

SURVIVAL AND SIGNALING CHANGES IN ANTIGEN PRESENTING CELL
SUBSETS AFTER RADIATION

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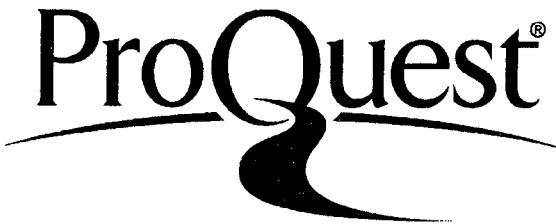
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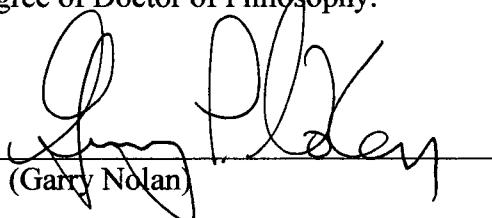
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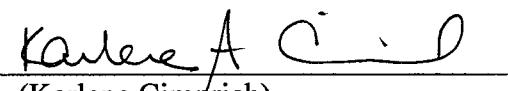
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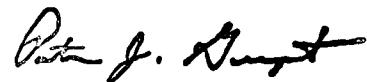
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ABSTRACT

Radiation therapy is a widely used cancer treatment that has the potential to influence anti-tumor immune responses. Both myeloablative and non-myeloablative radiation are often used as part of preparatory regimens for hematopoietic stem cell transplantation, in combination with other chemotherapy or immuno-modulatory (e.g. Anti-thymocyte globulin (ATG)) therapies for both cytotoxic and immune modulatory purposes. However, the mechanisms responsible for the effect of radiation on antigen presenting cell (APC) responsiveness and radioresistance are poorly understood.

The first studies described in this thesis were designed to identify and characterize early radiation-induced signaling changes in antigen presenting cells and to determine the effects of these signaling changes on APC receptor expression and function. The NF κ B pathway in antigen presenting cells was chosen for study because it is activated by radiation in a wide range of other cell types and plays a vital role in the maintenance and regulation of the immune system. The effects of therapeutically relevant doses radiation (2 and 20 Gy) were compared at various timepoints in the human monocytic cell line (U937) using phospho-flow cytometry staining methods and cytometric analysis. These studies demonstrated that radiation-induced changes in the phosphorylation state of NF κ B family members that were p53 independent. However, these changes were dependent upon activation of ATM in response to single or double-stranded breaks in DNA, as shown in experiments using an inhibitor of ATM and ATM siRNA knockdown U937 cells. In addition, studies examining the effect of radiation on co-stimulatory receptors with and without inhibition of the NF κ B pathway via phospho-flow cytometry

revealed that radiation-induced phosphorylation of NEMO promoted the activation and functional maturation of U937 cells. Furthermore, functional studies using both phospho-flow cytometry and/or mixed lymphocyte reactions to examine co-stimulatory receptor activation, pro-inflammatory cytokine release, and T cell proliferation with and without radiation and inhibition of the NF κ B pathway, demonstrated that NEMO is necessary for the activation, maturation, and enhanced responsiveness of human subsets of antigen presenting cells that occur after radiation. These findings provided insight into the mechanism of action of radiation-enhanced promotion of the antigen presenting cell responses. The methods of analysis employed can be used for monitoring immune changes that impact immune modulation in transplantation and tumor vaccines studies. Furthermore, NF κ B pathway proteins have the potential to serve as biomarkers for optimal anti-tumor responses. The NBD peptide may also have usefulness as a therapeutic agent for inhibition of graft versus host disease (GVHD) in patients who have undergone transplantation.

While the first set of experiments focused on antigen presenting cell responsiveness, the second set of experiments were designed to enhance our understanding of why antigen presenting cells, specifically monocytes and dendritic cells, are more radioresistant than conventional T cells. Flow cytometric analysis of various surface markers and intracellular signaling markers were used to examine the mechanisms behind the radioresistance of antigen presenting cells. The experiments described here showed a hierarchy of radiosensitivity among T cells, with naïve CD8 T cells being the most radiosensitive and CD4 memory T cells being the most radioresistant. Antigen presenting cells were found to be significantly more

radioresistant than T cell subsets (<10 fold decrease after radiation), and among APC, monocytes were more radiosensitive than either total or conventional dendritic cells. Furthermore APC expressed lower levels of Bax after radiation than T cells, and APC subsets that expressed high levels were also more sensitive to radiation induced cell death. These results demonstrate that T cell and APC subsets are dying by apoptosis after radiation, and that the differential level of Bax expression is an important determinant of the relative radiosensitivity of these immune cell subsets. Again, these findings are clinically relevant to transplant patients and patients with tumors receiving radiation therapy since APC survival may have importance for the generation of anti-tumor immunity and post-transplantation immune sequelae such as GVHD. In addition, elucidation of the mechanism of death of APC and T cell subsets, as described in chapter 3, provides potential markers of cell death that can be correlated to good graft versus tumor (GVT) effects versus bad (tumor recurrence and persistence) GVT effects. Thus, understanding the mechanistic basis for radiation-induced changes in APC and the effect of these changes on survival and function is essential for optimizing the use of radiation in transplantation and tumor vaccine treatment protocols.

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CHAPTER 1

INTRODUCTION

Ionizing Radiation Cellular Effects

Ionizing radiation has many cellular effects which ultimately lead to cell death (apoptosis), effects on cell cycle, activation of DNA repair mechanisms, changes in gene expression, activation of signal transduction pathways, and mutations which leads to genomic instability (Halperin et al., 2008). Exposure of tumor cells to ionizing radiation causes DNA damage and mitochondrial-dependent generation of reactive oxygen species which cause DNA-damage and result in DNA breaks (Kitagawa and Kastan 2005). This DNA damage causes changes in chromatin which results in autophosphorylation of Ataxia Telangiectasia Mutant (ATM) dimers and in turn, results in the release of phosphorylated and active ATM monomers. These monomers can phosphorylate other substrates (Kitagawa and Kastan 2005; Xu 2006). DNA-dependent protein kinase (DNA-PK) is activated early by DNA damage while ATM and Rad3-related protein (ATR) is activated later. Activation of these sensor proteins in turn promotes the activation of receptors and intracellular signaling pathways and stimulates cell cycle checkpoints, p53 activity, and DNA repair mechanisms (Meyn et al. 1996; Robles et al. 2001). P53 plays a role in cell cycle arrest and is necessary for DNA repair. It also initiates apoptosis through both mitochondrial and death receptor pathways by upregulating Bax, Bad, and death receptor (CD95) ligand expression ((Burns, Bernhard, and El-Deiry 2001; Guan et al. 2001; Herr et al. 1997; Sheard et al. 1997; Zhou and Elledge 2000)).

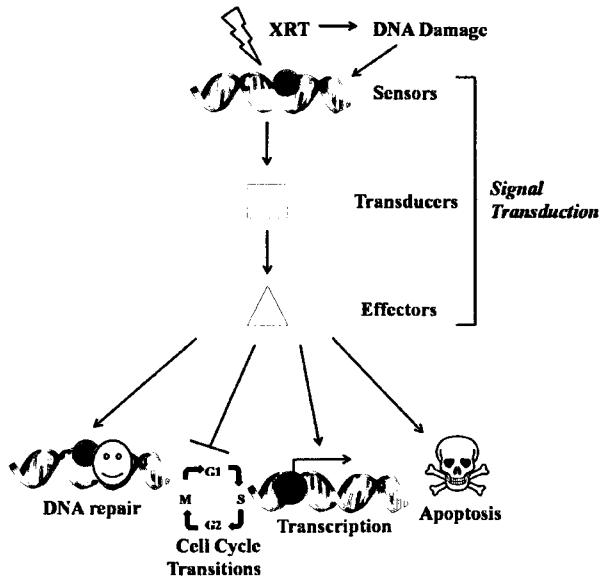


Figure 1. Four outcomes of DNA damage

Radiation-induced apoptosis can also occur via the c-Jun N-terminal kinase signaling pathway (Chen, Meyer, and Tan 1996; Chen et al. 1996; Verheij et al. 1998; Zanke et al. 1996) which is initiated by activation of acidic sphingomyelinase and increased the production of ceramide (generated from free-radical damage to lipid membrane) (Cock et al. 1998; Cremesti et al. 2001; Herr et al. 1997; Radford 1999; Tepper et al. 1999). Ceramide release leads to activation of receptor and nonreceptor tyrosine kinases and the activation of downstream signal transduction pathways. The generation of ROS and ceramide production also contribute to the other radiation-mediated effects.

used in allogeneic hematopoietic stem cell transplantation (HSCT) protocols for treatment of various hereditary and malignant diseases for decades (Main and Prehn 1955). HSCT is an important cornerstone of leukemia and lymphoma treatment, especially for high risk recurrent disease. However, the morbidity and mortality associated with myeloablative radiation used in stem cell transplantation has been a barrier to using HSCT as adoptive immunotherapy for most hematologic and other diseases.

Radiotherapy and Immunotherapy Combined

Total lymphoid irradiation (TLI) (radiation that selectively irradiates the primarily lymph node bearing regions and spleen), was the first utilized for treatment of Hodgkin's disease and non-Hodgkin's lymphoma (Hoppe 1987); Kaplan 1980). TLI was the first non-myeloablative host-conditioning regimen shown to induce tolerance and mixed chimerism following MHC-mismatched bone marrow transplantation in various animals (Slavin et al. 1978; Slavin et al. 1979; Strober et al. Transplantation tolerance after total lymphoid irradiation 1979; Strober et al. Allograft tolerance after total lymphoid irradiation (tli) 1979; Waer et al. 1990). During and immediately after TLI there are alterations in the proportions of T-cell subsets in the peripheral lymphoid system (Field and Strober 2001). Furthermore, when TLI is used with anti-thymocyte globulin (ATG), there are graft versus leukemic or lymphoma (GVL) benefits without the risk of acute graft versus host disease (GVHD). This is due to an increase in regulatory NKT cells, which act as "natural suppressors" that are present immediately post-transplantation (Lan et al. 2003; Lan et al. 2001). The same concept of TLI+ATG in preventing acute GVHD

Ionizing Radiation as a Cytotoxic Agent

Ionizing radiation has cytotoxic effects or the ability to kill cancer cells, thereby making it an important modality in the treatment of cancer and eradication of tumors. Different cell types and tissues possess different sensitivities to radiation. In general, actively proliferating cells are most sensitive to radiation since the mitotic activity of all cells decreases with maturation and cellular radiosensitivity tends to be inversely related to cell differentiation. Furthermore, the sensitivity of an organ depends on the relative sensitivity of the tissues and cells that make up the organ. Thus some tumors, depending on their cellular and tissue composition, are more sensitive to radiation than others (Perez and Brady 1998). Though ionizing radiation results in the elimination of the more radiosensitive fraction of cancer cells present within a tumor at the time of radiation exposure, no significant correlation exists between responsiveness of a tumor to irradiation and radiocurability (Halperin et al. 2008).

Lymphoid organs and bone marrow are among the most sensitive tissues in the body to direct radiation injury (Halperin et al. 2008). Total-body irradiation leads to a drastic fall in the number of circulating B and T lymphocytes whereas macrophages which give rise to monocytes, are less radiosensitive than lymphocytes (Hall and Giaccia 2006). The sensitivity of lymphoid organs and bone marrow to radiation has been exploited, with radiation being used both a cytotoxic agent and to suppress the immune response (e.g. to inhibit effects of antigen presentation to immunologically competent cells). Thus, the cytotoxic and immunosuppressive properties of radiation have been

and promoting GVL after HSCT was tested in humans. Lowsky, initial reported results from thirty-seven patients with malignant lymphoid diseases or acute leukemia treated with a regimen of TLI+ATG, which was followed by an infusion of HLA-matched peripheral-blood mononuclear cells from related or unrelated donors. Only two of these patients had acute GVHD and anti-tumor effects of GVL contributed to the increase in the complete remission rate (2005). Furthermore, radiation for lymphodepletion and/or myeloablation also has utility for the induction of solid organ allograft tolerance (Fuks and Slavin 1981; Scandling et al. 2008), and autoimmune diseases (Fuks and Slavin 1981; Strober 1987).

Immunotherapy clinical applications for cancer have progressed to include: the adoptive transfer of anti-tumor reactive T cells (cytotoxic T lymphocytes) (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005; Paulos et al. 2007); the use of therapeutic vaccines(Antonia, Mule, and Weber 2004; Luznik et al. 2003); strategies based on dendritic cells, strategies based on cytokines; and antibodies that promote tumor-specific T cell activation (Demaria et al. Immune-mediated inhibition of metastases after treatment with local radiation and cta-4 blockade in a mouse model of breast cancer 2005). Though there are currently immunotherapy trials in the clinic focused on eradicating advanced tumors in patients, radiation therapy also has a possible role of enhancing overall tumor immunogenicity.

Radiation is also being combined with different modalities to enhance the anti-tumor response. Examples of preclinical studies of radiotherapy combined with immunotherapy are described here. One example involves combined radiotherapy and immunotherapy strategies that are based upon dendritic cells. Fms-like tyrosine kinase

receptor 3 ligand (Flt3-L) is a growth factor which stimulates production of dendritic cells and has been shown to induce antitumor immunity to mouse tumors in combination with radiation (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005; Lynch et al. 1997; Maraskovsky et al. 1996). The first study that tested the combination of Flt3-L and local high-dose (60Gy) radiation therapy used the Lewis lung model mice of metastatic carcinoma and found that lung metastases were inhibited and disease free-survival was enhanced as compared to mice that were treated with radiation therapy or Flt3-L alone (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005). Another study showed that local tumor irradiation could be combined with IL-2 (cytokine that promotes T cell proliferation) and/or adoptive transfer of tumor-infiltrating lymphocytes to obtain a synergistic anti tumor effect (Cameron, Spiess, and Rosenberg 1990; Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005). Other cytokines have also been studied in combination with radiation such as IL-3, IL-12, and TNF α (Antonia, Mule, and Weber 2004; Chiang et al. 2000; Chiang et al. 1997; Eder, Geissler, and Ganser 1997; Oh et al. 2004). There are also strategies that target Toll-like receptors (TLR's). TLR's can result in dramatic immune responses including the induction of cytokines, activation of natural killer cells, and elicitation of strong T cell responses when dendritic cells and B cells are activated. Oligodeoxynucleotides that contain CpG motifs and bind to TLR 9 have been shown to enhance anti-tumor immunity (Krieg 2004). Administration of CpG oligodeoxynucleotides actually has a synergistic effect with local radiation and was able to control tumor growth in solitary tumors of immunogenic sarcoma (Fsa) in mice. Finally, studies have examined the use of antibody-mediated

blockade of the CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) molecules. CTLA-4 molecules are expressed on the surface of Helper T cells and transmit an inhibitor signal to T cells. Blockade of CTL-4 alone did not affect primary tumor growth and metastatic spread in a mouse 4T1 adenocarcinoma (metastatic breast cancer) model. However, combined radiation therapy with CTL-4 blockade induced a CD8+ T cell-mediated antitumor response. This combination inhibited metastases outside the irradiated field and increased the overall survival of the mice (Antonia, Mule, and Weber 2004).

The use of radiotherapy as a component of tumor vaccine strategies is an active area of preclinical investigation. In the past, tumor vaccines alone often did not generate a sufficient immune response to be efficacious and most tumors escaped immune destruction. Radiation therapy in combination with vaccines may be a useful strategy for enhancing tumor immunogenicity. In fact, a clinical study reported on the use of a recombinant cancer vaccine combined with radiotherapy in patients with localized prostate cancer (Gulley et al. 2005; Gulley, Madan, and Arlen 2007) in which patients who received radiation therapy between their fourth and sixth vaccinations showed evidence of de novo generation of T cells to prostate-associated antigens not present in the vaccine. This provided indirect evidence of the induction of immunity to patient tumor antigens (Gulley et al. 2005; Gulley, Madan, and Arlen 2007). Local radiation therapy has also been strategically used as a component of a vaccine strategy in combination with intratumoral CpG with some success (Brody et al. 2009). Recently, there has also been interest in combining radiation therapy and immunotherapy in vaccine protocols.

Therefore, effects of radiation on tumor responses and tumor immunity can be related, and immune modulation is required for successful HSCT and to enhance the efficacy of anti-tumor therapy treatments.

Ionizing Radiation and Antigen Presenting Cells

The danger model of immunity proposed by Matzinger is relevant to the study of radiation effects. This model suggests that antigen presenting cells are activated by danger or alarm signals from injured cells , (e.g. in response to exposure to pathogens, toxins, or mechanical damage) (Matzinger 1994, 2002). Furthermore, these “danger signals” emitted from tissues comprise cytokines, chemokines, inflammatory mediators, and heat-shock proteins which induce maturation and activation of antigen presenting cells (Gallucci and Matzinger 2001; McBride et al. 2004; McLellan, Brocker, and Kampgen 2000).

Radiation eliciting “danger signals” can affect innate and adaptive immunity (comprised of humoral and cell immunity). Innate immunity is mainly antigen nonspecific and is comprised of physical barriers, soluble factors (complement and chemokines), and effector cells which include natural killer cells, monocytes, and macrophages (Clark et al. 2000; Delves and Roitt The immune system. First of two parts 2000, The immune system. Second of two parts 2000). In contrast adaptive immunity is antigen-specific and involves antigen recognition and capture, processing, presentation, lymphocyte function, and immunologic memory (Delves and Roitt The immune system. First of two parts 2000, The immune system. Second of two parts 2000). Antigen-

presenting cells serve as the critical link between innate and adaptive immunity (Hartgers, Figdor, and Adema 2000).

Antigen presenting cells are vital to the induction and regulation of antigen-specific cellular immune responses. Dendritic cells (DC) are bone marrow-derived mononuclear cells found in blood and many peripheral tissues (O'Neill, Adams, and Bhardwaj 2004). DC are a heterogeneous population of cells produced in the bone marrow in response to growth and differentiation factors. There are three stages of differentiation for all DC subtypes: DC precursors, immature DC, and mature DC (O'Neill, Adams, and Bhardwaj 2004; Shortman and Liu 2002). In human blood, immature DC and DC precursors are lineage-negative ($CD3^-CD14^-CD19^-CD56^-$) HLA-DR⁺ mononuclear cells (Banchereau et al. 2000) and are divided into 2 populations that are considered conventional or non-conventional. These populations are separated by staining with antibodies to CD11c and CD123 (interleukin 3 receptor α). CD11c⁺CD123⁻ DC have a monocyteoid appearance and are called myeloid DC (MDC), whereas CD11c⁻CD123⁺ DC have morphologic features similar to plasma cells and are called plasmacytoid DC (PDC). Although commonly used, this nomenclature is somewhat misleading. Experiments in mice indicate that both DC populations can be derived from Flt3-expressing myeloid and lymphoid progenitors (D'Amico and Wu 2003; Karsunky et al. 2003; O'Neill, Adams, and Bhardwaj 2004). The plasmacytoid DC are important in innate and antiviral immunity, are found primarily in blood and lymphoid organs, and are the principal interferon α (IFN α)-producing cells in the body. Plasmacytoid DC can activate antitumor and antiviral antigen responses (Fonteneau et al. 2004; Salio et al. 2003) but they are difficult to obtain in sufficient quantities so their role in immune

modulation and immunotherapy has not been well studied. Myeloid DC are found in tissues besides blood and lymphoid organs and are capable of capturing and presenting antigens and producing cytokines, primarily TNF (Iwasaki and Medzhitov 2004).

Dendritic cells capture antigens best when they are “immature”. When they undergo differentiation and become mature, they become cells specialized for T cell stimulation. Immature DC capture bacteria, viruses, dead or dying cells, proteins, and immune complexes through phagocytosis, endocytosis, and pinocytosis. Dendritic cells process the captured proteins that are loaded onto major histocompatibility complex class I (MHC Class I) molecules expressed by most nucleated cells and major histocompatibility complex class II (MHC class II) molecules expressed by monocytes, macrophages, dendritic cells and B cells molecules. These peptide-MHC complexes are transported to the cell surface for recognition by antigen-specific T cells. The molecules MHC I and MHC II are recognized by CD8⁺ and CD4⁺ T cells, respectively (Guermonprez et al. 2002). Antigens can be acquired endogenously, meaning they are derived from intracellular proteins which are then processed and loaded onto MHC I (Thery and Amigorena 2001; Watts and Amigorena 2000). Interaction of the MHC molecule with the TCR allows cytotoxic lymphocytes, in the presence of IFN γ and IL-2 secretion from helper T cells, to proliferate and attack cells expressing particular antigen recognized by these cells. The killing of these target cells takes place through the perforin/granzyme-B or death receptor (Fas/FasL) mediated apoptosis (O'Neill, Adams, and Bhardwaj 2004). Antigens acquired exogenously or from the extracellular environment are processed onto MHC II. Peptides are also processed by the exogenous pathway which utilizes extracellular antigen taken up by APC. These proteins are

processed into peptide fragments and loaded onto MHC Class II molecules (Delamarre, Holcombe, and Mellman 2003). This antigen MHC complex is presented on the APC surface where it is recognized by an antigen-specific TCR of a CD4+ helper T cell which drives the T cell to secrete cytokines.

There is also a pathway called cross presentation that allows DC to process exogenous antigens on MHC Class I molecules. An alternative pathway also exists whereby DCs process exogenous antigens onto MHC I. This pathway allows APC to induce CD8⁺ and CD4⁺ T-cell responses to exogenous antigens like apoptotic or necrotic tumor cells, virus-infected cells, heat shock proteins, DNA or RNA encoded antigens, organisms like bacteria, viruses, virus like particles, exosomes, and soluble proteins (Fonteneau, Larsson, and Bhardwaj 2002; Guermonprez et al. 2003; Guermonprez et al. 2002). Cross-presentation is the primary way tumor antigens are presented to T cells (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005). Furthermore, in order for T cells to become activated, engagement of the T cell receptors with antigen bound to MHC class molecules and co-stimulatory signals is required. During APC maturation there is increased expression of MHC Class II, CD40, CD80, and CD86. The interaction of CD28 on the cell with CD80 and CD86 provides for this interaction (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005).

Ionizing Radiation Effects on the Immune System

As above, radiation affects the immune system by eliciting the “danger response” by playing a role in the process of antigen presentation and APC maturation, and thereby immune activation. More specifically radiation induces cellular expression of HLA-molecules (MHC Class I and Class II) and co-stimulatory molecules CD80 and CD86 (Liu et al. 2001; Morel et al. 1998; Sojka et al. 2000; Vereecque et al. 2000). Furthermore, radiation induces expression of immunomodulatory cytokines such as IL-1 β , IL-12, and TNF α which activate APC and cause proliferation of APC (Hallahan et al. 1989; Hong et al. 1999; Ishihara et al. 1993; Rieser et al. 1997; Rovere et al. 1998). Radiation also upregulates heat shock protein such as gp96 and hsp70 on tumors, which may have an effect on APC maturation and activation, as well as antigen transfer to APC (Abdulkarim et al. 2000; Feng et al. 2001; Melkonyan, Ushakova, and Umansky 1995; Somersan et al. 2001; Stammmer and Volm 1996); death receptors (CD95) and MHC class I on tumors recognized by APC (Abdulkarim et al. 2000; Albanese and Dainiak 2000; Belka et al. 1998; Jones et al. 2001; Nishioka et al. 1999; Ogawa and Ohashi 1997; Sheard 2001). Radiation also induces inflammatory mediators such as COX-2 and Prostaglandin E2 (PGE2), which enhance the overall “danger milieu” with secondary APC activation/maturation (Chen et al. 2001; Rieser et al. 1997; Steinauer et al. 2000). Without these signals, immature APC present antigen to T cells in the absence of necessary cytokines and costimulatory molecules, resulting in antigen-specific immunologic tolerance. Since radiation has such important effects on the way APC’s respond, and thereby how T cells act, understanding the mechanisms responsible for

radiation mediated modulation of the immune response is necessary for optimizing transplantation and anti-tumor protocols that use radiation. Radiation's elicitation of these "danger signals" in the microenvironment, not only results in activation and maturation of dendritic cells, increased antigen presentation, and enhanced tendency of tumor cells to either die or be recognized (Friedman 2002; Teitz-Tennenbaum et al. 2008), but it facilitates homing of both antigen-presenting and effector T cells to the tumor by chemokine release from the tumor cells (Ganss et al. 2002; Nikitina and Gabrilovich 2001; Zong et al. 2006). Radiation also causes upregulation of adhesion molecules such as CD31, CD54, CD62E, CD62P, CD144, CD106 on tumors which promote leukocyte adhesion chemotaxis.

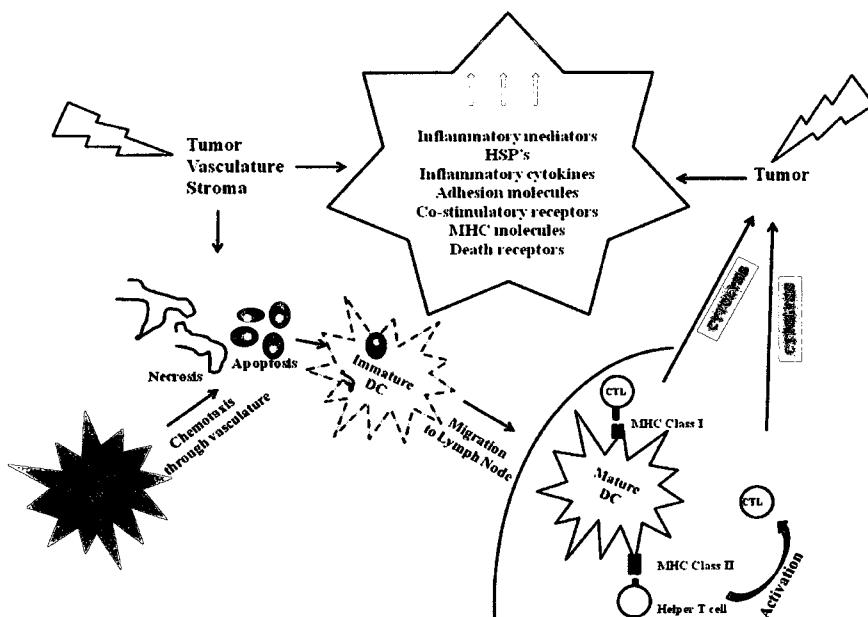


Figure 2. Overview of radiation's modulation of the immune system.

Radiation damage to tumor, vasculature, and stroma results in cellular apoptosis and necrosis. Radiation upregulates expression of inflammatory mediators, heat shock

proteins, inflammatory cytokines, adhesion molecules, costimulatory receptors, death receptors, and MHC molecules which comprises the “danger” microenvironment. Immature DC traffic to this area and phagocytose apoptotic and necrotic debris and mature DC migrate to lymph nodes where processed antigen is presented to T cells via exogenous or cross-presentation antigen processing pathways. CD4+ T helper cells are activated after interacting with the MHC Class II/antigen complex and subsequently induce CD8+ cytotoxic T cells (CTL’s) to promote cytolysis. CTL’s also interact with MHC Class I/antigen complex to induce an antigen-specific cytolytic attack.

