

Effects of Gamma Radiation on Fc ϵ RI and TLR-Mediated Mast Cell Activation¹

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Ionizing gamma radiation has several therapeutic indications including bone marrow transplantation and tumor ablation. Among immune cells, susceptibility of lymphocytes to gamma radiation is well known. However, there is little information on the effects of gamma radiation on mast cells, which are important in both innate and acquired immunity. Previous studies have suggested that mast cells may release histamine in response to high doses of gamma radiation, whereas other reports suggest that mast cells are relatively radioresistant. No strong link has been established between gamma radiation and its effect on mast cell survival and activation. We examined both human and murine mast cell survival and activation, including mechanisms related to innate and acquired immune responses following gamma radiation. Data revealed that human and murine mast cells were resistant to gamma radiation-induced cytotoxicity and, importantly, that irradiation did not directly induce β -hexosaminidase release. Instead, a transient attenuation of IgE-mediated β -hexosaminidase release and cytokine production was observed which appeared to be the result of reactive oxygen species formation after irradiation. Mast cells retained the ability to phagocytose *Escherichia coli* particles and respond to TLR ligands as measured by cytokine production after irradiation. In vivo, there was no decrease in mast cell numbers in skin of irradiated mice. Additionally, mast cells retained the ability to respond to Ag in vivo as measured by passive cutaneous anaphylaxis in mice after irradiation. Mast cells are thus resistant to the cytotoxic effects and alterations in function after irradiation and, despite a transient inhibition, ultimately respond to innate and acquired immune activation signals. *The Journal of Immunology*, 2007, 179: 3276–3286.

Tissue mast cells, which have been traditionally studied in the context of allergic inflammation, are now documented to play a role in both innate and acquired immunity. Similarly, they appear to contribute to tissue repair and maintenance of homeostasis (1). Because of these many functions and in a time where gamma radiation is used both therapeutically and as a biological threat, it is of interest to begin to understand how mast cells respond to gamma radiation. Although reports are few, it is known that although lymphocytes are sensitive to radiation-induced cytotoxicity, mast cells appear to be relatively resistant (2–4). In apparent contrast, since the 1960s, mast cells have been studied for a possible contribution to acute radiation syndrome (ARS)⁴ which occurs when a large surface area of the body is exposed to high doses of ionizing radiation (5–9). These historical reports have suggested that histamine, possibly from mast cells, is elevated in ARS contributing to decreased cerebral blood flow and resulting in

incapacitation within 30 min of ionizing radiation exposure (10). In addition, several studies measured serum histamine levels in mice exposed to high doses of gamma radiation, but no link between radiation, mast cells, and histamine release was ever clearly established (11, 12). Furthermore, when gamma radiation treatment has been used for pain management in patients with mastocytosis, there has been no evidence of mast cell mediator release (13, 14).

Despite the controversy of gamma radiation-induced mast cell activation and survival, there are no systematic studies on the effects of gamma radiation on mast cell survival, degranulation and cytokine secretion. To explore these questions, we monitored mast cell survival after gamma radiation both in vitro and in vivo. We similarly monitored mediator release after gamma radiation exposure and determined the ability of mast cells to be activated by IgE-dependent mechanisms both in vitro and in vivo. Finally, we determined whether mast cells could be induced to secrete cytokines in vitro after gamma radiation exposure and stimulation with TLR ligands. As will be shown, these studies revealed that mast cells are resistant to gamma radiation-induced cytotoxicity and, despite an initial transient inhibition, preserve their acquired and innate immune functions following irradiation.

Materials and Methods

Cell culture

Mouse bone marrow-derived mast cells (BMMC) were cultured from femoral marrow cells of C57BL/6 mice (The Jackson Laboratory). Cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 25 mM HEPES, 1.0 mM sodium pyruvate, nonessential amino acids (BioSource International), 0.0035% 2-ME, and 300 ng/ml recombinant mouse IL-3 (PeproTech). BMDCs were used after 4–6 wk of culture. Human-derived mast cells (HuMC) were cultured as described (15, 16). In brief, peripheral blood CD34⁺ progenitor cells were collected from healthy donors after informed consent and affinity column apheresis. CD34⁺ cells were cultured in StemPro-34 SFM (Invitrogen Life Technologies) in the presence of rIL-3 (first week only), rIL-6 and recombinant human stem cell factor (Peprotech). HuMC cultures were maintained up to 10 wk at 37°C and 5% CO₂.

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⁴ Abbreviations used in this paper: ARS, acute radiation syndrome; BMMC, bone marrow-derived mast cell; HuMC, human-derived mast cell; HSA, human serum albumin; poly(I:C), polyinosinic-polycytidylic acid; ROS, reactive oxygen species; DCF, dichlorofluorescein; PCA, passive cutaneous anaphylaxis; LDH, lactate dehydrogenase; BMT, bone marrow transplantation.

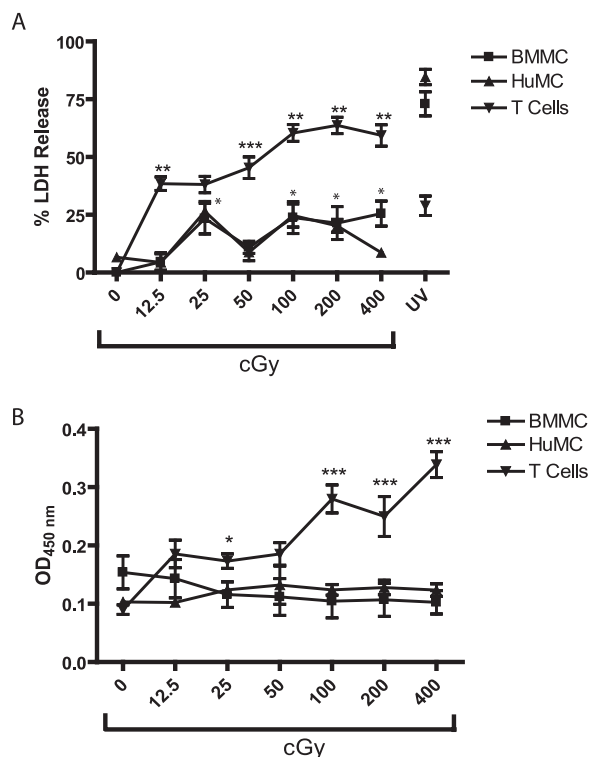


FIGURE 1. BMMC and HuMC are resistant to gamma radiation-induced cytotoxicity and apoptosis. Cytotoxicity and apoptosis were determined by examining release of lactate dehydrogenase and DNA fragmentation respectively. *A*, HuMCs, BMMCs, and T cells were exposed to a range of doses of gamma radiation or UV gamma radiation (302 nm wavelength, 5 min of exposure) followed by measurement of LDH release 72 h postirradiation. *B*, Using a TUNEL assay to determine induction of apoptosis, HuMCs, BMMCs, and T cells were exposed to a 12.5- to 400-cGy dose of gamma radiation, and DNA fragmentation was measured 72 h postirradiation. Data represent the average $\pm$ SEM of three independent experiments performed in triplicate. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

One half of the cytokine-supplemented medium was replaced weekly. T cells were prepared from single-cell suspensions obtained from spleens of C57BL/6 mice. Following lysis of mouse erythrocytes, T cells were isolated using mouse CD3⁺ T cell enrichment columns (R&D Systems).

