Molecular Pharmacology Fast Forward. Published on May 1, 2007 as doi:10.1124/mol.107.035394

MOL#35394

5-Androstenediol promotes survival of gamma-irradiated human hematopoietic progenitors through induction of NF-kappa B activation and G-CSF expression

Authors: Mang Xiao, Cynthia E. Inal, Vaishali I. Parekh, Cheng-Min Chang and Mark H. Whitnall.

Affiliation: Radiation Countermeasures Program, Armed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences, Bethesda, MD 20889-5603

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2007		**00-00-2007 to 00-00-2007**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
5- Androstenediol induces NFkB activation and cell survival	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Armed Forces Radiobiology Research Institute,Radiation Countermeasures Program,8901 Wisconsin Avenue,Bethesda,MD,20889**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

5-Androstenediol stimulates hematopoiesis and enhances survival in animals exposed to ionizing radiation (IR), suggesting this steroid may act on hematopoietic progenitor cells. We used γ-irradiated primary human CD34+ hematopoietic progenitor cells to show 5-AED protects hematopoietic cells from IR damage, as shown by enhanced cell survival clonogenicity, proliferation, and differentiation. Unlike in tumor cells, IR did not induce NF-κB (NFkB) activation in primary progenitors. However, IR stimulated IkBβ release from NFkB/IkB complexes and caused NFkB1 (p50) degradation. 5-AED stabilized NFkB1 in irradiated cells, as well as inducing NFkB gene expression and NFkB activation (DNA binding). 5-AED stimulated interleukin-6 (IL-6) and granulocyte colony-stimulating factor (GCSF) secretion. The survival-enhancing effects of 5-AED on clonogenic cells were abrogated by siRNA inhibition of NFkB gene expression, and also by neutralization of G-CSF with antibody. The effects of 5-AED on survival and G-CSF secretion were blocked by the NFkB inhibitor MG132. 5-AED had no effect on accumulation of the pro-apoptotic factor p53 after IR, as determined by Western blot. The results indicate that NFkB1 degradation after IR may be responsible for the radiation sensitivity of CD34+ cells, as compared to tumor cells. 5-AED exerts survival-enhancing effects on irradiated human hematopoietic progenitor cells via induction, stabilization, and activation of NFkB, which results in increased secretion of hematopoietic growth factor G-CSF.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**44**	

Running title:
5- Androstenediol induces NFkB activation and cell survival

Author correspondence information:
Mang Xiao
AFRRI
8901 Wisconsin Ave
Bethesda, MD 20889-5603
tel: 301-295-2597
fax: 301-295-3000
email: xiao@afrri.usuhs.mil

Number of text pages: 37

Number of figures: 6

Number of references: 40

Number of words: Abstract = 214

Introduction = 608

Discussion =1230

Nonstandard abbreviations:

5-AED, 5-androstenediol;

IR, ionizing radiation;

NFkB, nuclear factor κB;

IkB, I κB;

G-CSF, granulocyte colony-stimulating factor;

IL-6, interleukin-6;

Abstract:

5-Androstenediol stimulates hematopoiesis and enhances survival in animals exposed to ionizing radiation (IR), suggesting this steroid may act on hematopoietic progenitor cells. We used γ-irradiated primary human CD34+ hematopoietic progenitor cells to show 5-AED protects hematopoietic cells from IR damage, as shown by enhanced cell survival, clonogenicity, proliferation, and differentiation. Unlike in tumor cells, IR did not induce NF-κB (NFkB) activation in primary progenitors. However, IR stimulated IkB_{β} release from NFkB/IkB complexes and caused NFkB1 (p50) degradation. 5-AED stabilized NFkB1 in irradiated cells, as well as inducing NFkB gene expression and NFkB activation (DNA binding). 5-AED stimulated interleukin-6 (IL-6) and granulocyte colony-stimulating factor (G-CSF) secretion. The survival-enhancing effects of 5-AED on clonogenic cells were abrogated by siRNA inhibition of NFkB gene expression, and also by neutralization of G-CSF with antibody. The effects of 5-AED on survival and G-CSF secretion were blocked by the NFkB inhibitor MG132. 5-AED had no effect on accumulation of the pro-apoptotic factor p53 after IR, as determined by Western blot. The results indicate that NFkB1 degradation after IR may be responsible for the radiation sensitivity of CD34+ cells, as compared to tumor cells. 5-AED exerts survival-enhancing effects on irradiated human hematopoietic progenitor cells via induction, stabilization, and activation of NFkB, which results in increased secretion of hematopoietic growth factor G-CSF.

Introduction:

5-Androstenediol (5-androstene-3β-17β-diol, 5-AED) a novel non-toxic radiation countermeasure, enhances survival in mice and monkeys exposed to whole-body γ-IR(Stickney et al., 2007; Stickney et al., 2006; Whitnall et al., 2005), and induces hematopoiesis and hematopoietic growth factor expression(Singh et al., 2005; Stickney et al., 2007; Stickney et al., 2006; Whitnall et al., 2000). 5-AED administration causes increases in circulating granulocytes, monocytes, NK cells, and platelets in irradiated animals (Whitnall et al., 2000; Stickney et al., 2007). 5-AED also displays beneficial effects after burn injury, trauma, and sepsis(Szalay et al., 2006). However, the mechanisms of action of 5-AED are not well understood. Injury after prompt IR of hematopoietic tissue is due to apoptosis in hematopoietic stem and progenitor cells occurring over a period of hours to days. The moderate dose range (1-7 Gy in humans) of exposures to ionizing radiation poses a risk of damage to the hematopoietic system(Coleman et al., 2004) and results in mortality due to opportunistic infection and hemorrhage. Hence investigation of the signaling pathways involved in IR-induced apoptosis in human primary hematopoietic cells, and the possible modulation of apoptotic pathways by radiation countermeasures, is central to understanding the mechanisms of action of these agents.

NFkB is a dimeric DNA-binding protein of the Rel/NFkB family, which consists of five members (c-Rel, p65/RelA, RelB, p50, and p52). The p65/p50 heterodimer is the most common form in mammalian cells, and this is what is commonly referred to as “NFkB” (Karin and Ben-Neriah, 2000; Pahl, 1999). The Rel/NFkB complexes are retained in cytoplasm by inhibitors of the IkB family. Various stimuli, such as ultraviolet radiation, IR, and free radicals,

induce IkB phosphorylation and degradation through ubiquitin/proteasome pathways. The released NFkB translocates into the nucleus, binds to target DNA, and initiates transcription. NFkB is known to promote the expression of target genes that regulate immune responses, stress responses, and cell growth or survival (Joyce et al., 2001; Thompson et al., 1995). NFkB expression has been found in human fetal blood hematopoietic stem and progenitor Lin-CD34+CD38- cells(Shojaei et al., 2004), and in CD34+CD19- bone marrow cells, and is required for CD34+ cell clonogenic function and survival(Pyatt et al., 1999). Recent studies indicated that tumor cells usually possess high levels of constitutive NFkB activity. Exposure of these cells to IR increases NFkB activity(Braun et al., 2006; Kim et al., 2005). This disordered constitutive NFkB activity plays an important role in radioresistance of malignant cells, therefore inhibiting NFkB activity has been proposed as a cancer therapy strategy (Luo et al., 2005; Magne et al., 2006). However, NFkB also modulates IR-induced damage in normal human tissue, and levels of this factor in human primary CD34+ cells are relatively low (Guzman et al., 2001; Pyatt et al., 1999; Romano et al., 2003). Furthermore, baseline NFkB expression levels are lower in human CD34+ cells than all mature hematopoietic cell lineages (Granelli-Piperno et al., 1995; Griffin et al., 1989; McDonald et al., 1997). NFkB is activated by cytokines and is known to regulate hematopoiesis. Hence low NFkB expression may be related to the high radiation sensitivity of hematopoietic progenitor cells (Besancon et al., 1998; Pyatt et al., 1999).

To examine the effects of IR on human stem and progenitor cells, to evaluate the utility of 5-AED as a radiation countermeasure for human cells, and to test hypotheses concerning signaling pathways mediating these effects, we administered 5-AED to γ-irradiated human

primary CD34+ cells, a population comprising pluripotent hematopoietic stem cells and lineage-committed hematopoietic progenitors. The present results demonstrate that 5-AED can act directly on cells from hematopoietic tissue, and that direct cellular targets of 5-AED include one or more of the hematopoietic subpopulations contained within CD34+ cells.

Materials and Methods:

Drug preparation. 5-AED (androst-5-ene-3β, 17β-diol) was purchased from Steraloids (Wilton, NH) and freshly prepared and administered at doses of 1 μg/ml based on our preliminary data. Since 5-AED is insoluble in aqueous media, 20 mg 5-AED was added to 2 ml DMSO, and sonicated in a 45^{o}C water bath until completely dissolved. The 5-AED/DMSO solution was diluted in 10% FBS to achieve the 100 x stock solution. Vehicle controls were used in all experiments (Whitnall et al., 2000).

Cell culture, cytokines, IR, and drug treatment. Human CD34+ cells were provided by the National Hematopoietic Cell Processing Core directed by Dr. Shelly Heimfeld (Fred Hutchinson Cancer Research Center, Seattle, WA) (Elagib et al., 2004). Thawed CD34+ cells were cultured in serum-free medium consisting of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with BIT 9500 (Stem Cell Technologies, Tukwila, WA) and penicillin/streptomycin. Recombinant human (rh) stem cell factor (SCF, 100 ng/ml), rh flt-3 ligand (FL, 100 ng/ml) and rh interleukin-3 (IL-3, 25 ng/ml) were added. All cytokines were purchased from PeproTech, Inc. (Rocky Hill, NJ). CD34+ cells were γ–irradiated at doses of 0, 2, 4, or 6 Gy (0.6 Gy/min) in the Armed Forces Radiobiology Research Institute (AFRRI) Cobalt facility 72 h after thawing. After IR, cells were washed with serum-free medium once and fresh culture medium with the above cytokines and factors was added. Incubations of cells in 5-AED were for the 24 h period before IR, the 24 h period after IR, or both before and after IR. MG132 (Calbiochem, La Jolla, CA) (0.1 to 0.5 μM) was added one h before IR to CD34+

cultures with and without 5-AED administration in indicated experiments (Guzman et al., 2001; Romano et al., 1999).

Flow cytometry and clonogenic assays. Cell expansion and viability (trypan blue-negative cells) from all groups were counted. Death and apoptotic markers and cell lineage-specific surface phenotypes were determined using BD FACS Caliber flow cytometry. All antibodies and dyes including anti- CD34, anti-CD11b, Annexin-V, and 7-aminoactinomycin D (7AAD) or propidium iodide (PI) were purchased from BD Biosciences (San Jose, CA) (Dooley et al., 2004; Xiao et al., 2001).