Monocytes and U937 Cell Line

While dendritic cells are the most potent of the antigen presenting cells, monocytes also play an important role in the induction and regulation of antigen-specific responses. Monocytes have important regulatory and effector roles in innate and adaptive immunity and are the precursors for macrophages in tissues (Dale, Boxer, and Liles 2008). Monocytes also serve as precursors to dendritic cells. Studies have found dendritic cells as the terminal stage of monocyte differentiation following stimulation (+ of GM-CSF/IL-4) (Palucka et al. 1998). Monocytes have Fc receptors and express the IgG receptor Fc_γRI (CD64) constitutively. Monocytes and their differentiated progeny play important regulatory and effector roles in innate and adaptive immunity (Medzhitov and Janeway 2000; Unanue 1980). Monocytes have three major functions which include presentation of antigens, phagocytosis, and immunomodulation. Activated monocytes and macrophages also release cytokines such as IL-1, IL-6, TNF, and INF- α/β that play a role

in regulating hematopoiesis. Monocytes are also subject to immune modulation by chemokines. Monocytes, macrophages, and dendritic cells express microbial pattern-recognition receptors that recognize and detect pathogen-associated molecular patterns, resulting in their activation. A well-known group of these receptors are the Toll-like receptors (Brightbill et al. 1999; Medzhitov and Janeway 1997; Trinchieri and Sher 2007). Upon binding of specific ligands, TLRs signal via a pathway to activate Nuclear Factor Kappa B (NF κ B) and stimulate proinflammatory cytokine production from monocytes and macrophages (Medzhitov et al. 1998).

There are also human in-vitro cell lines which function as monocytes/macrophages. U937 cells, derived from a histiocytic lymphoma, exhibit monocyte morphology, and function as APC's (Sundstrom and Nilsson 1976). U937 cells have been shown to be activated and pushed to terminal monocytic differentiation by: phorbol 12-myristate 13-acetate (PMA) (Harris and Ralph 1985; Kaneko, Ikeda, and Nakanishi 1999), supernatants from human mixed lymphocyte cultures, phorbol esters, vitamin D3, gamma interferon, tumor necrosis factor (TNF) and retinoic acid (Koren, Anderson, and Larrick 1979; Olsson et al. 1983). They also express a basal level of major histocompatibility complex (MHC) class I and II and co-stimulatory molecules (CD86) as shown via FACS, with upregulation of MHC class II and I and co-stimulatory molecules after stimulation (Buggins et al. 1999; Passmore, Lukey, and Ress 2001). U937 cells also express adhesion molecules ICAM-1 (CD54), LFA-1 (CD11a), and Mac-1 (CD11b) (Buggins et al. 1999). Furthermore, they have the ability to phagocytose and present mycobacterial antigens to HLA-I class matched cytotoxic T lymphocytes (Passmore, Lukey, and Ress 2001).

An in-depth understanding of how radiation affects APC is crucial since these cells are responsible for the induction and regulation of antigen-specific responses. To date, the molecular mechanisms by which radiation affects immune responses have not been well studied. This is particularly important for early events in specific APC subsets that ultimately affect antigen presentation and APC maturation/activation.

The NF κ B pathway is one of the signaling pathways activated by radiation in tumor cells. It plays an important role in the apoptotic response (whether or not this role is anti-apoptotic or pro-apoptotic depends on the cell type) as well as in the maintenance and regulation of the immune system.

Nuclear Factor Kappa B

One of the primary roles of NF κ B is in the immune system. The transcriptional gene products that are characteristic of early events in the immune responses often result in the activation and modulation of NF κ B. Therefore, NF κ B mediates several critical changes that are characteristic of innate and adaptive immune responses (Hayden, West, and Ghosh 2006). NF κ B's role in innate immunity is mediated through induction of genes encoding cytokines, chemokines, enzymes, and antimicrobial peptides (Ghosh and Karin 2002; Schmitz, Bacher, and Dienz 2003; Zhang and Ghosh 2000). In addition, NF κ B's roles in adaptive immunity include its antiapoptotic effects (controls survival of B cells, T cells, monocytes, and dendritic cells (Steinauer et al. 2000), induced expression of MHC proteins, costimulatory molecules such as B7.1 (CD80), and cytokines such as IL-2, IL-12 and IFN- γ . Thus, NF κ B effects cell differentiation, DC development, and

cell proliferation (Hayden, West, and Ghosh 2006), making it an important pathway for the study of APC function/responsiveness after radiation .

The NF κ B family is composed of five related transcription factors: p50, p52, RelA (aka p65), c-Rel and RelB. These transcription factors share a highly conserved Rel Homology Domain (RH), which contains sequences for dimerization allowing them to form homodimers or heterodimers and bind to a variety of target DNA sequences, also known as kB sites, to modulate gene expression (Hayden, West, and Ghosh 2006; Magne et al. 2006). RelA, c-Rel, and RelB also contain C-terminal transcription activation domains (TADs), which enable them to activate target gene expression. In the classical or canonical pathway, NF κ B complexes are inactive in most cells. They reside in the cytoplasm in a complex with inhibitory I κ B proteins (in particular I κ B α). When the pathway is activated, NF κ B activation is mediated by an I κ B kinase complex (IKK). The IKK complex consists of two catalytically active kinases (IKK α and IKK β) and a regulatory scaffold protein, NF κ B Essential Modulator (NEMO). Phosphorylation and activation of NEMO is critical to phosphorylation and activation of IKK α and IKK β . Activation of this complex leads to phosphorylation of I κ B α . I κ B α is in turn degraded, NF κ B is phosphorylated and enters the nucleus to modulate target gene expression. (Hayden, West, and Ghosh 2006; Magne et al. 2006; Scheidereit 2006). In the canonical pathway, IKK β and NEMO are required for the activation of the p50/p65 complex whereas IKK α is relatively dispensable. In the non-canonical pathway, IKK α solely controls the activation of complexes that are inhibited by the I κ B protein p100. Both pathways are activated by distinct sets of stimuli and target distinct genes.

In addition, NF κ B is an important regulator of apoptosis (Magne et al. 2006). This was demonstrated in several studies where inactivation of NF κ B or NEMO resulted in increased apoptotic sensitivity of cells to various stimuli or stressors (Bottero et al. 2001; Van Antwerp et al. 1996; Wang, Mayo, and Baldwin 1996) or cell killing . NF κ B also regulates anti-apoptotic genes by interfering with apoptotic signaling. A well known example is in the TNF receptor I signaling pathway. In this pathway TNF receptor is coupled to the caspase cascade via the FADD adaptor. However, TNF also activates NF κ B via TRADD and TRAF adaptors. NF κ kB induces transcription of genes that code for anti-apoptotic proteins like c-IAP1 and 2 which block caspase function directly or indirectly (Deveraux and Reed 1999). NF κ B also plays a role in the mitochondrial-mediated apoptotic pathway as it transcribes the genes coding for several anti-apoptotic proteins (Chen et al. 2002; Deveraux and Reed 1999). NF κ B has been shown to interfere with the induction of bax in carcinomas (Bentires-Alj et al. 2001). It can also promote or amplify cell death by inducing genes that code for the death receptor Fas or its ligand FasL. This is the case during elimination of activated T cells in the Activation-Induced Cell Death response (Ravi, Bedi, and Fuchs 1998).

NF κ B and p53 can have opposite functions as was shown in a study where Oncogenic Ras initiated a p53-independent apoptotic response that was suppressed through the activation of NF κ B (Bellas, Lee, and Sonenshein 1995; Mayo et al. 1997). These two transcription factors seem to compete for interaction with transcriptional co-activators (Ravi, Bedi, and Fuchs 1998; Wadgaonkar et al. 1999). However, another study showed that p53 can be induced by NF κ B and that activation of NF κ B correlated

with the ability of p53 to induce apoptosis (Ryan et al. 2000). Overall, the interaction between NF κ B and p53 appears to be quite complex.

Importantly, NF κ B is activated by radiation in tumors. For example, radiation has been reported to induce activation of NF κ B and enhance DNA binding (p65 and p50) approximately four fold in human breast cancer MCF-7 cells shown by electrophilic mobility shift assay (EMSA) (Wang et al. [the nuclear factor kappa b activation: The key step of cell proliferation in estrogen receptor-negative breast cancer cells] 2005). In another study, total body irradiation induced NF κ B activation in intestinal epithelial cells in a dose and time-dependent manner in gel supershift assays (Wang et al. 2004).

Inhibition of NF κ B has been studied as a means of radiosensitizing tumor cells (Pajonk, Pajonk, and McBride 1999; Russell et al. 2002; Yang et al. 2000). In one study HD-My-Z Hodgkin cells were treated with the proteasome inhibitor MG-132, which induced apoptosis in HD-My-Z cells and sensitized them to ionizing radiation (Pajonk, Pajonk, and McBride 1999). In another study of a head and neck carcinoma cell line, dexamethasone significantly decreased NF κ B DNA-binding activity and enhanced baseline apoptosis and radiosensitivity (Didelot et al. 2001). In contrast, the blockade of NF κ B activation with a super-repressor did not improve cell death of irradiated prostate carcinoma or Hodgkin lymphoma cell lines (Magne et al. 2006).

Overall, NF κ B's role in regulation and development of the immune system and its activation by genotoxic stress (radiation) in tumors make this pathway a prime candidate for exploring acute radiation-induced signaling changes in APC. I hypothesized that early events in the NF κ B pathway functionally modify certain immune cells, particularly radioresistant APC (that survive weeks longer than T cells after radiation), and performed

experiments to elucidate the poorly understood role of the NFκB pathway in radiation-induced changes in APC.

The goal of this research (presented in chapter 2) was to characterize acute radiation induced changes in a human monocytic cell line and human antigen presenting cell subsets at the molecular and cellular level, and to identify markers associated with effective immune modulation. Thus, the specific aims were to:

- 1) Utilize phospho-flow cytometry to identify phospho-specific changes resulting from cell stress conditions in mutant p53 and wild-type p53 U937 cells.
- 2) Elucidate the mechanism of action for radiation-induced phosphor-specific changes identified.
- 3) To determine the physiological and functional significance of radiation-induced phosphor-specific signaling changes in antigen presenting cells from healthy donor peripheral-blood mononuclear cells (PBMC).

In attempting to understand how radiation affects the immune system and how radiation modifies APC, it was important to determine what was responsible for the greater radioresistance of antigen presenting cells as compared to T cells. Furthermore,

dendritic cells are particularly clinically relevant since they survive and are present in the early post-transplant period.

Although it is known that APC are relatively radioresistant, it is not known why antigen presenting cells, in particular monocytes and dendritic cells are more radioresistant than conventional T cells (e.g. role of the mitochondrial-mediated apoptotic pathway in this radioresistance has not been studied). I hypothesized that the selective difference (i.e. differences in sensitivity to radiation) in T cell subsets after radiation would be due to variation in the expression of Bax and there would be a selective difference in APC subsets after radiation due to varying Bax expression as well. The primary objectives of the experiments described in chapter 4 were to:

- 1) Further elucidate radiation-induced changes in the balance of human T cell subsets including CD4+ and CD8+ total T cells, CD4-CD8- T cells, CD4+ naïve and memory T cells, and CD8 naïve and memory T cells.
- 2) Determine if there is a selective difference in human APC subsets including monocyte and dendritic cells after radiation.
- 3) Analyze the role of Bax and Bcl-2 expression in the relative radioresistance of antigen presenting cells.

In the past, changes at the molecular level have been difficult to elucidate using standard biochemical analysis techniques due to low numbers of PBMC subsets after

radiation treatment. As a result, I used the phospho-flow cytometry technique for the majority of human immune cell subset studies. Phospho-flow cytometry allows for rapid and accurate analysis of the phosphorylation state of proteins within immune cellular subsets in complex, heterogenous populations, post-irradiation, to be analyzed accurately and rapidly. In addition, pFCS provides information about the functional responses of these cells to radiation (Krutzik et al. 2004).

The results presented here, provide important and clinically relevant insight into how radiation affects the immune system at the level of signal transduction pathways, and has near term translational potential.

CHAPTER 2

CHARACTERIZATION OF RADIATION-INDUCED IMMUNE SIGNALING CHANGES IN ANTIGEN PRESENTING CELLS

Abstract

Radiation therapy is a widely used cancer treatment and pre-transplantation conditioning regimen that has the potential to influence anti-tumor and post-transplantation immune responses. Although conventional fractionated radiation doses suppress certain types of immune responses by depleting lymphocyte subsets, we proposed that we could identify radiation-induced signaling changes in antigen presenting cells that enhance the function of specific subsets of antigen presenting immune cells. Using phospho-flow cytometry analysis of human monocytic cell line (U937), we identified novel radiation-induced changes in the phosphorylation state of NF κ B family members which are known in other cell types to play a vital role in the maintenance and regulation of the immune function. We found that although these phosphorylation changes were p53 independent, they were strongly dependent upon activation of ATM in response to single or double-stranded breaks in DNA. We demonstrated that radiation promotes the activation and functional maturation the antigen presenting cells, and that radiation-induced phosphorylation of NF κ B Essential Modulator (NEMO) is necessary for this functional effect. Furthermore, we found that NEMO is necessary for the activation, maturation, and responsiveness of human subsets of human antigen presenting

cells, such as monocytes and conventional dendritic cells from peripheral blood mononuclear cells. In summary, we identified changes in the NF κ B pathway that are required for radiation-induced antigen presenting cell activation and function, and the methods of our analysis are especially well suited to the study of functional changes in immune cell subsets. These findings provide insight into the mechanism of action of radiation-enhanced promotion of the antigen presenting cell response, and these methods are especially useful for monitoring immune changes that impact immune modulation in transplantation and tumor vaccine studies.

Introduction

It is well known that irradiation of tumors can directly kill cancer cells as well as tumor endothelial cells and other cells within the tumor stroma, resulting in vascular damage, particularly to small vessels within tumors, which can have secondary effects on tumor cell viability (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005; Watters 1999). Radiation can also have important effects on tumor infiltrating lymphocytes and antigen presenting cells within tumors (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005; Watters 1999) by promoting the recruitment and homing of antigen presenting and effector T cells to the irradiated tumor site (Ganss et al. 2002; Nikitina and Gabrilovich 2001). For example, tumor antigens released from tumor cells killed by radiation can serve as a source of tumor antigens for dendritic cell (DC) uptake and presentation of these antigens to T cells within the tumor microenvironment (Larsson, Fonteneau, and Bhardwaj 2001). Tumor immunity has been induced by exogenously administrating dendritic cells intravenously, subcutaneously, or intratumorally following local tumor radiation (Demaria and Formenti 2007; Kim et al. 2004; Nikitina and Gabrilovich 2001; Teitz-Tennenbaum et al. 2003). In other studies, administration of Fms-like tyrosine receptor 3 ligand (Flt3-L) enhanced DC mediated induction of anti-tumor T cell responses when used in combination with tumor irradiation (Chakravarty et al. 1999; Teitz-Tennenbaum et al. 2003). Other studies have demonstrated indirect activation of DC by radiation via LPS released from the gut following total body irradiation, with subsequent toll-like receptor activation, that enhanced the effectiveness of adoptively transferred tumor-specific CD8+ T cells, and resulted in tumor regression (Paulos et al. 2007). More recently, local radiation therapy

has been strategically used as a component of a vaccine strategy in combination with intratumoral CpG with some success (Brody et al. 2009).

Radiation can induce innate immune stimulatory signals (Matzinger 2002; McBride et al. 2004), including the release of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-2) (Hallahan et al. 1989; Hong et al. 1999; Ishihara et al. 1993; Rieser et al. 1997), and upregulation of co-stimulatory molecules, heat shock proteins, death receptors, and major histocompatibility complex molecules (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005; Friedman 2002; McBride et al. 2004; Teitz-Tennenbaum et al. 2008). These “danger signals” can lead to maturation and activation of antigen presenting cells (APC), which allow APC to process and present ingested antigen to T cells (Gallucci and Matzinger 2001; McLellan, Brocker, and Kampgen 2000) released by damaged and dead cells in irradiated tumors.

The molecular mechanisms of the radiation-mediated induction of these “danger signals” have not been studied (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005; McBride et al. 2004). Therefore, the goal of the studies described here, was to elucidate or identify early radiation-induced signaling changes in antigen presenting cells and elucidate the effects of these signaling changes on APC receptor expression and function. For our initial experiments, we used the U937 cell line (Sundstrom and Nilsson 1976) derived from a histiocytic lymphoma, that exhibits monocyte morphology and functions as an APC. The model was selected because it provides a homogenous population of cells, arrested in a pliant state of maturation (Harris and Ralph 1985). Thereafter, we used fresh peripheral blood

mononuclear cells (PBMC) from normal individuals to confirm the results obtained with the monocytic cell line and assess the influence of irradiation on human APC.

We focused on the NF κ B pathway since it is activated after radiation in many cell types, regulates cell death mechanisms, and has well-known regulatory roles in the immune system (Hayden, West, and Ghosh 2006). This pathway is activated by radiation in tumors and modulates the expression of various apoptotic and anti-apoptotic genes (Verheij and Bartelink 2000). It is also a critical regulator in the development and maintenance of the immune system, controls the survival of T cells, B cells, and APC (Magne et al. 2006), and regulates immune events, such as the cytokine transcription, microbial phagocytosis, cell differentiation, and cell proliferation (Hayden, West, and Ghosh 2006; Scheidereit 2006). In the classical or canonical pathway, NF κ B complexes are inactive in most cells and reside in the cytoplasm in a complex with inhibitory I κ B proteins (in particular I κ B α). When the pathway is activated, NF κ B activation is mediated by an I κ B kinase complex (IKK). The IKK complex consists of two catalytically active kinases (IKK α and IKK β) and a regulatory scaffold protein, NF κ B Essential Modulator (NEMO). Phosphorylation and activation of NEMO is critical to phosphorylation and activation of IKK α and IKK β , and activation of this complex leads to phosphorylation of I κ B α , which is then degraded and releases NF κ B, allowing it to be phosphorylated and enter the nuclear to modulate target gene expression (Baldwin 1996; Scheidereit 2006). We hypothesize that radiation-induced effects on early events in the NF κ B pathway, may activate or functionally modify certain immune cells, particularly radioresistant APC. The experiments described here were designed to elucidate the poorly understood role of the NF κ B pathway in radiation-induced changes in APC,

which are relevant to better understanding the mechanism by which radiation can enhance tumor immunity.

Materials and Methods

Cells and Culture

Human monocytic cells U937 (obtained from Laboratory of Dr. G. Nolan, Stanford University, Stanford, California, originally from ATCC repository) were cultured in RPMI-1640 medium supplemented with 5% fetal calf serum, 1mM glutamine, penicillin/streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C with normal oxygen content or under hypoxic conditions (2% O₂).

Human peripheral blood mononuclear cells (PBMC) (buffy coats of healthy donors obtained from the Stanford Blood Center, Palo Alto, California) were cultured (RPMI-1640 medium supplemented with heat-inactivated 10% human serum, 2mM glutamine, 100U penicillin/ml, 100µg streptomycin/ml) in a humidified atmosphere containing 5% CO₂ at 37°C with normal oxygen content or under hypoxic (2% O₂) conditions.

Irradiation

Cells were irradiated with γ -rays by a ¹³⁷Cs source emitting at a fixed dose rate of 148 cGy/min or 531 cGy/min.

Monoclonal Antibodies and Chemical Reagents

Anti-HLA-DR-APC, Anti-CD3-FITC, Anti-CD4-PE, Anti-Lineage Negative Cocktail-FITC, Anti-Alexa Fluor 700-CD11b, Anti-CD11c-V450, Anti-CD14-APC, Anti-CD80-PE-Cy5, Anti-CD86-PE, Anti-IL-4-PE, Anti-FACS stain buffer, and Anti-Mouse IgK/Negative Control (FBS) Compensation Particles Set were purchased from BD Pharmingen (San Diego, CA). Anti p-IKK γ (NEMO) (S376)-FITC, Anti-pNFkB p65

(S536)-Alexa Fluor 488, Anti pIκB α (Ser32) Rabbit mAb, and Anti- γ H2AX-FITC were purchased from Cell Signaling Technology Corp. (Danvers, CA). LIVE/DEAD fixable dead cell stain kit in Aqua was purchased from Invitrogen, Inc. (Camarillo, CA).

Flow Cytometry Analysis

U937 cells were harvested and washed with PBS and stained for viable cell populations using LIVE/DEAD Fixable Green Dead Cell Stain or Ethidium Monoazide (EMA)(5 μ g/ml; Invitrogen Camarillo, CA). U937 cells were stained for surface markers and/or fixed with 1.5% paraformaldehyde and permeabilized with 4°C 100% methanol while vortexing, washed with FACS stain buffer, and stained with appropriate antibodies to detect intracellular proteins. Thresholds for staining (surface and intracellular) were determined using the isotype-matched mAbs. For flow cytometry on whole blood, whole blood is stained for viable cell populations, surface markers to identify monocytes and dendritic cells, lysed with BD Lysing solution (1X) and fixed and permeabilized before staining with appropriate antibodies to detect intracellular proteins.

Monocyte/Macrophage Isolation

Human PBMC layer was isolated from buffy coats using Ficoll-Hypaque density gradient centrifugation. Buffy coats (8-10 mls) were diluted with phosphate-buffered saline (PBS). Diluted blood was layered onto Ficoll-Hypaque and centrifuged at 2,000 x rpm. Mononuclear layer was removed and washed three times with PBS at 1200 x rpm and viability was assessed with Trypan Blue or whole blood was washed with PBS and then cells were either negatively selected with Untouched Human CD4 T cell Dynabeads,

Flow Comp CD14 Dynabeads, or Human DC Enrichment Dynabeads (Invitrogen, Camarillo, CA). Following each selection, the cells were counted, and viability, and purity were assessed by flow cytometry. Cells were aliquoted for treatment with 0 or 2Gy, and incubated for various times. The cells were stained with LIVE/DEAD fixable green dead cell stain kit (1:1000 in PBS) prior to final surface marker staining and analysis to exclude dead cells. For phospho-flow cytometry (intracellular staining), cells were first incubated with LIVE/DEAD aqua stain, washed with PBS, and stained with the appropriate anti-surface receptor mAbs in FACS stain buffer. Cells were fixed with 1.5% paraformaldehyde and permeabilized with 4°C 100% methanol, washed with FACS stain buffer, and incubated with intracellular antibodies at room temperature for 30 minutes. This intracellular staining method varied for human intracellular cytokine staining (kit by BD Biosciences). Cells were activated with leukocyte activation cocktail (10µl/1-2 x10⁶ cells/ml) and monensin (4µl/6mls culture) and incubated for 4 hours at 37°C, 5% CO₂ before staining for cytokines. Thresholds for staining (surface and intracellular) were determined using the isotype-matched control mAbs. All analyses were performed using the LSRII in the Shared FACS Facility (Center for Molecular and Genetic Medicine at Stanford University). Resultant data was analyzed using FlowJo software (TreeStar, Ashland, OR).

Wildtype p53 Transfection

pCMV-Neo-Bam p53 wt and pCMV-Neo-Bam empty control plasmids were purchased from Addgene Co. (Cambridge, MA). Plasmid DNA was purified by isopropanol precipitation by centrifugation using Plasmid Midi Kit (Qiagen, Valencia, CA). U937

cells were fed with fresh medium one day prior to transfection. The next day, cells were collected and suspended in Electroporation Medium (10 µl BioBrene Plus per 100 ml and 0.2% (w/v) Glucose in PBS). The DNA was suspended in 0.3 ml of Electroporation Medium at room temperature and mixed with 0.5 ml of the cell suspension (final volume was 0.8 ml) in an electroporation cuvette. The mixture was electroporated at 960 microfarads, 300 V, left in the cuvettes at room temperature for 5 min, and then transferred to 1 ml of medium in a 15 ml tube and incubated for 5 to 10 min at room temperature. Cells were resuspended in 4 ml of medium, and added to 10 ml of culture medium + G418 in a T-75 flask (15 ml total volume). To maintain a stable transfection culture, medium and G418 were replaced every two days. Cells were assayed for expression two days after transfection.

Cytoplasmic Extract Preparation and Western Blot Analysis

Cells were lysed in cell extraction buffer purchased from Invitrogen. (Camarillo, CA), supplemented with 1mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Nuclei were separated by centrifugation and sonicated cytoplasmic extracts were obtained. The protein concentration in each sample was determined using the method of Bradford Protein Assay. Samples, each containing 20 µg of cell lysate in loading buffer, were electrophoresed in 12% polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. Then immunoblotting was performed with various antibodies (Phospho-p53 (Ser15), phospho-ATM (S1981), ATM, Phospho-IKK-γ (Ser376), IKK-γ, DNA-PK, ATR, and actin) from Cell Signaling Technology Corp. (Danvers, MA). After washing the blots with TBS (1X)-Tween (1%), blots were incubated with anti-mouse IgG, or anti-rabbit IgG HRP-linked antibody.

Immunoreactivity of the blots was detected by ECL Western Blot Detection Reagent (Amersham Biosciences, Pittsburg, PA).

Assay for quantitative determination of GSH and GSSG levels

Intracellular GSH (reduced glutathione) and GSSG (glutathione disulfide) levels were measured using the GSH reductase recycling assay as previously described (Rahman, Kode, and Biswas 2006). Concentrations of GSH and GSSG were expressed as nmol/mg of protein and the ratios of GSSG/(GSH+GSSG) were calculated. GSH levels of cells were also measured by flow cytometry with Thiol Tracker Violet (Glutathione Detection Reagent) from Invitrogen, Camarillo, CA) following incubating the cells with the dye for 30 min at 37°C.

Transient siRNA Transfection of U937 Cells

These experiments used HP Validated (by quantitative RT-PCR) ATM siRNA from Qiagen (Valencia, CA), AllStars Negative (nonsilencing) Control siRNA (5nmol), HiPerfect transfection reagent and RNase free water, and were performed in an RNase-free environment with 20 μ M siRNA solutions. The day before the transfection, cells were seeded at 8 x 10⁴ cells per well in a 24 well plate with complete RPMI-1640 medium and allowed to incubate under normal growth conditions. On the day of transfection, siRNA at a final concentration of 5nM HiPerFect Transfection Reagent was added to siRNA and vortexed. The siRNA samples were incubated at room temperature for 5-10 minutes and added drop-wise to the cells while swirling. Cells were incubated under normal growth medium and allowed to incubate for 48 hours before use.

Culture for Mixed Lymphocyte Reaction

CD4+ T cells from human peripheral blood mononuclear cells (PBMC) were purified using indirect magnetic labeling for negative isolation of CD4 T cells with CD4+ T cell isolation kits (Miltenyi Biotech, Auburn, CA). 100,000 CD4+ T cells from a healthy donor were combined and incubated 1:10 (APC:T) with unirradiated or irradiated (2 or 20 Gy) U937 cells for up to 6 days in a humidified 37°C, 5% CO₂ incubator. U937 cells were irradiated and allowed to incubate for 48 hours before combining with CD4+ T cells.

Similar experiments were set-up with CD4+ T cells isolated from PBMC or whole blood using purified CD4 T cell Dynabeads (Invitrogen, Camarillo, CA) in which 500,000 CD4+ T cells were combined 1:5 (APC:T) with unirradiated or irradiated (2 Gy) CD14+ cells purified using CD14 Dynabeads (Invitrogen, Camarillo, CA) or purified Lineage – DC (CD3⁺CD14⁺CD16⁺CD19⁺CD20⁺CD56⁻) isolated from PBMC or whole blood by negative isolation using Human DC Enrichment Kit (Invitrogen, Camarillo, CA).