Irradiation of cells

Murine and human mast cells were grown in culture as above and then prepared as described for each assay. Cells were irradiated in suspension in either a 96-well plate or a small culture flask using an Eldorado 8 ⁶⁰Co teletherapy unit (Theratronics International, formerly Atomic Energy of Canada) at dose rates between 200 and 220 cGy/min. Cells were irradiated to a total dose ranging from 2 to 5000 cGy as called for in the experimental design for each experiment. Decay corrections were done monthly, and full electron equilibrium was ensured for all irradiations. The radiation doses used in these experiments were chosen to cover a wide range which included very low doses (2 cGy) to large doses (400 cGy) which is approximately one-half of the LD₅₀ for humans to very high doses (5000 cGy) which is uniformly lethal to humans. This range includes the dose typical of a daily radiation treatment (200 cGy) in a radiation oncology clinic.

Cell viability and apoptosis

Cell viability was determined by trypan blue staining and lactate dehydrogenase (LDH) release (17). The percentage of LDH release was measured in supernatants of BMMCs or freshly isolated splenic T cells 24–72 h after gamma radiation with doses from 12.5 to 400 cGy or 5 min of exposure to UV (302 nm; BioVision). Untreated cells were used as a negative control and 1% Triton X-100 (Sigma-Aldrich)-treated BMMCs were used as a positive control, representing 100% LDH release. Apoptosis was deter-

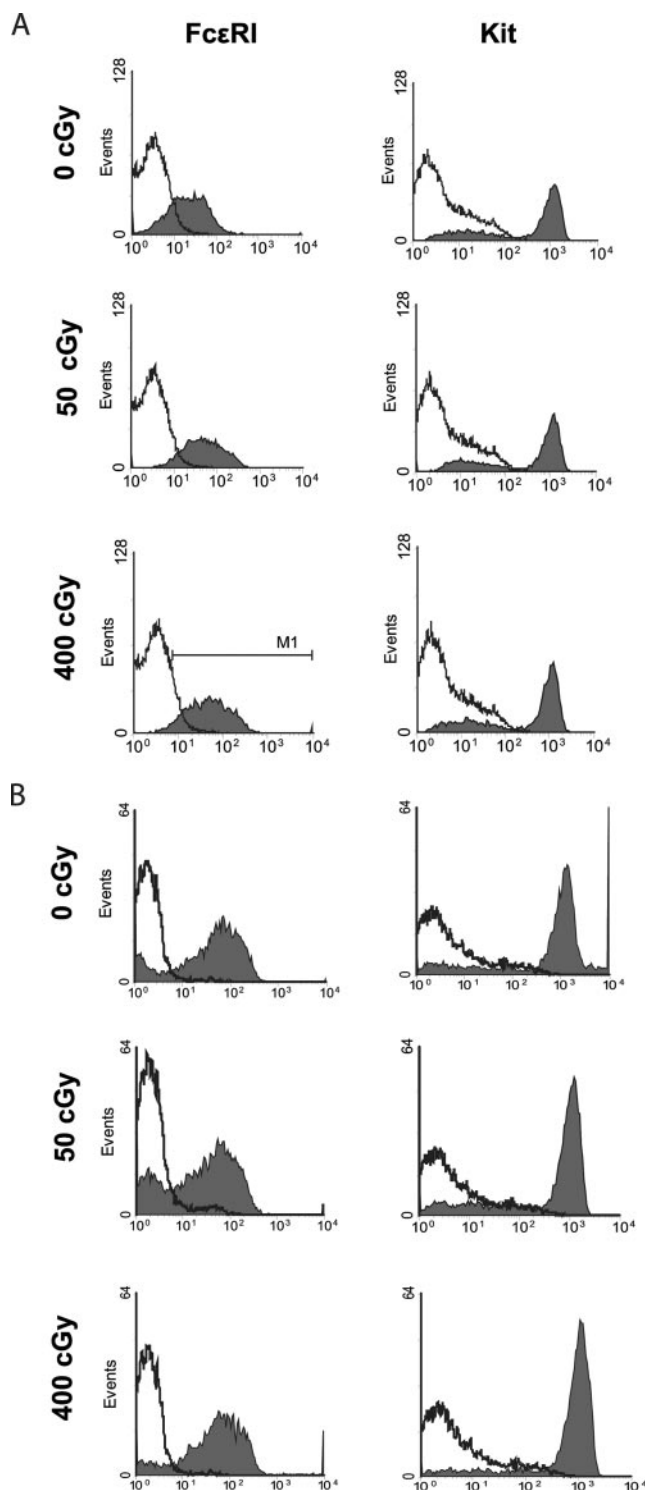


FIGURE 2. IgE and Kit expression are not altered by exposure to gamma radiation. FcεRI and Kit expression by BMMCs were measured at 30 min (*A*) or 24 h (*B*) postirradiation at doses of 0, 50, and 400 cGy. Values are representative examples of three separate experiments.

mined by DNA fragmentation using a TiterTacs TUNEL assay (R&D Systems; Ref. 18). Equal numbers of cells (1×10^5 /well) were plated in a 96-well plate and then exposed to a 12.5- to 400-cGy dose of gamma radiation. DNA fragmentation was measured 24–72 h after irradiation. Nontreated cells constituted the negative control, and a DNA nuclease-generated sample served as a positive control. Experiments were repeated three times. The reported values are mean OD values from each treatment.

Table I. Mast cell mediator release after ionizing gamma irradiation

	Mediator Release		<i>p</i>
	0 cGy	400 cGy	
BMMC			
β -Hexosaminidase ^a	4.2 $\pm$ 1.4%	3.9 $\pm$ 1.2% ^b	0.047
TNF- α ^c	33.0 $\pm$ 5.8 pg/ml	52.1 $\pm$ 6.2 pg/ml	0.04
IL-6 ^c	882.2 $\pm$ 64.8 pg/ml	1027 $\pm$ 86.8 pg/ml	0.199
IL-13 ^c	157.2 $\pm$ 36.6 pg/ml	176.0 $\pm$ 29.6 pg/ml	0.69
HuMC			
IL-8 ^c	26.3 $\pm$ 7.6 pg/ml	14.2 $\pm$ 7.3 pg/ml	0.234
GM-CSF ^c	7.0 $\pm$ 0.2 pg/ml	4.7 $\pm$ 0.7 pg/ml	0.037

^a Represents 30 min postirradiation.^b Also performed out to 5000-cGy dose, resulting in 5.5 $\pm$ 1.0% β -hexosaminidase release.^c Represents 24 h postirradiation.