Committed hematopoietic progenitors in the CD34+ population were quantitated in standard semisolid cultures in triplicate using 1 ml of Methocult GF+ (Stem Cell Technologies), which consists of 1% methylcellulose in IMDM, 30% FBS, 1% BSA, 2 mM L-glutamine, 10^{-5} M 2-mercaptoethanol, 50 ng/ml SCF, 20 ng/ml GM-CSF, 20 ng/ml G-CSF, 20 ng/ml IL-3, and 3 U/ml erythropoietin. Cells from liquid culture were washed twice with IMDM before assays and seeded with 1-5 x 10^3 cells/dish in 35 cm cell culture dishes (from BD). Plates were scored for erythroid, granulocyte-macrophage, and mixed-lineage colonies after culturing for 14 days at 37°C, 5% CO_2 (Elagib et al., 2004).

Quantitative Real-Time PCR (QRT-PCR). Total RNA was extracted from 1 x 10^4 total cultured cells using RNAqueous-4PCR Kits from Ambion (Austin, TX) and was reverse transcribed using random hexamers according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Gene sequences were obtained from GeneBank. Primers and probes for all

target gene sequences were designed using the computer program Beacon Designer (Premier, Palo Alto, CA). Multiplex QRT-PCR assays were carried out using cDNA, primers (human IL-6, G-CSF, NFkBp65 and p50, as well as 18S rRNA subunit which was used as an internal control), fluorogenic probes, and iQ Supermix (Bio-Rad, Hercules, CA). The flurochromes used in this study were FAM (6-carboxyfluorescein), HEX (hexachloro-6-carboxyfluorescein), Cy 5, and Texas Red. Quadruplex PCR reactions were run in triplicate on a Bio-Rad iQ5 using 5'-fluorogenic nuclease TaqMan (TM) methodology according to the manufacturer's instructions. Negative controls with no RNA template were included in every analysis, and all samples were normalized with 18S RNA. Results were analyzed using amplification curves and threshold cycles collected from PCR data analysis. PCR primers and probe sequences were as follows:

IL-6 F: 5'-GGTCCAGTTGCCTTCTCC-3';

Il-6 R: 5'-TGTCAATTCGTTCTGAAGAGG-3';

IL-6 probe: 5'-CGCGATCTGGTGTTGCCTGCTGCCTTCCGATCGCG-3';

G-CFS F: 5'-GATGGGTGAGTGTCTTGG-3';

G-CSF R: 5'-ACTGGGTGCCTTTAATCC-3';

G-CSF probe: 5'-CGCGATCCTGTCACACCAGCCTCCCTCCCGATCGCG-3';

NFkBp65F: 5'-GTTCACAGACCTGGCATCC-3';

NFkBp65 R: 5'-TGTCACTAGGCGAGTTATAGC-3';

NFkBp65 probe: 5'-CGCGATCCCACACAACTGAGCCCATGCTGAGATCGCG-3';

NFkBp50 F: 5'-AATGACAGAGGCGTGTATAAGG-3';

NFkBp50 R: 5'-GAGCTGCTTGGCGGATTAG-3';

NFkBp50 probe: 5'-CGCGATCGCAAATAGGCAAGGTCAGGGTGCAGATCGCG-3';

18S RNA F: 5' AGG-AAT-TCC-CAG-TAA-GTG-CG-3'

18S RNA R: 5'-GCC-TCA-CTA-AAC-CAT-CCA-A-3'

18S RNA probe: 5'-TEXASRED-TCCCTGCCCTTTGTACACACCGCC-BHQ2-3'

NFkB siRNA transfection. NFkBp65 siRNA from siGENOME SMARTpool (Dharmacon Inc., Lafayette, CO) was transfected into CD34+ cells using a Nucleofector II (amaxa Inc., Gaithersburg, MD) according to the manufacturer's protocol. In brief, 10^6 CD34+ cells were resuspended in 100 μl of human CD34 cell Nucleofector solution (Human CD34 cell Nucleofector Kit, Cat No. VPA-1003, amaxa Inc.) with 1.5 μg of NFkBp65 siRNA-siGENOME SMARTpool and/or 1.5 μg of maxGFP siRNA (positive control provided in the siRNA Test Kit , amaxa, Inc.). Samples were transferred into an amaxa certified cuvette and nucleotransfered with program A-27 using the Nucleofector II. After transfection, cells were immediately transferred into fresh, prewarmed, cytokine-supplemented CD34+ culture medium with or without 5-AED. These cells then were cultured in a 37°C incubator until irradiation on the next day (24 h after siRNA transfection). Western blots and colony assays were performed 24 h post IR (48 h after siRNA transfection).

Immunoprecipitation and Immunoblotting. Immunoprecipitation kits from Sigma (Saint Louis, Missouri) were used as follows: 1-5 x 10^6 cells from each sample were harvested, washed, and lysed with 0.5 ml lysis buffer, 1-5 µg of purified primary antibody, 1x IP buffer (provided in kit), and protease inhibitor cocktail. Components were added to a spin column and incubated overnight at 4^{o}C with inversion. Precleared protein G beads (20-30 µl) were added to the column and incubated overnight at 4^{o}C. Beads were washed several times at 4^{o}C and the effluent discarded. After the last wash, the supernatant was carefully removed, and 50 µl 1x Laemmli sample buffer was added to the pellet. After being vortexed and heated to 90-100^{o}C for 5 min, samples were spun at 10,000 g for 5 min, supernatants were collected for SDS-PAGE, and proteins were analyzed by immunoblotting as follows: Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were preblocked and probed with primary antibodies (for NFkBp65 and p50, IkB_α and IkB_β, p53, and loading controls), per the manufacturer's instructions, followed by the appropriate horseradish peroxidase-conjugated secondary antibody (all antibodies were from Santa Cruz Biotechnology, Inc., Santa Cruz, California). Signal detection used an enhanced chemiluminescence kit (Amersham Biosciences, Rochester, NY) and Kodak X-ray film (Elagib et al., 2004).

Nuclear extract preparation. Nuclear extracts were prepared using a nuclear extraction kit (Panomics Inc., Redwood City, CA) according to the manufacturer's protocol. Briefly, cells were washed and resuspended with Prepare Buffer A mix (10 mM HEPES, 10 mM KCL and 10 mM EDTA with DTT, protease inhibitor cocktail and IGEPAL). After incubation on ice for

10 min, samples were centrifuged at maximum speed for 3 min at 4^{o}C. The supernatant was discarded and the pellet resuspended in Buffer B Mix (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA and 10% glycerol with protease inhibitor cocktail and DTT). Eppendorf tubes containing sample were laid horizontally on ice and shaken on a rocking platform at 200 rpm for 2 h. Samples were then centrifuged at maximum speed in an Eppendorf centrifuge for 5 min at 4^{o}C and supernatants (nuclear extracts) were collected.

Transbinding assay. An NFkB1 (p50) probe was used with the Transbinding ELISA kit from Panomics (Redwood City, CA), which employs an oligonucleotide-containing NFkB consensus binding site immobilized on a 96-well plate. Activated NFkB from cell nuclear extracts specifically binds to this oligonucleotide. Complexes bound to the oligonucleotide were detected by antibody directed against the p50 subunit and a secondary HRP-conjugated antibody which was quantified by spectrophotometry. Cold control probe (consensus oligonucleotide) was used as a competitor to control for nonspecific binding (Lu and Wahl, 2005).

Cytokine antibody array and ELISA for cytokines in culture medium. To determine endogenous hematopoietic factor synthesis in cultures, serum-free culture medium from indicated samples was subjected to cytokine antibody array analysis using the Ray Bio Human Cytokine Antibody Array VII kit (Ray Biotech, Inc. Norcross, GA) according to the manufacturer's instructions. The kit provided antibodies for detection of 60 cytokines, chemokines, growth factors and soluble receptors of cytokines. In brief, the array membrane coated with cytokine antibodies was first blocked with blocking buffer and then incubated with

one ml of pooled serum-free culture medium from three individual experiments overnight. After washing and incubation with biotin-conjugated second antibody for 2 h at room temperature, the membranes were washed again and incubated with HRP-conjugated streptavidin. The membrane was developed using enhanced chemiluminescence solution and exposed to x-ray film. Soluble cytokines G-CSF and IL-6 released from serum-free cultured cells were also measured by ELISA kits (R&D Systems, Minneapolis, Minnesota) after normalizing the protein concentration in every sample, according to the company's instructions (Singh et al., 2005).

Modulation of intercellular signaling with neutralizing antibodies. Neutralization of IL-6 and G-CSF bioactivity was performed as described in the manufacturer's instructions (R&D Systems). Briefly, neutralizing antibody (1 μg/ml), or control nonspecific IgG from the same species, was added to the culture medium 1 h before cell addition. Following this preincubation period, 5 x 10^5 CD34+ cells were added with or without 5-AED administration, and cells were exposed to γ-radiation at the indicated doses. Antibodies were maintained in the cultures after IR. Twenty-four h after IR, cells were used for colony assays or other indicated assays to determine the effects of IL-6 and G-CSF neutralization.

Statistical Methods. Differences between means were compared by ANOVA and by Student's *t* tests. $P<0.05$ was considered statistically significant. Results are presented as means ± standard deviations.

Results

Effects of 5-AED on human CD34+ cell survival, differentiation, and clonogenicity after IR. In pilot studies, 5-AED up to 10 µg/ml had no toxicity, and the optimal dose for enhancing cell survival was 1 µg/ml. The effects of 5-AED were then tested in CD34+ cells adding the steroid before, after, and both before and after IR. The optimal effect was obtained when 5-AED was administered both before and after IR (data not shown). In the next series of experiments, CD34+ cells were cultured with or without 5-AED for 24 h before IR (2, 4, or 6 Gy). Following IR, CD34+ cells were immediately transferred to fresh serum-free culture medium supplemented with cytokines and growth factors, with or without 5-AED. Twenty-four h after IR, apoptotic cell death was dramatically increased ($p<0.01$, compared to unirradiated controls) and significantly related to radiation dose ($p<0.05$, 2 Gy compared to 4 Gy, and 4 Gy compared to 6 Gy) as determined by annexin-V and PI or 7AAD staining using flow cytometry (Figure 1A). We further analyzed IR-induced apoptosis in both CD34+ and differentiated CD34- subpopulations in these cultured cells. The percentage of annexin-V and 7AAD-positive apoptotic cells was markedly higher in CD34+ cells than CD34- differentiated cells at all IR doses (Figure 1A, $p<0.01$). 5-AED-induced frequencies of Annexin-V and 7AAD positive cells decreased in CD34+ cells with 2 and 4 Gy, and in CD34- cells with 4 and 6 Gy exposures, suggesting that 5-AED protects hematopoietic cells from apoptosis occurring within 24 h after IR (Figure 1A, $p<0.05$).

