radiation and stimulation

As indicated earlier, although radiation with 0.6–2.4 Gy enhances T cell proliferation and function, it is also likely to cause T cell death. T cell apoptosis was assessed in an experiment identical to the proliferation assay shown in Fig. 1C. Apoptosis rate, as expected, increased with radiation dose and was higher in the CD3/CD28 bead-stimulated compared with unstimulated cultures (Fig. 2A). Although the results represent a snapshot of apoptotic events at a given time point, the proliferation assay measures all proliferation that happened in 4 d. To understand the net combined outcome, the absolute numbers of T cells were counted in these cultures (Fig. 2B). Whereas the numbers of unstimulated T cells decreased in a radiation dose-dependent manner (Fig. 2B, black bars), the number of cells in the stimulated and irradiated cultures (Fig. 2B, gray bars) remained stable and even displayed a small gain that, however, did not reach statistical significance. The results indicate that radiation-induced cell loss is compensated by enhanced proliferation of stimulated T cells at lower doses of radiation.

Similar proliferation and net cell number profiles by different T cell subsets following irradiation

Although we have demonstrated that 2 Gy in vitro radiation has a preferential negative effect on CD45RA+ T cells (17), the results on Fig. 2C versus Fig. 2D indicate that it is only true if resting T cells are the recipients of the radiation (black bars: T cells without stimulation). When T cells were stimulated by TCR cross-linking, CD45RA− and CD45RA+ cells displayed no radiation-related preferential loss of CD45RA+ cells (Fig. 2C versus Fig. 2D, gray bars). Both subsets displayed stable cell numbers as a net outcome of stimulation and radiation, with small increases at 1.2 and 2.4 Gy radiation. Furthermore, the same pattern of proliferation of the two subsets was also observed in a CFSE assay (Fig. 2E). These results confirm the observation shown in Fig. 1B that irradiated T cells, receiving stimulation, react with an enhanced functional response that is not due to preferential elimination of less differentiated T cells. To demonstrate that it is also not due to removal of immunosuppression represented by the Treg subset, we determined the frequency and the absolute numbers of CD25high Foxp3+CD4+ T cells in this experiment (Fig. 2F). No significant changes were found due to irradiation, further confirming that the observed functional gain is a direct effect of radiation on T cells.
Radiation alone or in combination with T cell activation triggers different signaling pathways

As differences in early T cell signaling may shed light on some of the cellular events following radiation, we studied signaling in T cells, purified from PBMC and divided into four groups, receiving 1.2 Gy or no radiation alone or in combination with CD3/CD28 cross-linking. The phosphokinase array we used detects relative levels of phosphorylation at 46 kinase phosphorylation sites. Radiation alone resulted in increased phosphorylation of cell cycle control proteins (CREB, p38, p53) and kinases associated with protein synthesis and metabolism, such as p70S6K, mTOR, and AMPK2α (Fig. 3A; IR). Increased phosphorylation in the irradiated and stimulated group of T cells (ionizing radiation [IR] + TCR) compared with stimulated T cells (TCR) was observed for Akt, ERK1/2, p27, phospholipase Cγ, Chk-2, p53, and paxillin (first block of heat map) and p70S6 kinases (fourth block, proteins 26–27). Activation of some of these proteins indicates DNA damage-induced cell cycle control (Chk-2, p53, p27), whereas others are involved in enhanced TCR-mediated signaling (Akt, ERK1/2, phospholipase Cγ). Levels of phosphorylation and the changes are also depicted in a column graph for the key proteins to indicate the magnitude of changes arising at these phosphorylation sites (Fig. 3B). The results of the phosphokinase array enabled us to select Akt, ERK1/2, and mTOR as potentially important kinases for further studies. Phosphoflow experiments shown on Figs. 3C and 4A confirm that Akt and ERK1/2 phosphorylation was significantly higher in the TCR plus IR group compared with the TCR group. mTOR phosphorylation was equally high in both groups.

Enhanced ERK1/2 phosphorylation in irradiated T cells following stimulation

The ERK1/2 pathway has the ability of fine-tuning TCR-mediated T cell activation either by lowering the activation threshold, as in rheumatoid arthritis (24), or causing T cell dysfunction when ERK signaling is suppressed, as demonstrated in tumor-infiltrating lymphocytes (25). In the phosphoflow experiment, shown in Fig. 4A, T cells were irradiated with 1.2 Gy and then stimulated with cognate peptide. Increased ERK1/2 phosphorylation was detected in irradiated T cells after peptide stimulation compared with peptide stimulation alone. To study whether irradiated T cells remain susceptible, T cell stimulation was carried out either together with irradiation or 22 h later. ERK1/2 phosphorylation was measured 10 min later. Radiation-mediated significant enhancement of phosphorylation was observed in both groups even at low peptide concentrations (20 ng/ml). IFN-γ production by T cells following simultaneous or delayed (16-h) stimulation with CD3/CD28 cross-linking (Fig. 4B) confirmed that irradiation, even when Ag stimulation was delayed, still promoted increased IFN-γ production—data drawing a parallel between the phosphorylation results and functional outcome.