Degranulation and cytokine release

BMMCs or HuMCs were seeded at 5×10^4 cells/well in 96-well flat-bottom plates. BMMCs were sensitized with 100 ng/ml mouse IgE anti-DNP (Sigma-Aldrich) for 24 h for degranulation experiments (19). HuMCs were sensitized overnight with 100 ng/ml biotinylated IgE (Sigma-Aldrich). BMMCs and HuMCs were irradiated to 12.5–400 cGy between 30 min and 24 h before the addition of 100 ng/ml DNP-human serum albumin (HAS; Sigma-Aldrich) or 100 ng/ml streptavidin (Sigma-Aldrich), respectively. Thirty min (at 37°C) after addition of DNP-HSA or streptavidin, *p*-nitrophenyl-*N*-acetyl- β -D-glucopyranoside was added to cell supernatants and lysates for 90 min as a chromogenic substrate for *N*-acetyl- β -D-hexosaminidase (Sigma-Aldrich; Ref. 19). The reaction was stopped with 0.2 M glycine. OD was measured at 405 nm using a GENios ELISA plate reader (ReTirSoft). β -Hexosaminidase release was expressed as the percentage of total cell content after subtracting background release from unstimulated cells. Finally, the effect of free radical scavengers on β -hexosaminidase release was tested by adding Trolox (3–300 μ M), cysteine (0.1–10 μ M), or tempol (0.1–10 μ M) to the culture medium 1 h before irradiation. The cells were washed, the medium was changed immediately before irradiation, and β -hexosaminidase release was measured as above. Cytokines were measured in cell culture supernatants of BMMCs or HuMCs seeded at 2×10^5 cells/well 24 h after gamma radiation to doses of 12.5–400 cGy. Additionally, cytokine production in BMMCs or HuMCs sensitized with 100 ng/ml IgE anti-DNP or 100 ng/ml biotinylated IgE, respectively (Sigma-Aldrich) was measured after addition of 100 ng/ml DNP-HSA or 100 ng/ml streptavidin (Sigma-Aldrich) at 30 min and 24 h postirradiation. DNP-HSA or streptavidin was added for a total of 8 h before supernatant collection for cytokine analysis. Additionally, cytokines were measured in supernatants of BMMCs exposed to radiation doses of 12.5–400 cGy with the addition of LPS (100 ng/ml; Alexis Biochemicals), Pam3Cys (1 μ g/ml; Alexis Biochemicals) or polyinosinic-polycytidylic acid (poly(I:C); 10 μ g/ml; Amersham Biosciences) 30 min postirradiation. Supernatants were collected 24 h after addition of LPS, Pam3Cys, or poly(I:C). Mouse TNF- α , IL-6, IL-13, and human GM-CSF and IL-8 were measured using DuoSet ELISA Development Systems (R&D Systems). IFN- α was measured using a mouse IFN ELISA kit (R&D Systems). Phagocytosis of fluorescent *E. coli* particles was determined using a Vybrant Phagocytosis Assay Kit (Molecular Probes). Briefly, BMMCs were seeded at 10,000 cells/well in a 96-well plate before addition of fluorescent *E. coli* particles. Thirty min after addition of particles, BMMCs were washed, and trypan blue was added to quench fluorescence of nonphagocytosed particles. Plates were read at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Net fluorescence was determined after subtraction of the negative control value, and fluorescence intensity was correlated to particle number according to manufacturer. In addition, a murine macrophage cell line (J774; American Type Culture Collection) was used as a positive control.

Detection of reactive oxygen species (ROS)

ROS were measured in a 96-well plate assay using the fluorescent probe dichlorofluorescein (DCF) (20). BMMCs (1×10^6 /ml) were incubated with DCF diacetate (20 μ M) in cell culture medium for 15 min at 4°C with rotation. Cells were then washed in HEPES buffer (10 mM HEPES supplemented with 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na₂HPO₄·7H₂O, 5.6 mM glucose, 1.8 mM CaCl₂·2H₂O, 1.3 mM MgSO₄·7H₂O; 10 ml) and seeded at 400,000 per well in a black opaque 96-well microplate. After gamma radiation to doses of 50 and 400 cGy, DCF fluorescence was then

monitored for 15 min using a GENios fluorescent plate reader (ReTirSoft) set at an excitation wavelength of 492 nm and emission wavelength of 535 nm. Fluorescence was expressed as relative fluorescent units. The kinetic data were collected using an XFlour4 macro within Microsoft Excel.

Flow cytometry

BMMCs were examined for expression of Fc ϵ RI and Kit (CD117) by flow cytometry 30 min and 24 h after 50- and 400-cGy doses of gamma radiation. Before irradiation, BMMCs were sensitized for 2 h at 37°C with mouse monoclonal IgE-anti DNP (Sigma-Aldrich) at 100 ng/ml in RPMI medium. After irradiation, BMMCs were washed twice with PBS-BSA and stained with DNP-FITC. Additionally, BMMCs were stained with PE anti-mouse CD117 Ab (BD Pharmingen) at 4°C for 30 min. Data were obtained on a FACScan flow cytometer (BD Biosciences) and analyzed with WinMDI 1.2 software (The Scripps Research Institute).

Animal irradiation

Female C57BL/6 mice were obtained from The Jackson Laboratory. The mice were ~20 wk of age at the time of study. All experiments were conducted under the aegis of a protocol approved by the National Cancer Institute Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council. Total body irradiation was accomplished by placing the mice in a round Lucite holder which allowed the animals to move. The Lucite holder was placed on a rotating platform to ensure exposure of the entire mouse to the radiation beam. This was confirmed by pre-experiment dosimetry measurements and calculations. A total of 2000 cGy were delivered to each mouse in equal doses during a 10-day period using an Eldorado 8 ⁶⁰Co teletherapy unit (Theratronics International; formerly Atomic Energy of Canada) at a dose rate of 11.6 cGy/min. Isolated head gamma radiation was accomplished by placing each individual mouse in a specially built Lucite jig so that the animal could be immobilized without the use of anesthetics. A Lucite cone was fitted to the jig to prevent head movement during the radiation exposure and lead shields ensured that only the head was irradiated. Single gamma radiation doses of 400 cGy were administered using a Therapax DXT300 x-ray irradiator (Pantak) at a dose rate of 190 cGy/min.

Detection of mast cells in skin of irradiated mice

C57BL/6 mice were irradiated as described above. On days 0, 1, 3, and 10 of irradiation, four animals were euthanized per day, and a skin sample was collected. Samples were fixed in fresh Carnoy's fixative, embedded in paraffin, and mounted on glass slides and then stained using eosin and toluidine blue (American Histolabs). Data represent the average number of mast cells from six random areas per skin section from each of the four mice per time point.

Passive cutaneous anaphylaxis (PCA)

The effects of gamma radiation on IgE-mediated mast cell degranulation were determined in vivo using the PCA reaction. C57BL/6 mice (16 wk old; *n* = 6 mice per group) received intradermal injections of 1 μ g of mouse monoclonal IgE anti-DNP in 25 μ l of PBS in the left ear and 25 μ l of PBS in the right ear as a control. After 24 h, the mice were irradiated as described above, and PCA was determined 30 min and 24 h postirradiation. After gamma radiation exposure, the mice were challenged with Ag by i.v. injection of 0.5 mg/ml DNP-HSA and 0.5% Evans blue in 100 μ l of saline into the tail vein. After inhalation of isoflurane, the mice were euthanized by CO₂