Next, we evaluated the effects of 5-AED on colony-forming potential. Results from one representative experiment (of a total of five) are shown in Figure 1B. Clonogenic assays starting with 5×10^3 cells/dish were plated 24 h after IR. Colony efficiencies for unirradiated

CD34+ cells ranged from 15% to 30%, and these efficiencies were not affected by 5-AED. For irradiated cells, BFU-E colonies in 5-AED-treated cultures increased from 163±17 (vehicle-treated) to 418±20 after 2 Gy ($p<0.01$), and from 59±15 to 118±11 after 4 Gy ($p<0.05$). CFU-GM colonies in 5-AED-treated cultures rose from 89±6 to 177±13 after 2 Gy ($p<0.01$), and from 27±3 to 51±3 cells after 4 Gy ($p<0.01$). 5-AED increased numbers of cells surviving 7 days after IR in these cultures starting with 7.5×10^5 cells per culture (Figure 1C). Seven days after IR, numbers of trypan blue-negative (live) cells from 5-AED-treated cultures were doubled after 2 Gy (from $1.5\pm0.20 \times 10^6$ without 5-AED to $3.0\pm0.29 \times 10^6$ with 5-AED, $p<0.01$), and a similar result was obtained after 4 Gy ($1.0\pm0.17 \times 10^6$ without 5-AED to $1.8\pm0.23 \times 10^6$ with 5-AED, $p<0.01$). In addition, 5-AED administration to irradiated CD34+ cell cultures was associated with a trend toward elevated frequency of CD11b+ cells (granulocytes, monocytes and NK cells) as determined by flow cytometry 7-21 days after IR (Figures 1D and 1E).

Role of NFkB in 5-AED effects. Because of the limited number of CD34+ cells after IR, we evaluated IR and 5-AED-mediated NFkB activation in CD34+ cells using a DNA-binding assay. The transbinding NFkB assay is more sensitive than EMSA, is comparable in specificity, and requires fewer cells per sample (Lu and Wahl, 2005). Results from nuclear extracts obtained 24 h after IR (Figure 2A) demonstrated that 5-AED but not IR stimulated NFkB activity in CD34+ cultures ($p<0.01$ at 0 and 2 Gy, $p<0.05$ at 4 Gy). Unirradiated, untreated samples (Figure 2A, 0 Gy, white bar) did not display detectable levels of NFkB activity in this assay (compare to 5-AED + cold probe).

To characterize the activation of NFkB as a transcriptional activator in CD34+ cells, and to verify that 5-AED-stimulated NFkB activation is associated with its radiation countermeasure function, we used peptide aldehyde MG132, a potent proteasome inhibitor that prevents degradation of the regulatory molecule IkB (Guzman et al., 2001; Lin and Kobayashi, 2003). Previous reports indicated 1.0 μM of MG132 has no toxicity in unstimulated normal human CD34+ cells (Guzman et al., 2001). Therefore, doses of MG132 from 0.1 to 0.5 μM were added one h before IR to CD34+ cultures with and without 5-AED administration. As shown in Figure 2B, NFkBp65 (RelA) subunit expression was detected by immunoblotting in nuclear extracts from unirradiated and irradiated samples. As described in Materials and Methods, all CD34+ cells were cultured in the presence of SCF, FL and IL-3 for 72 h before IR, which might explain the baseline expression of NFkBp65. IR (2 or 4 Gy) had no effect on NFkBp65 levels. In the absence of 5-AED, MG132 treatment at concentrations as low as 0.1 μM dramatically downregulated NFkBp65 expression, suggesting that NFkB translocation from cytoplasm to nucleus was inhibited by MG132 (Figure 2B). Addition of 5-AED did not upregulate NFkBp65 expression. However, it blocked NFkBp65 downregulation in MG132-treated nuclear extracts. Separate samples from the same experiment were cultured for 7 days after IR, and trypan blue-negative cells were counted. Figure 2C shows that MG132 inhibited cell survival and expansion in a dose-dependent fashion. The effects of 5-AED on survival of irradiated CD34+ cells were abrogated by even the lowest dose of MG132. Consistent with results from the immunoblotting assay, 5-AED partially protected cells from the lower dose (0.1 μM) of MG132, whereas 0.5 μM of MG132 completely inhibited CD34+ cell growth.

MG132 is a general proteasome inhibitor, which affects a wide variety of cellular processes. Therefore, we also evaluated the effect of a more specific NFkB inhibitor. NFkB siRNA was transfected into CD34+ cells before 5-AED administration and IR using Nucleofector technology as described in Materials and Methods. NFkB siGENOME SMARTpool (Dharmacon Inc.) contains a mixture of four siRNAs targeting one human NFkBp65 gene, which silences gene expression at the mRNA level by at least 75%. Western blots and colony assays were performed 48 h post-NFkBp65 siRNA and/or positive control siRNA (maxGFP siRNA) transfection with or without 5-AED administration, and 24 h after irradiation. Results from Western blot (figure 2D) showed NFkB protein levels markedly decreased after NFkBp65 siRNA transfection. In contrast, control siRNA-transfected cells expressed NFkB at the same level as non-transfected samples. Colony efficiencies were dramatically inhibited by NFkB gene knockdown in both erythroid (BFU-E and CFU-E) and myeloid (CFU-GM) lineages compared with control siRNA-transfected samples (figure 2E). The effect of 5-AED induced clonogenicity at different doses of IR was completely blocked in NFkB siRNA-transfected samples (figure 2E).

Ionizing radiation but not 5-AED induced IkB$_\beta$ release from NFkB/IkB complexes.

NFkB is normally sequestered in the cytoplasm of unstimulated cells in a complex with IkB. NFkB can be rapidly released by degradation of IkB and can enter the nucleus without a requirement for de novo protein synthesis (Joyce et al., 2001; Thompson et al., 1995). The overall activation of NFkB consists of two overlapping phases, a transient phase mediated by IkB$_\alpha$ degradation, and a persistent phase mediated through IkB$_\beta$ degradation. Although our data showed NFkBp65 expression in irradiated cell nuclear extracts, there was no evidence of

IR-induced NFkB activation in these cultured CD34+ cells as determined by the DNA-binding assay. Due to the relatively low level of IkBs in CD34+ cells, IkB expression and phosphorylation were undetectable in total cell lysates by Western blot. Therefore, to address the question of whether IR and/or 5-AED induces IkB release from NFkB/IkB complexes, immunoprecipitation (IP) was used to evaluate NFkB/IkB protein interaction. Cell lysates were subjected to IP with an NFkBp65 antibody. After SDS-gel separation, protein levels were assessed by immunoblotting using antibodies to NFkBp65, NFkBp50, IkB_{α}, and IkB_{β}. Figure 3 shows that, 4 h after IR, IkB_{α} signal was not detectable in any sample, presumably due to baseline activation of the NFkB pathway by the cytokines added to the culture medium. IkB_{β} was detectable in NFkB/IkB complexes but was attenuated by IR in a dose-dependent fashion. Compared with unirradiated cells, IkB_{β} present in NFkB/IkB complexes was similar after 2 Gy, lower after 4 Gy, and undetectable after 6 Gy (Figure 3). Twenty-four h after IR, levels of IkB_{β} were barely detectable in any sample (data not shown). 5-AED did not change patterns of IkB_{β} disappearance from NFkB/IkB complexes.

NFkB gene and protein expression in cultured CD34+ cells with and without IR and 5-AED. Since 5-AED significantly stimulated NFkB activity in CD34+ cells, but we did not detect an effect of 5-AED on NFkB translocation or IkB degradation, we decided to assess 5-AED-induced NFkB expression. First, NFkB gene expression was measured using multiplex QRT-PCR, which allowed us to assay 18S rRNA and NFkB subunits p65 and p50 simultaneously in the same sample. Gene expression was expressed as a relative quantity (RQ) normalized to 18S rRNA. Figure 4A shows that NFkB gene expression was unchanged 4 h after IR or sham-IR, with or without 5-AED treatment, whereas 5-AED enhanced NFkBp65

mRNA levels 5-fold 24 h after sham-IR, and 6-fold 24 h after 4 Gy, compared with vehicle-treated cultures. Subunit NFkBp50 mRNA expression was also induced by 5-AED (to 3 times control levels) 24 h after 4 Gy IR. NFkBp50 gene expression was not increased 24 h after IR alone (Figure 4A).

Secondly, we used Western blots to determine NFkBp65 and NFkBp50 protein levels in whole cell lysates from cultured CD34+ cells. NFkBp65 levels displayed no differences between treatments (Figure 4B). NFkBp50 antibody was then used on the same membranes, after anti-NFkBp65 antibody was stripped. IR caused protein degradation and low molecular weight fragments, in a dose-dependent manner (Figure 4B). IR-induced NFkBp50 damage could directly affect NFkB dimerization, DNA binding, and transcriptional activity. Stability of the Rel homology domain (RHD) is critical for NFkBp50 generation.(Carlsen et al., 2004; Lin et al., 2000; Lin and Kobayashi, 2003) The RHD consists of two structurally similar subdomains, sd1 and sd2, linked by a short loop. In Figure 4B, small fragments with the molecular weight of sd1(Lin and Kobayashi, 2003) are evident after IR at doses of 4 and 6 Gy. Addition of 5-AED before or after IR decreased the appearance of this fragment, and the fragment was undetectable when 5-AED was administered both before and after IR. These effects of 5-AED were consistent in all experiments (n=9).

G-CSF and IL-6 production induced by 5-AED. Our previous *in vivo* studies demonstrated that 5-AED induced the hematopoietic growth factors IL-6 and G-CSF in mice (Singh et al., 2005). To test our hypothesis that 5-AED acts via initiation of a cytokine cascade in hematopoietic cells, secreted cytokines and chemokines were assayed in serum-free medium

from CD34+ cells 24 h post IR with or without 5-AED, using a cytokine antibody array. In Figure 5A, results from three individual experiments' samples pooled demonstrated significant G-CSF elevations after 4 Gy, with and without 5-AED. Next, we quantitated IL-6 and G-CSF expression at the mRNA and protein levels using QRT-PCR and ELISA. 5-AED elevated IL-6 mRNA levels 2-fold in CD34+ cells without IR, and 10-fold 4 h after 4 Gy compared to vehicle-treated cultures (Figure 5B). Twenty-four h after sham-irradiation or irradiation, 5-AED-induced IL-6 mRNA levels were still higher than in vehicle-treated cultures (Figure 5B). The G-CSF mRNA level increased 17-fold in 5-AED-treated cultures 4 h after 4 Gy (Figure 5C) and returned to baseline levels 24 h after IR (data not shown). Consistent with these results, 5-AED elevated IL-6 secretion (Figure 5D) from CD34+ cell cultures 6-fold 24 h after 4 Gy, and 10-fold after 6 Gy measured by ELISA. After 4 days in culture, IL-6 protein levels were elevated by 5-AED treatment (1 μg/ml) in both unirradiated and irradiated cultures. Observations of G-CSF secretion from CD34+ cell cultures were consistent with the cytokine antibody array results. 5-AED elevated G-CSF levels after 0 Gy (from 0.6 ± 0.31 vehicle control to 1.2 ± 0.4 pg/ml), 2 Gy (from 1.8 ± 0.22 to 2.7 ± 0.25 pg/ml, $p<0.05$), and 4 Gy (from 0.96 ± 0.25 to 2.0 ± 0.2 pg/ml, $p<0.05$) (Figure 5E). Irradiation alone (2 Gy) induced an increase in G-CSF protein level (Figure 5E).