To define whether there was a causal relationship between enhanced activation of the ERK1/2 pathway and elevated T cell responses to cognate peptide stimulation, T cells isolated from PBMC were pretreated with UO126, a MEK inhibitor blocking ERK1/2 phosphorylation, or FR180204, a downstream inhibitor blocking ERK-RSK-p70S6K activation (26), for 1 h before irradiation. The inhibitors were not removed during Ag stimulation, which was carried out with CD3+ cells pulsed with a HCMV-IE1 peptide. Increased T cell death was not detected due to treatment with the inhibitors. Ag-specific IFN-γ-producing T cell frequencies were measured 6 d later by ICCS (Fig. 4C). This model was chosen for assessing the consequences of blocking the ERK1/2 pathway as it reflects the effects of radiation on Ag-specific immune responses from complex T cell populations over time. Complete inhibition of T cell activation was observed with UO126 (10 μM), whereas FR180204 (10 μM) abrogated the advantage gained by radiation, confirming the role of pERK1/2 in radiation-induced functional enhancement.

Following activation, there is a greater increase of glucose uptake by irradiated than nonirradiated T cells

T cells require ATP and metabolic precursors for proliferation. Switching to glycolysis ensures that these demands are rapidly met.

![FIGURE 2](http://www.jimmunol.org/)
As the phosphokinase array indicated that in irradiated and activated T cells several key players of the glycolytic metabolic pathway (mTOR, p70S6K, ERK, Akt) are highly activated, experiments were carried out to determine glucose uptake by T cells as an indicator of
metabolic changes. Growing T cells following irradiation and
stimulation in glucose-containing media and changing it to isotope-
labeled DG-containing media allowed us to assess the intensity of
glucose uptake in the cells. As DG cannot undergo glycolysis, it
becomes trapped in the cells and the amount of isotope-labeled DG
can be compared between groups. As early as 12 h after CD3/CD28
cross-linking, both irradiated (1.2 Gy) and nonirradiated T cells
displayed elevated DG uptake, but it was significantly higher in
irradiated T cells (Fig. 4D), and it also remained significantly higher
at later time points (24 and 48 h). Furthermore, when T cells were
stimulated with CD3/CD28 cross-linking and DG was added to the
cultures from the beginning of stimulation, IFN-γ production 12 h
after stimulation was significantly inhibited in irradiated T cells
by DG at any dose of radiation studied compared with that in
nonirradiated T cells (Fig. 4E). These results confirm that T cell
function is associated with elevated glucose uptake in irradiated
cells, indicating an accelerated switch to glycolysis in these T cells.

Radiation lowers T cell activation threshold and broadens Th1
cytokine responses

The ERK1/2 signaling experiments indicated that T cells that receive
radiation may respond better to lower doses of cognate peptide. To
test it in a functional assay, PBMC received 0 or 1.8 Gy radiation and
were pulsed with increasing concentrations of CEF peptides (0, 0.2,
or 2 μg/ml). IFN-γ production was measured by flow cytometry in
T cells. A response was determined as positive when cytokine re-
sponse was 2-fold above that by unstimulated T cells; that is, the
stimulation index (SI) was >2. In the cultures without radiation,
positive T cell responses were observed only with 2 μg/ml peptide stimulation for both CD8+ and CD4+ T cells (Fig. 5A, 5B; 3.9-fold and 3.0-fold above background). Frequencies of IFN-γ-producing T cells in the irradiated group were 29.6-fold or 16-fold higher than background for CD8+ and CD4+ T cells, respectively. The IFN-γ response also became positive in the irradiated group of T cells when stimulated with 10-fold less peptide (0.2 μg/ml) (6.9- and 2.9-fold, respectively). Furthermore, upon irradiation, a broader Th1 cytokine production was observed, as T cells produced not only IFN-γ but also IL-2 and TNF-α. Some of the irradiated T cells produced a combination of two (IL-2 + IFN-γ or IFN-γ + TNF-α) or all three cytokines at ≥2-fold above background; this was not observed in the absence of radiation (Fig. 5C).

Enhancement of T cell responses by radiation is Ag specific

The experiment above (Fig. 5A, 5B) indicates that the effect of 1.8 Gy radiation does not cause background T cell stimulation (DMSO control). To study more systematically the potential of radiation to generate nonspecific T cell responses, two HLA-A2+ healthy donors with known HCMV serostatus were selected and T cell stimulation was carried out with a HCMV-IE1 peptide. Irradiation of PBMC from the HCMV-seropositive donor significantly increased the frequency of the responding CD8+ T cells (Fig. 5D) compared with that without irradiation. The highest response (5.7-fold above that by nonirradiated T cells) was observed with 2.4 Gy radiation. Frequencies of IFN-γ-producing T cells without peptide stimulation remained low after radiation. Radiation and peptide stimulation together did not generate IE1 peptide-specific T cell responses from the seronegative donor, demonstrating that the radiation-induced enhancement of T cell function to a cognate Ag is Ag specific.