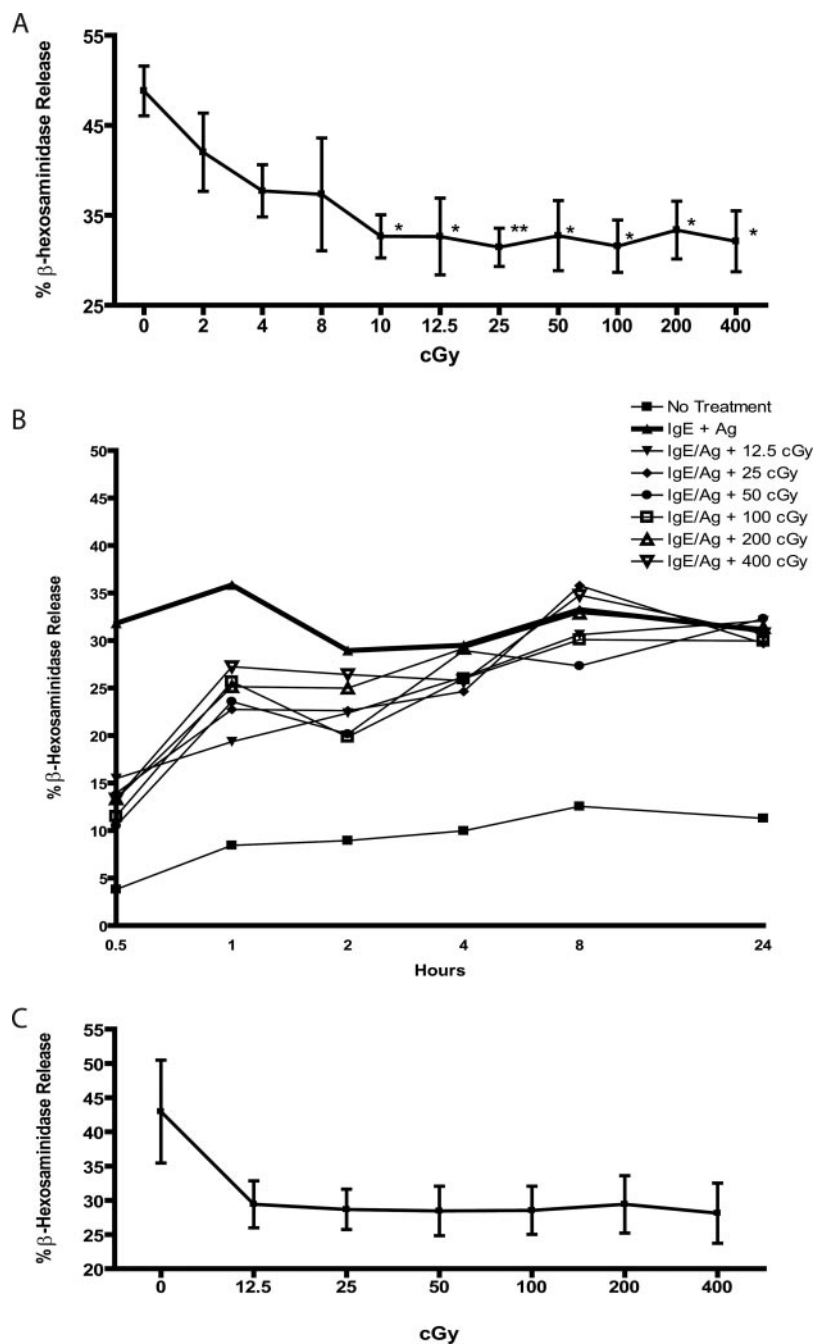


FIGURE 3. Irradiation of mast cells transiently attenuates IgE-mediated β -hexosaminidase release in vitro. *A*, IgE-mediated β -hexosaminidase release by BMMCs was measured 30 min after irradiation at doses ranging from 2 to 400 cGy. *B*, IgE-mediated β -hexosaminidase release by BMMCs after gamma radiation to doses of 12.5–400 cGy was examined during a time course of 30 min–24 h. *C*, IgE-mediated β -hexosaminidase release by HuMCs 30 min postirradiation at doses of 12.5–400 cGy. Each experiment was performed in triplicate. *A* and *C*, average $\pm$ SEM of three independent experiments performed in triplicate. *B*, representative example of three separate experiments. *, $p \leq 0.05$; **, $p \leq 0.01$.

asphyxiation 30 min after injection of Ag, and the ears were removed and incubated in 200 μ l of formamide at 55°C for 24 h. Extravasation of Evans blue was quantitated by spectrophotometric analysis at 620 nm. Sample concentration was determined by comparison to a standard curve of Evans blue. The net microgram amount of Evans blue was determined by subtraction of the amount of Evans blue in the IgE-treated ear minus the PBS-treated ear.

Statistics

Statistical analysis used the software package PRISM, version 4 (Graph-Pad). Differences between untreated and radiation-treated samples were assessed using one-way ANOVA with the Bonferroni posttest. The area under the curve was calculated for ROS measurements using PRISM. All values are reported as the mean $\pm$ SEM.

Results

Mast cells are resistant to radiation-induced cytotoxicity

Ionizing gamma radiation is commonly used as a method of decreasing lymphocyte numbers via cytotoxicity for the purpose of

bone marrow transplantation (BMT) and, to a lesser degree, for inflammatory conditions such as rheumatoid arthritis (21–24). To compare the relative sensitivity of mast cells with that of T cells after gamma radiation, we followed the release of LDH and measured DNA fragmentation as markers of cytotoxicity and apoptosis, respectively. As determined by LDH release 72 h postirradiation, both HuMCs and BMMCs exhibited minimal radiation-induced cytotoxicity compared with mouse T cells (Fig. 1A). Comparable LDH release was not detected from mast cells until a dose of 3200 cGy (data not shown). However, in contrast to exposure to gamma radiation, UV irradiation of HuMCs and BMMCs did result in significant LDH release as reported (Fig. 1A). In addition to LDH release, DNA fragmentation, as measured by an in vitro TUNEL assay, was not significantly elevated 72-h after gamma radiation in HuMCs and BMMCs as compared with nonirradiated cells (Fig. 1B). However, DNA fragmentation in mouse T cells exposed to