Levels of IL-6 and G-CSF were measured 48 h after NFkB inhibitor MG132 (0.1 μM) addition to CD34+ cells with and without 5-AED treatment and IR. Figure 5F shows that MG132 upregulated IL-6 release from irradiated cells ($p<0.01$), and this effect of MG132 was correlated with radiation dose. In contrast, 5-AED-induced G-CSF secretion was blocked by MG132 administration, as shown in Figure 5G. After both 2 Gy and 4 Gy, G-CSF levels were

significantly above vehicle control levels after 5-AED alone, but not after 5-AED plus MG132 (Figure 5G).

5-AED effects mediated by G-CSF, not IL-6 or p53. To evaluate the potential role of G-CSF and IL-6 in the effects of 5-AED, neutralizing antibodies were used. Anti-human G-CSF or anti-human IL-6 antibody (1 μg/ml) was added to culture medium 1 h before 5-AED administration and 25 h before IR, and maintained in the cultures after IR with or without 5-AED addition. As shown in Figures 6A and 6B, addition of anti-G-CSF antibody inhibited colony formation in irradiated cells, and impaired the effect of 5-AED on progenitor cell survival. Anti-IL-6 antibody did not inhibit colony efficiency: 5-AED administration significantly increased colony numbers in irradiated CD34+ cells both with and without IL-6 neutralization (Figure 6C).

The tumor suppressor gene p53 plays an important role in apoptosis and cell death (Fei and El-Deiry, 2003). The p53 protein is tightly regulated and remains at low levels in unstressed cells, but is rapidly activated (stabilized) by various types of cellular stresses, including IR. To test whether the effects of 5-AED in irradiated CD34+ cells correlated with p53 signaling, p53 protein expression was determined by Western blot (Figure 6D). Expression of p53 in unirradiated cells was undetectable. Four h after IR (4 Gy), p53 was clearly induced, and levels had declined by 24 h after IR but were still present. 5-AED had no effect on p53 levels.

Discussion

We showed previously that 5-AED (HE2100, the active principal ingredient of NEUMUNE®) induces production of G-CSF(Singh et al., 2005), stimulates hematopoiesis (Stickney et al., 2007; Stickney et al., 2006; Whitnall et al., 2000), and enhances survival in mice(Whitnall et al., 2000) and monkeys (Stickney et al., 2007) exposed to whole-body γ-IR. However, the intracellular signaling pathways that mediate these beneficial effects of 5-AED were unknown. Moreover, it was not known whether 5-AED can act directly on hematopoietic tissue, or whether its actions were dependent on indirect effects on other tissues. The present results demonstrate that 5-AED acts directly on cells from hematopoietic tissue.

In this report, we used *in vitro* approaches to evaluate the radiation countermeasure effects of 5-AED in primary human hematopoietic CD34+ cells. Our results showed that 5-AED improved CD34+ cell survival, proliferation, and differentiation into functional hematopoietic lineages after IR. 5-AED induced NFkB activation in cultured CD34+ cells, as confirmed using a DNA-binding assay. In contrast, γ–radiation did not stimulate the activity of NFkB in these cells. To characterize the activation of NFkB as a transcriptional activator in CD34+ cells, and also to verify that 5-AED-stimulated NFkB activation is associated with its radiation countermeasure function, a proteasome inhibitor, MG132, was used to inhibit NFkB activation. Our results indicate that 5-AED-induced cell survival of irradiated CD34+ cells is inhibited by MG132. However, as noted, MG132 affects a wide variety of cellular processes. Therefore, we evaluated the effect of 5-AED in NFkBp65 gene knockdown cells using colony-forming assays. NFkBp65 siRNA transfection significantly inhibited NFkBp65 protein expression and clonogenicity of CD34+ cells, and completely blocked the effect of 5-AED on

clonogenic cell survival after irradiation. These results support our hypothesis that 5-AED promotes survival of γ-irradiated human hematopoietic progenitors through induction of NFkB activation.

In the present study, IR-induced IkB release from NFkB/IkB complexes was confirmed by IP. IkB_β but not IkB_α levels in NFkB/IkB complexes were attenuated by radiation in a dose-dependent fashion, suggesting that this persistent phase regulator of NFkB activation played a key role in radiation-induced NFkB translocation to the nucleus of CD34+ cells. The major difference between the IkB_α and IkB_β isoforms lies in their responses to different inducers of NFkB activity and their different mechanisms of NFkB regulation (Malek et al., 2001; Russell and Tofilon, 2002; Thompson et al., 1995). A previous report (Basu et al., 1998) demonstrated that IR, but not TNF_α, induced DNA-dependent protein kinase (DNA-PK) activity. IkB_α was a poor substrate, whereas IkB_β was strongly phosphorylated by DNA-PK in two distinct regions after IR-induced DNA damage. Therefore, activation of NFkB by DNA-PK following DNA damage may proceed through direct phosphorylation of IkB_β. Those results indicated that IR induced the activity of NFkB beginning 2-4 h after exposure. In contrast, TNF_α-mediated activation of NFkB occurs with peak activation at 30 min (Russell and Tofilon, 2002). These observations were consistent with our observations, in which IkB_β was released from NFkB/IkB complexes within 4 h after IR. Surprisingly, although IR induced IkB_β degradation and release from NFkB/IkB complexes, it did not induce NFkB activation (DNA binding). In contrast, 5-AED did not increase NFkB translocation and IkB release in irradiated cells, but it significantly stimulated NFkB activity. Therefore, 5-AED-mediated NFkB activation in

CD34+ cells was not through acceleration of IkB phosphorylation and ubiquitination. Our results indicate that NFkB activation may involve instead upregulation of NFkB expression and stabilization of the p50 subunit.

To investigate the mechanisms of 5-AED induced NFkB activation, we assessed NFkB expression. Evidence of 5-AED-induced NFkBp65 mRNA expression was obtained 24 h after IR or sham-IR, whereas NFkBp50 mRNA expression was enhanced by 5-AED only in irradiated cells. Protein levels of NFkBp65 showed the same expression patterns in all samples. For NFkBp50, the small molecular weight fragment appearing in irradiated samples needs further definition, but the disappearance of this fragment in 5-AED-treated cultures was consistently observed in all experiments. Functional NFkBp65/p50 dimers bind specific kappa B sites on target DNA sequences. The 3D structure of a p65/p50 dimer bound to DNA reveals that NFkB proteins adopt a specific and unique conformation to recognize DNA using loops from both subunits, and not alpha helixes like other transcription factors (Magne et al., 2006; Jacobs and Harrison, 1998). Each subunit contacts one half of the specific binding sites on DNA, therefore activity of NFkB needs both p65 and p50 subunits. We propose that NFkBp50 degradation after IR may be partly responsible for the radiation sensitivity of CD34+ cells, as compared to tumor cells. Although IR induced IkB release from NFkB complexes, resulting in NFkB translocation into the nucleus, IR-induced NFkBp50 protein degradation may block NFkB activation in CD34+ cells. We could not observe an IR-induced decrease in NFkB activity, because unirradiated, untreated samples did not display detectable levels of NFkB activity. 5-AED stimulates NFkB gene expression and stabilizes the p50 subunit, resulting in NFkB activation, which protects CD34+ cells from IR injury.

In this study, 5-AED-induced IL-6 and G-CSF production was evident at the mRNA and protein levels after IR. The results of our G-CSF neutralization experiments showed that 5-AED-induced G-CSF release significantly promoted survival of hematopoietic progenitor cells. The IL-6 and G-CSF gene promoters have binding sites for multiple transcription factors including AP-1, NFkB, CREB, and CCAAT enhancer binding proteins. Previous reports indicated that the NFkB-binding site is crucial for the activation of the IL-6 and G-CSF promoters (Dunn et al., 1994; Vanden Berghe et al., 2000). Therefore, G-CSF and IL-6 function in response to many stress stimuli, including IR, most likely is under NFkB regulation. In the present study, levels of G-CSF were inhibited by MG132. The results suggest that G-CSF is a survival factor downstream of NFkB activation induced by 5-AED. Surprisingly, our data showed that 5-AED-induced IL-6 expression is not regulated by NFkB. Other studies have also shown increased IL-6 production after MG132 administration (Pritts et al., 2002), and in NFkB1 (p50) knockout mice (Zhou et al., 2001). Neutralizing IL-6 with an antibody did not reduce clonogenicity in CD34+ cells and did not block the effects of 5-AED, indicating that the radiation countermeasure effects of 5-AED in this experimental system are not dependent on IL-6. In addition, although IR dramatically induced p53 expression in CD34+ cells, 5-AED had no effect on p53 levels, suggesting that the effects of 5-AED on CD34+ cells are p53-independent. In summary, our results demonstrate that IR stimulated IkB release from NFkB/IkB complexes in CD34+ cells. However, we observed IR-induced NFkB1 (p50) degradation in CD34+ cells, which may explain their high radiosensitivity. 5-AED rescued CD34+ progenitor cells from IR through stabilizing NFkB1 and stimulating NFkB expression and activation, resulting in downstream production of the hematopoietic survival factor G-CSF.

In the present report, 5-AED protected CD34+ cells from 4 Gy IR. The moderate dose range (1-7 Gy in humans) poses a risk of damage to the hematopoietic system and results in mortality due to opportunistic infection and hemorrhage(Coleman et al., 2004). We hope that 5-AED will be useful at doses higher than 4 Gy, although significant decreases in mortality would be expected even if that were the limit, since the LD50 in humans is about 3.5 Gy. In addition, although we demonstrated direct effects of 5-AED on progenitor cells here, the beneficial effects of 5-AED in vivo may partially be mediated indirectly, i.e. via actions on other cell types or other tissues. We are presently investigating these issues.

References

Basu S, Rosenzweig KR, Youmell M and Price BD (1998) The DNA-dependent protein kinase participates in the activation of NF kappa B following DNA damage. *Biochem Biophys Res Commun* **247**:79-83.

Besancon F, Atfi A, Gespach C, Cayre YE and Bourgeade MF (1998) Evidence for a role of NF-kappaB in the survival of hematopoietic cells mediated by interleukin 3 and the oncogenic TEL/platelet-derived growth factor receptor beta fusion protein. *Proc Natl Acad Sci U S A* **95**:8081-8086.

Braun T, Carvalho G, Coquelle A, Vozenin MC, Lepelley P, Hirsch F, Kiladjian JJ, Ribrag V, Fenaux P and Kroemer G (2006) NF-kappaB constitutes a potential therapeutic target in high-risk myelodysplastic syndrome. *Blood* **107**:1156-1165.

Carlsen H, Alexander G, Austenaa LM, Ebihara K and Blomhoff R (2004) Molecular imaging of the transcription factor NF-kappaB, a primary regulator of stress response. *Mutat Res* **551**:199-211.