Cultures were incubated for up to 6 days in a humidified 37°C, 5% CO₂ incubator. Monocytes and DC were irradiated and allowed to incubate for 12 hours before combining with CD4+ T cells. In addition, CD4+ T cells isolated from whole blood were combined with CD4+ T cell depleted unirradiated and irradiated whole blood. Both U937 and human APC subset MLR experiments were set-up in 96 well plates to assess cell proliferation, cytokine production, and/or co-stimulatory molecule upregulation. Co-stimulatory molecule (CD80 and CD86) upregulation was assessed in APC subset MLRs only.

Cell Proliferation ELISA BrdU Assay

BrdU incorporation was measured using a kit supplied by Roche Applied Science (Indianapolis, IN). In this assay, 100,000 CD4+T cells were seeded with U937 cells at a 1:10 (APC:T) ratio and 500,000 CD4+ T cells were seeded with monocytes or dendritic cells at 1:5 (APC:T) ratios in 96-well culture dishes from human PBMC and from whole blood. The assay was prepared by incubating the cells for 24 hrs at 37°C with 10 µl of working BrdU stock in each well, followed by air drying at 60°C for 1hr, after which time 100 µl FixDenat solution was added for 30 min at room temperature. The FixDenat solution was removed and 100 µl Anti-BrdU-POD working solution was allowed to incubate at 37°C for 60 min. Plates were washed with PBS 3 times for 15 min each at room temperature. Finally, 100 µl of TMB substrate solution was incubated with the cells for 15 min at room temperature and 25 µl stop solution (H₂SO₄) was added before reading the plate. Absorbance was measured spectrophotometrically between 450 and 595nm after 4 days incubation for cultures of both U937 cells and monocytes and DC. Background (no BrdU label) was subtracted from measured absorbances. The mean absorbance±S.E.M. was calculated.

ELISA Cytokine Supernatant Assay

Single Analyte ELISArray Kits (SA Biosciences, Frederick, MD) for IL-4, TNF- α and IFN- γ were used to analyze cytokine production from MLRs per manufacturer's instructions using the standard sandwich technique. Cells were removed by centrifugation for 10 minutes at 1000 \times g. Supernatants were aliquoted and stored at \leq -20 °C until further use.

Co-stimulatory Molecules Assay

CD4+T cells were combined 1:5 (APC:T) with unirradiated and irradiated (2 Gy) monocytes and allowed to incubate for 12 and 48 hours at 37°C, 5% CO₂, as above. Monocytes were combined immediately with CD4+ T cells after being irradiated. Cells from the MLR were removed at the timepoints described above and stained for CD80 and CD86 expression. The same experimental design was used for CD4+T cells:DC MLR experiments.

Reagents

The following reagents were used in experiments as follows: 100% Methanol (Stanford Biosciences) and 16% Paraformaldehyde (Electron Microscopy Sciences, Hartfield, PA) used at 1.5% for intracellular staining Ficoll-Paque Plus (GE Healthcare Amersham Biosciences ,Piscataway, NJ); N-acetyl-L-cysteine, and L-Buthionine-sulfoximine, were purchased in solid form from Sigma-Aldrich (St. Louis, MO); IKK γ NEMO Binding Domain (NBD) Inhibitory Peptide Set including IKK γ NEMO Binding Domain (NBD) Inhibitor Peptide: 2 x 1 mg (lyophilized) (Imgenex, San Diego, CA); and ATM inhibitor, Morpholin-4yl-6-thianthren-1-yl-pyran-4-one (Ku55933) (Calbiochem, San Diego, CA).

Statistical Analysis Tool

To accurately compare changes in phosphorylation profiles of the NF κ B pathway after radiation, the median fluorescence intensity (MFI) was calculated using FlowJo software from Treestar Corp (Ashland, OR). Two-tailed Student's t-test (were used to calculate p-

values (Predictive Analytics SoftWare (PASW)) and GraphPad Sofware, San Diego, CA). Standard error of the mean (S.E.M) was calculated using GraphPad Sofware, San Diego, CA. For all tests, p value of 0.05 or less was considered significant.

Results

Radiation (XRT) induces phosphorylation of NF κ B members

To determine how XRT affects immunologically important signaling proteins in the U937 line, cells were irradiated with 2 or 20 Gy and allowed to incubate for various timepoints. Phospho-specific antibodies were used to identify XRT induced-changes in NF κ B pathway family members. XRT induced phosphorylation of NF κ B (Figure 1A) and NF κ B essential modulator (NEMO) (Figure 1B) in a dose-dependent manner, with the greatest activation of both at 4hr after 20 Gy, which subsequently declined over 16 hrs to near baseline levels. Representative histograms of phosphorylation of NEMO and NF κ B in U937 cells after 2 and 20 Gy at 4hr and 16hrs are shown in Supplementary Figure 1.

Since XRT leads to DNA damage from single and double stranded breaks we hypothesized that XRT-induced NF κ B changes were due to DNA damage. In order to test this hypothesis, experiments were performed to determine if DNA-damaging agents resulted in NF κ B pathway phosphorylation profiles similar to those obtained with XRT. For these experiments, Doxorubicin at two different doses, 0.1 μ g/ml and 0.25 μ g/ml, was used because it is known to cause DNA damage (which is sensed by ATM,) and to activate NF κ B (Alderton 2007). In the U937 cells, Doxorubicin at 0.1 μ g/ml does not induce phosphorylation of NF κ B or NEMO (data not shown). However, at 0.25 μ g/ml, Doxorubicin induced phosphorylation of NF κ B and NEMO after 3hr in a dose-dependent manner. Initial phosphorylation of NF κ B and NEMO was seen at 1 hour, with the

greatest activation of both occurring at 4 hours, which then declined and reached a plateau by 16 hours (Figure 1C and 1D).

ROS levels contribute to XRT-induced NEMO phosphorylation

XRT not only leads to direct DNA damage (including double stranded breaks), but also produces ROS which has been shown to indirectly cause DNA damage and can also directly activate the NF κ B pathway in certain systems (Ho, Chen, and Bray 1999; Nordberg and Arner 2001; Siebenlist, Franzoso, and Brown 1994; Verhasselt et al. 1999). Next we studied the effect of radiation on the redox status of the cell, as measured by the ratio of GSH (reduced):GSSG (disulfide), and demonstrated the importance of ROS in producing XRT-induced NF κ B changes.

The GSSG:total GSH (GSH_t) ratio in U937 cells was the highest 1hr after irradiation with 20 Gy, compared to cells irradiated with 2 or 0 Gy, and declined over time in a dose-dependent manner to near baseline at 16 hrs(Figure 2A). We examined the effect of radiation on the redox status of the cell by enhancing ROS production by treating cells with either L-Buthionine sulphoximine (BSO; which irreversibly inhibits γ -glutamylcysteine synthase, leading to depletion of GSH) or N-acetyl cysteine (NAC; a potent antioxidant that increases the levels of GSH and indirectly reduces ROS). In the absence of radiation, increasing oxidative stress (with BSO) or reducing oxidative stress (with NAC) had no direct effect on NEMO or NF κ B phopshorylation. However, when the effects of BSO or NAC on radiation-induced changes were examined, we found that cells treated with BSO for 3 hrs had significantly lower levels of GSH at 1 ($p=0.0392$) to

16 hrs ($p=0.0037$) compared to untreated cells (Supplemental Figure 2A). In contrast, treatment with NAC for 3 hrs significantly increased GSH levels at 1 ($p=0.01548$) to 16 hrs ($p=0.0039$) compared to untreated cells (Supplemental Figure 2B). When U937 mutant cells were pre-treated with 1.95 μ M BSO, irradiated at 0, 2, or 20 Gy, and allowed to incubate for 1, 4, 8, or 16 hrs, there was no additional activation of pNEMO above baseline as compared to XRT alone, and no significant difference between median fluorescence intensity (MFI) values for cells treated with XRT alone compared to cells treated with BSO+XRT (data not shown). With 3.95 μ M BSO, there was a statistically significant increase in activation at 1hr after 2 ($p=0.0280$) and 20 Gy ($p=0.0452$) when compared to the 0 Gy control, that did not occur at 4, 8, or 16 hrs after 2 or 20 Gy (Figure 2B). Representative histograms of 3.95 μ M BSO+ XRT versus XRT alone changes in pNEMO are shown in Supplementary Figure 2C.

In similar experiments, 1mM NAC did not inhibit XRT-induced activation of pNEMO in U937 mutant cells. There was no significant difference between MFI values for cells treated with XRT alone or NAC+XRT (data not shown). In contrast, when 10mM NAC was used, there was a statistically significant partial inhibition of activation of pNEMO by XRT at 1 and 4 hrs ($p=0.0416$), with the greatest inhibition occurring at 1hr after 20 Gy ($p=0.0411$), that declined over time (Figure 2C). Representative histograms of 10mM NAC+ XRT changes versus XRT alone in pNEMO are shown in Supplementary Figure 2D. These results suggest that ROS may have a modest contribution to XRT induced phosphorylation of NEMO, but that other factors must play a more important role in producing the XRT induced changes described above.

XRT induced phosphorylation of NEMO requires ATM

Since XRT leads to DNA damage from double stranded breaks, we hypothesized that XRT-induced NF κ B changes were due to DNA damage. In order to test this hypothesis, we used an inhibitor of ATM (Ataxia Telangiectasia Mutated) kinase, Ku55933, that selectively inhibits ATM-dependent cellular phosphorylation following XRT ($IC_{50}=13\text{nM}$), and does not inhibit DNA-Protein Kinase (DNA-PK; $IC_{50}=16.6\mu\text{M}$) or ATM and rad3 related Protein Kinase (ATR; $IC_{50}>100\mu\text{M}$). Kinetics for CHK2, the downstream target of ATM, CHK1, downstream target of ATR, and DNA-PK were analyzed via phospho-flow cytometry and confirm this specificity (Supplementary Figure 3). In these experiments, U937 cells were treated with $10\mu\text{M}$ Ku55933 for 60 minutes before irradiation with 0, 2, or 20 Gy. Ku55933 significantly inhibited ($p=<0.05$) XRT-mediated induction of pNEMO in U937 mutant cells at 1, 4, 8, and 16 hrs after 2 Gy and 20 Gy. Though significant, inhibition was less at 24 hrs (Figure 3A). Since the greatest activation of phospho (p) NEMO was seen at 4hr after 20 Gy, we used this dose to confirm these results by western blot. We found that NEMO and ATM were not phosphorylated in untreated U937 cells. Although radiation alone phosphorylated both ATM and NEMO, pre-treatment with Ku55933 significantly inhibited this phosphorylation. The requirement of ATM for phosphorylation of NEMO was confirmed using an ATM deficient cell line (G0536). Radiation alone phosphorylated NEMO but not ATM, and pre-treatment with Ku55933 again significantly inhibited phosphorylation of NEMO. The amount of total NEMO, DNA-PK, and ATR, were consistent in all three treatment groups (Figure 3B).

To further demonstrate that DNA-damage is the main mechanism of action responsible for the observed XRT-induced changes, U937 cells were treated with/without siRNA for ATM or negative control siRNA. In ATM knocked down cells, NEMO and ATM were not phosphorylated after 20 Gy. Similar amounts of total NEMO protein and ATM were observed in all groups with the exception of ATM knocked-down cells that did not express ATM. ATM siRNA did not affect expression of other PIKK family kinases DNA-PKcs and ATR (Figure 3C).

In order to determine whether other DNA-damaging treatments (i.e. chemotherapeutic cytotoxic agents such as doxorubicin) also depend on ATM activation for phosphorylation of NEMO, cells treated with U937 cells were also treated with 10 μ M Ku55933 for 60 minutes before incubation with 0.25 μ g/ml Doxorubicin for 3 hours in order to determine if NEMO activation would be inhibited, and produce similar phosphorylation profiles to those seen with Ku55933 inhibition of NEMO after XRT. Ku55933 significantly inhibited ($p=<0.05$) doxorubicin-mediated induction of pNEMO in U937 mutant cells at 1, 4, 8, and 16 and 24 hrs after doxorubicin treatment (Figure 3D).

γ H2AX and NEMO phosphorylation patterns correlate after XRT

To further study the relationships between induction of DNA damage by radiation and phosphorylation of NEMO, flow cytometry was used to detect phosphorylated H2AX (the fluorescent intensity correlates to the amount of DNA damage present), since phosphorylation of histone H2AX (γ H2AX) occurs rapidly in response to the presence of DNA double-strand breaks (Olive and Banath 2004). U937 cells were treated with 0, 2,

and 20 Gy and allowed to incubate at 1, 4, 8, and 16 hrs prior to measuring γ H2AX phosphorylation. Cells stained with phospho (p) NEMO were run in parallel. Results demonstrated that γ H2AX was phosphorylated after XRT in a dose-dependent manner. An initial increase in phosphorylation was seen at 1hr after 2 and 20 Gy, which increased further at 4hrs after 2 Gy and was highest at 4hr after 20 Gy. After phosphorylation peaked at 4hrs, it declined at 8hrs and further decreased at 16hrs to near baseline levels seen at 0 Gy. This phosphorylation pattern was highly correlated (correlation coefficient= 0.95955) to the kinetics of NEMO in U937 cells after XRT. The correlation of kinetics for γ H2AX and NEMO phosphorylation suggested that XRT-induced changes in the NF κ B pathway are due to DNA damage (Supplementary Figures 4A). The magnitude of increase in γ H2AX and NEMO is similar at the different time points, but was higher for γ H2AX is higher at 1hr than NEMO, suggesting that activation of γ H2AX is occurring earlier (Supplementary Figure 4B).

DNA-damage can lead to activation of receptors and intracellular signaling pathways that in turn stimulate p53 activity along with cell cycle checkpoints and DNA repair mechanisms. Since U937 cells possesses a mutant, functionally inactive p53 gene, we transfected U937 cells with wildtype p53 to determine the role of p53 in the acute-XRT induced changes observed in U937 cells. Western blot analysis confirmed that the p53 transfected gene was functionally activated, because p21, a protein directly downstream of p53, was upregulated in a dose-dependent manner after treatment with varying doses of Doxorubicin (0.5, 1, and 2 μ g/ml), which is a known p53 inducer. No upregulation of p21 occurred in empty vector control cells (Supplementary Figure 5A).

We found that XRT-induced phosphorylation of NEMO and NF κ B was not changed by the presence of p53 (Supplementary Figure 5B and 5C). Representative histograms of the phosphorylation of NEMO and NF κ B in U937 and p53+ U937 cells after 2 and 20 Gy at 4hr and 16hr are shown in Supplementary Figure 5D and 5E. Importantly, phosphorylation profiles for NEMO and NF κ B in mutant p53 and p53+ cells were not altered by vector alone (Supplementary Figure 5F and 5G). The presence of p53 also did not alter activation of NEMO and NF κ B because Doxorubicin induced phosphorylation changes in NEMO and NF κ B in U937 cells at 1, 4, 8, and 16 hrs that were striking similar to those seen in p53 mutant U937 cells (Supplementary Figure 6A and 6B). Furthermore, changes in redox status of the cell after radiation as detected by GSSG: GSH_t ratio, BSO+XRT and NAC+XRT were not p53 dependent (data not shown).

NF κ B pathway is necessary for maturation of U937 cells by XRT

We next studied the effect of XRT on the levels of expression of APC activation and maturation markers, including MHC and co-stimulatory molecules- CD40 and CD86, MHC Class II (HLA-DR), but not CD80, were upregulated in a dose-dependent fashion at 48 hrs. Since U937 cells do not express a basal level of CD80, it was not surprising that this costimulatory molecule was not upregulated after XRT (Figure 4A). Representative histograms of CD40, CD80, CD86, HLA-DR expression, and PMA (positive control) are shown in Supplementary Figure 8A.

In order to determine if the NF κ B pathway is responsible for the maturation of U937 cells, a IKK γ (NEMO) Binding Domain Inhibitory Peptide (NBD) was utilized. NBD inhibits formation of the complex between NEMO and IKK α /IKK β and thereby prevents phosphorylation of I κ B α , and subsequent phosphorylation of NF κ B (Strickland and Ghosh 2006). These effects were not p53 dependent (data not shown). When cells were either mock-treated with XRT, treated with XRT alone, or pre-treated with the control or NBD peptide for 12 hrs prior to XRT, NBD peptide significantly inhibited upregulation of CD40, CD86, and MHC Class II when compared to XRT +control peptide and XRT alone (Figure 4A). NBD treatment with 2 and 20 Gy prevented upregulation of CD40 when compared to 2 or 20 Gy+control peptide (p-values=0.0457 and 0.0040) and 2 or 20 Gy alone (p-values=0.0085 and 0.0003) (Figure 4A). Similarly, inhibition of CD86 upregulation by 2 or 20 Gy+NBD peptide was statistically significant when compared to 2 or 20 Gy+control peptide (p-values=0.0003 and 0.0001) and 2 or 20 Gy alone (p-values=0.0012 and 0.0005) (Figure 4A). MHC Class II upregulation was significantly inhibited by 2 or 20 Gy+NBD peptide when compared to 2 or 20 Gy+control peptide (p-values=0.0084 and 0.0001) and 2 or 20 Gy alone (p-values=0.0077 and 0.0001) (Figure 4A). These changes were not p53 dependent (data not shown). No changes were seen in CD80 expression with XRT alone, XRT+control peptide, or XRT+NBD peptide. Control peptide values were very similar to XRT alone (data not shown).

In addition, to confirm that the NBD peptide inhibits activation of the NF κ B pathway, cells were irradiated with 20 Gy at 0 and 48 hrs. XRT induced phosphorylation of I κ B α at 48hrs, when compared to mock-treated (0 Gy) controls at the same timepoints.

When cells were treated with the non-specific control peptide+XRT, MFI values were the same as those from cells treated with XRT alone. In irradiated cells pre-treated with the NBD peptide, phosphorylation of I κ B α was inhibited at 48hrs when compared to levels in irradiated cells pre-treated with the control peptide (Supplementary Figure 7B).

NBD Peptide Decreases U937 Cell Responsiveness

It was vital to determine whether the observed changes in the NF κ B pathway after XRT had any measurable effect on U937 function. Allogeneic MLR was chosen for this purpose because it is a fundamental technique used to assess human lymphocyte biological responses to various stimuli like APC (Muul et al. 2008). Human U937 cells can be induced to undergo terminal monocytic differentiation in mixed lymphocyte cultures and to express higher levels of CD14 and CD11b (Koren, Anderson, and Lerrick 1979). Experiments were then performed to study the potential effect of changes in specific cellular activities and activation of the NF κ B pathway after XRT on U937 cellular responsiveness/function. Cells were either treated with 0, 2, or 20 Gy XRT with or without pre-incubation with the control or NBD peptide for 12 hrs. Following irradiation, cells were incubated for 48 hrs before adding purified CD4+ T cells (isolated from PBMC from healthy donors), in a 1(APC):10(T) ratio for 4 days. T cell proliferation was measured spectrophotometrically to quantitate the incorporation of BrdU into the cell populations. As shown in Figure 8, control groups consisted of responder cells alone (purified CD4+ T cells), stimulator cells alone (irradiated U937 cells), and CD4+T cells combined with unirradiated U937 cells in each treatment group.

CD4+ T cells combined with irradiated U937 cells or U937 cells pre-incubated with non-specific control peptide, resulted in dose-dependent T cell proliferation when compared to CD4+ T cells incubated with unirradiated U937 cells. However, when cells were treated with the NBD peptide, T cell proliferation decreased 2 fold and 2.39 fold at 2 (p=0.0129) and 20 Gy (p=0.0016), respectively, when compared to XRT alone. T cell proliferation was decreased 1.97 fold and 2.32 fold at 2 (p=0.0323) and 20 Gy (p=0.0132), respectively, when compared to cells in control peptide treatment group (Figure 4B).

XRT activates and enhances U937 cell response: Cytokine production increases in T cells after XRT

Pro-inflammatory cytokine production is another indicator of APC activation and cytokine production. U937 cells were either treated with 0, 2, or 20 Gy XRT with or without pre-incubation with the control or NBD peptide for 12 hrs. Following irradiation, cells were incubated for 48 hours before adding purified CD4+ T cells (isolated from PBMC from healthy donors), in a 1(APC):10(T) ratio and were allowed to incubate for up to 6 days. Cells were removed from MLR on days 1, 2, 4, and 6 and supernatants were collected for assays of IL-4, TNF- α , and IFN- γ cytokine production via ELISA. TNF- α , and IFN- γ cytokine production (pg/ml), increased after 1 day and peaked after incubation for 2 days, with TNF- α and IFN- γ at about 700 pg/ml and 1000 pg/ml, respectively. Cytokine production then declined by day 6 to approximately 400 pg/ml and 200 pg/ml for TNF- α and IFN- γ , respectively in unirradiated cultures. This same

phenomenon was also seen in cells irradiated with 2 Gy with enhanced cytokine production after irradiation. TNF- α concentrations increased from approximately from 690 to 950 pg/ml ($p=<0.0001$) and IFN- γ concentration increased from approximately 1000 to 1500 pg/ml ($p=<0.0001$) after 2 days incubation (Figure 5A). Similarly, in unirradiated cells, IL-4 production increased after 1 day and peaked at 2 days (at about 35 pg/ml) with subsequent decline by day 6 (to approximately 13 pg/ml). The magnitude of enhanced IL-4 production was significantly less than that of TNF- α and IFN- γ , and actually declined after 2 Gy (Figure 5B). IL-4 production at 2 days was 18 pg/ml after 2 Gy versus 35 pg/ml in unirradiated control cultures ($p=0.0031$). Production of cytokines TNF- α , IFN- γ , and IL-4 in unirradiated cells is consistent with activation and function of U937 cells in the MLR. Increased levels of TNF- α and IFN- γ in cultures of irradiated cells further indicates that radiation enhanced U937 responsiveness. In other experiments, U937 cells were pre-treated with the control peptide (non-specific to NEMO) prior to irradiation and coculture with CD4+ T cells. There was no difference in TNF- α , IFN- γ , or IL-4 concentrations at 1, 2, 4, or 6 hrs in supernatants from cultures irradiated as compared to unirradiated cells (Figure 5C and 5D). Supernatants from similar experiments using a NBD inhibitory peptide demonstrated significant inhibition of TNF- α and IFN- γ levels by the peptide (Figure 5E). The kinetics of cytokine induction were similar, but in cultures pretreated with the inhibitor, the day 2 TNF- α cytokine concentration was 31 pg/ml and IFN- γ was 32 pg/ml, which is more than 30 fold less than observed in control cultures. TNF- α and IFN- γ cytokine production also increased after 2 Gy, in cultures pretreated with the NBD inhibitor, with peaks levels of 48 and 52 pg/ml at 2 days, respectively. However, again these levels were 30 fold less than in cultures of

irradiated cells without the inhibitor. The difference between TNF- α and IFN- γ cytokine production at 0 and 2 Gy was still significant ($p \leq 0.002$) (Figure 5E). Pretreatment with the NBD peptide largely obviated this difference in IL-4 cytokine production, with levels of 35 and 20 pg/ml after 0 and 2 Gy, respectively, although the decrease in IL-4 after radiation was still significant ($p = 0.0021$) (Figure 5F). These results suggest that activation of NF κ B is important for U937 cell function/responsiveness and increased responsiveness after radiation, and that IL-4 production is independent of NF κ B activation by radiation.

Next, intracellular cytokine production was examined in order to determine the cellular source of cytokine production. At 48 hours, pooled unirradiated and irradiated (2 Gy) U937 and CD4+ T cells from MLR cultures were activated with Leukocyte Activation Cocktail and Monensin (BD Biosciences) for 4 hours before surface marker and intracellular cytokine staining. Cells were stained with CD3, CD4, CD14, CD11b, and cytokine markers. The results demonstrated that IL-4, IFN- γ , and TNF- α were produced by CD4+ T cells. The percentage of cytokine positive CD4+ T cells is shown in two-dimensional plots of cytokine versus CD4 (Supplementary Figure 8A) and in bar-graph representation (Supplementary Figure 8B).

XRT enhances APC function which is dependent upon activation of NEMO

Since the experiments described above demonstrated that XRT induces functionally significant effects in a monocytic cell line via NEMO and NF κ B phosphorylation, experiments were next performed to determine the importance of the

NF κ B pathway in APC responsiveness and the role of the NF κ B pathway in APC function/responsiveness after radiation. To do so, APC subsets, monocytes and dendritic cells, were used for a variety of functional experiments. First, the effect of radiation on the function of monocytes, was studied by combining either unirradiated or irradiated monocytes (2 Gy) ($CD14^+$) with $CD4^+$ T cells in a 1:5 (T:APC) ratio. Cells were removed and stained for subset surface markers as well as CD80 and CD86 at 12 and 48 hours. Figure 6A shows representative examples of two-color flow cytometric analyses of CD80 and CD86 expression from gated $CD14^+CD11b^+$ cells before and after irradiation at these time points. At 12 hours after irradiation, $CD86^+$ monocytes increased from 2.86% to 6.21% and after 48 hours, increased from 2.02% to 5.11% (Figure 6A). In contrast, there was a substantial increase in CD80 expression at 12 hours from 0.43% to 30.4%, but only a very small increase at later time points (Figure 6A). This can be explained by the large increase (93.1%) in $CD86^+ CD80^+$ monocytes at 48 hours, although at 12 hours CD86/CD80 expression had increased from 0.32% to 56.4% (Figure 6A). Similar results were obtained with monocytes pre-treated with the control peptide before irradiation (data not shown). Not only did radiation enhance expression of co-stimulatory molecules CD80 and CD86 or monocytes, but inhibition of NEMO by the NBD peptide, which inhibits $I\kappa B\alpha$ activation (Supplementary Figure 9A), inhibited this increased expression.

Experiments were then performed using the same experimental design as above with monocytes treated with the NBD peptide for 12 hours before irradiation and cocultured with $CD4^+$ T cells. Figure 6B shows representative examples of two-color flow cytometric analyses of CD80 and CD86 expression from gated $CD14^+CD11b^+$ cells

before and after treatment with NBD peptide+XRT at 12 and 48 hours. With the inhibitor, there was only a negligible difference in CD86 expression between any of the groups, and a small increase in expression of CD80 from 0.61% to 6.61% at 12 hours and 0.21% to 11.3% at 48 hours (Figure 6B). The greater increase in CD80 expression at 48 hours in NBD treated cells compared with those treated with XRT alone can be explained by the presence of fewer double positive CD80/86 cells. NEMO inhibition significantly suppressed radiation-induced CD80/86 expression ($\leq 3.6\%$) at all timepoints ($p=<0.0001$). The mean CD80/86 expression percentages from unirradiated and irradiated monocytes at 12 and 48 hours are shown in Figure (Supplementary Figure 9B). These results suggest that activation of the NF κ B pathway is vital to enhanced monocyte response/function after radiation.