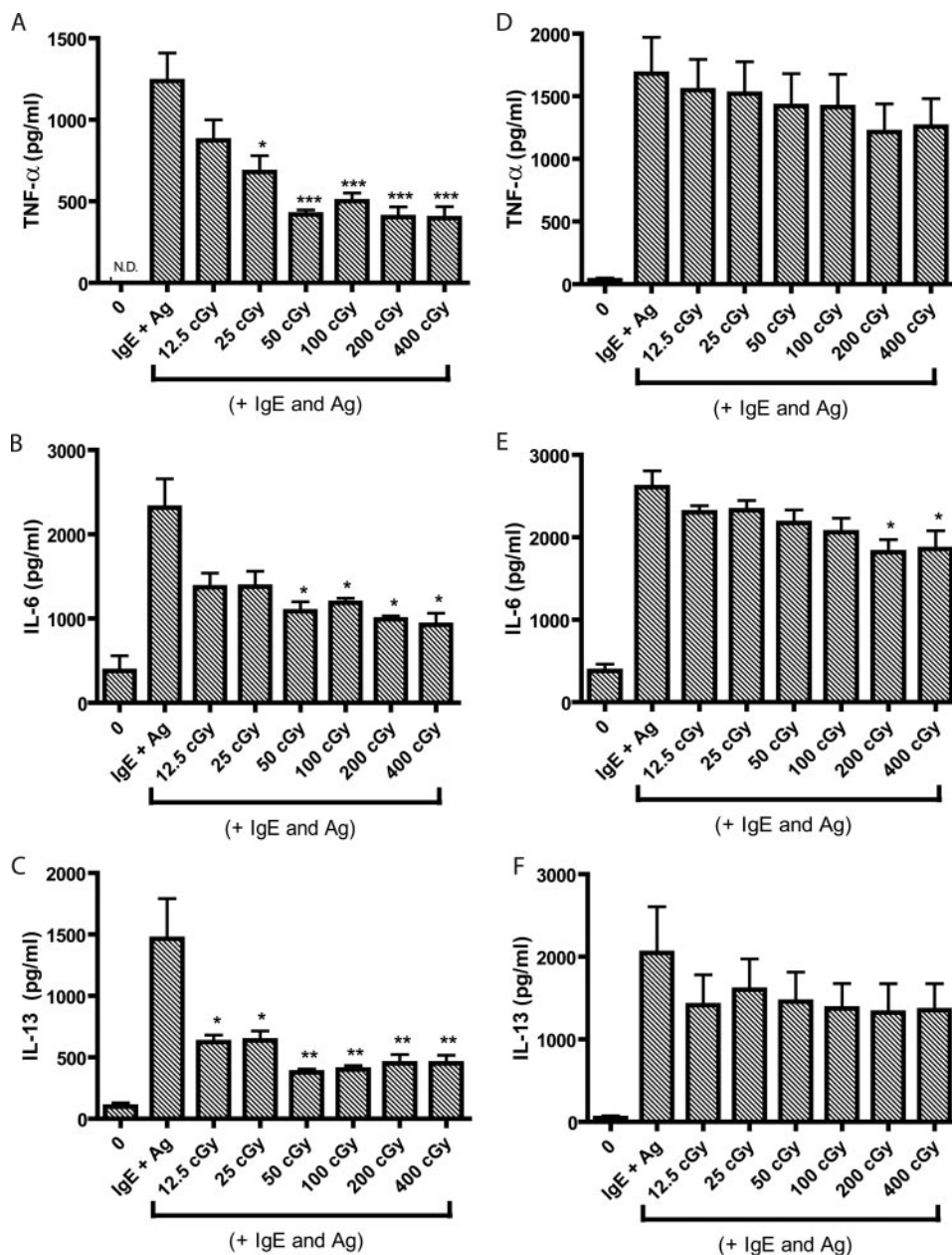


FIGURE 4. Irradiation of BMMCs attenuates IgE-mediated cytokine release. Thirty minutes after gamma radiation to doses of 12.5–400 cGy, BMMC were activated via FcεRI cross-linking with Ag and production of TNF-α (A), IL-6 (B), and IL-13 (C) were measured 8 h after addition of Ag. Similarly, IgE-mediated production of TNF-α (D), IL-6 (E), and IL-13 (F) were examined 24 postirradiation. Values are the average $\pm$ SEM of three independent experiments (where bone marrow was cultured from three separate mice) each performed in triplicate. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. N.D., Not detected.

gamma radiation was significantly elevated compared with nonirradiated T cells. These observations demonstrate that although some mast cell cytotoxicity occurred after irradiation, 1) there was no correlation with radiation dose, and 2) the degree of cytotoxicity was significantly less than that seen in T cells. Although the LDH assay suggested the presence of some cytotoxicity, the TUNEL assay did not reveal a significant change from unirradiated cells. Because of this, the amount of cytotoxicity was thought to be minimal and clearly demonstrates that mast cells are relatively resistant to gamma radiation-induced cytotoxicity when compared with lymphocytes.

Radiation does not alter FcεRI or Kit expression and does not directly induce mast cell degranulation

It has been suggested that some symptoms associated with ARS, such as decreased cerebral blood flow, are related to the release of histamine from mast cells, although data are lacking to substantiate this (10). Therefore, a series of experiments was initiated to assess the ability of gamma radiation exposure to directly induce degran-

ulation of BMMCs and HuMCs. First, the expression levels of FcεRI and Kit (CD117) were examined 30 min and 24 h postirradiation. As shown in Fig. 2A, gamma radiation did not alter the expression of FcεRI or Kit (CD117) at either time point. Exposure to gamma radiation ranging from 12.5 to 5000 cGy did not directly induce HuMCs or BMMCs to degranulate as measured by release of β -hexosaminidase 30 min after irradiation (Table I). Additionally, 24 h after exposure, gamma radiation by itself minimally induced production of TNF-α, whereas IL-6 and IL-13 production from BMMCs was not altered. Also, gamma radiation did not induce IL-8 and minimal GM-CSF production was detected from HuMCs.

Radiation transiently inhibits FcεRI-mediated mast cell degranulation and cytokine production

As reported, gamma radiation did not directly induce mast cell degranulation or cytokine production. We thus determined whether gamma radiation would alter FcεRI-mediated degranulation. BMMCs (sensitized with IgE anti-DNP) were radiated and then

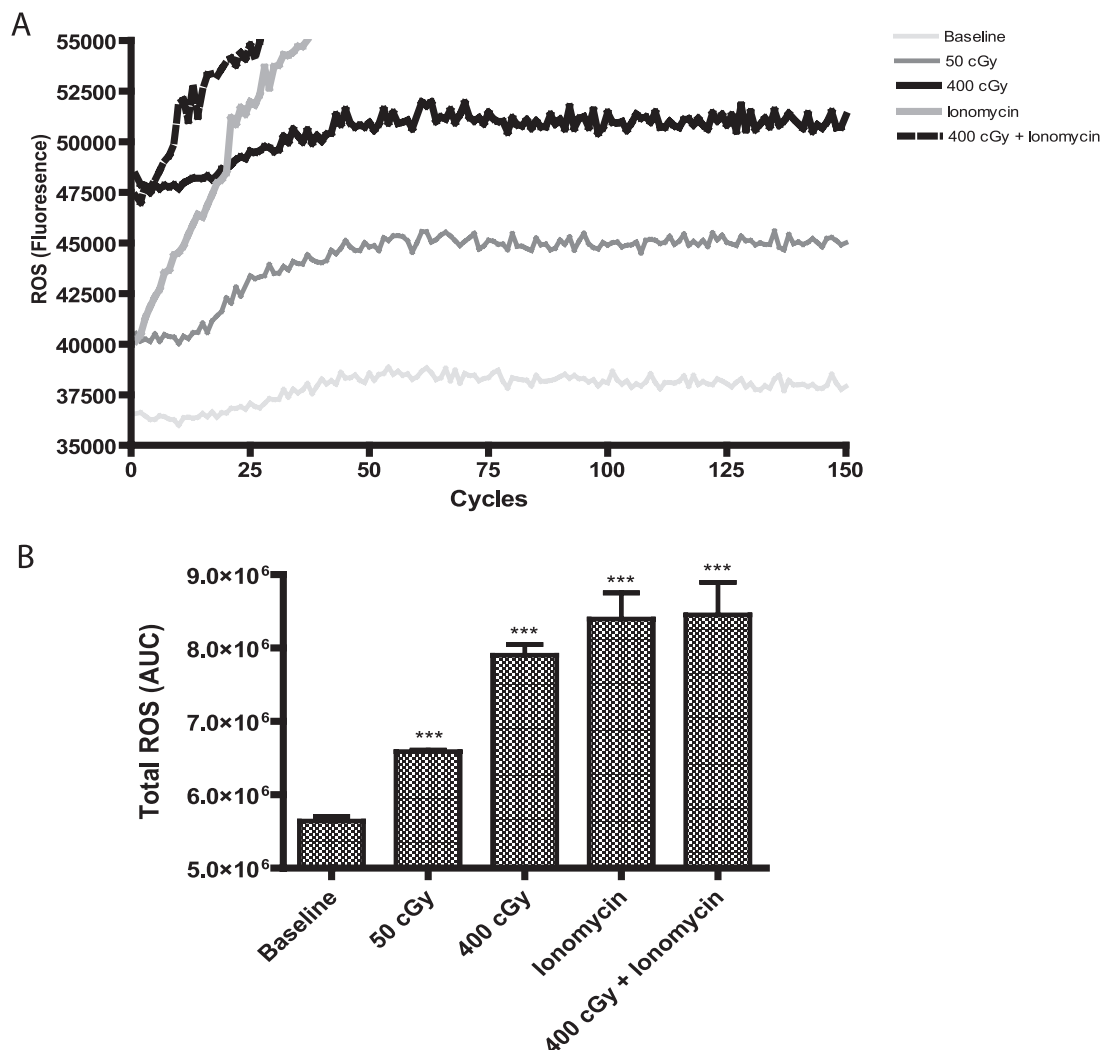


FIGURE 5. Ionizing gamma radiation induces ROS production in BMMC. *A*, ROS production in BMMC exposed to 50 and 400 cGy of gamma radiation. BMMC were loaded with the fluorescent probe DCF, and intracellular ROS was measured immediately after exposure of BMMC to gamma radiation. *B*, Area under the curve (AUC) representation of total ROS production in irradiated BMMCs as compared with nonirradiated cells. Ionomycin was used to generate a positive control. Values are the average $\pm$ SEM of three independent experiments performed in triplicate. $p \leq 0.001$.

activated by addition of DNP-HSA between 30 min and 24 h postradiation. A radiation dose-dependent decrease in Fc ϵ RI-mediated β -hexosaminidase release from BMMCs was observed within 30 min of exposure to gamma radiation (Fig. 3*A*). The inhibition of degranulation after radiation exposure was transient and recovered within 4 h (Fig. 3*B*). A similar effect is seen in HuMCs, with Fc ϵ RI-mediated degranulation reduced within 30 min after irradiation (Fig. 3*C*) and recovery observed within 24 h (data not shown).