Coleman CN, Stone HB, Moulder JE and Pellmar TC (2004) Medicine. Modulation of radiation injury. *Science* **304**:693-694.

Dooley DC, Oppenlander BK and Xiao M (2004) Analysis of primitive CD34- and CD34+ hematopoietic cells from adults: gain and loss of CD34 antigen by undifferentiated cells are closely linked to proliferative status in culture. *Stem Cells* **22**:556-569.

Dunn SM, Coles LS, Lang RK, Gerondakis S, Vadas MA and Shannon MF (1994) Requirement for nuclear factor (NF)-kappa B p65 and NF-interleukin-6 binding

elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. *Blood* **83**:2469-2479.

Elagib KE, Xiao M, Hussaini IM, Delehanty LL, Palmer LA, Racke FK, Birrer MJ, Shanmugasundaram G, McDevitt MA and Goldfarb AN (2004) Jun blockade of erythropoiesis: role for repression of GATA-1 by HERP2. *Mol Cell Biol* **24**:7779-7794.

Fei P and El-Deiry WS (2003) P53 and radiation responses. *Oncogene* **22**:5774-5783.

Granelli-Piperno A, Pope M, Inaba K and Steinman RM (1995) Coexpression of NF-kappa B/Rel and Sp1 transcription factors in human immunodeficiency virus 1-induced, dendritic cell-T-cell syncytia. *Proc Natl Acad Sci U S A* **92**:10944-10948.

Griffin GE, Leung K, Folks TM, Kunkel S and Nabel GJ (1989) Activation of HIV gene expression during monocyte differentiation by induction of NF-kappa B. *Nature* **339**:70-73.

Guzman ML, Neering SJ, Upchurch D, Grimes B, Howard DS, Rizzieri DA, Luger SM and Jordan CT (2001) Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood* **98**:2301-2307.

Jacobs MD and Harrison SC (1998) Structure of an IkappaBalpha/NF-kappaB complex. *Cell* **95**:749-758.

Joyce D, Albanese C, Steer J, Fu M, Bouzahzah B and Pestell RG (2001) NF-kappaB and cell-cycle regulation: the cyclin connection. *Cytokine Growth Factor Rev* **12**:73-90.

Karin M and Ben-Neriah Y (2000) Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* **18**:621-663.

Kim BY, Kim KA, Kwon O, Kim SO, Kim MS, Kim BS, Oh WK, Kim GD, Jung M and Ahn JS (2005) NF-kappaB inhibition radiosensitizes Ki-Ras-transformed cells to ionizing radiation. *Carcinogenesis* **26**:1395-1403.

Lin L, DeMartino GN and Greene WC (2000) Cotranslational dimerization of the Rel homology domain of NF-kappaB1 generates p50-p105 heterodimers and is required for effective p50 production. *Embo J* **19**:4712-4722.

Lin L and Kobayashi M (2003) Stability of the Rel homology domain is critical for generation of NF-kappa B p50 subunit. *J Biol Chem* **278**:31479-31485.

Lu Y and Wahl LM (2005) Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NF-kappa B activity in lipopolysaccharide-activated human primary monocytes. *J Immunol* **175**:5423-5429.

Luo JL, Kamata H and Karin M (2005) IKK/NF-kappaB signaling: balancing life and death--a new approach to cancer therapy. *J Clin Invest* **115**:2625-2632.

Magne N, Toillon RA, Bottero V, Didelot C, Houtte PV, Gerard JP and Peyron JF (2006) NF-kappaB modulation and ionizing radiation: mechanisms and future directions for cancer treatment. *Cancer Lett* **231**:158-168.

Malek S, Chen Y, Huxford T and Ghosh G (2001) IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells. *J Biol Chem* **276**:45225-45235.

McDonald PP, Bald A and Cassatella MA (1997) Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils. *Blood* **89**:3421-3433.

Pahl HL (1999) Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* **18**:6853-6866.

Pritts TA, Hungness ES, Hershko DD, Robb BW, Sun X, Luo GJ, Fischer JE, Wong HR and Hasselgren PO (2002) Proteasome inhibitors induce heat shock response and increase IL-6 expression in human intestinal epithelial cells. *Am J Physiol Regul Integr Comp Physiol* **282**:R1016-1026.

Pyatt DW, Stillman WS, Yang Y, Gross S, Zheng JH and Irons RD (1999) An essential role for NF-kappaB in human CD34(+) bone marrow cell survival. *Blood* **93**:3302-3308.

Romano MF, Lamberti A, Bisogni R, Garbi C, Pagnano AM, Auletta P, Tassone P, Turco MC and Venuta S (1999) Amifostine inhibits hematopoietic progenitor cell apoptosis by activating NF-kappaB/Rel transcription factors. *Blood* **94**:4060-4066.

Romano MF, Petrella A, Bisogni R, Turco MC and Venuta S (2003) Effect of NF-kappaB/Rel inhibition on spontaneous vs chemotherapy-induced apoptosis in AML and normal cord blood CD34+ cells. *Leukemia* **17**:1190-1192.

Russell JS and Tofilon PJ (2002) Radiation-induced activation of nuclear factor-kappaB involves selective degradation of plasma membrane-associated I(kappa)B(alpha). *Mol Biol Cell* **13**:3431-3440.

Shojaei F, Gallacher L and Bhatia M (2004) Differential gene expression of human stem progenitor cells derived from early stages of in utero human hematopoiesis. *Blood* **103**:2530-2540.

Singh VK, Shafran RL, Inal CE, Jackson WE, 3rd and Whitnall MH (2005) Effects of whole-body gamma irradiation and 5-androstenediol administration on serum G-CSF. *Immunopharmacol Immunotoxicol* **27**:521-534.

Stickney DR, Dowding C, Authier S, Garsd A, Onizuka-Handa N, Reading C and Frincke JM (2007) 5-androstenediol improves survival in clinically unsupported rhesus monkeys with radiation-induced myelosuppression. *Int Immunopharmacol* **7**:500-505.

Stickney DR, Dowding C, Garsd A, Ahlem C, Whitnall M, McKeon M, Reading C and Frincke J (2006) 5-androstenediol stimulates multilineage hematopoiesis in rhesus monkeys with radiation-induced myelosuppression. *Int Immunopharmacol* **6**:1706-1713.

Szalay L, Shimizu T, Suzuki T, Hsieh YC, Choudhry MA, Schwacha MG, Bland KI and Chaudry IH (2006) Androstenediol administration after trauma-hemorrhage attenuates inflammatory response, reduces organ damage, and improves survival following sepsis. *Am J Physiol Gastrointest Liver Physiol* **291**:G260-266.

Thompson JE, Phillips RJ, Erdjument-Bromage H, Tempst P and Ghosh S (1995) I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. *Cell* **80**:573-582.

Vanden Berghe W, Vermeulen L, De Wilde G, De Bosscher K, Boone E and Haegeman G (2000) Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. *Biochem Pharmacol* **60**:1185-1195.

Whitnall MH, Elliott TB, Harding RA, Inal CE, Landauer MR, Wilhelmsen CL, McKinney L, Miner VL, Jackson WE, Loria RM, Ledney GD and Seed TM (2000) Androstenediol

stimulates myelopoiesis and enhances resistance to infection in gamma-irradiated mice. *Int J Immunopharmacol* **22**:1-14.

Whitnall MH, Villa V, Seed TM, Benjack J, Miner V, Lewbart ML, Dowding CA and Jackson WE, 3rd (2005) Molecular specificity of 5-androstenediol as a systemic radioprotectant in mice. *Immunopharmacol Immunotoxicol* **27**:15-32.

Xiao M, Oppenlander BK and Dooley DC (2001) Transforming growth factor-beta(1) induces apoptosis in CD34(+)CD38(-/low) cells that express Bcl-2 at a low level. *Exp Hematol* **29**:1098-1108.

Zhou D, Yu T, Chen G, Brown SA, Yu Z, Mattson MP and Thompson JS (2001) Effects of NF-kappaB1 (p50) targeted gene disruption on ionizing radiation-induced NF-kappaB activation and TNFalpha, IL-1alpha, IL-1beta and IL-6 mRNA expression in vivo. *Int J Radiat Biol* **77**:763-772.

Footnotes:

Research Support: NIAID Interagency Agreement Y1–AI–3809–01 to AFRRI, Henry M. Jackson Foundation award 52762 to MW, and DoD Peer Reviewed Medical Research Program grant PR054180 to MW

Legends for figures

Figure 1. Effects of IR and 5-AED on human CD34+ cells. (A) Twenty-four h after IR or sham IR, apoptotic cell death was determined by annexin-V and 7AAD staining in a total of 3 experiments. Significant differences: a: CD34+ vs. CD34- cells, ($p<0.01$); b: radiation dose response ($p<0.05$, 2 Gy vs 4 Gy and 4 Gy vs 6 Gy); c: 5-AED vs. control ($p<0.05$). Means ± SD. (B) Cells were plated for clonogenic assays 24 h after IR or sham IR. 5-AED (1 μg/ml) was added to cultures for a 48 h period starting 24 h before IR or sham IR. Colonies were counted 14 days later. CFU-MIX: multipotential progenitors. CFU-GM: granulocyte-macrophage progenitors. CFU-E: very early erythroid precursor cells. BFU-E: earliest known erythroid precursor cells. Colony generation in all lineages was inhibited by IR, and this inhibition was ameliorated by 5-AED. Means ± SD. *$p<0.05$, **$p<0.01$ (5-AED vs. vehicle, for BFU-E and CFU-GM). (C) In separate cells from the same experiment, 5-AED enhanced survival (Trypan Blue assay) of CD34+ cells 7 days after IR. The starting cell number was 7.5 X 10^5 per culture as shown by the horizontal line. Means ± SD. **$p<0.01$ (5-AED vs. vehicle). Results (B and C) were from one representative experiment of a total of 5 independent experiments, and each experiment was performed in triplicate. (D, E) 5-AED administration was associated with a trend toward elevated frequency of CD11b+ cells as determined by flow cytometry from two independent experiments 7 (D) and 21 (E) days after IR.

Figure 2. 5-AED but not IR stimulated NFkB activation in CD34+ cells. (A) Activation of the anti-apoptotic transcription factor NFkB was analyzed with a DNA-binding ELISA assay as described in Materials and Methods. 5-AED (1μg/ml) was added to cultures for a 48 h period starting 24 h before IR. Background levels for the assay are shown by

the “5-AED + cold probe” values. Results were from a total of three experiments and each experiment was performed in triplicate. Means ± SD. *p<0.05, **p<0.01 (5-AED vs. vehicle control) (B) Western blot shows MG132 treatment (1 h, 0.1 μM) downregulated NFkBp65 levels in CD34+ cell nuclear extracts, suggesting NFkB relocation from cytoplasm to nucleus was inhibited. 5-AED blocked this MG132 effect. (C) Different doses of MG132 from 0.1 to 0.5 μM were added to CD34+ cultures 1 h before IR. 5-AED (1 μg/ml) or vehicle was added to cultures for a 48 h period starting 24 h before IR. Survival of CD34+ cells seven days after IR was assayed using Trypan Blue. MG132 concentrations are shown in the legend. MG132 treatment at every concentration caused significant decreases in numbers of surviving cells (p<0.01). The beneficial effects of 5-AED were abrogated by all concentrations of MG132. Data from a total of three experiments. (D) Western blot shows NFkBp65 and β-actin (loading control) expression in control, NFkBp65-siRNA transfected, maxGFP-siRNA transfected, and NFkBp65+maxGFPsiRNA co-transfected samples. (E) Cells were plated for clonogenic assays 48 h post-siRNA transfection and 24 h after IR. 5-AED (1 μg/ml) was added to cultures for a 48 h period starting 24 h before IR. Means ± SD. *p<0.05, **p<0.01 (5-AED treatment vs. control), # p<0.05, ## p<0.01 (NFkBp65-siRNA transfected cells vs. maxGFP-siRNA transfected cells, with or without 5-AED treatment).