NBD peptide decreases Monocyte and Dendritic Cell responsiveness

Next we wanted to study the effects of radiation-induced activation of the NF κ B pathway on APC function. MLR cultures were set up as before and T cell proliferation was measured by incorporation of BrdU into the cells and readout as absorbance. As shown in Figure 6C, control groups consisted of responder cells alone (purified CD4+ T cells), stimulator cells alone (irradiated monocytes), and CD4+T cells combined with unirradiated monocytes in each treatment group. CD4+ T cells combined with irradiated monocytes or monocytes (1:5) pre-incubated with non-specific control peptide, resulted in increased T cell proliferation, from 0.1084 to 0.6204 with XRT alone and from 0.110 to 0.609 with control peptide+XRT when compared to CD4+ T cells incubated with

unirradiated monocytes. However, when monocytes were treated with the NBD peptide, T cell proliferation decreased by about 4 fold ($p=0.0003$) when compared to XRT alone, and T cell proliferation was decreased by about 4 fold ($p=<0.0001$) when compared to cells in control peptide treatment group. These results show that radiation enhances monocyte function, which seems to be dependent upon NEMO activation or NF κ B pathway function.

Similar results were seen with dendritic cells (Figure 6D). T cell proliferation increased from 0.141 to 0.726 with XRT alone and from 0.141 to 0.716 with control peptide+XRT when compared to CD4 $^{+}$ T cells incubated with unirradiated dendritic cells. However, in cultures with monocytes pretreated with the NBD peptide, T cell proliferation decreased by about 5 fold ($p=<0.0001$) when compared to XRT alone, and T cell proliferation was decreased by about 5 fold ($p=<0.0001$) when compared to cells in control peptide treatment group. Therefore radiation enhanced dendritic cell function, which was dependent upon NEMO activation or NF κ B pathway function.

In addition, we confirmed these results in whole blood samples. When examining APC responsiveness in whole blood via MLR cultures to readout T cell proliferation, similar results were seen to those with human PBMC. Monocytes (Figure 7A) and dendritic cells (Figure 7B) isolated from whole blood were combined with CD4 $^{+}$ T cells isolated from whole blood. T cell proliferation increased from 0.105 to 0.478 and from 0.113 to 0.586 in MLR with monocytes and dendritic cells, respectively. No difference was seen in T cell proliferation when APC were pre-incubated with control peptide before XRT in MLR cultures. However, in cultures were monocytes and dendritic cells

were pretreated with the NBD peptide, T cell proliferation decreased by about 3.5 fold ($p=0.0002$) and 5 fold ($p=<0.0001$), respectively compared to XRT and control peptide+XRT.

XRT activates and enhances Monocyte and Dendritic Cell response in a NEMO dependent manner

Pro-inflammatory cytokine production was measured from MLR cultures of unirradiated or irradiated (2 or 20 Gy) monocytes ($CD14^+$). Cells were removed from MLR cultures on days 1, 2, 4, and 6 and supernatants were collected for measurement of IL-4, TNF- α , and IFN- γ cytokine concentration peaked at 900 pg/ml and 880 pg/ml, respectively, on day 2, and declined by day 6 to approximately 330 pg/ml and 175 pg/ml for TNF- α and IFN- γ , respectively in unirradiated cultures. This same phenomenon was seen after 2 Gy, although the magnitude of cytokine induction was greater after irradiation. TNF- α increased from approximately from 900 to 1000 pg/ml ($p=0.0006$) and IFN- γ increased from approximately 880 to 1300 pg/ml ($p=<0.0001$) after 2 days (Figure 8A). In unirradiated control cultures, IL-4 production increased after 1 day and peaked at 2 days at 30 pg/ml. In contrast, IL-4 levels decreased following 2 Gy (20 pg/ml after 2 Gy versus 30 pg/ml after 0 Gy at day 2 ($p=0.024$) (Figure 8A). The induction of these cytokines in the MLR demonstrates monocyte activation and functioning. Elevated levels of TNF- α and IFN- γ after 2 Gy indicates that radiation enhanced monocyte responsiveness increased. IL-4 cytokine production after radiation suggests that IL-4 may play an anti-inflammatory role. Results from control peptide

(non-specific to NEMO) treated monocytes were not significantly different from those treated with radiation alone (Figure 8B).

Pre-treatment with the NBD inhibitory peptide inhibited TNF- α and IFN- γ production (Figure 8C). At day 2, TNF- α and IFN- γ cytokine concentrations were about 30 pg/ml which is over a 30 fold decrease in production from that seen after 0 Gy alone. Although there was a relative increase in TNF- α and IFN- γ cytokine production ($p\leq 0.03$) after 2 Gy, levels of TNF- α and IFN- γ were approximately 20 fold and 25 fold lower with the NBD inhibitory peptide than with radiation alone (Figure 8C). In contrast, the NBD peptide had no effect on radiation-induced IL-4 production (Figure 8C).

Similar results were seen for dendritic cell cytokine production as well. TNF- α and IFN- γ cytokine production rose after 1 day, peaked at 2 days and then started to decline (Figure 8D). IL-4 production in unirradiated cells followed the same trend, but declined after radiation at day 2 from 62.5 pg/ml to 46 pg/ml ($p=0.0006$) (Figure 8D). The control peptide had no effect on cytokine production (Figure 8E). As before, the NBD inhibitory peptide significantly inhibited TNF- α and IFN- γ production in MLRs by over 30 fold (Figure 8F) in cultures with and without irradiated dendritic cells. Again, the level of IL-4 cytokine production was not significantly affected by cells pre-treated with the NBD peptide (Figure 8F).

Effect of allogeneic MLR on cytokine positive T Cells, Monocytes and Dendritic Cells

Intracellular cytokine production in monocytes was examined in order to determine which cells, APC and/or T cells were producing the cytokines, using the same methodology used for U937 cells and were stained with CD3, CD4, CD14, CD11b, and cytokine markers. Data again confirms that cytokines IL-4, IFN- γ and TNF- α were produced by CD4 $^{+}$ T cells. The percentage of cytokine positive CD4 $^{+}$ T cells is shown in two-dimensional plots of cytokine versus CD4 (Supplementary Figure 10A). Supplementary Figure 10B shows bar-graphs representative of cytokine positive CD4 $^{+}$ T cells. The percentage of cytokine positive monocytes is shown in two-dimensional plots of cytokine versus CD4 and in bar-graph form (Supplementary Figure 10C and D). Similar experiments were performed with MLRs of dendritic cells and CD4 $^{+}$ T in which cells were stained with CD3, CD4, Lin $^{-}$ and cytokine markers. The results demonstrated that IL-4, IFN- γ , and TNF- α were again produced by CD4 $^{+}$ T cells (Supplementary Figures 11A and 11B). With both monocytes and dendritic cells, only small changes in the percent of cytokine positive CD4 $^{+}$ T cells occurred after irradiation.

Discussion

Previous studies have shown that radiation has important effects beyond cytotoxicity on immune cells, such as enhanced antigen presentation (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005; Friedman 2002), pro-inflammatory cytokine release (TNF- α , IL-1 β , IL-2) (Hallahan et al. 1989; Hong et al. 1999; Ishihara et al. 1993; Rieser et al. 1997), and DC activation, which can augment T cell mediated anti-tumor effects (Paulos et al. 2007). Radiation can also stimulate dendritic cell maturation by prostaglandin E2 (PEG-2) and cyclooxygenase-2 (COX-2) (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005; McBride et al. 2004; McLellan, Brocker, and Kampgen 2000), which enhance the overall “danger milieu.” Other clinically relevant effects of radiation on the tumor microenvironment (Lan et al. 2001) include stimulation of APC homing to irradiated sites (Steinauer et al. 2000) by chemokine release (e.g. SDF-2) from irradiated tumor cells (Teitz-Tennenbaum et al. 2008), and altered adhesion molecule expression by irradiated endothelial cells, which promote leukocyte adhesion chemotaxis (Cameron, Spiess, and Rosenberg 1990; Ishihara et al. 1993; Trinchieri 2003; Zong et al. 2006). Few direct immune enhancing effects of radiation have been described. Furthermore, it is not well understood at the molecular level how radiation enhances tumor immunity, especially with regard to important signaling changes that occur in APC. Thus the goal of the experiments described here was to elucidate or identify early radiation-induced signaling changes in antigen presenting cells and to elucidate the effects of these signaling changes on APC responsiveness and function.

We first examined radiation-induced signaling changes in U937 cells and the underlying mechanism (s) responsible for these changes. This cell line was chosen for initial studies because (a) it has been widely used as a model for monocytes/macrophage in differentiation vitro (Kim et al. 2004; Nikitina and Gabrilovich 2001) and (b) has been used as a model for monocytic cells (Chakravarty et al. 1999; Magne et al. 2006; Verheij and Bartelink 2000). U937 cells can be induced to undergo terminal monocytic differentiation in mixed lymphocytes cultures, as well as by PMA, Vitamin D3, IFN- γ , and TNF- α (Baldwin 1996; Hayden, West, and Ghosh 2006). U937 cells display hallmark characteristics of APC, and express basal levels of major histocompatibility complex (MHC) class II and I as well as co-stimulatory molecules. For example, FACS studies have shown upregulation of MHC class II and I and co-stimulatory molecules after stimulation by PMA (Buggins et al. 1999). Differentiated U937 cells also phagocytose bacteria efficiently and are able to present bacterial antigen to CD8+ T cells (Passmore, Lukey, and Ress 2001). U937 cells have a functionally inactive p53 (Scheidereit 2006), which allowed us to manipulate the p53 activity in these cells to determine the role of p53 in radiation-induced changes in the NF κ B signal transduction pathway. The data indicated that radiation and doxorubicin induced NF κ B pathway activation in U937 cells in a p53 independent manner. Furthermore, ATM inhibition prevented radiation and doxorubicin, both known DNA damage inducers, from phosphorylating and activating NEMO. In addition, radiation altered the redox status of the cell as indicated by changes in the GSH:GSSG ratio, and lead to NF κ B pathway activation. However, with redox modifying agents, the effects of ROS on the NF κ B pathway were minimal. Based upon these findings, a model was developed to explain

the underlying etiology of XRT-induced effects on the NF κ B pathway in U937 cells, in which XRT induced double-strand breaks, resulting in phosphorylation of ATM, and subsequent activation and phosphorylation of NEMO. Phosphorylation of NEMO activates a downstream signaling cascade that results in phosphorylation of NF κ B. XRT also produces ROS that indirectly contributes to for activation of the NF κ B pathway in U937 cells, but are not an absolute requirement for activation of this pathway. ROS can damage DNA, result in double-strand breaks, and thereby phosphorylate ATM and activate the NF κ B signaling pathway. It is conceivable that ROS may also directly phosphorylate NEMO, but this would be a minor pathway compared with the DSB-mediated ATM pathway, as indicated by results showing little effect of redox status on radiation (ROS) –induced changes on the NF κ B pathway (Figure 9). The model developed is based upon radiation-induced phosphorylation changes in the NF κ B pathway that were p53 independent in the human monocytic cell line. Though it is known that radiation induced induction of downstream genes in the DNA damage pathway which control cell cycle arrest (Canman et al., 1994) and programmed cell death are dependent upon wildtype 53 of which whose function may or may not require ATM., (though in most cases has been shown to require activation of ATM) (Morgan and Kastan 1997), evidence in other studies have shown that NF κ B’s interaction with p53 depdends on the cell type (Bellas, Lee, and Sonenshein 1995; Mayo et al. 1997; Ravi et al. 1998; Ryan et al. 2000; Wadgaonkar et al. 1999). In this case, ATM induced activation of p53 was not required for NF κ B pathway activation. Future inhibition studies in human PBMC could examine the relationship between ATM and p53 and how it affects radiation-induced cativaiton of the NF κ B pathwayThough other studies have shown the

radiation activates NF κ B in a variety of cell types, it was important to show that radiation does activate NF κ B in a human monocytic cell line, since this line was used for the initial functional radiation APC studies. Importantly, other studies have not examined the functional significance of the NF κ B pathway activation after radiation on immune modulation or more specifically on the enhancement of immune responses as it relates to APC activation. We did so using human PBMC from healthy donors to determine the relevance of our observations in U937 cells to mixed cell populations found in human PBMC. In the past, changes at the molecular level have been difficult to elucidate in PBMC because of exquisite radiosensitivity of some of the subpopulations present in PBMC, resulting in very low numbers of viable cells following irradiation. However, we were able to use phospho-flow cytometry (pFCS) to accurately and rapidly assess the phosphorylation state of proteins within specific immune cellular subsets in complex, heterogeneous cell populations following irradiation (Wang et al. Co-activation of erk, nf- κ pab, and gadd45beta in response to ionizing radiation 2005). Importantly, pFCS also provided information about the functional responses of these cells to radiation, which has not been possible until very recently.

Our results demonstrate that the NF κ B pathway is functionally important for modulation of U937 cells and APC subsets after radiation. Initial studies showed that inhibition of NEMO, a molecule upstream of NF κ B that initiates the signaling cascade, diminished U937 cell responsiveness after radiation (as shown by decreased co-stimulatory and MHC molecules expression), decreased T cell proliferation in MLR, and decreased TNF- α and IFN- γ cytokine production by T cells in MLR. Decreased IL-4 cytokine production by T cells after radiation was not changed by inhibition of NEMO,

and it is not clear why radiation decreases IL-4 production. We hypothesize that IL-4 production is not regulated by NF κ B, and that IL-4 might be able to suppress NF κ B activation, so inhibition of the NF κ B pathway would have no effect on IL-4 production. This is consistent with data from others suggesting that IL-4 can function as an inhibitor of NF κ B activation (Ahn and Aggarwal 2005). Overall, our results demonstrate that activation of the NF κ B pathway is necessary for U937 responsiveness and/or enhanced responsiveness after radiation. Of note, U937 cells have a 0.6% risk of contamination with other cell lines, so a small amount of caution should be used in interpreting these results.

In order to really determine the functional significance of the radiation-induced NF κ B pathway activation in immune modulation or enhancement of the immune response, we examined the role of radiation-induced NF κ B pathway activation in APC subsets from healthy human PBMC. Cytokine production, co-stimulatory molecule expression, and T cell proliferation were used as indicators of APC activation/responsiveness, and demonstrated that the NF κ B pathway activation is vital for enhanced monocyte and dendritic cell responses after radiation. Radiation increased co-stimulatory molecule expression and T cell proliferation in monocytes and dendritic cells in MLR. Radiation also increased cytokine production of TNF- α by monocytes (which could also be produced by T cells) and IFN- γ by T cells, while decreasing IL-4 production by T cells in supernatants from MLR. However, inhibition of NEMO altered, and in fact inhibited, the responsiveness of APC after radiation. This was shown by inhibitory effects on co-stimulatory molecule expression and increased T cell proliferation in MLR in which APC were pretreated with the inhibitor. Increased

cytokine production of TNF- α and IFN- γ was also inhibited by the NEMO inhibitor, while there was no effect on IL-4 production. Inhibition of TNF- α and IFN- γ cytokine production indicates that activation of NF κ B is important for monocyte and dendritic cell function/responsiveness and for increased responsiveness after radiation. The observation that IL-4 production was not altered by inhibition of NF κ B suggests that IL-4 production does not depend on NF κ B activation with or without radiation. Overall, these results strongly support the hypothesis that NF κ B is vital for enhanced APC responsiveness after radiation.

The role of radiation as an independent immune modulatory agent is an area of active investigation. These results demonstrate an important mechanism by which radiation enhances APC response and could directly enhance tumor immunity. Future experiments will study the effect of local tumor irradiation on NF κ B pathway signaling changes in APC to further confirm these findings.

The results presented here provide important insight into how radiation affects APC at the level of signal transduction pathways. Furthermore, these findings potentially have important clinical relevance, since modulation of APC responsiveness through signaling pathways such as NF κ B, may allow for optimization of current immunotherapy protocols that use radiation. Here we have demonstrated that the NF κ B pathway is activated by radiation and is necessary for U937 and PBMC APC responsiveness after radiation. In addition, we have shown that activation of the NF κ B pathway by radiation is necessary for APC co-stimulatory molecule upregulation, APC maturation and activation, pro-inflammatory cytokine expression/release, and enhanced T cell

proliferation following APC irradiation. These findings provide insight into the mechanism(s) of radiation enhanced tumor immunity and have relevance for optimizing the use of radiation for the purposes of immune modulation.

Prior to the work described in this thesis, only a few studies have tried to characterize direct immune enhancing effects of radiation or determined functionally relevant radiation-induced intracellular signaling changes at the molecular level that occur in APC. Therefore, little has been known about why APC are relatively radioresistant (compared to T cells) at the molecular level. This is clinically relevant since these changes could alter clinical outcomes in patients' receiving radiotherapy. The experiments performed in chapter 3 help to begin to elucidate these effects, which could have near term translational potential.

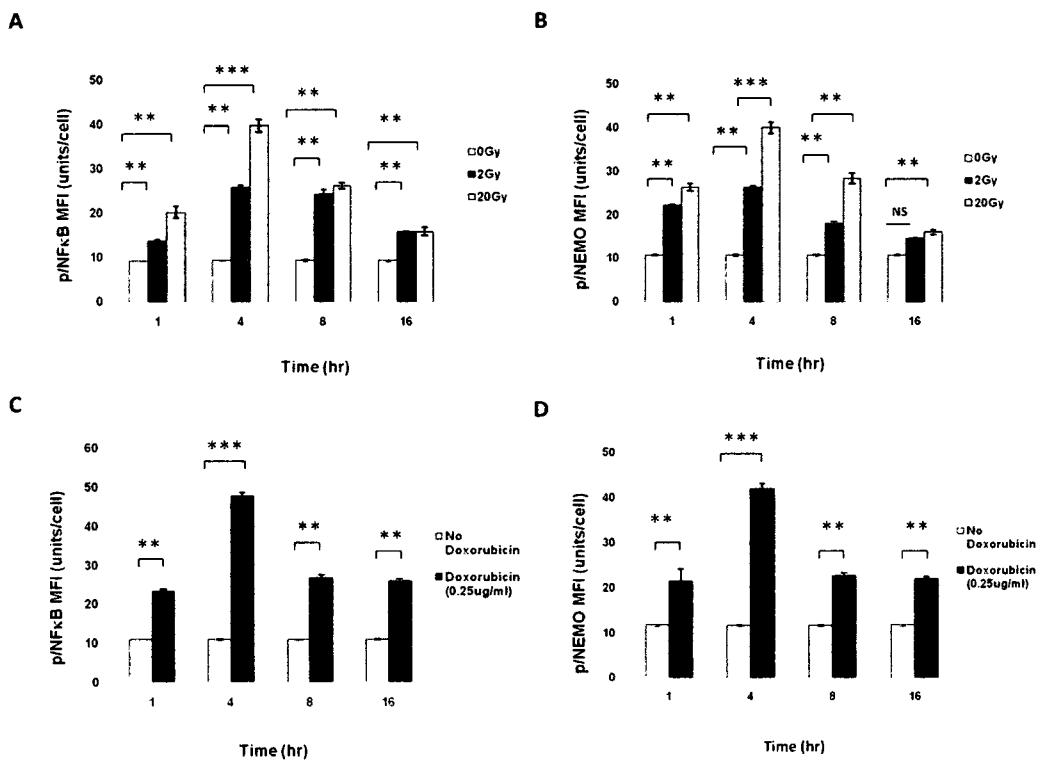


Figure 1. Phospho-flow cytometry determines phospho-specific changes in NF κ B pathway resulting from XRT and Doxorubicin in U937 cells.

(A) Treatment of U937 cells with XRT showed increased levels of NF κ B phosphorylation in U937 and (B) NEMO phosphorylation 1-16 hrs after irradiation with 0, 2, and 20 Gy. (C) NF κ B phosphorylation in U937 after 3 hrs with 0.25 μ g/ml of Doxorubicin. (D) NEMO phosphorylation in U937 after 3 hrs with 0.25 μ g/ml Doxorubicin. (A-D) *(p=<0.05), **(p=<0.01), ***(p=<0.001); and NS, not significant. For all experiments, data is shown as normalized average MFI \pm S.E.M from three independent experiments unless otherwise specified.

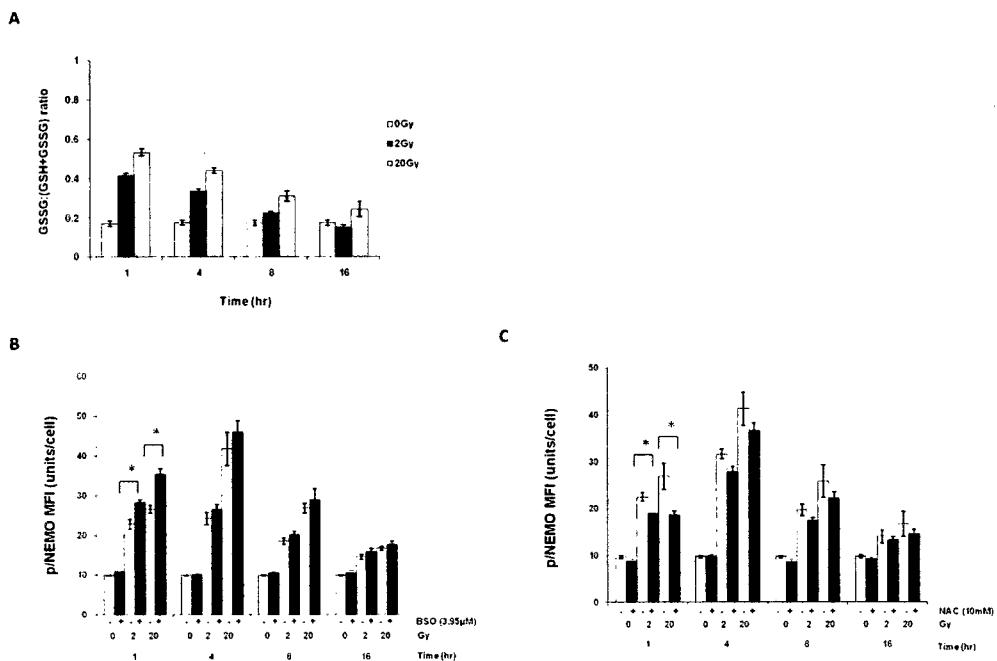


Figure 2. ROS levels modestly contribute to XRT-induced phosphorylation of NEMO.

(A) Ratio of GSSG to GSH+GSSG (total GSH) in U937 cells 1-16 hrs after irradiation with 0, 2, and 20 Gy. Data are shown as the average ratio +/- standard error from three independent experiments. (B) Phosphorylation of NEMO phosphorylation at 1-16 hrs after (+) pre-treatment with 3.95 μ M BSO for 3 hrs prior to 0, 2, or 20 Gy irradiation or (-) radiation alone in U937 mutant cells (C) Phosphorylation of NEMO at 1-16 hrs after pre-treatment with 10mM NAC for 3 hrs prior to 0, 2, or 20 Gy irradiation in U937 mutant cells. (B-C) *(p=<0.05).

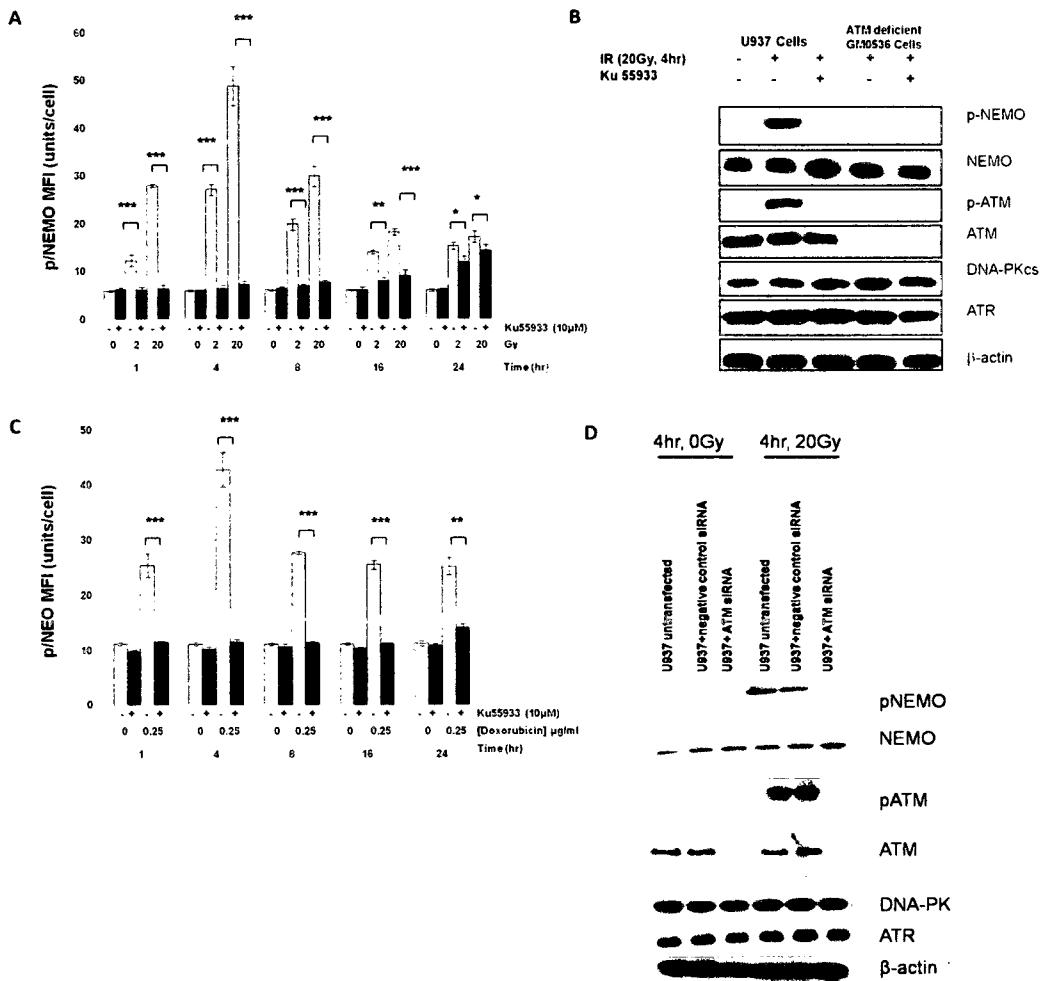


Figure 3. Phosphorylation of NEMO after XRT and Doxorubicin requires ATM.

(A) U937 cells treated with 10μM Ku55933 (+) versus no inhibitor (-) prior to irradiation (0, 2, or 20 Gy) at 1-24 hrs. (B) Western blot analysis of NEMO and ATM phosphorylation in U937 and ATM deficient GM0536 cells. β-actin served as a loading control. (C) Phosphorylation of NEMO and ATM were measured by western blot analysis in untransfected U937 and U937+negative control siRNA cells. NEMO and ATM were phosphorylated after treatment with 20 Gy but not in untreated (0 Gy) U937

cells. Radiation did not phosphorylate ATM or NEMO in ATM knocked down cells. β -actin served as a protein loading control. (D) U937 cells treated with 10 μ M Ku55933 (+) versus no inhibitor (-) prior to 0.25 μ g/ml Doxorubcin at 1-24 hrs. *(p=<0.05), **(p=<0.01), ***(p=<0.001); and NS: not significant.