In addition to degranulation, Fc ϵ RI-mediated stimulation of mast cells induces production of several cytokines. Irradiated BMMCs were sensitized with IgE and stimulated with Ag for 8 h to induce cytokine production. As demonstrated with β -hexosaminidase release, when Fc ϵ RI stimulation was initiated 30 min postirradiation, TNF- α , IL-6, and IL-13 production was transiently inhibited (Fig. 4, *A–C*). However, when Fc ϵ RI stimulation was initiated 24 h postirradiation, cytokine production had recovered to levels seen in nonirradiated BMMCs (Fig. 4, *D–F*).

Radiation induces ROS production which mediates transient inhibition of mast cell degranulation

The production of ROS has been implicated in mediating many of the effects observed after gamma radiation exposure (25–32). The

production of ROS by BMMCs was measured after irradiation to 50 or 400 cGy (Fig. 5*A*). Measurement of ROS production was initiated within 1 min after gamma radiation exposure but had already reached a plateau level that was dependent on the dose of gamma radiation. Fig. 5*B* shows that total ROS production, measured as area under the curve, immediately following 400 cGy irradiation of BMMCs was significantly increased as compared with nonirradiated cells.

The role of ROS in the transient inhibition of the degranulation response in irradiated BMMCs was examined using trolox, a vitamin E analog that scavenges peroxy radicals; cysteine, a potent radioprotector which uses a sulfhydryl group to scavenge free radicals; and tempol, a nitroxide free radical that acts as a superoxide dismutase mimic. Fig. 6 demonstrates protection against gamma radiation-induced inhibition of IgE-mediated degranulation in BMMCs with trolox and cysteine 30 min postirradiation. The apparent inhibition of degranulation by 10 μ M cysteine resulted from cytotoxicity at that dose (see Fig. 6 legend). Tempol did not protect against the attenuation of degranulation, which may be due to free radical activity of the compound itself. These results demonstrate that gamma radiation induces ROS production in BMMC, which can be inhibited with several antioxidants. Further, by inhibiting

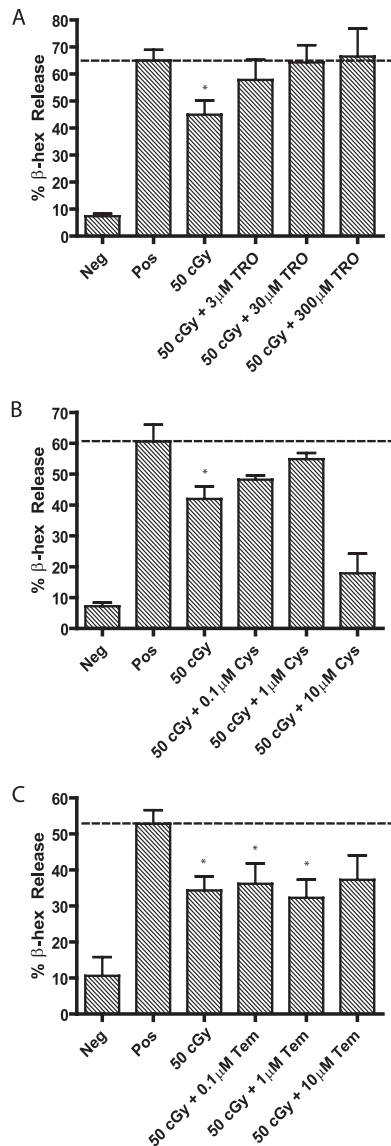


FIGURE 6. Free radical scavengers prevent attenuation of degranulation in irradiated BMMC. The effect of three free radical scavengers on BMMC degranulation was tested. Trolox (TRO; A), cysteine (B), and Tempol (Tem; C) were added to BMMCs at several concentrations to BMMCs before irradiation to a dose of 50 cGy to block gamma radiation-induced ROS production. IgE-mediated release of β -hexosaminidase was then measured 30 min after irradiation. Under all conditions, cell viability was assessed by trypan blue staining, and at the end of each experiment it was >95% with the exception of 10 μ M cysteine where viability dropped to <50%. Values are the average $\pm$ SEM of three independent experiments (where bone marrow was cultured from three separate mice) each performed in triplicate. *, $p \leq 0.05$. Pos, Positive; Neg, negative.

gamma radiation-induced ROS production, the transient inhibition of mast cell degranulation seen within 30 min after irradiation is prevented.

TLR-mediated cytokine production and phagocytosis is not inhibited in mast cells by exposure to gamma radiation

Mast cells are involved in the response to pathogens through recognition of bacterial and viral components via TLRs (33–38). The ability of BMMCs to respond to TLR ligands was examined after irradiation. In contrast to observations of IgE-mediated degranulation in BMMCs, this innate immune function of mast cells was not impaired after gamma radiation exposure ranging from 12.5 to

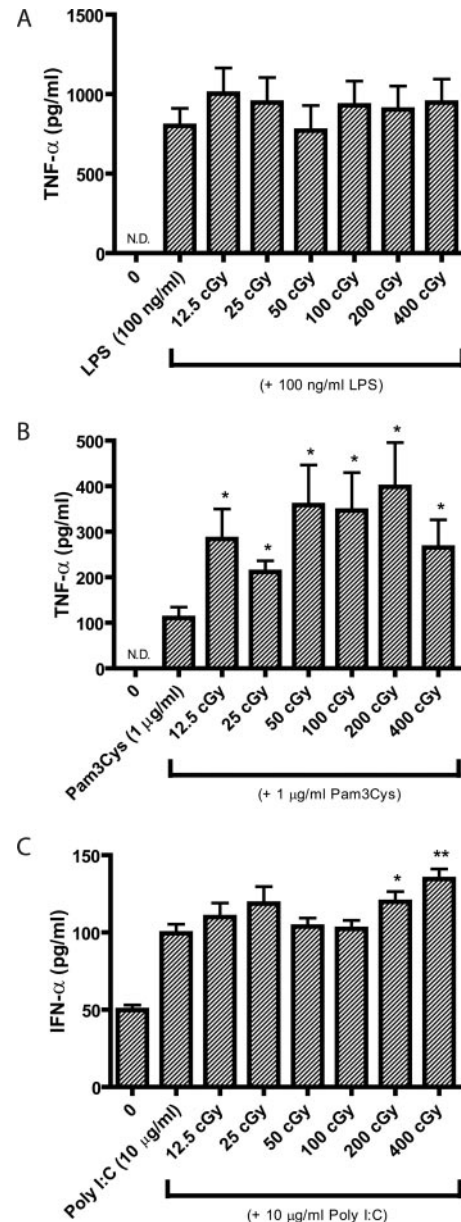


FIGURE 7. TLR-mediated cytokine release is not inhibited by exposure to gamma radiation. TLR-mediated cytokine production by BMMC was examined after exposure to gamma radiation. LPS-induced TNF- α (A), Pam3Cys-induced TNF- α (B), and poly(I:C)-mediated IFN- α production (C) were measured 30 min after irradiation at doses of 12.5–400 cGy. Values are the average $\pm$ SEM of three independent experiments (where bone marrow was cultured from three separate mice) each performed in triplicate. *, $p \leq 0.05$; **, $p \leq 0.01$.