Figure 3. **IR but not 5-AED induced IkB$_\beta$ release from NFkB/IkB complexes.** CD34+ cell lysates collected at 4 h after IR with or without 5-AED were subjected to immunoprecipitation using an NFkBp65 antibody. After SDS-gel separation, proteins were analyzed by immunoblotting using anti-NFkBp65, NFkBp50, IkB$_\alpha$, and IkB$_\beta$ antibodies.

Figure 4. Effect of 5-AED on NFkB expression in CD34+ cells. (A) NFkB gene expression was measured using multiplex QRT-PCR, and 18S rRNA as a control to calculate the relative quantity (RQ) of gene expression 4 and 24 h after 0 or 4 Gy IR with and without 5-AED administration. Means ± SD. *p<0.05, **p<0.01 (5-AED vs. vehicle). (B) Western blot determination of NFkBp65 and NFkBp50 subunit expression in whole cell lysates. NFkBp65 levels were similar after all treatments. Immunoblot using anti-NFkBp50 antibody on the same membrane after anti-NFkBp65 antibody was stripped showed NFkBp50 protein degradation and a low molecular weight fragment (27-32 kD, tentatively identified as “small fragment”, small arrows on gels) after IR (4 and 6 Gy). Addition of 5-AED before or after IR decreased levels of the low molecular weight fragment, and when 5-AED was administered both before and after IR (“dual”), the small fragment disappeared.

Figure 5. Effects of 5-AED on IL-6 and G-CSF expression in irradiated CD34+ cells. (A) A cytokine antibody array was used with pooled serum-free culture medium from CD34+ cultures with and without IR and 5-AED. (B, C) 5-AED (1 μg/ml) was added to cultures for a 48 h period starting 24 h before IR. IL-6 (B) and G-CSF (C) gene expression was determined by QRT-PCR. (D, E) 5-AED stimulated IL-6 (D) and G-CSF (E) secretion as determined by ELISA. Means ± SD. *p<0.05, **p<0.01 (5-AED vs. vehicle, and unirradiated vs. 2 Gy for G-CSF level). (F, G) Levels of IL-6 (F) and G-CSF (G) were measured 48 h after IR. The NFkB inhibitor MG132 (0.1 μM) was added 1 h before IR. 5-AED or vehicle (1 μg/ml) was added for a 48 h period starting 24 h before IR. Means ± SD. *p<0.05, **p<0.01 (vs. vehicle-treated control)

Figure 6. **The beneficial effects of 5-AED are dependent on G-CSF but not IL-6 or changes in p53 levels.** G-CSF (A, B) or IL-6 (C) neutralizing antibody (1 μg/ml), or nonspecific IgG from the same species, was added to the culture medium 1 h before cell addition. Following this preincubation period, 5 x 10^5 CD34+ cells were added, with or without 5-AED administration, and cells were irradiated 24 h later. 24 h after IR, cells were plated for colony assays. Means ± SD. *$p<0.05$, **$p<0.01$ (5-AED vs. vehicle) (D) p53 protein levels were determined by Western blot. p53 in unirradiated cells was undetectable. In contrast, IR (4 Gy) dramatically induced p53 expression in CD34+ cell cultures after 4 and 24 h. 5-AED had no effect on p53 levels. NFkBp65 and β-actin protein levels (used as controls) were assayed on the same membrane after p53 antibody was stripped.

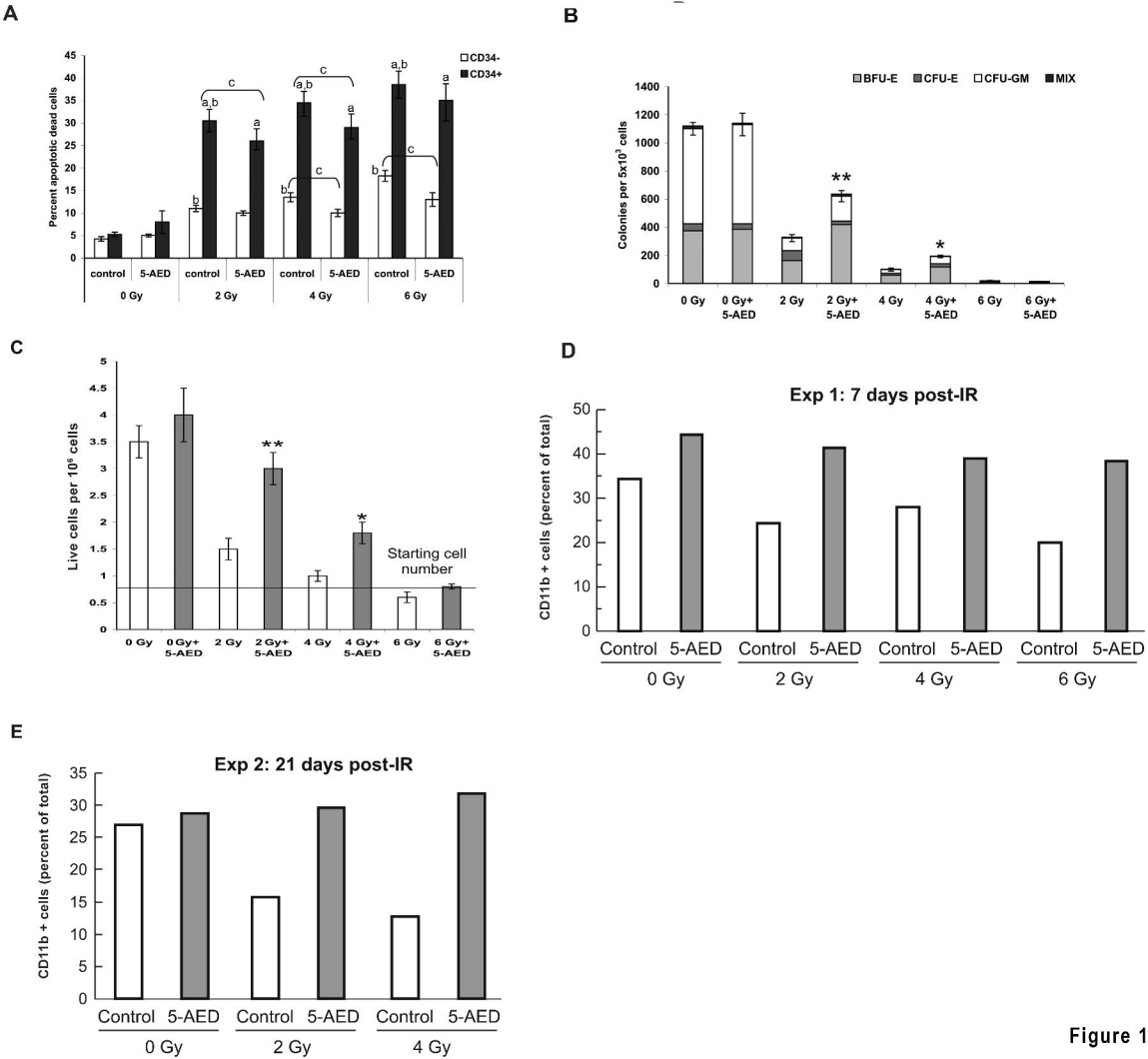

A
Percent apoptotic dead cells
CD34-
CD34+
control
5-AED
0 Gy
2 Gy
4 Gy
6 Gy
B
BFU-E
CFU-E
CFU-GM
MIX
Colonies per 5x10^3 cells
0 Gy
0 Gy+ 5-AED
2 Gy
2 Gy+ 5-AED
4 Gy
4 Gy+ 5-AED
6 Gy
6 Gy+ 5-AED
C
Live cells per 10^6 cells
Starting cell number
D
Exp 1: 7 days post-IR
CD11b + cells (percent of total)
Control
5-AED
E
Exp 2: 21 days post-IR

Figure 1

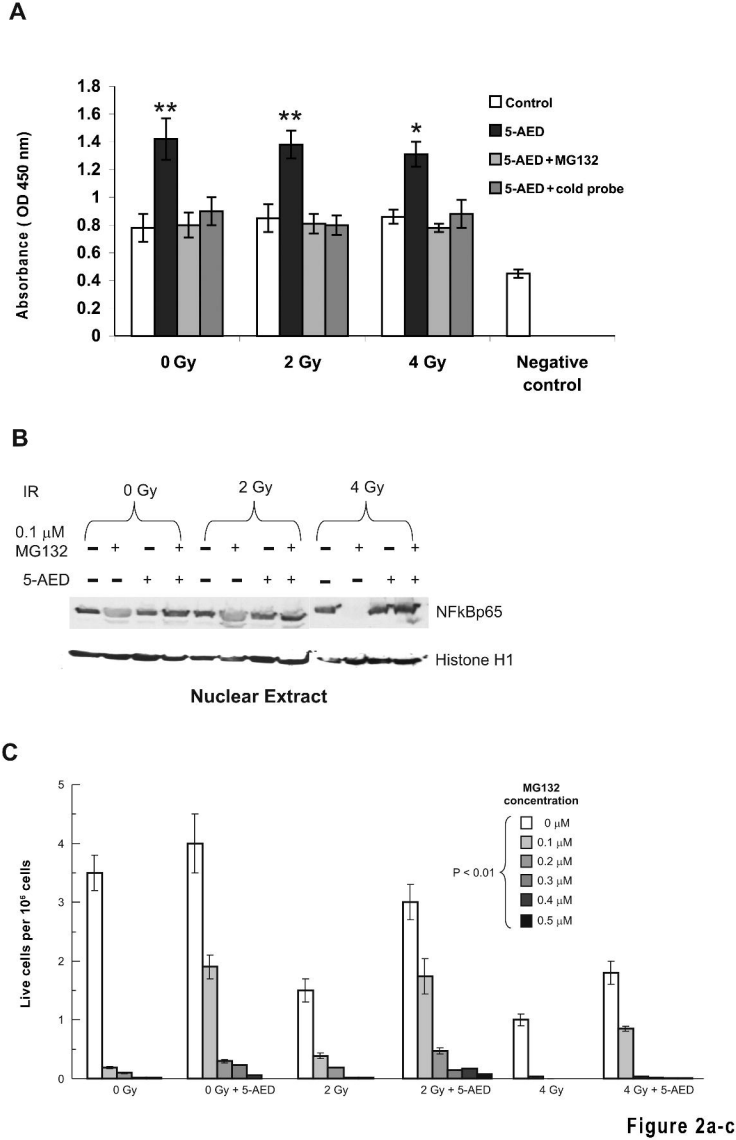
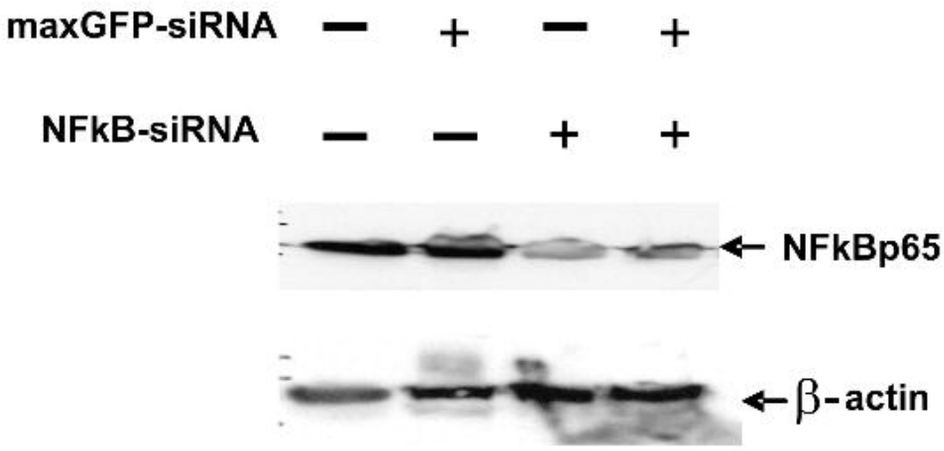