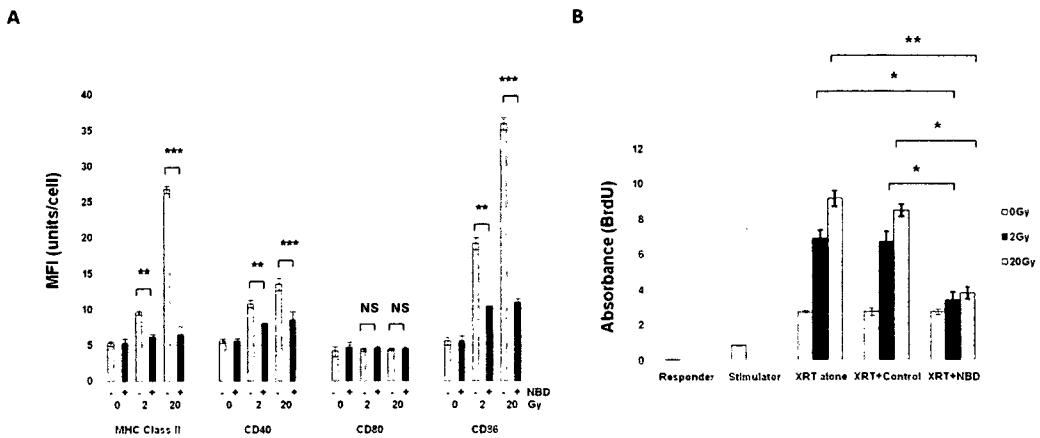


Figure 4. XRT-induced maturation and responsiveness of U937 cells requires

NF κ B.

(A) Inhibition of CD40, CD86, and MHC Class II phosphorylation at 48 hrs by NBD peptide (+) versus no peptide (-) after 2 or 20Gy XRT compared to CD40, CD86, and MHC Class II phosphorylation after XRT alone. NS=not significant, **(p=<0.01), and ***(p=<0.001). T cell proliferation was measured following combination of CD4+ T cells with irradiated (2 or 20 Gy) U937 cells and U937 pretreated with non-specific control peptide increased T cell proliferation. Irradiated U937 cells pre-treated with NBD peptide showed reduced T cell proliferation. Responder cells alone (purified CD4+ T cells), stimulator cells alone (irradiated U937 cells), and CD4+T cells were combined with unirradiated U937 cells and served as negative controls. Shown is the average absorbance (from triplicates) \pm S.E.M. from three independent experiments. *(p=0.05) and **(p=<0.01).

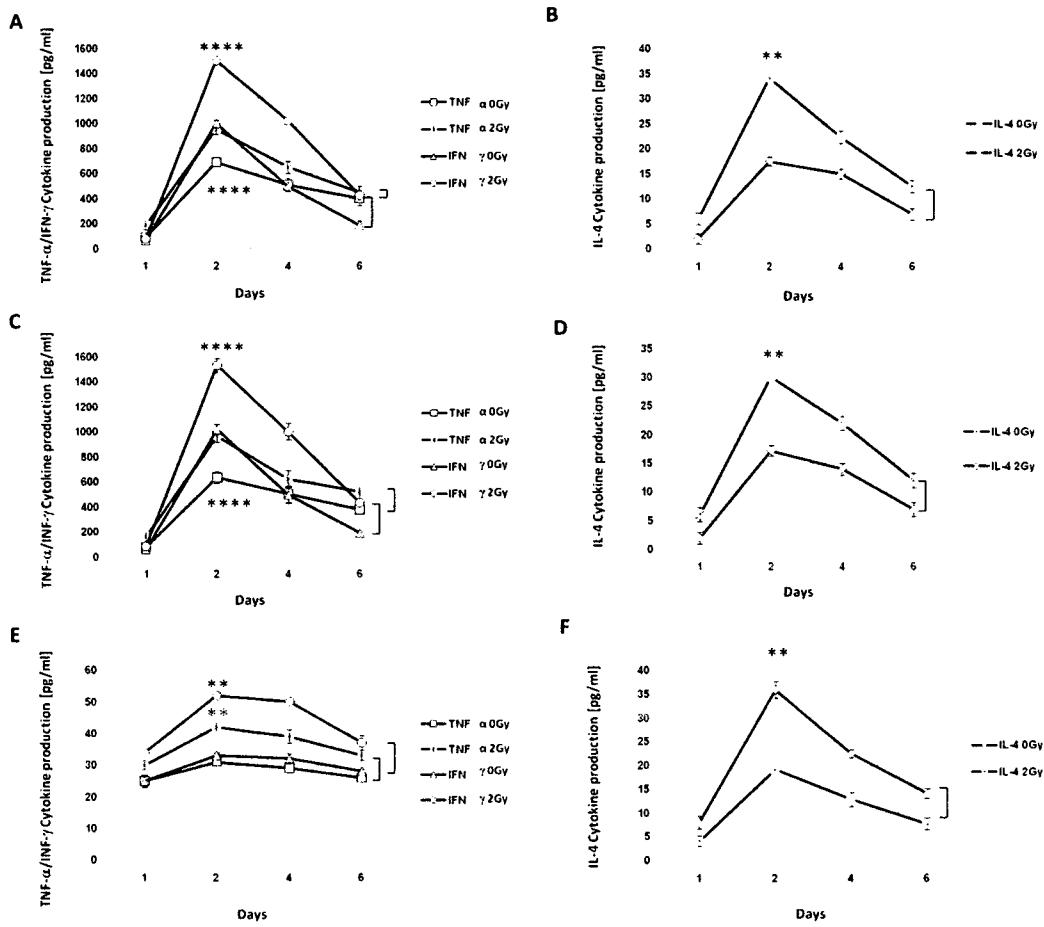


Figure 5. Supernatants from allogeneic MLR demonstrate XRT enhances TNF- α and IFN- γ cytokine production, but not IL-4.

Unirradiated and irradiated U937 cells were combined with purified CD4 $^{+}$ T cells, in a 1(APC):10(T) ratio and on days 1, 2, 4, and 6, supernatants were used to assay IL-4, TNF- α , and IFN- γ cytokine production via ELISA. Concentrations of cytokines were measured as pg/ml. (A) TNF- α and IFN- γ cytokine production after 0 Gy and 2 Gy. (B) IL-4 cytokine production before and after radiation (2 Gy). (C) Supernatant TNF- α and IFN- γ cytokine concentrations from MLR where U937 cells were pre-treated with control

peptide before 0 or 2 Gy). (D) IL-4 cytokine concentration from supernatants of the same MLR as Figure 5C. (E) Shows inhibition of TNF- α and IFN- γ cytokine production where U937 cells were pre-treated with NBD peptide before mock-irradiation or irradiation and combining with CD4 $^{+}$ T cells. (F) Unchanged IL-4 cytokine production from supernatants of the same MLR as Figure 5E. (A-F) Shown is the average cytokine production (from triplicates) \pm S.E.M. from three independent experiments. **(P=<0.01) and ***(p=<0.0001).

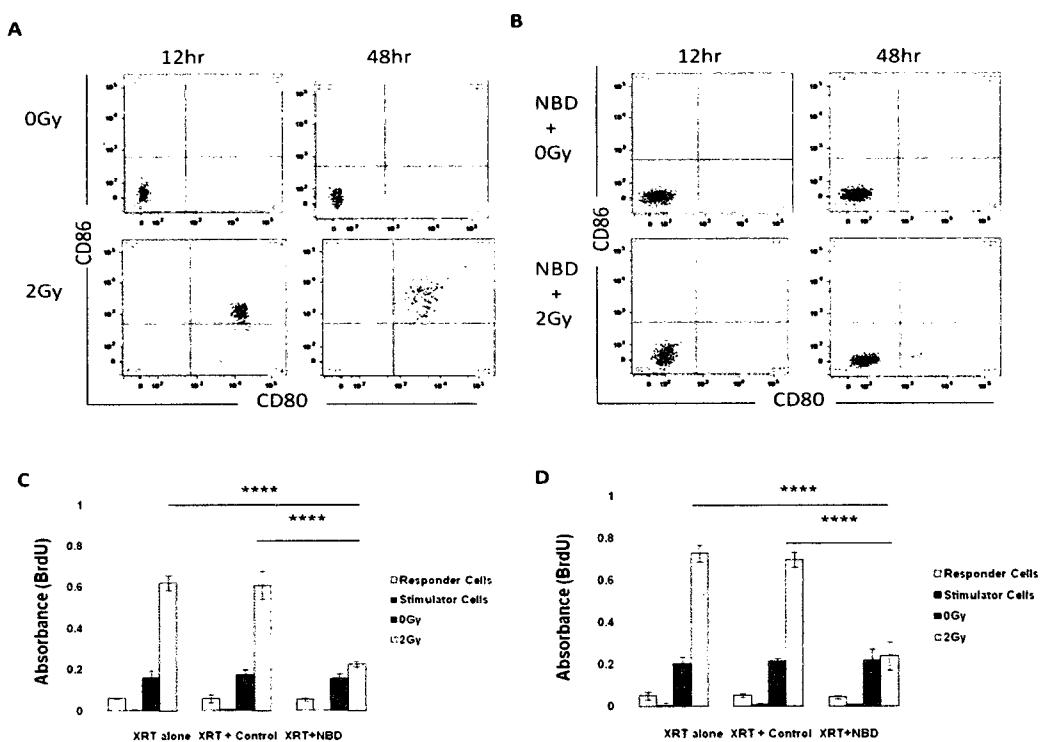


Figure 6. APC increased CD80 and CD86 expression and responsiveness after XRT is dependent on NEMO activation in allogeneic MLR.

Co-stimulatory molecule expression from monocytes was measured in monocytes combined with CD4 $^{+}$ T cells in MLR. CD80, CD86, and CD80/86 expression from

gated CD14⁺CD11b⁺ cells before (top row) and after 2 Gy (bottom row) at 12 and 48 hours, shown as FACS plots without NBD peptide (A) and with (B) NBD peptide pre-treatment. (C-D) NBD peptide inhibits T cell proliferation in MLR after XRT compared to control MLR cultures. Cells were either treated with 0, or 2 Gy XRT alone or pre-incubated with the control or NBD peptide for 12 hrs before adding CD4⁺ T cells. Controls groups consisted of responder cells alone (purified CD4+ T cells), stimulator cells alone (irradiated monocytes or dendritic cells), and CD4+T cells combined with unirradiated monocytes or unirradiated dendritic cells in each treatment group. (C) Increased T cell proliferation after XRT alone and control peptide+XRT by CD4+ T cells incubated with irradiated monocytes when compared to CD4+ T cells incubated with unirradiated monocytes from PBMC. NBD peptide+XRT resulted in decreased T cell proliferation by CD4+ T cells incubated with irradiated monocytes when compared to XRT alone and control peptide+XRT treatment groups. (D) Increased T cell proliferation after XRT alone and control peptide+XRT by CD4+ T cells incubated with irradiated conventional dendritic cells when compared to CD4+ T cells incubated with unirradiated conventional dendritic cells from PBMC. NBD peptide+XRT resulted in decreased T cell proliferation by CD4+ T cells incubated with irradiated conventional dendritic cells when compared to XRT alone and control peptide+XRT treatment groups. (C-D) Shown is the average absorbance (from triplicates) ±S.E.M. from five independent experiments. **** (p=<0.0001).

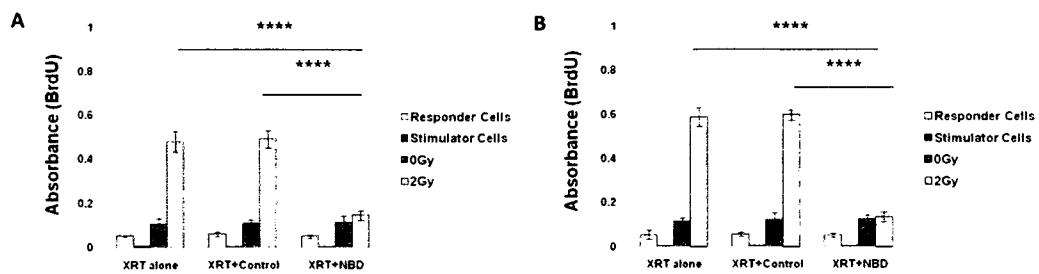


Figure 7. Responsiveness after XRT is dependent on NEMO activation in allogeneic MLR Cultures from Whole Blood.

NBD peptide inhibits T cell proliferation in MLR after XRT compared to control MLR cultures. Cells were either treated with 0, or 2 Gy XRT alone or pre-incubated with the control or NBD peptide for 12 hrs before adding CD4⁺ T cells. Controls groups consisted of responder cells alone (purified CD4+ T cells), stimulator cells alone (irradiated monocytes or dendritic cells), and CD4+T cells combined with unirradiated monocytes or unirradiated dendritic cells in each treatment group. (A) Increased T cell proliferation after XRT alone and control peptide+XRT by CD4+ T cells incubated with irradiated monocytes when compared to CD4+ T cells incubated with unirradiated monocytes from whole blood. NBD peptide+XRT resulted in decreased T cell proliferation by CD4+ T cells incubated with irradiated monocytes when compared to XRT alone and control peptide+XRT treatment groups. (B) Increased T cell proliferation after XRT alone and control peptide+XRT by CD4+ T cells incubated with irradiated conventional dendritic cells when compared to CD4+ T cells incubated with unirradiated conventional dendritic cells from whole blood. NBD peptide+XRT resulted in decreased T cell proliferation by CD4+ T cells incubated with irradiated conventional dendritic cells when compared to XRT alone and control peptide+XRT treatment groups. (A-B) Shown is the average

absorbance (from triplicates)±S.E.M. from three independent experiments. ***
($p=<0.001$) and **** ($p=<0.0001$).

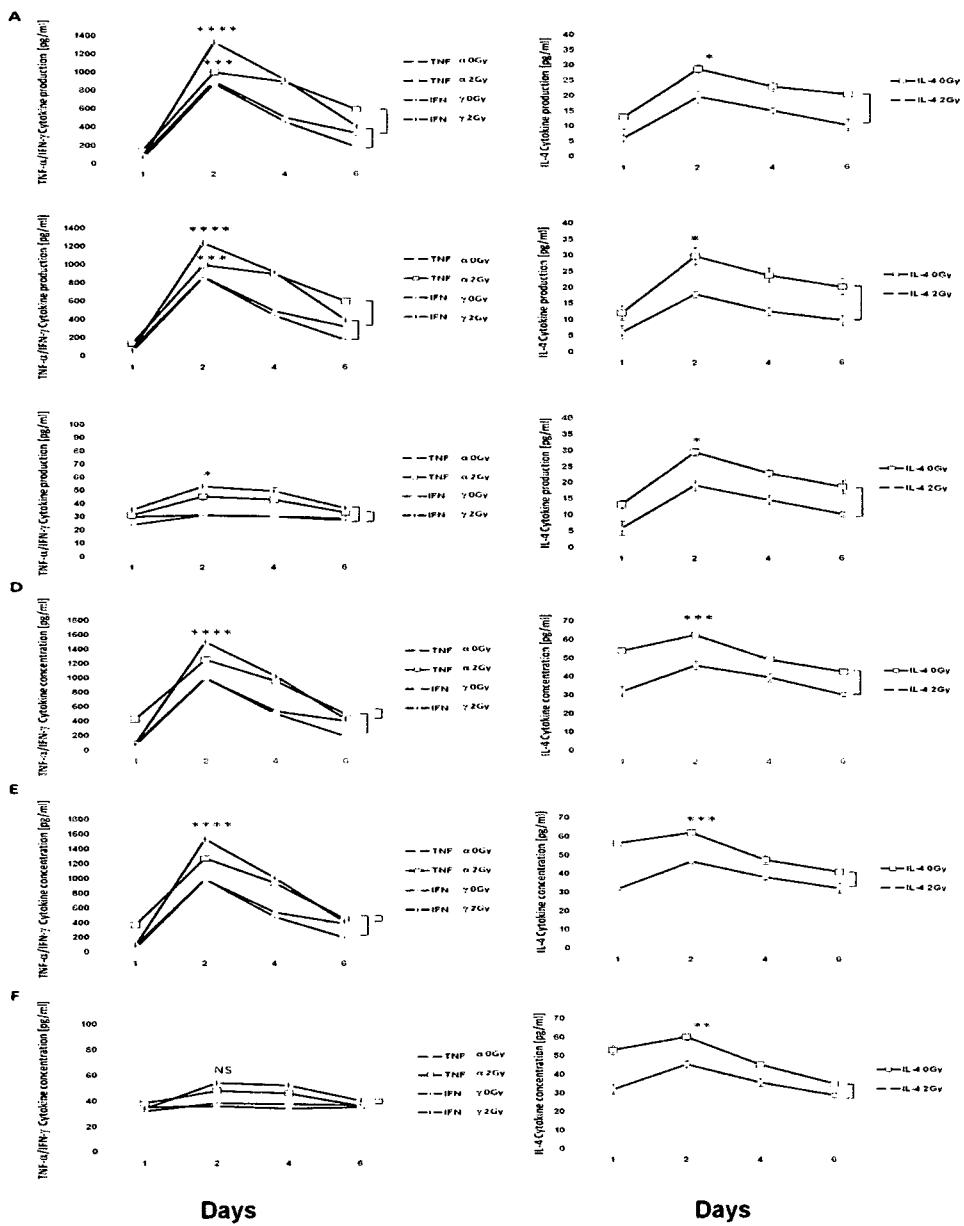


Figure 8. XRT enhances Monocyte and Conventional Dendritic Cell response: supernatants from allogeneic MLR demonstrate XRT enhances TNF- α and IFN- γ cytokine production in a NEMO dependent manner, but not IL-4.

(A-C) Monocytes ($CD14^+$) were either treated with 0, 2, or 20 Gy XRT alone or pre-incubated with the control or NBD peptide for 12 hrs. Following irradiation, cells were

incubated for 12 hrs before adding CD4⁺ T cells for the MLR. (A) TNF- α , IFN- γ , and IL-4 cytokine production [pg/ml] before and after radiation without peptide. (B) with control peptide, and (C) with NBD peptide

(D-F) Dendritic cells (Lin $^-$) were either treated with 0, 2, or 20 Gy XRT alone or pre-incubated with the control or NBD peptide for 12 hrs before adding CD4⁺ T cells (D) TNF- α , IFN- γ , and IL-4 cytokine production before and after radiation without peptide, (E) with control peptide, and (F) with NBD peptide (A-F) *(p=<0.05), ***(p=<0.001) and ****(p=<0.0001). Shown is the average cytokine production (from triplicates) \pm S.E.M. from five independent experiments.

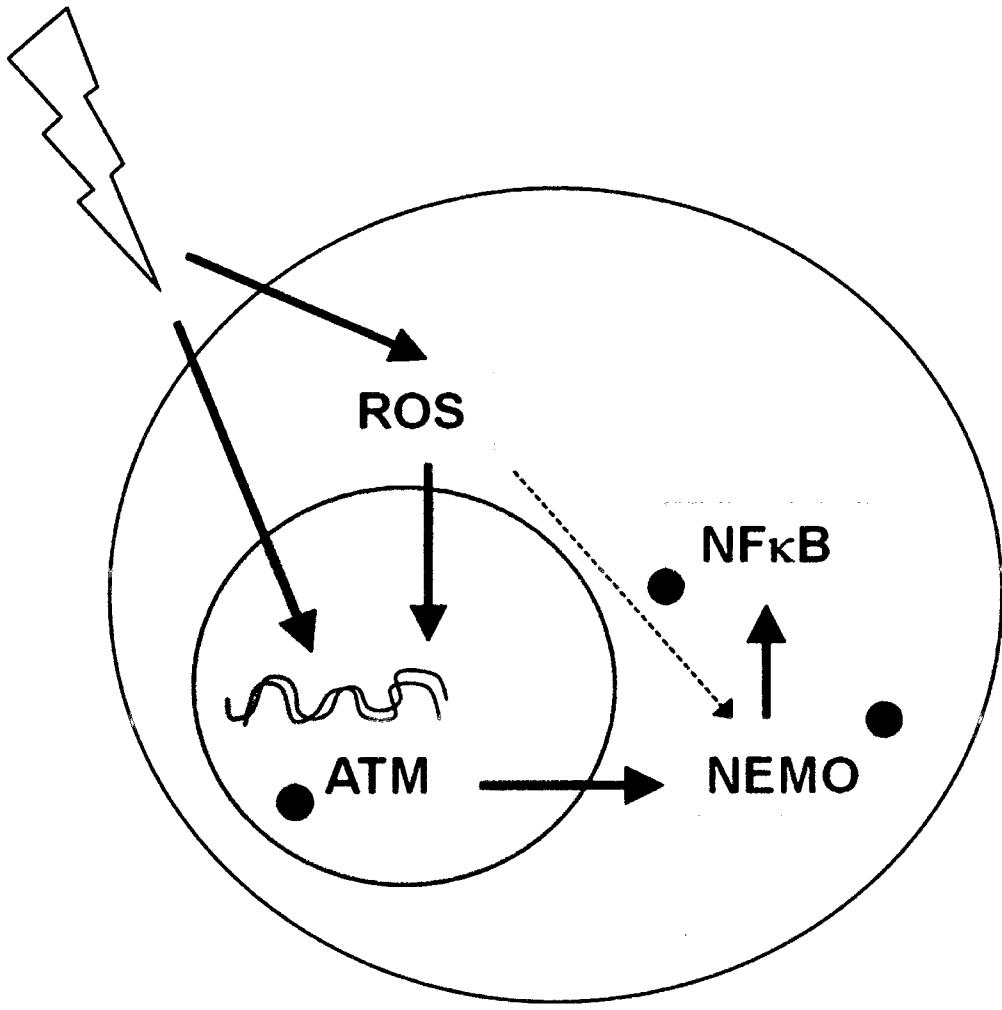


Figure 9. XRT-induced activation of NF κ B pathway requires ATM, but not ROS in U937 cells.

Schematic of the mechanism of action for XRT-induced NF κ B changes in U937 cells, in which XRT induced double-strand breaks, results in phosphorylation of ATM, and subsequent activation and phosphorylation of NEMO. ROS may also directly phosphorylate NEMO, but this would be a minor pathway.

References

- Alderton, G. 2007. DNA damage is stressful. *Nat Rev Cancer* 7: 226-227. *J*
- Ahn, K. S. and B. B. Aggarwal. 2005. Transcription factor nf-kappab: A sensor for smoke and stress signals. *Ann N Y Acad Sci* 1056: 218-33. *J*
- Baldwin, A. S., Jr. 1996. The nf-kappa b and i kappa b proteins: New discoveries and insights. *Annu Rev Immunol* 14: 649-83. *J*
- Bellas, R. E., J. S. Lee, and G. E. Sonenshein. 1995. Expression of a constitutive nf-kappa b-like activity is essential for proliferation of cultured bovine vascular smooth muscle cells. *J Clin Invest* 96, no. 5: 2521-7. *J*
- Brody, J. D., M. J. Goldstein, D. K. Czerwinski, and R. Levy. 2009. Immunotransplantation preferentially expands t-effector cells over t-regulatory cells and cures large lymphoma tumors. *Blood* 113, no. 1: 85-94. *J*
- Buggins, A. G., N. Lea, J. Gaken, D. Darling, F. Farzaneh, G. J. Mufti, and W. J. Hirst. 1999. Effect of costimulation and the microenvironment on antigen presentation by leukemic cells. *Blood* 94, no. 10: 3479-90. *J*
- Cameron, R. B., P. J. Spiess, and S. A. Rosenberg. 1990. Synergistic antitumor activity of tumor-infiltrating lymphocytes, interleukin 2, and local tumor irradiation. Studies on the mechanism of action. *J Exp Med* 171, no. 1: 249-63. *J*

Chakravarty, P. K., A. Alfieri, E. K. Thomas, V. Beri, K. E. Tanaka, B. Vikram, and C. Guha. 1999. Flt3-ligand administration after radiation therapy prolongs survival in a murine model of metastatic lung cancer. *Cancer Res* 59, no. 24: 6028-32. J

Canman, C. E., A. C. Wolff, C. Y. Chen, A. J. Fornace, Jr., and M. B. Kastan. 1994. The p53-dependent g1 cell cycle checkpoint pathway and ataxia-telangiectasia. J
Cancer Res 54, no. 19: 5054-8.

Demaria, S., N. Bhardwaj, W. H. McBride, and S. C. Formenti. 2005. Combining radiotherapy and immunotherapy: A revived partnership. *Int J Radiat Oncol Biol Phys* 63, no. 3: 655-66. J

Demaria, S. and S. C. Formenti. 2007. Sensors of ionizing radiation effects on the immunological microenvironment of cancer. *Int J Radiat Biol* 83, no. 11-12: 819-25. J

Friedman, E. J. 2002. Immune modulation by ionizing radiation and its implications for cancer immunotherapy. *Curr Pharm Des* 8, no. 19: 1765-80. J

Gallucci, S. and P. Matzinger. 2001. Danger signals: Sos to the immune system. *Curr Opin Immunol* 13, no. 1: 114-9. J

Ganss, R., E. Ryschich, E. Klar, B. Arnold, and G. J. Hammerling. 2002. Combination of t-cell therapy and trigger of inflammation induces remodeling of the vasculature and tumor eradication. *Cancer Res* 62, no. 5: 1462-70. J

- Hallahan, D. E., D. R. Spriggs, M. A. Beckett, D. W. Kufe, and R. R. Weichselbaum. 1989. Increased tumor necrosis factor alpha mrna after cellular exposure to ionizing radiation. *Proc Natl Acad Sci U S A* 86, no. 24: 10104-7. J
- Harris, P. and P. Ralph. 1985. Human leukemic models of myelomonocytic development: A review of the hl-60 and u937 cell lines. *J Leukoc Biol* 37, no. 4: 407-22. J
- Hayden, M. S., A. P. West, and S. Ghosh. 2006. Nf-kappab and the immune response. *Oncogene* 25, no. 51: 6758-80. J
- Ho, E., G. Chen, and T. M. Bray. 1999. Supplementation of n-acetylcysteine inhibits nfkappab activation and protects against alloxan-induced diabetes in cd-1 mice. *FASEB J* 13, no. 13: 1845-54. J
- Hong, J. H., C. S. Chiang, C. Y. Tsao, P. Y. Lin, W. H. McBride, and C. J. Wu. 1999. Rapid induction of cytokine gene expression in the lung after single and fractionated doses of radiation. *Int J Radiat Biol* 75, no. 11: 1421-7. J
- Ishihara, H., K. Tsuneoka, A. B. Dimchev, and M. Shikita. 1993. Induction of the expression of the interleukin-1 beta gene in mouse spleen by ionizing radiation. *Radiat Res* 133, no. 3: 321-6. J
- Kim, K. W., S. H. Kim, J. G. Shin, G. S. Kim, Y. O. Son, S. W. Park, B. H. Kwon, D. W. Kim, C. H. Lee, M. Y. Sol, M. H. Jeong, B. S. Chung, and C. D. Kang. 2004. Direct injection of immature dendritic cells into irradiated tumor induces efficient antitumor immunity. *Int J Cancer* 109, no. 5: 685-90. J

Koren, H. S., S. J. Anderson, and J. W. Lerrick. 1979. In vitro activation of a human macrophage-like cell line. *Nature* 279, no. 5711: 328-31. 

Lan, F., D. Zeng, M. Higuchi, P. Huie, J. P. Higgins, and S. Strober. 2001. Predominance of nk1.1+tcr alpha beta+ or dx5+tcr alpha beta+ t cells in mice conditioned with fractionated lymphoid irradiation protects against graft-versus-host disease: "Natural suppressor" Cells. *J Immunol* 167, no. 4: 2087-96. 