400 cGy. As shown in Fig. 7, within 30 min of irradiation, a time at which mast cell IgE-mediated degranulation was impaired, BMMCs were able to recognize and respond to the TLR ligands LPS, Pam3Cys, and poly(I:C) as measured by the production of cytokines. Although LPS-mediated production of TNF- α remained at normal levels after irradiation, Pam3Cys- and poly(I:C)-mediated TNF- α and IFN- α production was actually enhanced after radiation exposure (Fig. 7, B and C). Additionally, phagocytosis of fluorescent *E. coli* particles by BMMCs was not altered after exposure to gamma radiation 30 min postirradiation (0 cGy = 16.55 ± 1.63 particles/cell vs 400 cGy = 16.14 ± 1.88 particles/cell; $p = 0.87$) or 24 h postirradiation (0 cGy = 16.55 ± 1.63 particles/cell vs 400 cGy = 16.53 ± 3.99 particles/cell; $p = 0.99$).

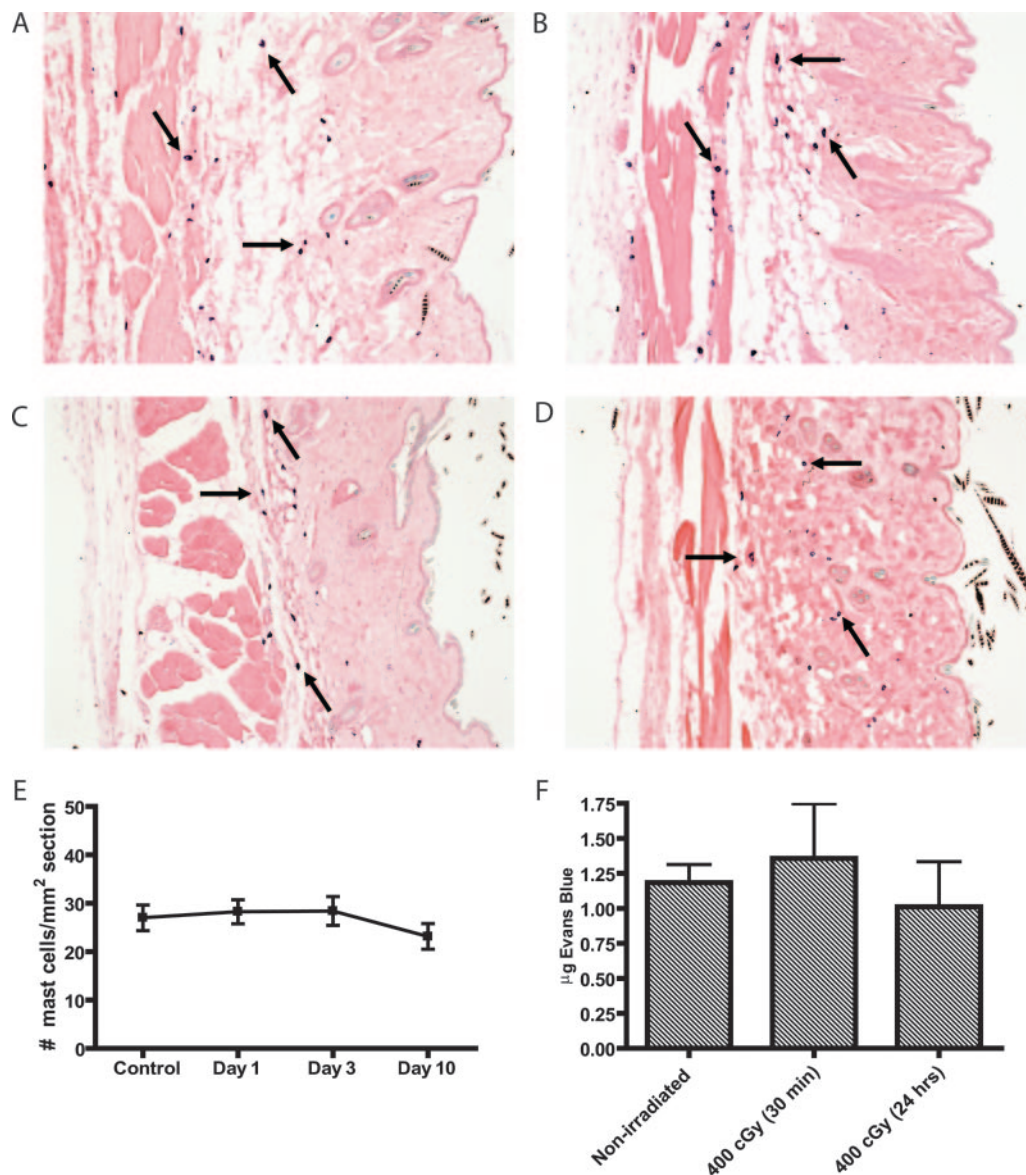


FIGURE 8. Mast cells in vivo do not decrease in number within skin and retain their ability to degranulate in response to Ag after irradiation. The number of mast cells present in the skin of C57BL/6 mice ($n = 4$ mice/group) after 200 cGy of daily irradiation was determined at baseline (A) and 1 day (B; 200 cGy total), 3 days (C; 600 cGy total) or 10 days (D; 2000 cGy total) after fractionated gamma radiation doses. Arrows indicate mast cells stained with toluidine blue. E, Number of mast cells in 6 random areas and is the average of 3 skin samples from 4 mice for each time point. (F) In vivo mast cell activation as measured by PCA. C57BL/6 mice ($n = 6$ mice/group) received 400 cGy of gamma radiation to the head, and PCA was measured in the ears of mice 30 min and 24 h postirradiation. *, $p \leq 0.05$.

These observations add weight to the conclusion that mast cell function is resistant to alteration after gamma radiation exposure and retain their ability to perform innate immune functions.

The number of mast cells and FcεRI-mediated degranulation remain normal in irradiated mice

As determined in vitro, mast cells are resistant to gamma radiation-induced cytotoxicity and FcεRI-mediated mast cell degranulation is only transiently inhibited after irradiation. To determine the effects of gamma radiation on mast cells in vivo, we examined skin mast cell numbers in mice after whole-body irradiation. In addition, the ability of mast cells to degranulate after irradiation as measured by PCA was examined. After the fractionated irradiation of mice to 2000 cGy during 10 days, there was a small but insignificant decrease in the number of mast cells present in the skin despite the induction of mild radiation-induced changes in the skin

connective tissue (Fig. 8). This is consistent with the finding in vitro that mast cells are relatively resistant to gamma radiation-induced cytotoxicity. PCA was used to determine the ability of mast cells to respond to Ag after irradiation of mice. Mice received ear injections of IgE anti-DNP and were challenged with DNP-HSA 30 min and 24 h postirradiation. Results of the PCA reaction demonstrated no significant change in mast cell function 30 min or 24 h after irradiation as compared with nonirradiated mice. These results demonstrate that in vivo, mast cells remain viable and retain their ability to degranulate via IgE following irradiation.