A
Absorbance (OD 450 nm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
*
Control
5-AED
5-AED + MG132
5-AED + cold probe
0 Gy
2 Gy
4 Gy
Negative control
B
IR
0 Gy
2 Gy
4 Gy
0.1 μM MG132
5-AED
NFkBp65
Histone H1
Nuclear Extract
C
Live cells per 10^5 cells
MG132 concentration
0 μM
0.1 μM
0.2 μM
0.3 μM
0.4 μM
0.5 μM
P < 0.01
0 Gy
0 Gy + 5-AED
2 Gy
2 Gy + 5-AED
4 Gy
4 Gy + 5-AED

Figure 2a-c

D



E

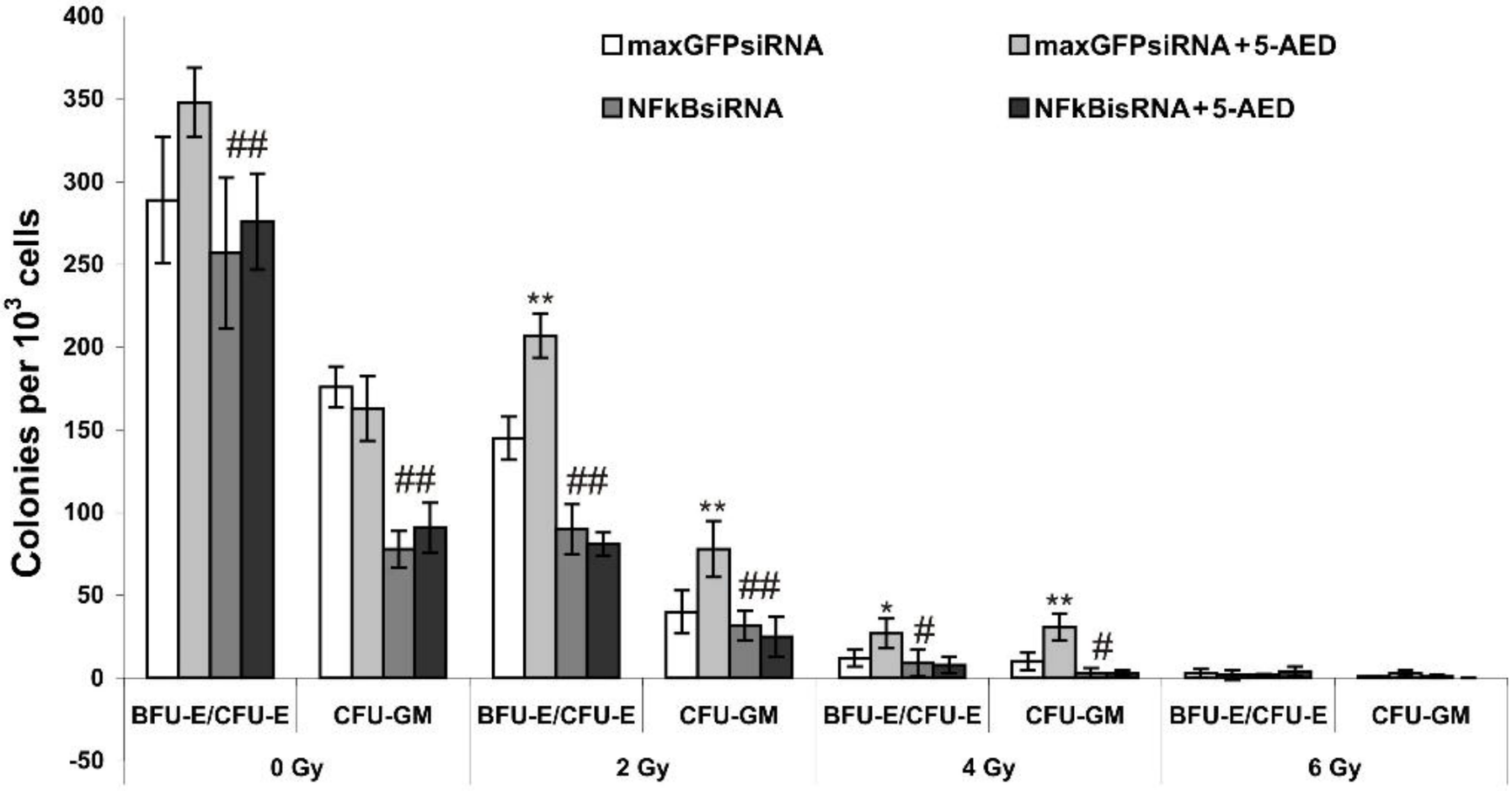
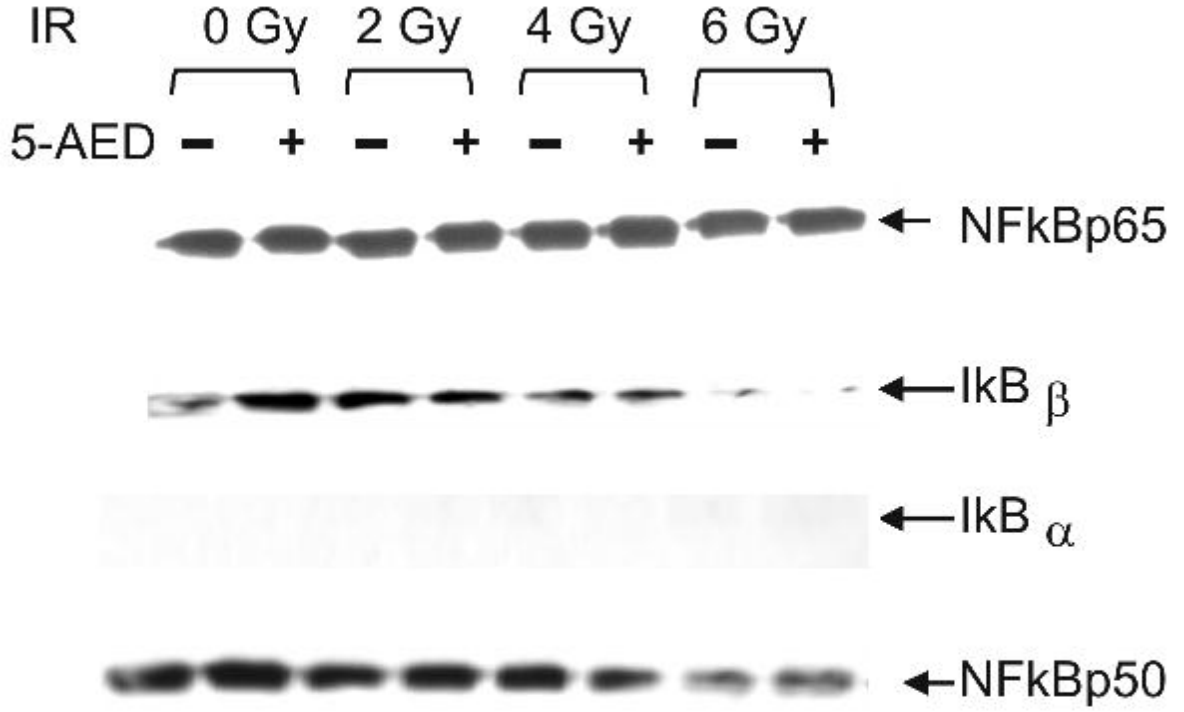