Larsson, M., J. F. Fonteneau, and N. Bhardwaj. 2001. Dendritic cells resurrect antigens from dead cells. *Trends Immunol* 22, no. 3: 141-8. 

Magne, N., R. A. Toillon, V. Bottero, C. Didelot, P. V. Houtte, J. P. Gerard, and J. F. Peyron. 2006. Nf-kappab modulation and ionizing radiation: Mechanisms and future directions for cancer treatment. *Cancer Lett* 231, no. 2: 158-68. 

Matzinger, P. 2002. The danger model: A renewed sense of self. *Science* 296, no. 5566: 301-5. 

Mayo, M. W., C. Y. Wang, P. C. Cogswell, K. S. Rogers-Graham, S. W. Lowe, C. J. Der, and A. S. Baldwin, Jr. 1997. Requirement of nf-kappab activation to suppress p53-independent apoptosis induced by oncogenic ras. *Science* 278, no. 5344: 1812-5. 

McBride, W. H., C. S. Chiang, J. L. Olson, C. C. Wang, J. H. Hong, F. Pajonk, G. J. Dougherty, K. S. Iwamoto, M. Pervan, and Y. P. Liao. 2004. A sense of danger from radiation. *Radiat Res* 162, no. 1: 1-19. 

McLellan, A. D., E. B. Brocker, and E. Kampgen. 2000. Dendritic cell activation by danger and antigen-specific t-cell signalling. *Exp Dermatol* 9, no. 5: 313-22. J

Morgan, S. E. and M. B. Kastan. 1997. P53 and atm: Cell cycle, cell death, and cancer.

Adv Cancer Res 71: 1-25. J

Muul, L. M., C. Silvin, S. P. James, and F. Candotti. 2008. Measurement of proliferative responses of cultured lymphocytes. *Curr Protoc Immunol Chapter 7: Unit 7 10 1-* J
7 10 24.

Nikitina, E. Y. and D. I. Gabrilovich. 2001. Combination of gamma-irradiation and dendritic cell administration induces a potent antitumor response in tumor-bearing mice: Approach to treatment of advanced stage cancer. *Int J Cancer* 94, no. 6: J
825-33.

Nordberg, J. and E. S. Arner. 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* 31, no. 11: 1287-312. J

Olive, P. L. and J. P. Banath. 2004. Phosphorylation of histone h2ax as a measure of radiosensitivity. *Int J Radiat Oncol Biol Phys* 58, no. 2: 331-5. J

Passmore, J. S., P. T. Lukey, and S. R. Ress. 2001. The human macrophage cell line u937 as an in vitro model for selective evaluation of mycobacterial antigen-specific cytotoxic t-cell function. *Immunology* 102, no. 2: 146-56. J

Paulos, C. M., C. Wrzesinski, A. Kaiser, C. S. Hinrichs, M. Chieppa, L. Cassard, D. C. Palmer, A. Boni, P. Muranski, Z. Yu, L. Gattinoni, P. A. Antony, S. A. Rosenberg, and N. P. Restifo. 2007. Microbial translocation augments the

function of adoptively transferred self/tumor-specific cd8+ t cells via tlr4
signaling. *J Clin Invest* 117, no. 8: 2197-204. J

Rahman, I., A. Kode, and S. K. Biswas. 2006. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat Protoc* 1, no. 6: 3159-65. J

Rieser, C., G. Bock, H. Klocker, G. Bartsch, and M. Thurnher. 1997. Prostaglandin e2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: Synergistic activation of interleukin 12 production. *J Exp Med* 186, no. 9: 1603-8. J

Ravi, R., A. Bedi, and E. J. Fuchs. 1998. Cd95 (fas)-induced caspase-mediated proteolysis of nf-kappab. *Cancer Res* 58, no. 5: 882-6. J

Ryan, K. M., M. K. Ernst, N. R. Rice, and K. H. Vousden. 2000. Role of nf-kappab in p53-mediated programmed cell death. *Nature* 404, no. 6780: 892-7. J

Scheidereit, C. 2006. Ikappab kinase complexes: Gateways to nf-kappab activation and transcription. *Oncogene* 25, no. 51: 6685-705. J

Siebenlist, U., G. Franzoso, and K. Brown. 1994. Structure, regulation and function of nf-kappa b. *Annu Rev Cell Biol* 10: 405-55. J

Steinauer, K. K., I. Gibbs, S. Ning, J. N. French, J. Armstrong, and S. J. Knox. 2000. Radiation induces upregulation of cyclooxygenase-2 (cox-2) protein in pc-3 cells. *Int J Radiat Oncol Biol Phys* 48, no. 2: 325-8. J

Strickland, I. and S. Ghosh. 2006. Use of cell permeable nbd peptides for suppression of inflammation. *Ann Rheum Dis* 65 Suppl 3: iii75-82. J

Sundstrom, C. and K. Nilsson. 1976. Establishment and characterization of a human histiocytic lymphoma cell line (u-937). *Int J Cancer* 17, no. 5: 565-77. *J*

Teitz-Tennenbaum, S., Q. Li, R. Okuyama, M. A. Davis, R. Sun, J. Whitfield, R. N. Knibbs, L. M. Stoolman, and A. E. Chang. 2008. Mechanisms involved in radiation enhancement of intratumoral dendritic cell therapy. *J Immunother* 31, no. 4: 345-58. *J*

Teitz-Tennenbaum, S., Q. Li, S. Rynkiewicz, F. Ito, M. A. Davis, C. J. McGinn, and A. E. Chang. 2003. Radiotherapy potentiates the therapeutic efficacy of intratumoral dendritic cell administration. *Cancer Res* 63, no. 23: 8466-75. *J*

Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 3, no. 2: 133-46. *J*

Verhasselt, V., W. Vanden Berghe, N. Vanderheyde, F. Willems, G. Haegeman, and M. Goldman. 1999. N-acetyl-l-cysteine inhibits primary human t cell responses at the dendritic cell level: Association with nf-kappab inhibition. *J Immunol* 162, no. 5: 2569-74. *J*

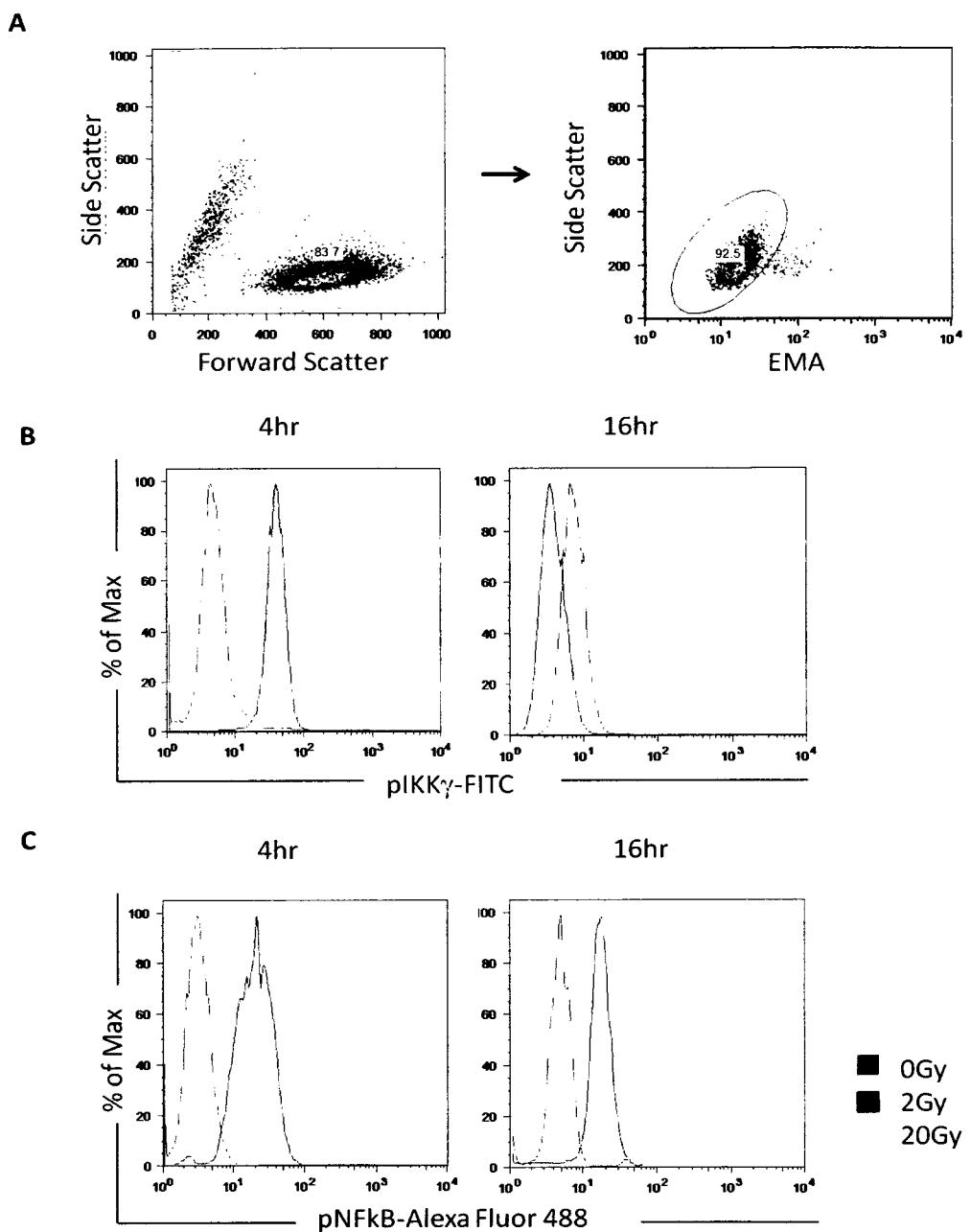
Verheij, M. and H. Bartelink. 2000. Radiation-induced apoptosis. *Cell Tissue Res* 301, no. 1: 133-42. *J*

Wang, T., Y. C. Hu, S. Dong, M. Fan, D. Tamae, M. Ozeki, Q. Gao, D. Gius, and J. J. Li. 2005. Co-activation of erk, nf-kappab, and gadd45beta in response to ionizing radiation. *J Biol Chem* 280, no. 13: 12593-601. *J*

Watters, D. 1999. Molecular mechanisms of ionizing radiation-induced apoptosis.

Immunol Cell Biol 77, no. 3: 263-71. 

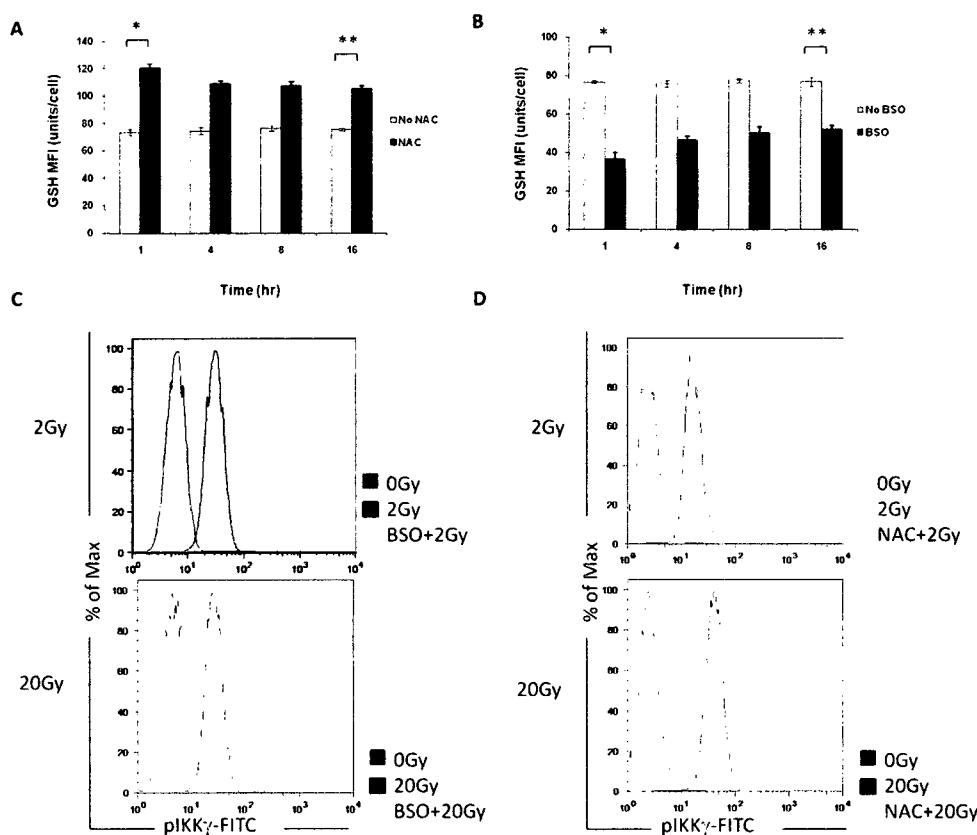
Zong, Z. W., T. M. Cheng, Y. P. Su, X. Z. Ran, N. Li, G. P. Ai, and H. Xu. 2006. Crucial role of sdf-1/cxcr4 interaction in the recruitment of transplanted dermal multipotent cells to sublethally irradiated bone marrow. *J Radiat Res (Tokyo)* 47, no. 3-4: 287-93. 



Supplemental Figure 1. XRT-induced phosphorylation of NEMO and NF κ B.

(A) Representative dot plots of unstained forward versus side scatter gated U937 cells and subsequent gating of live cells using Ethidium Monoazide (EMA) in unirradiated

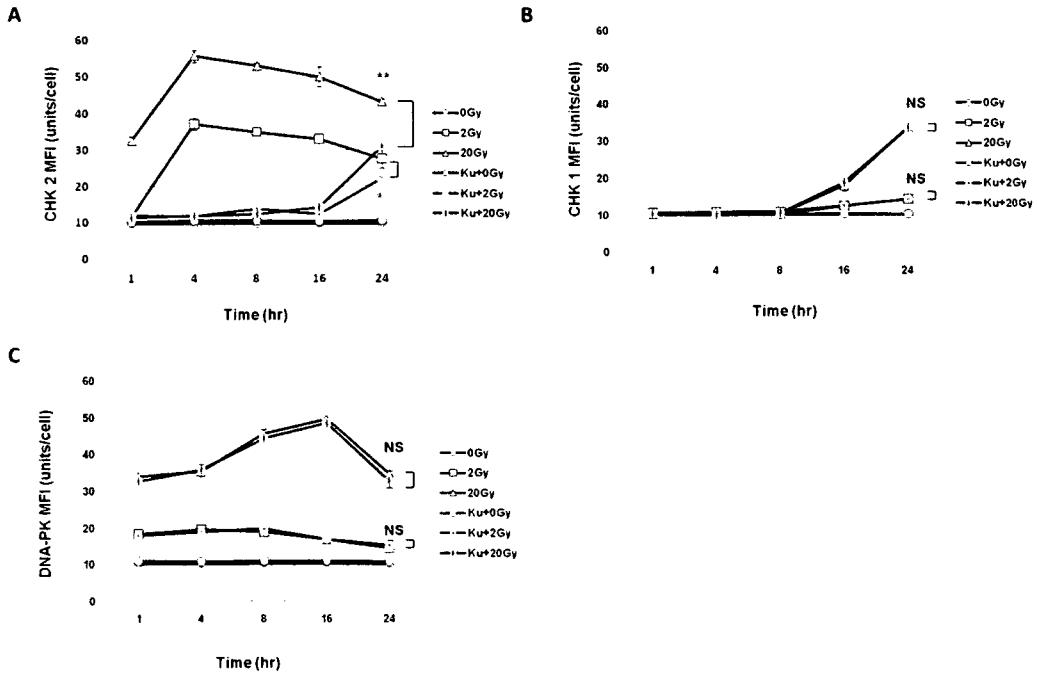
sample. Gated histograms of cells stained with NEMO or NF κ B. Shown are representative histograms of U937 p53 mutant cells at 4hr and 16hr phosphorylation of (B) NEMO and (C) NF κ B before and after 2 and 20 Gy.



Supplemental Figure 2. Redox-modulatory agents BSO and NAC effect on GSH levels and histograms of BSO and NAC on XRT-induced phosphorylation of NEMO.

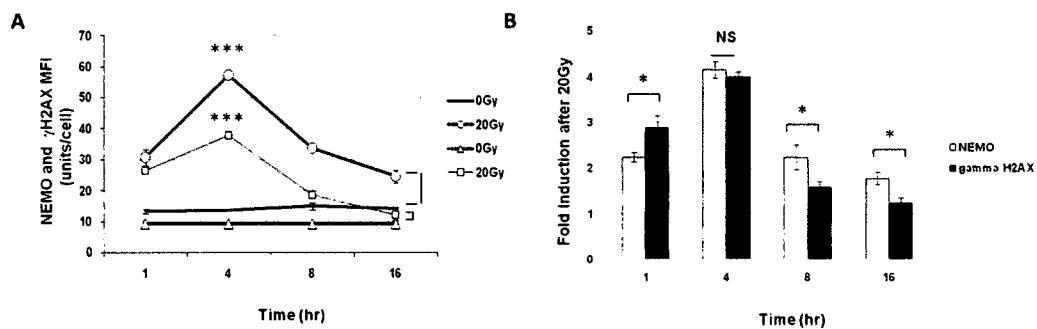
(A) GSH levels at 1-16hrs after treatment with 3.95 μ M BSO for 3 hrs or 10mM NAC treatment for 3 hrs in U937 mutant cells. *($p=<0.05$) and **($p=<0.01$). For all experiments, data is shown as the normalized average MFI \pm S.E.M. for three independent experiments unless otherwise noted. (B) Representative histograms of 3.95 μ M BSO+XRT versus XRT alone changes in phosphorylation of NEMO at 1hr. (C)

Representative histograms of 10mM NAC+ XRT versus XRT alone changes in phosphorylation of NEMO at 1hr.



Supplemental Figure 3. Kinetics of Ku 55933 effects on XRT-induced phosphorylation of CHK2, CHK1, and DNA-PK.

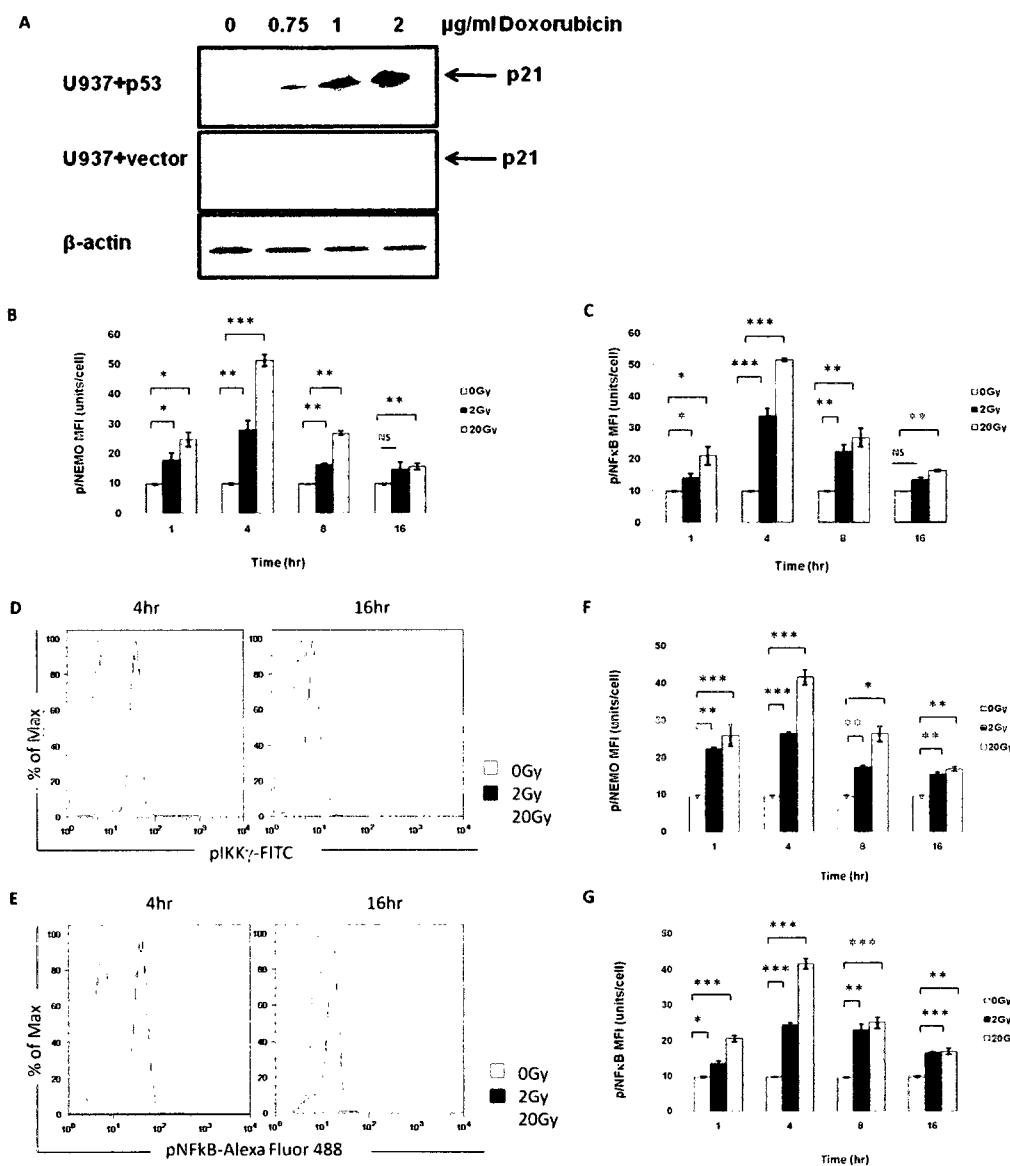
(A) Phosphorylation of CHK2 in U937 cells 1-24 hrs after irradiation with 0, 2, and 20 Gy or with ATM inhibitor (Ku 55933) pre-treatment before irradiation. (B) Phosphorylation of CHK1 in U937 cells 1-24 hrs after irradiation with 0, 2, and 20 Gy or with ATM inhibitor (Ku 55933) pre-treatment before irradiation. (C) Phosphorylation of DNA-PK in U937 cells 1-24 hrs after irradiation with 0, 2, and 20 Gy or with ATM inhibitor (Ku 55933) pre-treatment before irradiation. (A-C) NS=not significant, *($p=<0.05$) and ** ($p=<0.01$). .



Supplemental Figure 4. Correlation between γ H2AX and NEMO phosphorylation profiles after XRT.

(A) Phosphorylation of γ H2AX (Δ and \square) and NEMO (\circ and \times) in U937 cells 1-16hrs after irradiation with 0, 2, and 20 Gy. *($p=<0.05$), ** ($p=<0.01$), and ***($p=<0.001$),.

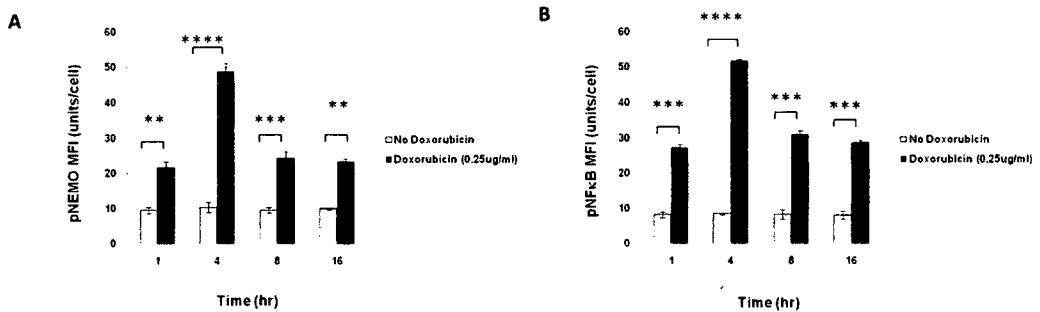
(B) Fold induction of γ H2AX and NEMO after 20 Gy XRT.



Supplemental Figure 5. XRT-induced phosphorylation of NEMO and NF κ B is p53 independent.

(A) Restoration of p53 function in U937 cells. Measurement of p21 by western blot analysis in doxorubicin-treated U937 cells transfected with p53 or an empty vector. The p53 transfected U937 cells exhibited dose-dependent p21 upregulation while the empty

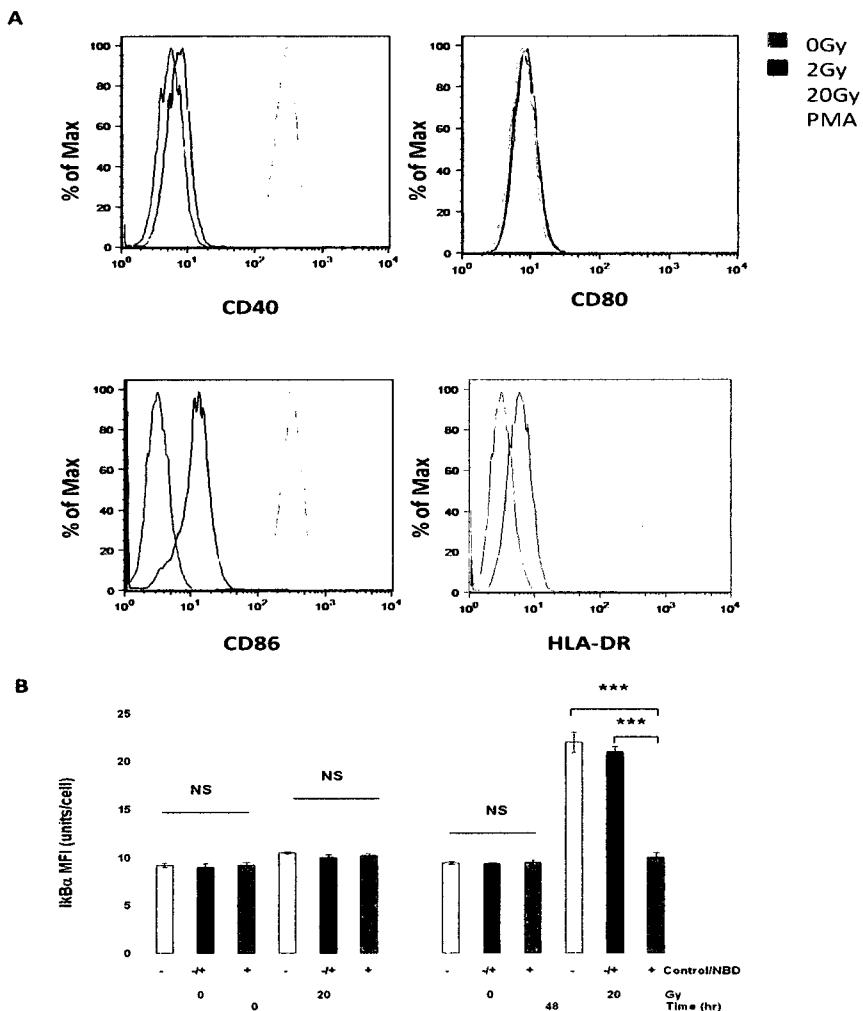
vector control cells did not. β -actin served as a protein loading control. (B) Treatment of U937 cells with XRT showed increased levels of NEMO phosphorylation in U937 p53 wildtype cells 1-16 hrs after irradiation with 0, 2, and 20 Gy and (C) levels of NF κ B phosphorylation in U937 p53 wildtype cells 1-16 hrs after irradiation with 0, 2, and 20 Gy. (B-C) NS=not significant, *(p=<0.05), **(p=<0.01), and ***(p=<0.001). (D-E) Shown are representative histograms of U937 p53 wildtype cells at 4hr and 16hr phosphorylation of (D) NEMO and (E) NF κ B before and after 2 and 20 Gy. (F-G) Empty vector cells do not alter XRT-induced changes of in NF κ B pathway in U937 p53 mutant cells. (F) Levels of NEMO phosphorylation in U937 empty vector cells 1-16 hrs after irradiation with 0, 2, and 20 Gy. (G) Levels of NF κ B phosphorylation in U937 empty vector cells 1-16 hrs after irradiation with 0, 2, and 20 Gy. (F-G) *(p=<0.05), **(p=<0.01), ***(p=<0.001).