T cells from cancer patients after single pelvic radiation treatment display enhanced ERK1/2 phosphorylation and cytokine production

Patients receiving one fraction of pelvic radiation donated blood before and 24 h after radiation treatment. Unseparated PBMC were treated with CD3/CD28 cross-linking; cytokine production and ERK1/2 phosphorylation levels were assessed. Stimulated PBMC produced more TNF-α and IFN-γ from five of six patients after radiation treatment than before, the difference in TNF-α production reaching significance (p = 0.0479) (Fig. 6A, 6B). TCR cross-linking also resulted in enhanced phosphorylation of ERK1/2 after radiation therapy (Fig. 6C). ERK1/2 phosphorylation decreased significantly only in one of nine patients and was unchanged or significantly upregulated in eight of nine patients (p = 0.0412 in the eight patients). The observed changes were not due to T cell subset alterations, as lymphocyte ratios remained stable in PBMC (data not shown) and the ratios of CD3+ cells in the lymphocyte gate and the frequencies of CD27+CD28+ T cells remained unchanged (Fig. 6D, 6E). These results demonstrate that the side effect of cancer radiotherapy on T cells is not negligible and, under the right circumstances, it may act as an adjuvant, boosting T cell function.

Discussion

The beneficial immunological consequences of high-dose cancer radiotherapy, such as tumor Ag cross-presentation from dying tumor cells or increased susceptibility of irradiated tumor cells to CTL killing, are well documented (27, 28). Whereas high-dose radiation is detrimental for lymphocytes, the impact of low-dose radiation on T cells, whether this occurs in a localized treatment or during low-dose whole-body irradiation, has not been addressed in a systematic way. In this study, we demonstrate that 0.6–2.4 Gy radiation enhances T cell function by increasing T cell proliferation and the ratios of CD3+ cells in the lymphocyte subset alterations, as lymphocyte ratios remained stable in PBMC significantly only in one of nine patients and was unchanged or radiation therapy (Fig. 6C). ERK1/2 phosphorylation decreased linking also resulted in enhanced phosphorylation of ERK1/2 after radiation treatment than before, the difference in TNF-α production reaching significance (p = 0.0479) (Fig. 6A, 6B). TCR cross-linking also resulted in enhanced phosphorylation of ERK1/2 after radiation therapy (Fig. 6C). ERK1/2 phosphorylation decreased significantly only in one of nine patients and was unchanged or significantly upregulated in eight of nine patients (p = 0.0412 in the eight patients). The observed changes were not due to T cell subset alterations, as lymphocyte ratios remained stable in PBMC (data not shown) and the ratios of CD3+ cells in the lymphocyte gate and the frequencies of CD27+CD28+ T cells remained unchanged (Fig. 6D, 6E). These results demonstrate that the side effect of cancer radiotherapy on T cells is not negligible and, under the right circumstances, it may act as an adjuvant, boosting T cell function.

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and Ag-specific cytokine production, while lowering the T cell activation threshold to cognate peptide stimulation. It is a direct effect on T cells, not influenced by the radiation treatment of CD3+ T cells serving as APCs. Our findings identify radiation-induced direct changes in T cell function at doses relevant in the treatment of hematological and other malignancies.

The question as to whether lower doses of radiation enhance T cell function directly or via enhancing Ag presentation was studied by irradiating separated CD3+ and CD3- cells. The effect of radiation on Ag presentation is expected to be dose dependent, with high doses being damaging. Our results partly correlate with a previous observation that radiation, in a single fraction of ≤2 Gy, does not affect Ag presentation (29), confirming that any positive effect of radiation on T cells is a direct effect. We used T cell lines as well as primary T cells purified from PBMC to study direct radiation effects. T cells were stimulated with recall peptides in either an Ag-specific manner or by TCR cross-linking in an Ag-nonspecific manner by CD3/CD28 Ab-coated beads. Regardless of the type of T cells or stimulation used, we were able to demonstrate the basic phenomenon of low-dose radiation-induced enhanced function in all experimental setups. We also revealed some unexpected effects of lower radiation doses on T cell function, such as decreased activation threshold to cognate peptide and the emergence of poly-functional T cell responses—these effects have not been reported before. There are several cellular and molecular changes that may account for these findings. Irradiation may remove nonactivated T cells or those with low-affinity TCR to cognate peptide via apoptosis in longer assays. The enhanced function may thus reflect enrichment of higher affinity T cells, similar to that described in a kinetic model with transgenic T cells (30). However, the enhanced cytotoxic activity detected in a 4-h assay and cytokine production in a 12-h assay point toward a direct augmentation of T cell effector function by radiation.