Figure 2d-e



IP: anti-NFkBp65

Figure 3

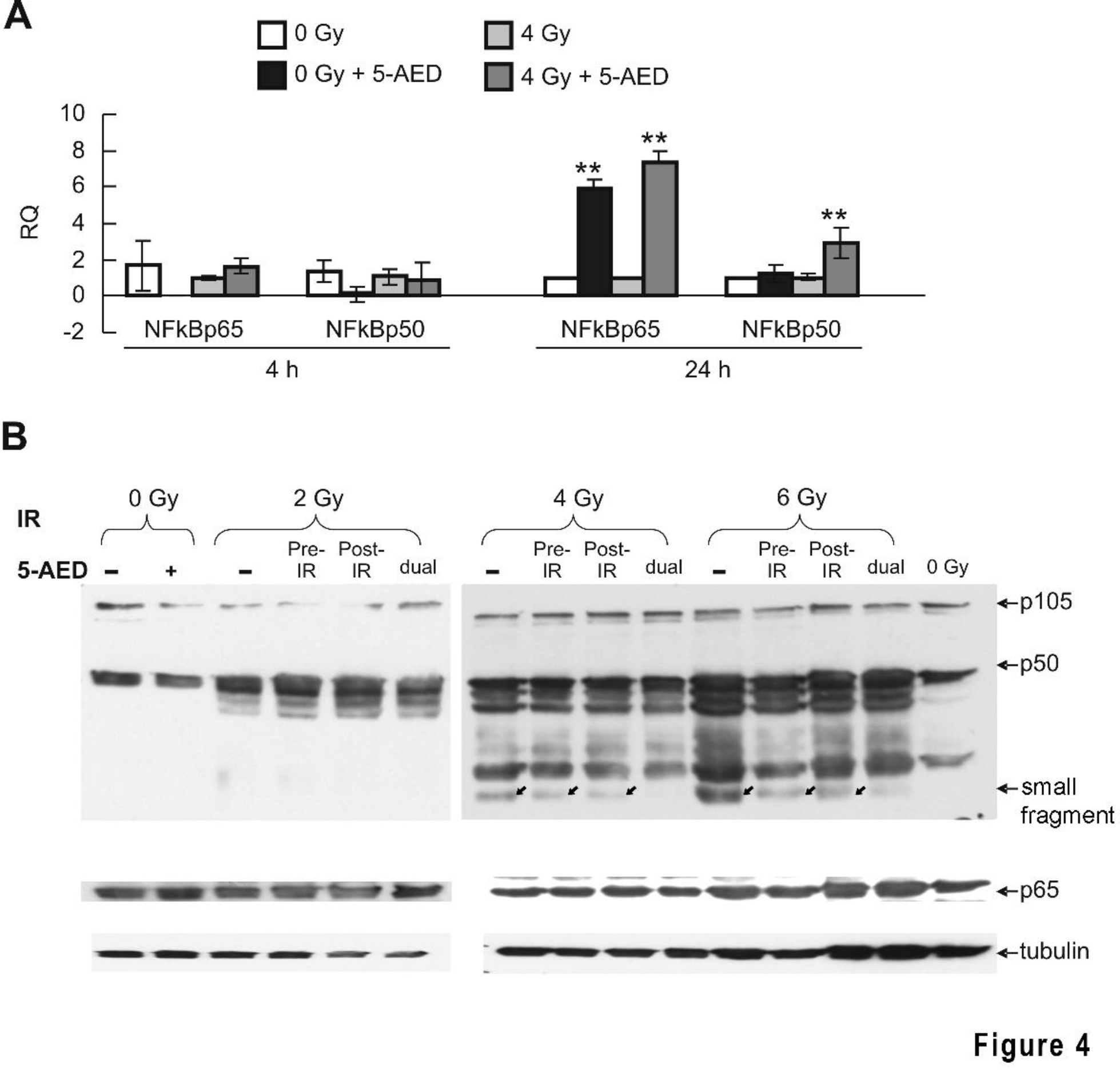

A
0 Gy
0 Gy + 5-AED
4 Gy
4 Gy + 5-AED
RQ
10
8
6
4
2
0
-2
**
**
**
NFkBp65
NFkBp50
NFkBp65
NFkBp50
4 h
24 h
B
IR
5-AED
0 Gy
2 Gy
4 Gy
6 Gy
Pre-IR
Post-IR
dual
0 Gy
p105
p50
small fragment
p65
tubulin

Figure 4

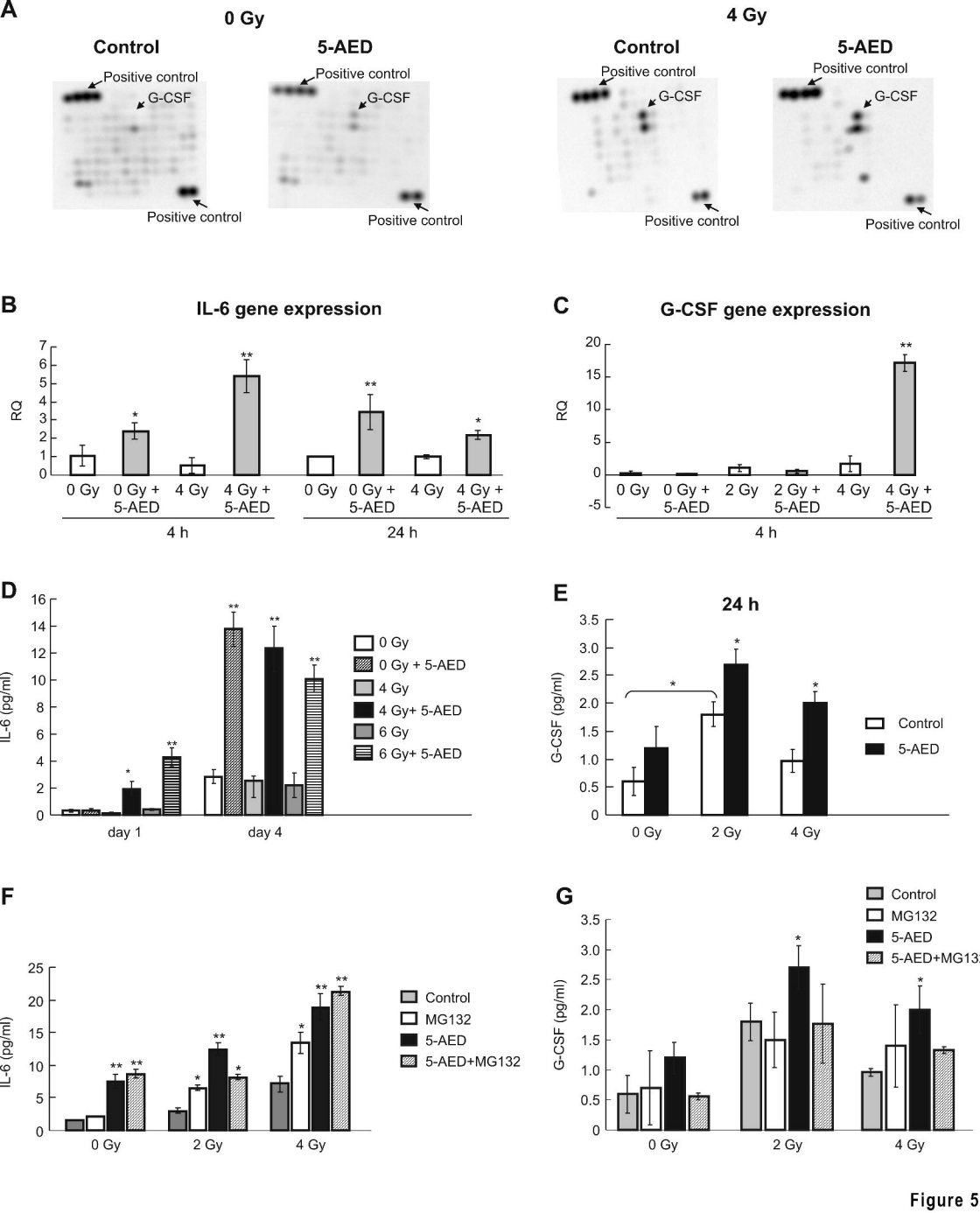

A
0 Gy
Control
5-AED
Positive control
G-CSF
Positive control
Positive control
G-CSF
Positive control
4 Gy
Control
5-AED
Positive control
G-CSF
Positive control
Positive control
G-CSF
Positive control
B
IL-6 gene expression
RQ
0 Gy
0 Gy + 5-AED
4 Gy
4 Gy + 5-AED
0 Gy
0 Gy + 5-AED
4 Gy
4 Gy + 5-AED
4 h
24 h
C
G-CSF gene expression
RQ
0 Gy
0 Gy + 5-AED
2 Gy
2 Gy + 5-AED
4 Gy
4 Gy + 5-AED
4 h
D
IL-6 (pg/ml)
0 Gy
0 Gy + 5-AED
4 Gy
4 Gy+ 5-AED
6 Gy
6 Gy+ 5-AED
day 1
day 4
E
24 h
G-CSF (pg/ml)
Control
5-AED
0 Gy
2 Gy
4 Gy
F
IL-6 (pg/ml)
Control
MG132
5-AED
5-AED+MG132
0 Gy
2 Gy
4 Gy
G
G-CSF (pg/ml)
Control
MG132
5-AED
5-AED+MG132
0 Gy
2 Gy
4 Gy

Figure 5

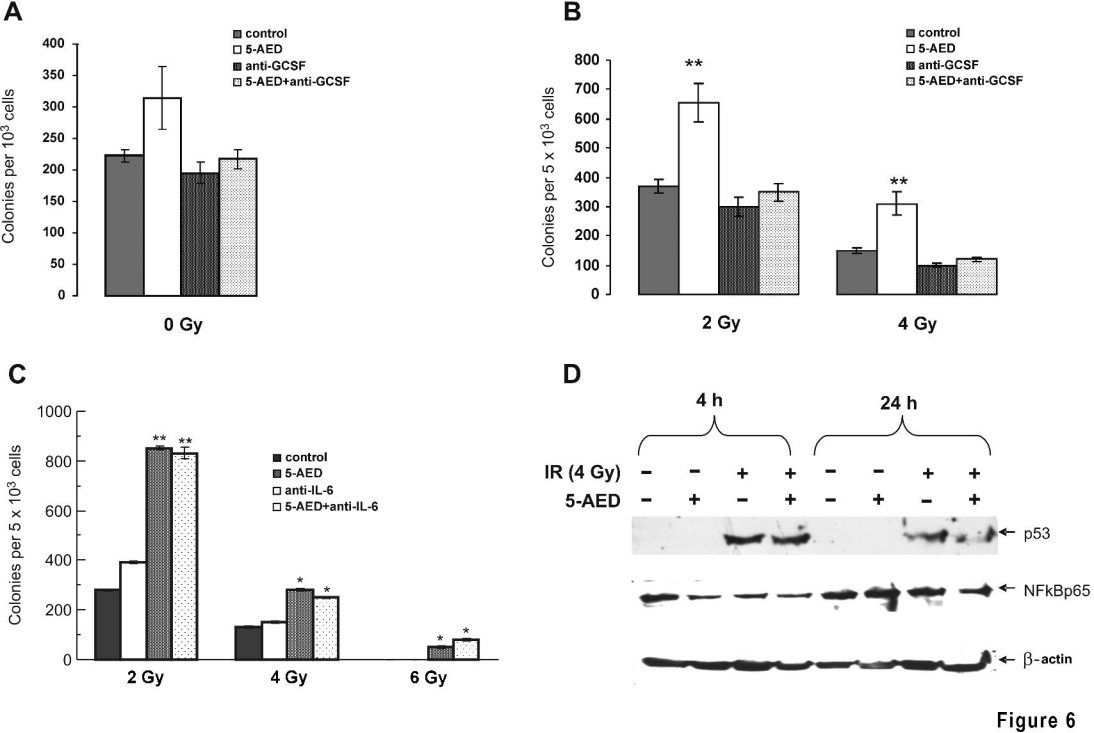

A
control
5-AED
anti-GCSF
5-AED+anti-GCSF
Colonies per 10^3 cells
400
350
300
250
200
150
100
50
0
0 Gy
B
control
5-AED
anti-GCSF
5-AED+anti-GCSF
Colonies per 5 x 10^3 cells
800
700
600
500
400
300
200
100
0
**
**
2 Gy
4 Gy
C
control
5-AED
anti-IL-6
5-AED+anti-IL-6
Colonies per 5 x 10^3 cells
1000
800
600
400
200
0
**
**
*
*
*
*
2 Gy
4 Gy
6 Gy
D
4 h
24 h
IR (4 Gy) - - + + - - + +
5-AED - + - + - + - +
p53
NFkBp65
β-actin

Figure 6