Supplemental Figure 6. Doxorubicin-induced phosphorylation of NEMO and NF κ B pathway is p53 independent.

(A) Levels of NEMO phosphorylation in wildtype p53 U937 cells 3 hrs after 0.25μg/ml Doxorubicin treatment 0, 2, and 20 Gy. (B) Levels of NF κ B phosphorylation in

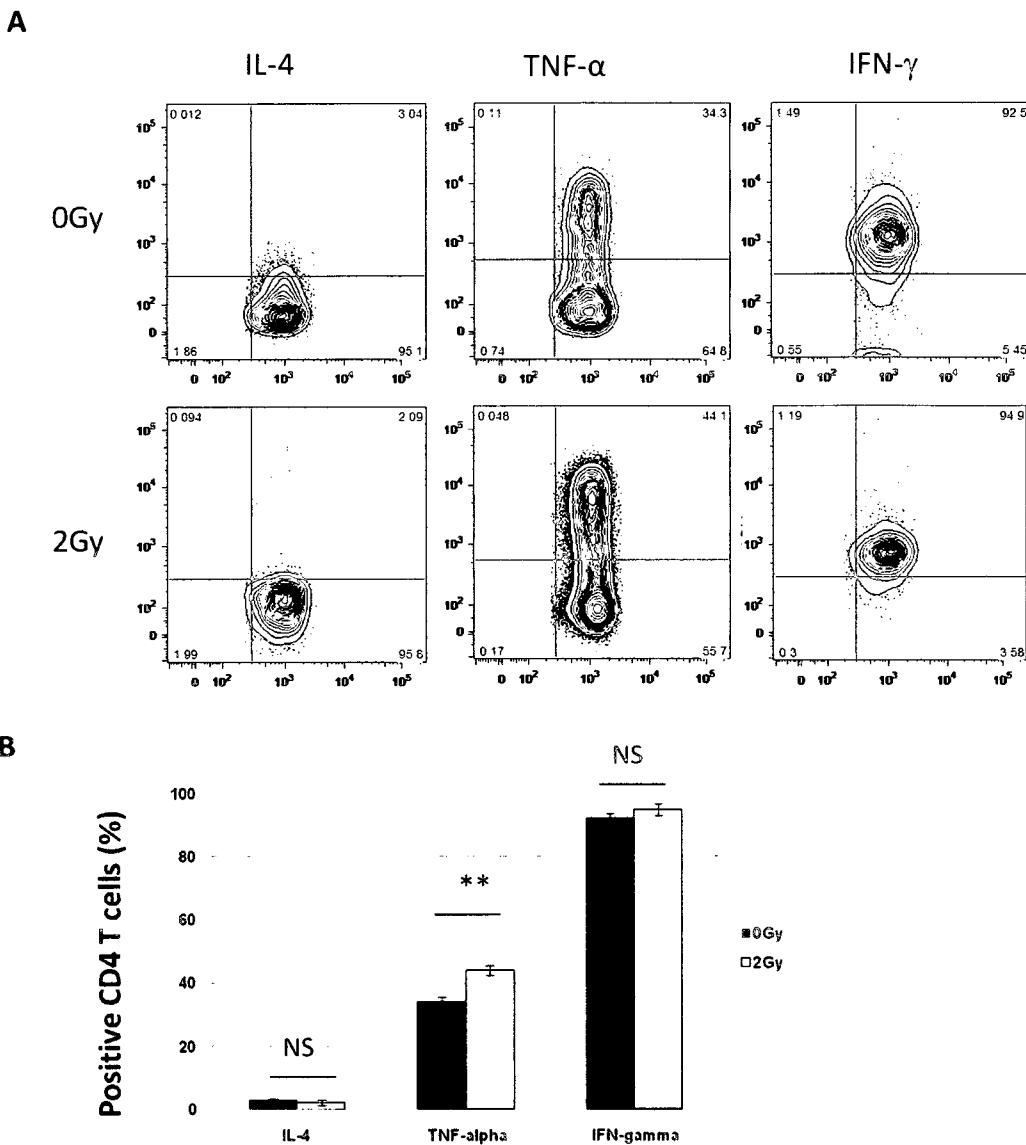
wildtype p53 U937 cells 3 hrs after 0.25 µg/ml Doxorubicin treatment 0, 2, and 20 Gy.
 (A-B) **(p=<0.01), ***(p=<0.001), and ****(p=<0.0001).



Supplemental Figure 7. XRT increases expression of co-stimulatory and MHC molecules on U937 cells.

(A) Shown are representative histograms of increased expression of CD40, CD86, HLA-DR, and PMA (positive control) before and after 48 hours XRT. (B) NBD inhibitory peptide does inhibit XRT-induced phosphorylation of NFκB pathway. Control

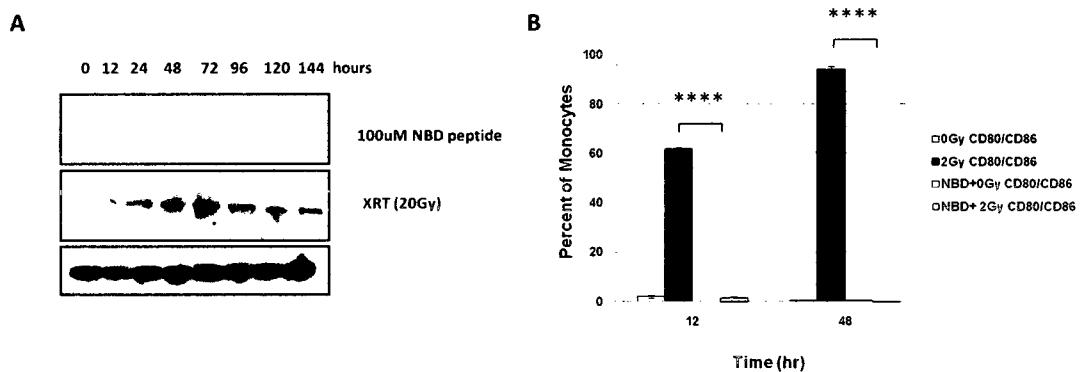
experiment shows level of I κ B α phosphorylation in U937 cells after irradiation with 0 or 20 Gy alone (-)and pre-treatment with control peptide (-/+) or NBD peptide (+) before 0 or 20 Gy at 0 and 48 hrs. NS=not significant, **(p=<0.01), and ***(p=<0.001).



Supplemental Figure 8. Intracellular cytokines TNF- α , IFN- γ , and IL-4 are produced by CD4+ T cells in allogeneic MLR.

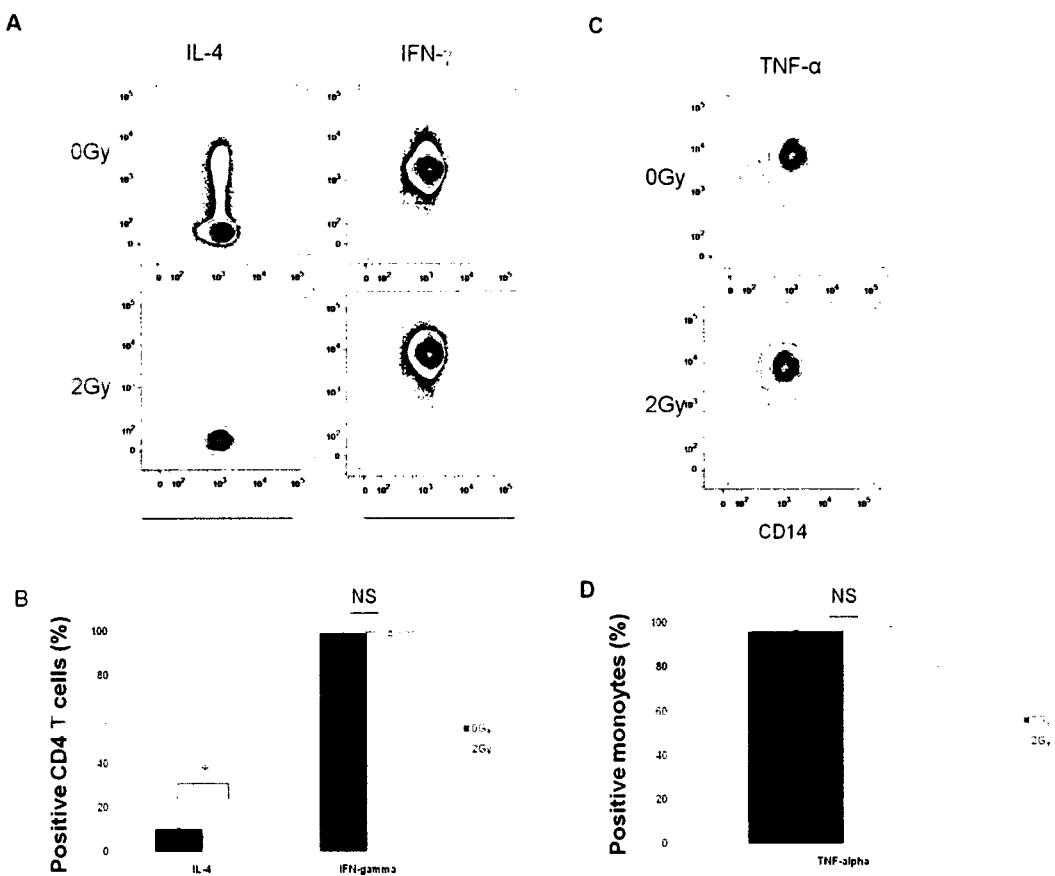
U937 cells and CD4+ T cells that were combined in MLR were pooled according to their radiation dose of 0 Gy or 2 Gy at 48 hours and activated with Leukocyte Activation Cocktail and Monensin for 4 hours before surface marker and intracellular cytokine

staining. (A) Two-dimensional plots of the percentage of cytokine positive CD4+ T cells versus CD4. (B) Shows bar-graph representation of the mean positive IL-4, TNF- α , and IFN- γ T cells. Shown are the average percentages of positive cells \pm S.E.M. from three independent experiments. NS=not significant and **(p=<0.01).



Supplemental Figure 9. Increased CD80 and CD86 expression by Monocytes is dependent on NEMO activation in allogeneic MLR.

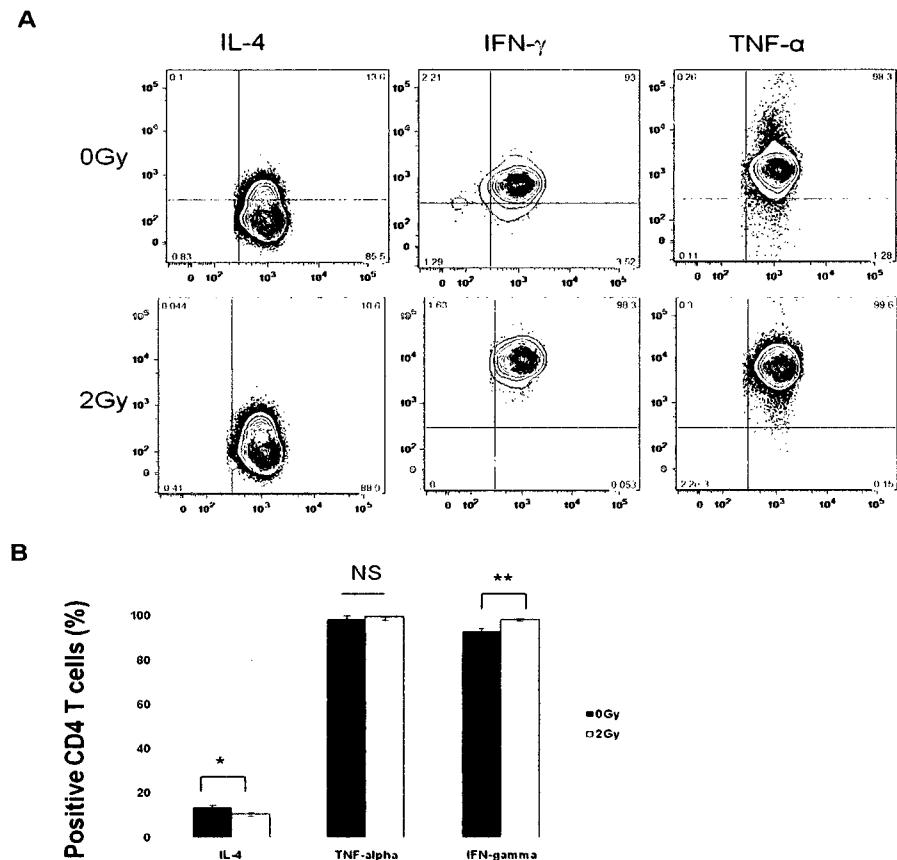
(A) NBD inhibitory peptide does inhibit activation of NF κ B pathway. Control experiment shows western blot of NBD peptide inhibition of I κ B α in monocytes that were pre-treated before XRT. (B) Co-stimulatory molecule expression from monocytes was measured in monocytes combined with CD4+ T cells in MLR. (B) The mean CD80/86 expression percentages from unirradiated and irradiated monocytes at 12 and 48 hours. Shown is the average percentage \pm S.E.M. from five independent experiments. ***(p=<0.0001).



Supplemental Figure 10. IFN- γ and IL-4 are produced by CD4+ T cells and TNF- α by monocytes in allogeneic MLR.

Monocytes and CD4+ T cells that were combined in MLR were pooled according to their radiation dose of 0 Gy or 2 Gy at 48 hours and activated to detect intracellular cytokine staining. (A) Two-dimensional plots of the percentage of cytokine versus CD4. (B) Shows bar-graph representation of the mean positive IL-4 and IFN- γ T cells. (C) Two-dimensional plots of the percentage of cytokine versus CD14. (D) Shows bar-graph representation of the mean positive TNF- α monocytes. Shown are the average

percentages of positive cells \pm S.E.M. from five independent experiments. NS=not significant and *(p=<0.05).



Supplemental Figure 11. TNF- α , IFN- γ and IL-4 are produced by CD4+ T cells in allogeneic MLR.

Dendritic Cells and CD4+ T cells that were combined in MLR were pooled according to their radiation dose of 0 Gy or 2 Gy at 48 hours and activated to detect intracellular cytokine staining. (A) Two-dimensional plots of the percentage of cytokine versus CD4. (B) Shows bar-graph representation of the mean positive IL-4, TNF- α , and IFN- γ T cells.

Shown are the average percentages of positive cells \pm S.E.M. from five independent experiments. NS=not significant, *($p=<0.05$), and **($p=<0.01$).

CHAPTER 3

DIFFERENTIAL BAX EXPRESSION IS A DETERMINANT OF RADIOSensitivity IN T CELLS AND ANTIGEN PRESENTING CELL SUBSETS

Abstract

Studies have shown that changes in host T cell immunity play an important role in graft versus host disease and graft versus leukemia effects. However, studies of the effect of radiation on the balance of immune cells have been primarily focused on T cell subsets. The mechanisms for the relative radioresistance of human memory T cells and APC subsets specifically monocytes and dendritic cells, are not well understood. The experiments described here demonstrate a hierarchy of radiosensitivity among T cells, with naïve CD8 T cells being the most radiosensitive and CD4 memory T cells being the most radioresistant. In addition, APC were found to be significantly more radioresistant (up to a 150 fold more) than T cell subsets, and among APC, monocytes were more radiosensitive than either total or conventional dendritic cells. T cells and APC both demonstrate varying propensities to undergo apoptotic death after radiation as indicated by differential levels of Bax expression. Although ultimate survival of the “radioresistant” cell subsets is also diminished, the short term shifts in subset ratios modify the post-radiation immune system in a manner that is relevant to immunomodulatory therapies.

Introduction

Both myeloablative and non-myeloablative radiation can be an important component of preparatory regimens for hematopoietic stem cell transplantation used to treat patients with hereditary and malignant diseases. For instance, some studies have used non-myeloablative techniques, such as total lymphoid irradiation, in which the major lymph node groups and spleen are irradiated to prevent rejection of the allogeneic donor cells by residual host immune cells (Field and Strober 2001; Mielcarek et al. 2003), and facilitate engrafted donor T cells mediated graft anti-tumor activity, and tumor cell eradication (Lan et al. 2003; Lan et al. 2001; Luznik et al. 2003; Maris et al. 2004).

Radiation causes significant lymphodepletion due to the marked sensitivity of lymphocytes to irradiation. Furthermore, radiation is known to increases antigen presentation (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005; Friedman 2002), causes pro-inflammatory cytokine release (TNF- α , IL-1 β , IL-2) (Hallahan et al. 1989; Hong et al. 1999; Ishihara et al. 1993; Rieser et al. 1997), stimulates “danger response” pathways (Steinauer et al. 2000), induces dendritic cell maturation by known inducers prostaglandin E2 (PEG-2) and cyclooxygenase-2 (COX-2) (Demaria et al. Combining radiotherapy and immunotherapy: A revived partnership 2005; Rieser et al. 1997; Steinauer et al. 2000), promotes anti-tumor effects of T cells via DC activation (Paulos et al. 2007; Ren et al. 2006) and stimulates APC homing to irradiated sites (Teitz-Tennenbaum et al. 2008). These effects are dependent

upon radiation-induced immune modulation, the understanding of which is rudimentary in terms of the effects of radiation on specific immune cell subsets and their functions.

More recent studies have shown that the T cell subset depletion by myeloablative or non-myeloablative radiation is not uniform, due to the differential sensitivity of immune cell subsets to radiation induced cell death (Anderson et al. 2004; Yao et al. 2009). As a result, the balance of T cell subsets is altered after non-myeloablative or myeloablative irradiation, with increased survival of regulatory T cells, including CD4+ natural killer (NK) T cells and CD4⁺CD25⁺ Treg cells, as compared to conventional (CD4 and CD8 T cells) (Fuks and Slavin 1981; Higuchi et al. 2002; Joffre et al. 2008; Pillai et al. 2007; Strober 1987). This has been observed in mice conditioned with non-myeloablative total lymphoid irradiation and anti-thymocyte globulin, in which there was a relative increase in regulatory NKT cells and CD4⁺CD25⁺ Treg cells in the immediately post-transplantation period (Edinger et al. 2003; Kohrt et al. 2009; Lan et al. 2003; Lan et al. 2001; Tamada et al. 1998). This change in the T cell subset balance provides graft versus leukemic (GVL) benefits without the risk of acute graft versus host disease (GVHD). This approach can result in successful engraftment of allogeneic bone marrow or peripheral stem cell transplants after nonmyeloablative irradiation, and is dependent in part on the survival of residual NK T cells and CD4⁺CD25⁺ Treg cells that suppress alloreactivity of residual host conventional T cells (Higuchi et al. 2002; Joffre et al. 2008).

Previous studies have shown that the selective effect of radiation on T cell subsets is partially due to the differential expression of anti-apoptotic proteins. NKT cells in mice have been reported to express high levels of anti-apoptotic proteins such as Bcl-2

constitutively, and to upregulate these proteins after exposure to irradiation or exposure to glucocorticoids (Seino, Harada, and Taniguchi 2004; Tamada et al. 1998; Yao et al. 2009). Other studies have demonstrated that the expression of Bcl-2 suppresses the p53 mitochondrial-mediated apoptotic pathway, and thymocytes from mice with an inactivated Bcl-2 gene are dramatically more sensitive to radiation induced cell death than thymocytes from wild type mice (Veis et al. 1993). In contrast, mice overexpressing Bcl-2 were markedly more resistant to radiation induced cell death (Domen, Gandy, and Weissman 1998). Other data suggests that irradiation of T cells results in upregulation of pro-apoptotic Bax expression, but without any alteration in the level of Bcl-2 expression (Ame, Spenlehauer, and de Murcia 2004):

Importantly, monocytes/macrophages, and dendritic cells have been found to be present immediately post stem cell transplantation, and are more radioresistant than T cells. Studies to date have not determined the etiology of the relative radioresistance of antigen presenting cells, in particular monocytes and dendritic cells, or the potential role of the p53 mitochondrial-mediated apoptotic pathway as a mediator of the radioresistance of these important immune cell subpopulations. The primary objectives of the experiments described here were to further elucidate radiation-induced changes in the balance of human T cell subsets including CD4+ and CD8+ total T cells, CD4-CD8- T cells, CD4+ naïve and memory T cells, and CD8 naïve and memory T cells, 2) determine if there is a selective difference in human antigen presenting cell subsets including monocyte and dendritic cells after radiation, and 3) study the role of Bax and Bcl-2 expression in the relative radioresistance of antigen presenting cells.

Materials and Methods

Cells and Culture

Human peripheral blood mononuclear cells (PBMC) (obtained from buffy coats by the Stanford Blood Center, Palo Alto, California) were cultured in human tissue cultured medium (RPMI-1640 medium supplemented with 10% human serum (heat-inactivated for 1hr at 55°C), 2mM glutamine, 100U penicillin/ml, 100µg streptomycin/ml) in a humidified atmosphere containing 5% CO₂ at 37°C with normal oxygen content or under hypoxic conditions (2% O₂).

Irradiation

Cells were irradiated with γ -rays using a ¹³⁷Cs source emitting at a fixed dose rate of 531 cGy/min.

Monoclonal Antibodies and Chemical Reagents

Anti-HLA-DR-PerCP, Anti-CD3-V450, Anti-CD3-FITC, Anti-CD4-PE, Anti-CD45RA-PE-Cy7, Anti-C62L-APC, Anti-Lineage Negative Cocktail-FITC, Anti-Alexa Fluor 700-CD11b, Anti-CD11c-V450, Anti-CD14-APC, Anti-Cleaved PARP-Alexa Fluor 647, Anti-IkBa-PE, Anti-BCL-2-FITC, Anti-Bax-PE, Anti-Bax-PerCP-Cy5.5, APO-Direct Kit (FTIC-dUTP), FACS stain buffer, and Anti-Mouse IgK/Negative Control (FBS) Compensation Particles Set (BD Pharmingen, San Diego, CA) and Anti-CD8-Qdot 655, and LIVE/DEAD fixable dead cell stain kit in Aqua (Invitrogen, Camarillo, CA). Chemical reagents were as follows: 70% ethanol for permeabilization with APO-Direct Kit, 100% Methanol (Stanford Biosciences), 16% Paraformaldehyde (Electron

Microscopy Sciences, Hartfield, PA) (used at 1.5%) for intracellular staining, and Ficoll-Paque Plus (GE Healthcare Amersham Biosciences, Piscataway, NJ).

Flow cytometry analysis

Human PBMC layers were isolated from buffy coats using Ficoll-Hypaque density gradient centrifugation. Buffy coats (8-10 mls) were diluted with phosphate-buffered saline (PBS). Diluted blood was layered onto Ficoll-Hypaque and centrifuged at 2,000 x rpm. Mononuclear layers were removed and washed three times with PBS at 1200 x rpm. Counted cells were aliquoted for treatment with 0 or 20 Gy, incubated for 0-96hrs, and viable cells were counted at various timepoints before staining. LIVE/DEAD fixable green dead cell stain (1:1000 in PBS) was added prior to surface marker staining and analysis to exclude dead cells.

To analyze intracellular IkB α , Bcl-2, Bax, and cleaved PARP, all cells were first incubated with LIVE/DEAD aqua stain, washed twice with PBS, and stained with the appropriate anti-surface receptor mAbs in FACS stain buffer. Cells were fixed with 1.5% paraformaldehyde and permeabilized with 4°C 100% methanol while vortexing, washed with FACS stain buffer twice and incubated with intracellular antibodies at room temperature for 30 minutes. For TUNEL (Anti-BrdU) staining by phospho-flow cytometry to detect fragmented DNA using the APO-Direct Kit (BD Biosciences, San Diego, CA per manufacturer's instructions. Cells were fixed with 1.5% paraformaldehyde and permeabilized with ice cold 70% ethanol, incubated with DNA labeling solution (reaction buffer, TdT enzyme, FITC-dUTP, and distilled water), washed with rinse buffer, and analyzed. Positive and negative control cells were provided for

TUNEL staining. Thresholds for staining (surface and intracellular) were determined using isotype-matched mAbs. All analyses were performed using the LSRII in the Shared FACS Facility (Center for Molecular and Genetic Medicine at Stanford University). Resultant data was analyzed using FlowJo software (TreeStar, Ashland, OR).

Statistical analysis

Difference in absolute number, percent and fold change of immunophenotypic populations of cells were analyzed using the two-tailed Student's *t*-test (GraphPad Software, San Diego, CA). For all tests, *p* value of 0.05 or less was considered significant.

Results

Effect of irradiation on different T cell subsets from healthy donor human peripheral blood mononuclear cells (PBMC)

In order to determine the effect of radiation on human PBMC, cells were treated with 0 or 20 Gy (2000 cGy) and stained for subset surface markers following 0, 24, 48, 72, and 96 incubation. 0 hour timepoint refers to cells that were immediately processed after irradiation. Processing time was 2-3 hours. Figure 1A shows representative examples (out of 5 experiments) of two-color flow cytometric analyses of gated CD3⁺ T cells from untreated and irradiated (20 Gy) human PBMC at 0 and 96 hours. When comparing untreated to irradiated cells at 0 hours (), there was a negligible difference in total CD4⁺ T cells, total CD8⁺ T cells, and CD3⁺CD4⁻CD8⁻ (DN) T cells. However, at 96 hours, there was a modest increase in total CD4⁺ T cells from 54.5% to 63.9% after irradiation and an associated decrease in the percentage of total CD8⁺ from 41.4% to 26.7%, with an associated increase in DN T cells from 4.37% to 8.89%. At 96 hours the ratio of CD4⁺:CD8⁺ cells changed from approximately 1:1 to about 3:1 following irradiation. The mean CD4⁺, CD8⁺, and DN T cell percentages from unirradiated and irradiated human PBMC at 96 hours are shown in Figure 1B.

The relative increase in the percentage of CD4⁺ T cells as compared to the decrease in the percentage of CD8⁺ T cells is explained by the greater decrease in the absolute number of CD8⁺ T cells compared to CD4⁺ T cells (Figure 1C). By 96 hours following 20Gy, the absolute number of total CD4⁺ T cells decreased by approximately

400 fold and the absolute number of CD8⁺ T cells decreased by about 1200 fold compared with unirradiated control cells ($p=<0.0001$) (Figure 1D).