The molecular mechanism behind the functional effects was studied by detecting changes in TCR-related signaling. Upon stimulation of T cells via the TCR, initial tyrosine phosphorylation is followed by activation of serine/threonine kinases such as PI3K-Akt, MAPK-ERK1/2, and AMPK. The timing of 1 h selected in this study may not be optimal for all kinases included in the array; nevertheless, the results represent a snapshot of activation comparative between the T cell groups. As heterogeneous populations of primary T cells were tested, the phosphorylation data represent an overall picture, whereas differences between subsets may exist. The 1.2 Gy radiation alone activated not only cell-cycle regulatory proteins (p27, CREB, p38, and p53) but also kinases involved in metabolic changes (mTOR, p70S6K, and AMPK). Radiation itself can activate mTOR as a survival pathway in tumor cells (31–33), but such an effect has not been demonstrated in T cells. AMPK activation by radiation in resting T cells is also a novel observation, correlating with reported AMPK phosphorylation in irradiated tumor cells (34, 35). Simultaneous radiation treatment and TCR stimulation resulted in the consistent and significant activation of ERK1/2 and Akt above the level observed by TCR stimulation alone. These changes are likely to contribute to the enhanced T cell function observed in short- and long-term functional assays. The results of stimulation of irradiated T cells with decreasing doses of cognate peptide correlate with those of Singh et al. (24), who demonstrated that augmented ERK1/2 activity increases T cell reactivity even to suboptimal Ag stimulation. Inhibition of the ERK1/2 pathway by UO126, which inhibits MEK-mediated ERK1/2 phosphorylation, completely abolished T cell function, whereas downstream inhibition with the FL180204 inhibitor only removed the radiation-associated functional gain. The results confirm the involvement of the ERK1/2 pathway in the regulation of irradiated T cell function at multiple levels—this correlates with observations on ERK1/2 controlling T cell activation via a switch-like mechanism (36). An enhanced downstream effect of ERK is the phosphorylation of paxillin, a scaffolding molecule in T cells. Paxillin is phosphorylated in the irradiated and stimulated T cell group above that observed with either radiation or TCR.

**FIGURE 6.** In vivo radiation enhances T cell TNF-α production. PBMC of prostate carcinoma patients before (RT0) and 24 h after (RT1) standard pelvic irradiation were stimulated with CD3/CD28 cross-linking. (A) TNF-α and (B) IFN-γ production were measured by cytokine bead array 24 h later (n = 6), whereas (C) ERK phosphorylation was measured by phosphoflow flow cytometry 20 min after treatment (n = 9). (D and E) PBMC characteristics from the same patients as in (A, B) (n = 6) before in vitro treatment for parameters, as indicated on the y-axes. The lines represent individual patients; the short horizontal lines indicate the means. Statistical differences between treatment groups were calculated using Wilcoxon matched-pairs signed-ranks test. *p < 0.05.
stimulation alone. Paxillin activation has been implicated in the cytotoxic capability of T cells by regulating the polarization of cytotoxic granules (37). It is a likely contributor to the observed enhanced cytotoxic activity of irradiated T cells. For Akt activation, phosphorylation of the T308 residue was higher than with TCR stimulation alone. Upon TCR and costimulation, T cells switch from catabolic to anabolic processes, such as glycolysis, generating not only ATP but also biosynthetic precursors. Glycolysis is associated with the increased uptake of glucose and glutamine triggered by Akt activation and regulated by the mTOR pathway (18, 19, 26, 38). In our study, irradiation alone did not increase glucose uptake; however, protein synthesis and ATP production may have increased in these T cells. This would agree with findings that synthesis of some proteins, such as survival- and DNA repair-related proteins, is activated by radiation in nontransformed cells (39). However, in the absence of CD3/CD28 activation, irradiated T cells are unlikely to be able to switch to glycolysis.

We confirmed that the results obtained in vitro are also valid when radiation is delivered in vivo. T cells from prostate cancer patients who received a single low-dose pelvic radiation displayed increased ERK1/2 phosphorylation and IFN-γ and TNF-α production in the majority of patients (TNF-α being significant) 24 h after radiation compared with T cells before radiation.

Although the immunological benefits of low clinical doses of RT (0.5, 2, and 5 Gy) are currently being investigated in clinical trials for the treatment of colorectal and pancreatic cancers (40), the data presented in this paper suggest that more benefit may be achieved if radiation and T cell stimulation are combined. Our findings are related to the cellular target of the proliferation-augmenting effect of chronic low-dose ionizing radiation in mice. Radiat. Res. 139: 47–52.