Discussion

This paper reports a systematic investigation into the effects of gamma radiation on mast cells. In this work, we demonstrate for the first time that mast cells are resistant to gamma radiation-induced cytotoxicity, do not degranulate in response to gamma

radiation, and retain specific innate and acquired immune functions after gamma radiation exposure. These results support the concept that mast cells survive and function after irradiation and despite a transient inhibition, ultimately retain an ability to undergo IgE-mediated activation, and may also contribute to innate immunity. Mast cells appear to be highly resistant to gamma radiation-induced cytotoxicity. These data are consistent with previous studies which have suggested mast cells are resistant to gamma radiation-induced cell death. For example, one study demonstrated that there is no significant decrease in choroidal mast cells following high-dose irradiation of rabbits (4). Other reports demonstrate the resistance or recurrence of mast cell sarcomas after treatment with gamma radiation (39–41). Additionally, our findings of mast cell survival following gamma radiation are consistent with veterinary studies examining the response of canine mast cell tumors to treatment with radiation. Failure rates of nearly 50% were seen using conventional dosing regimens suggesting an inherent resistance to the effects of gamma radiation (42). This is in direct contrast to the use of gamma radiation to treat lymphoma which often responds completely and quickly to low doses and few fractions (32, 43–46). Consistent with this, we found murine T cells to be highly susceptible to gamma radiation-induced cytotoxicity. However, not all lymphocytes are susceptible to gamma radiation. A recent report demonstrates that B-1 B cells also exhibit resistance to cytotoxicity which is conferred by cross-linking of the BCR with Ags (47). In these cells, this results in the increased phosphorylation of STAT3, possibly leading to up-regulation of prosurvival genes such as *bcl-2*, *bcl-x_L*, and *mcl-1* (47). Although the mechanism that imparts resistance to gamma radiation in mast cells is not known, it will be interesting to study whether similar prosurvival genes are involved.

In addition to the importance of mast cell resistance to gamma radiation-induced cytotoxicity, it is also important to address the effects of gamma radiation on mast cell degranulation, as it has been hypothesized that mast cell degranulation and histamine release play a role in altering cerebral blood flow in primates leading to symptoms seen in acute radiation syndrome (48). However, in contrast to data suggesting that gamma radiation can induce mast cell degranulation, mastocytosis patients receiving radiation therapy exhibit no signs of mast cell degranulation such as flushing or elevation in histamine levels (13, 14). In the current study, we could not detect mast cell degranulation or cytokine production resulting directly from gamma radiation exposure. Thus, despite the historical assertion that histamine release from mast cells is an underlying cause of ARS, we did not find that radiation alone was a sufficient stimulus for either directly activating mast cells or altering their phenotype in such a way as to make them more susceptible to activation by environmental stimuli. It remains possible that histamine from non-mast cell sources, such as enterochromaffin cells or neutrophils, may be more important in the pathophysiology underlying ARS (49, 50).

Mast cells have been studied extensively for their role in allergic disease, and the activation of mast cells by cross-linking of the IgE receptor is well elucidated (51). The effects of gamma radiation on the IgE-dependent activation of mast cells in response to allergen has not been examined. Here, we report that gamma radiation exposure induces a transient inhibition of IgE-mediated mast cell degranulation and cytokine production that recovers to normal levels within several hours. This transient inhibition of IgE-mediated mast cell degranulation was itself mediated by the production of ROS resulting from irradiation, and treatment of the cells with several antioxidants prevented this inhibition of degranulation after irradiation. Although IgE-mediated activation, but not TLR-mediated activation, induces small physiological amounts of ROS

in mast cells (Swindle, E. J., unpublished data), these results clearly demonstrate that superphysiological amounts of ROS produced after irradiation can lead to transient inhibition of mast cell degranulation. The generation of intracellular ROS by gamma radiation is an area of intense research (26, 52, 53). It has been clearly established that gamma radiation induces free radical formation in living cells, and free radicals are believed to be responsible for much of the damage that results after irradiation (27, 32, 54, 55). Although the mechanism by which gamma radiation-induced ROS inhibits mast cell degranulation is not yet known, several possibilities exist including direct interaction with phosphorylation/dephosphorylation of signaling proteins and alterations in the proteasome/ubiquitination system (56).

In addition to the well-characterized IgE-mediated adaptive immune function, mast cells also participate in innate immunity through several mechanisms including TLR-induced activation (33–37). It is also known that mast cells have phagocytic properties and are capable of intracellular killing (57–60). To determine the effect of radiation on this function of mast cells, TLR ligands were used to stimulate cells after exposure to gamma radiation. Unlike IgE-mediated activation, TLR stimulation does not induce mast cell degranulation but does trigger cytokine production (33, 36, 37). Results indicate that, unlike the attenuation of function observed with IgE-mediated stimulation, irradiation did not modify TLR-induced cytokine production. In fact, with Pam3Cys and poly(I:C) stimulation, enhancement of cytokine production was observed. In addition, mast cells retained their phagocytic capabilities after irradiation as demonstrated by phagocytosis of fluorescent *E. coli* particles. These findings are important because the resistance of mast cells to gamma radiation-induced cytotoxicity permits continued innate immune responsiveness of mast cells that could help protect the host after radiation exposure whether from therapeutic uses or acts of terrorism and war. However, it should be recognized that other cell types should be considered in providing innate host defense such as neutrophils and mononuclear phagocytes. However, neutrophils are reported to be sensitive to radiation, and sublethal irradiation of mice induces profound neutropenia (61).

Mast cells are extremely abundant in the skin and serve as a first line of defense against pathogens. In the current study, mast cell numbers did not decrease in the skin of mice after fractionated doses of gamma radiation out to 10 days. This is unlike other immune cells, which decrease rapidly in number after exposure to even low doses of radiation (32). Similar to the 24-h postirradiation *in vitro* results, the PCA reaction revealed no significant change in the ability of mast cells to degranulate in response to Ag after irradiation as compared with nonirradiated mice. Although this differs from the *in vitro* results at 30 min postirradiation, we found several molecules such as trolox and cysteine were radioprotective *in vitro*, and equivalent protective mechanisms possibly exist *in vivo*. These results are consistent with an older study that found no differences in mast cell numbers and PCA reactivity in irradiated mice (2). Overall, the *in vivo* results demonstrate that mast cells remain present and able to function after irradiation. Importantly, these data suggest that patients receiving radiation therapy for BMT may retain an ability to undergo IgE-mediated mast cell activation.

Overall, immune cells are susceptible to radiation-induced damage and readily undergo apoptosis in response to small doses of radiation. However, in this study, we demonstrate that mast cells are resistant to both apoptotic and mitotic cell death after exposure to gamma radiation. Furthermore, irradiated mast cells retained their innate immune function and despite an initial transient inhibition, also responded to IgE-mediated activation after irradiation.

These findings may become important in the situation where normal host responses become severely compromised due to radiation exposure. One of the severe complications of BMT is infection. Thus, mast cells surviving radiation could be critical for protection during this period of impaired immune function. For example, most of the infections in the first month after radiation therapy for BMT are opportunistic, whereas overwhelming bacterial sepsis is much less frequent (62–67). Because the role of mast cells in immune responses during the postradiation recovery period has not been examined, there are no recommendations regarding the use of medications that affect mast cell function in the pre- and posttransplantation period. Because mast cells may perhaps facilitate recovery after gamma radiation therapy for BMT, the use of medications affecting mast cell number and function should at least be considered in patients who can tolerate the resulting increase in mast cell activity. In addition, as the prevalence of allergic diseases increases in the population, more medications are expected to be developed that are aimed at reducing the function and number of mast cells, and these may have unanticipated adverse effects in the event of radiation exposure whether for therapeutic purposes or inadvertent exposure.

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Disclosures

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