Radiation changes the T cell subset balance to favor effector memory CD4⁺ T cells

Figure 2A shows representative examples of two-color flow cytometric analyses for naïve (CD45RA⁺CD62L⁺) and effector memory (CD45RA⁻CD62L⁻) cells of gated CD4⁺T cells from untreated and irradiated (20 Gy) human PBMC at 0 and 96 hours. At 0 hours there was a small decrease in naïve cells from 72.7% to 62.6% and a small increase in effector memory cells from 8.55% to 11.4% compared to unirradiated control cells. There was a substantial decrease in naïve cells after irradiation from 57.6% to 7.02% and corresponding increase in effector memory cells from 19.5% to 45.2% at the 96 hour timepoint. Figure 2B shows representative examples of two-color flow cytometric analyses for naïve (CD45RA⁺CD62L⁺) and effector memory (CD45RA⁻CD62L⁻) cells of gated CD8⁺T cells from untreated and irradiated (20 Gy) CD3⁺ T cells at 0 and 96 hours. Immediately following irradiation, there was a small decrease in CD8⁺T naïve cells from 67.4% to 57.3% and a small increase in effector memory cells from 7.63% to 16.3%. There was a substantial decrease in naïve cells after irradiation from 46.2 to 12.3% and a substantial associated increase in effector memory cells from 21.3% to 35.8% at 96 hours, with a change in the naïve CD4⁺:CD8⁺ ratio from about 1:1.5 to about 1:2, while the CD4⁺:CD8⁺ ratio stayed at 1:1 for effector memory cells. The mean percentages for naïve CD4⁺ and CD8⁺ cells and effector memory CD4⁺ and CD8⁺ from unirradiated and irradiated human PBMC at 96 hours are shown in Figure 1C. Overall, after irradiation

there are more effector memory cells than naïve cells, although there is a greater percentage of CD4⁺ than CD8⁺ effector memory cells (Figure 2C).

The higher percentage of naïve CD8⁺ T versus CD4⁺ T cells after irradiation can be explained by a greater decrease in the absolute number of naïve CD4⁺ T cells. Furthermore, the greater increase in effector memory CD4⁺ T cells can be explained by a smaller decrease in the absolute number of effector memory CD4⁺ T cells versus effector memory CD8⁺ T cells as shown in Figure 2D. Overall, there is a greater decrease in naïve T cells compared to effector memory T cells after irradiation. When unirradiated cells were compared to irradiated cells at 96 hours, the absolute number of naïve CD4⁺ T cells decreased by approximately 1500 fold and the absolute number of naïve CD8⁺ T cells decreased by about 1800 fold ($p=<0.0001$). In addition, the absolute number of effector memory CD4⁺ T cells decreased by approximately 60 fold whereas the effector memory CD8⁺ T cells decreased by approximately 90 fold ($(p=0.006)$) (Figure 2E).

Effect of irradiation on different APC subsets from healthy donor human PBMC

Figure 3A shows representative examples of two-color flow cytometric analyses for monocytes (HLA-DR⁺CD11b⁺CD14⁺) from untreated and irradiated (20 Gy) human PBMC at 0 and 96 hours. Immediately following irradiation, there was a small decrease in monocytes from 72.5% to 69.6% (0 hours) and a small decrease in monocytes from 71.8% to 51.4% at the 96 hour timepoint. Figure 3B shows representative examples of one-color flow cytometric analyses for total dendritic cells (Lin-, Side Scatter) from untreated and irradiated (20 Gy) human PBMC at 0 and 96 hours. There was a small increase in total dendritic cells from 2.37% to 2.39% at 0 hours and an increase from

2.6% to 4.35% at 96 hours after radiation. The ratio of total dendritic cells increased from 1:1 to 1:2 after radiation. Figure 3C shows representative examples of two-color flow cytometric analyses for conventional dendritic cells (HLA-Dr+, CD11c+) from untreated and irradiated (20 Gy) gated lin- cells at 0 and 96 hours. Initially radiation resulted in a small decrease in conventional dendritic cells from 78% to 73.1% and after 96 hours a significant increase in conventional dendritic cells from 74.7% to 91.9%. The mean percentages for monocytes, total dendritic cells, and conventional dendritic cells from unirradiated and irradiated human PBMC at 96 hours are shown in Figure 3D. The absolute number of monocytes was larger (Figure 1E) than dendritic cells (Figure 3F and 3G), but decreased more than dendritic cells after radiation. At 96 hours following irradiation the absolute number of monocytes decreased by approximately 8 fold and the absolute number of total dendritic cells decreased by approximately 2 fold ($p=<0.0001$). In addition, the absolute number of conventional dendritic cells decreased by approximately 2 fold (Figure 3H).

Increased cleaved PARP activity indicates T cells die by apoptosis, and their radiosensitivity could be due to lack of I κ B α activity.

We next studied the potential role of I κ B α activation, an upstream target of NFkB that is necessary for translocation of NFkB to the nucleus to transcribe genes and its potential interaction with PARP (downstream target of the caspase-mediated apoptotic pathways) following irradiation in CD8 $^{+}$ and CD4 $^{+}$ T cells, in order to try to better understand the etiology of the relative radiosensitivity of CD8 $^{+}$ T cells. Cells were treated with 0 Gy and 20 Gy (2000cGy) and stained for subset surface makers, as well as

intracellular I κ B α and cleaved PARP at 0, 1, 4, 8, 24, 48, 72, and 96 hours. PARP was chosen for study because the catalytic activity of PARP is increased in cells following DNA damage and plays an important role in normal cellular response to DNA damage. In addition it is the target of the caspase protease activity associated with apoptosis and is cleaved by Caspase 3 during apoptosis (Ame, Spenlehauer, and de Murcia 2004; Darzynkiewicz et al. 1997). Representative examples of two-color flow cytometric analyses of I κ B α $^+$ and PARP $^+$ in unirradiated and irradiated gated CD4 $^+$ T cells and CD8 $^+$ T cells at 0 and 96 hours are shown in Figure 4A and 4B, respectively. Initially, there was no difference in the percentage of cleaved PARP $^+$ CD4 $^+$ T or CD8 $^+$ T cells or I κ B α $^+$ CD4 $^+$ T or CD8 $^+$ T cells. However, at 96 hours post irradiation, the level of cleaved PARP $^+$ CD4 $^+$ T cells increased from 0.55% to 45.5%, with an increase in cleaved PARP $^+$ CD8 $^+$ T cells from 0.28% to 49.5% at 96 hours. At 96 hours there was no significant difference in the ratio of cleaved PARP $^+$ CD4 $^+$: cleaved PARP $^+$ CD8 $^+$, although the mean percentage of cleaved PARP $^+$ CD8 $^+$ T cells was greater than cleaved PARP $^+$ CD4 $^+$ T cells ($p=0.042253$) (Figure 4C), which can be explained by the larger absolute number of cleaved PARP $^+$ CD8 $^+$ T cells (Figure 4D) after 20 Gy radiation than CD4 $^+$ T cells (Figure 4E). At 96 hours following irradiation, the absolute number of cleaved PARP $^+$ CD4 $^+$ T cells increased by approximately 180 fold and the absolute number of cleaved PARP $^+$ CD8 $^+$ T cells increased by about 220 fold ($p=0.0055$) (Figure 4F). There was no increase in the absolute number of I κ B α $^+$ CD4 $^+$ T or CD8 $^+$ T cells after irradiation at the 96 hour timepoint (Figure 4F). There was no evidence of interaction between I κ B α and PARP, since I κ B α expression did not increase with PARP.

Decreased PARP activity and increased I κ B α activity may contribute to APC radioresistance.

Next radiation-induced cleaved PARP and I κ B α expression were studied in APC subsets. Cells were treated with 0 Gy and 20 Gy (2000cGy) and stained for subset surface makers, as well as intracellular for I κ B α and cleaved PARP at 0, 1, 4, 8, 24, 48, 72, and 96 hours. Representative examples of two-color flow cytometric analyses of I κ B α $^+$ and PARP $^+$ in unirradiated and irradiated (20 Gy) gated monocytes and lin $^-$ dendritic cells at 0 and 96 hours are shown in Figure 5A. Initially, there were no cleaved PARP $^+$ monocytes or conventional dendritic cells and only 0.78% I κ B α $^+$ conventional dendritic cells. There was also no increase in cleaved PARP $^+$ monocytes or conventional dendritic cells after irradiation at 96 hours (Figure 5A and 5B). However, there was a dramatic increase in I κ B α $^+$ monocytes from 0.008% to 75.6% and in conventional dendritic cells from 0% to 83.7% after irradiation at the 96 hour timepoint (Figure 5A and 5B). However, at 96 hours there was no significant difference in the ratio of I κ B α $^+$ monocytes: I κ B α $^+$ conventional dendritic cells, although the mean percentage of I κ B α $^+$ conventional dendritic cells was greater than I κ B α $^+$ monocytes ($p=0.017$) following radiation (Figure 5C). In addition, though cleaved PARP $^+$ cells did not increase after irradiation, there was an increase in I κ B α $^+$ PARP $^+$ cells. The I κ B α $^+$ PARP $^+$ cells increased in monocytes from 0% to 24.4% (Figure 5A) and increased slightly less from 0% to 16.3% in conventional dendritic cells (Figure 5B) after irradiation at 96 hours. The mean percentage of I κ B α $^+$ PARP $^+$ monocytes was greater than the mean percentage of I κ B α $^+$ PARP $^+$ conventional dendritic cells (Figure 5C) which can be explained by the larger absolute number of I κ B α $^+$ PARP $^+$ monocytes (Figure 5D) than conventional

dendritic cells (Figure 5E). At 96 hours following irradiation, the absolute number of I κ B α^+ monocytes had increased by approximately 55 fold and the absolute number of I κ B α^+ conventional dendritic cells increased by about 20 fold ($p=<0.0001$) (Figure 5F). The absolute number of I κ B α^+ PARP $^+$ monocytes increased by approximately 21 fold and the absolute number of I κ B α^+ PARP $^+$ conventional dendritic cells increased by about 4 fold ($p=0.0003$) (Figure 5F). When unirradiated cells were compared to irradiated cells at 96 hours, there was no increase in the absolute number of PARP $^+$ monocytes or PARP $^+$ conventional dendritic cells (Figure 5F).

CD8 $^+$ T cells are more sensitive to radiation-induced apoptosis than CD4 $^+$ T cells.

Next, the level of Bax and TUNEL expression in T cells were measured following irradiation. Cells were treated with 0 Gy and 20 Gy (2000cGy) and stained for subset surface makers as well as intracellular Bax and TUNEL at 0, 1, 4, 8, and 24 hours. Figure 6A shows representative examples of two-color flow cytometric analyses of Bax $^+$ and/or TUNEL $^+$ gated CD4 $^+$ T cells and CD8 $^+$ T cells from untreated and irradiated (20 Gy) human PBMC at 0 and 24 hours. Compared to untreated cells at 0 hours, there was a small increase in the percentage of Bax $^+$ CD4 $^+$ T cells from 0.1% to 0.62% and in CD8 $^+$ T cells from 0.96% to 0.98% following irradiation. The change in TUNEL $^+$ CD4 $^+$ T cells or CD8 $^+$ T cells was negligible. Bax $^+$ CD4 $^+$ T cells decreased from 85% to 67.9% after irradiation and there was a significantly greater decrease in the percentage of Bax $^+$ CD8 $^+$ T cells from 93.2% to 16.2% at the 24 hours. The decrease in Bax $^+$ cells corresponded with an increase in TUNEL $^+$ and Bax $^+$ TUNEL $^+$ cells at the 24 hour timepoint. There was a greater increase in TUNEL $^+$ CD8 T from 0.07% to 51.4% than TUNEL $^+$ CD4 $^+$ T cells

(0.007% to 12.3%) at 24 hours. Bax⁺TUNEL⁺ CD4⁺ T cells and Bax⁺TUNEL⁺ CD8⁺ T cells increased from 0.2 to 16.4% and 1.2% to 28.1% at 24 hours, respectively. At 24 hours the ratio of Bax⁺CD4⁺:Bax⁺CD8⁺ changed from 1:1 to 4:1; the ratio of TUNEL⁺CD4⁺:TUNEL⁺ CD8⁺ cells increased from 1:1 to 1:5; and the ratio of Bax⁺TUNEL⁺CD4⁺: Bax⁺TUNEL⁺CD8⁺ decreased from 1:5 to 1:2 following irradiation (Figure 6A). Overall, the mean percentage of Bax⁺CD8⁺ T cells was less than Bax⁺CD4⁺ T cells ($p=0.0045$), but the mean percentage of TUNEL⁺ CD8⁺ T cells was significantly greater than CD4⁺ T cells, and the mean percentage of Bax⁺TUNEL⁺ CD8⁺ T cells was greater than Bax⁺TUNEL⁺ CD4⁺ T cells after irradiation at 24 hours (Figure 6B)

Overall, the decrease in the percentage of Bax⁺ T cells at 24 hours after irradiation can be explained by the increased absolute number of TUNEL⁺ and Bax⁺TUNEL⁺ T cells. Decreased numbers of Bax⁺ CD8⁺ T cells (Figure 6C) relative to CD4⁺ T (Figure 6D) cells can be attributed to the larger number of TUNEL⁺ CD8⁺ T cells (Figure 6C) than CD4⁺ T cells (Figure 6D) present 24 hours after irradiation. By 24 hours after irradiation, the absolute number of Bax⁺ CD4⁺ T cells was approximately 2 fold lower than in unirradiated cells, while the absolute number of Bax⁺ CD8⁺ T cells was 10 fold less ($p=<0.0003$) (Figure 6E). In contrast, the absolute number of TUNEL⁺ CD4⁺ T cells increased by 58 fold and the absolute number of TUNEL⁺ CD8⁺ T cells increased by approximately 145 fold ($p=<0.0001$). There was also a 57 fold increase in the absolute number of Bax⁺TUNEL⁺ CD4⁺ T cells compared with a 13 fold increase in Bax⁺TUNEL⁺ CD8⁺ T cells ($p=<0.00022$) after irradiation (Figure 6E). Overall, there were more apoptotic CD8⁺ T cells than CD4⁺ T cells.

Conventional dendritic cells are slightly more radioresistant than monocytes.

APC were more resistant to radiation-induced apoptosis than T cells. In order to better understand this relative radioresistance, Bax and TUNEL activation was assessed in monocytes and conventional dendritic cells treated with 0 Gy and 20 Gy (2000cGy) and stained for subset surface makers, as well as intracellular Bax and TUNEL at 0, 1, 4, 8, and 24 hours. Figure 7A shows representative examples of two-color flow cytometric analyses of Bax⁺ and/or TUNEL⁺ gated monocytes and conventional dendritic cells from untreated and irradiated (20 Gy) human PBMC at 0 and 24 hours. There was no significant difference in the percentage of Bax⁺ or TUNEL⁺ monocytes or conventional dendritic cells before or after irradiation at 0 hours. By 24 hours after irradiation there was an increase in Bax⁺ monocytes (were 11%) and Bax⁺ conventional dendritic cells (7.5%). However, there was only 2.1% TUNEL⁺ monocytes and 1.5% conventional dendritic cells. The ratio of Bax⁺ monocytes:Bax⁺ conventional dendritic cells changed slightly from 1:1 to 1.5:1.following irradiation (Figure 7A). At 24 hours, the mean percentage of Bax⁺ monocytes was greater than Bax⁺ conventional dendritic cells, but this difference was not statistically significant ($p=0.153$). The mean percentage of TUNEL⁺ monocytes was greater than TUNEL⁺ conventional dendritic cells ($p=0.156$) and the mean percentage of Bax⁺TUNEL⁺ monocytes cells was greater than Bax⁺TUNEL⁺ conventional dendritic cells, although neither difference was statistically significant ($p=.201$) (Figure 7B).

The absolute number of Bax⁺, TUNEL⁺ and Bax⁺TUNEL⁺ monocytes after irradiation at 24 hours (Figure 7C) was greater than the absolute numbers for conventional dendritic cells (Figure 7D). Comparison of untreated versus irradiated

cells at 24 hours, resulted in a increase in the absolute number of Bax⁺ monocytes by approximately 2 fold, whereas the absolute number of Bax⁺ dendritic cells increased by about 1 fold ($p=0.04$) after irradiation (Figure 7E). There was a 26 fold increase in the absolute number of TUNEL⁺ monocytes cells compared to a 15 fold increase in the absolute number of TUNEL⁺ conventional dendritic cells ($p=0.001$), and a 7 fold increase in the absolute number of Bax⁺TUNEL⁺ monocytes compared to a 2 fold increase in Bax⁺TUNEL⁺ conventional dendritic cells ($p<0.126$) after radiation at 24 hours (Figure 7E). Overall, monocytes expressed higher levels of Bax and TUNEL than conventional dendritic cells after irradiation but the magnitude of Bax⁺, TUNEL⁺, and Bax⁺TUNEL⁺ APC after irradiation is significantly less than that observed in T cells.

Radiation increased expression of Bax in T cell subsets while Bcl-2 expression remained relatively constant.

Next, we compared the level of intracellular expression of Bcl-2 and Bax in T cells subsets after irradiation at 0, 1, 4, 8, and 24 hours. Bax and Bcl-2 expression has been shown in previous studies to peak with quantitative PCR in immune cells before 24 hours (Yao et al. 2009). Shown are representative examples of one-color flow cytometric analyses of Bcl-2⁺ versus Side Scatter gated CD4⁺ T cells from untreated and irradiated (20 Gy) human PBMC at 0 and 24 hours (Supplemental Figure 1A). Similar analyses were performed for gated CD8⁺ T cells (Supplemental Figure 1B). Supplemental Figure 1C shows flow cytometric analyses of Bax⁺ versus Side Scatter gated CD4⁺ T cells and gated CD8⁺ T cells (Supplemental Figure 1D) from untreated and irradiated (20 Gy) human PBMC at 0 and 24 hours. Isotype controls at 24 hours after 20 Gy showed no

detectable Bax⁺ and/or Bcl-2⁺ CD4⁺ or CD8⁺ T cells. Immediately after radiation there was a small decrease in the percentage of Bcl-2⁺ CD4⁺ T cells from 78.1% to 76.3% and at 24 hours there was a small decrease in Bcl-2⁺ CD4⁺ T cells from 68.4% to 64.5% (Supplemental Figure 1A). There was a minimal decrease in Bcl-2⁺ CD8⁺ T cells from 71.4% to 70.4% immediately after radiation and a small decrease from 66.6% to 63.6% at the 24 hour timepoint (Supplemental Figure 1B). Because the magnitude of these changes was so small, there were no significant changes in the ratio of Bcl-2⁺CD4⁺: Bcl-2⁺CD8⁺ cells. The percentage of Bax⁺ CD4⁺ T cells only increased from 17.1% to 18.9% at 0 hours, but by 24 hours there was an increase in Bax⁺ CD4⁺ T cells from 19.6% to 32.7% (Supplemental Figure 1C). Bax⁺ CD8⁺ T cells increased from 23.2% to 23.8% at 0 hours and from 24.6% to 50.2% at 24 hours (Supplemental Figure 1D). Therefore, the ratio of Bax⁺CD4⁺:Bax⁺CD8⁺ increased from 1:1 to 1:1.5, and the relative proportion of Bax⁺ CD8 cells doubled by 24 hours (Supplemental Figure 1C and 1D) . Mean percentages of Bcl-2 lo and hi CD4⁺ and CD8⁺ T cells and Bax lo and hi CD4⁺ and CD8⁺ T cells at 24 hours are shown in Figure 1E and Figure 1F, respectively.

The difference in absolute number of Bcl-2 lo CD4⁺ or Bcl-2 hi CD4⁺ T cells before and after irradiation at 24 hours was not statistically different. Similar results were obtained with CD8⁺ T cells (Supplemental Figure 1G). However, the absolute number of Bcl-2 hi CD4⁺ T cells was greater than Bcl-2 hi CD8⁺ T cells, and there was not a statistically significant difference in the absolute numbers of Bax lo CD4⁺ T cells after irradiation. However, absolute number of Bax hi CD4⁺ T cells after irradiation increased ($p=0.0004$) at 24 hours. The lower percentage of Bax lo CD8⁺ T cells after irradiation can be explained by a lower absolute number of Bax lo CD8⁺ T cells ($p=0.002$). The

higher absolute number of Bax hi CD8⁺ T cells is a reflection of the higher absolute number of Bax hi CD8⁺ T cells after irradiation at 24 hours ($p=0.003$) (Supplemental Figure 1H).

At 24 hours, the absolute number of Bcl-2 lo CD4⁺ T and CD8⁺ T cells both increased by approximately 1 fold after irradiation ($p=.195$), while the absolute number of Bcl-2 hi CD4⁺ T and Bcl-2 hi CD8⁺ T cells decreased by about 1 and 1.5 fold respectively ($p=0.058$) (Supplemental Figure 1I). Bax lo CD4⁺ T and CD8⁺ T cells decreased by about 1.5 and 2 fold, respectively ($p=.434$). In contrast, the absolute number of Bax hi CD4⁺ T cells increased by about 3 fold and the absolute number of Bax hi CD8⁺ T cells increased by approximately 6 fold ($p=<0.0011$) (Supplemental Figure 1J). Though there is a slight difference in absolute Bcl-2 hi numbers of CD4 and CD8 T cells after radiation, proportionally it is minimal when compared to the magnitude of change of Bax expression in the cell subsets.

Effect of radiation on Bax and Bcl-2 expression in APC subsets.

Intracellular expression of Bax and Bcl-2 were examined in APC subsets as well. As above, cells were treated with 0 Gy and 20 Gy (2000cGy) and stained for subset surface makers as well as intracellular Bax and Bcl-2 at 0, 1, 4, 8, and 24 hours. Represented is one-color flow cytometric analyses of Bcl-2⁺ versus Side Scatter gated monocytes (Supplemental Figure 2A), Bcl-2⁺ versus Side Scatter gated conventional dendritic cells (Supplemental Figure 2B), Bax⁺ versus Side Scatter gated monocytes (Supplemental Figure 2C), and Bax⁺ versus Side Scatter gated conventional dendritic cells (Supplemental Figure 2D) from untreated and irradiated (20 Gy) human PBMC at 0

and 24 hours. Isotype controls at 24 hours after 20 Gy showed no detectable Bax⁺ and/or Bcl-2⁺ monocytes or conventional dendritic cells. At 0 and 24 hours there were very small decreases in the percentage of Bcl-2⁺ monocytes (89.4% to 88.8% and 85.2% to 82.7%, respectively) after radiation (Supplemental Figure 2A). Similarly, there were also very small decreases in Bcl-2⁺ conventional dendritic cells from 94.1% to 90.2% immediately after radiation and a small decrease from 93.2% to 86.5% at 24 hours (Supplemental Figure 2B). In addition, there was no significant change in the ratio of Bcl-2⁺monocytes: Bcl-2⁺conventional dendritic cells (1:1) 24 hours following radiation (Supplemental Figure 2A and Figure 2B). Immediately after radiation there was a very small increase in the percentage of Bax⁺ monocytes from 7.11% to 7.59%, but at 24 hours Bax⁺ monocytes increased from 7.59% to 18.4% (Supplemental Figure 2C). Bax⁺ conventional dendritic cells increased from 4.27% to 8.14% at 0 hours, with a much more marked increased from 4.51% to 17.3% at 24 hours (Supplemental Figure 2D). The ratio of Bax⁺monocytes:Bax⁺conventional dendritic cells again remained at 1:1 24 hours after radiation, although the proportion of Bax⁺ monocytes and conventional dendritic cells after irradiation increased (Supplemental Figure 2C and 2D) . At 24 hours the mean percentage of Bcl-2 lo monocytes and conventional dendritic cells increased slightly and the mean percentage of Bcl-2 hi monocytes and conventional dendritic cells decreased slightly after irradiation, though the decrease in Bcl-2 hi conventional dendritic cells was not statistically significant (Supplemental Figure 2E). Although the mean percentage of Bcl-2 lo monocytes was greater than Bcl-2 lo conventional dendritic cells after irradiation ($p=0.055$) and the mean percentage of Blc-2 hi monocytes was less than Bcl-2 hi conventional dendritic cells 24 hours after irradiation ($p=0.281$), with no statistically

significant differences (Supplemental Figure 2E). In addition, the mean percentage of Bax lo monocytes and conventional dendritic cells decreased after irradiation, while the mean percentage of Bax hi monocytes and conventional dendritic cells increased after irradiation at 24 hours. The mean percentage of Bax lo monocytes was less than Bax lo conventional dendritic cells ($p=0.552$) and the mean percentage of Bax hi monocytes was greater than Bax hi conventional dendritic cells ($p=0.692$), but again these differences were not statistically different (Supplemental Figure 2F).

At 24 hours, the difference in absolute number of Bcl-2 lo monocytes before and after irradiation was not statistically different ($p=.1830$). The slightly lower percentage of Bcl-2 hi monocytes corresponded to a lower absolute number of Bcl-2 hi monocytes after irradiation ($p=0.0022$) (Supplemental Figure 2G). After irradiation, there was a higher absolute number of Bcl-2 lo conventional dendritic cells, which was negligible ($p=0.059$). The difference in absolute number of Bcl-2 hi conventional dendritic cells before and after irradiation was not statistically significant ($p=0.361$) (Supplemental Figure 2G). In addition, the difference in absolute number of Bax lo monocytes before and after radiation was not statistically significant ($p=0.132$). There was a negligible decrease in the absolute number of Bax hi monocytes cells at 24 hours ($p=0.214$). The change in absolute number of Bax lo conventional dendritic cells before and after radiation was not statistically significant ($p=0.051$). The higher percentage of Bax hi conventional dendritic cells after irradiation can be explained by a higher absolute number of Bax hi conventional dendritic cells after irradiation ($p=0.009$) (Supplemental Figure 2H).

In comparison at 24 hours, the absolute number of Bcl-2 lo monocytes decreased by about 2 fold, while Bcl-2 lo conventional dendritic cells increased by approximately 1 fold ($p=0.021$) after radiation. In addition, the absolute number of Bcl-2 hi monocytes decreased by about 1.5 fold and Bcl-2 hi conventional dendritic cells absolute number decreased by about 1 fold ($p=0.054$) (Supplemental Figure 2I). When compared, the absolute number of Bax lo monocytes decreased by about 3 fold and the absolute number of Bax conventional dendritic cells decreased by approximately 2 fold ($p=.005$). Also, the absolute number of Bax hi monocytes increased by about 1 fold and the absolute number of Bax hi conventional dendritic cells also increased by approximately 1 fold ($p=<0.0002$) (Supplemental Figure 2J). There were more Bax⁺ monocytes than conventional dendritic cells.

Increase in Bax expression as compared to Bax/Bcl-2 expression after irradiation makes CD8 T cells more radiosensitive than CD4 T cells.

Next, experiments were performed to determine how radiation affected the number of Bax⁺ versus Bax⁺/Bcl-2⁺ cells after irradiation. Cells were treated with 0 Gy and 20 Gy (2000cGy) and stained for subset surface makers as well as intracellular Bax and Bcl-2 at 0, 1, 4, 8, and 24 hours. Representative examples of two-color flow cytometric analyses of Bcl-2⁺ versus Bax⁺ gated CD4⁺ T cells (Supplemental Figure 3A) and CD8⁺ T cells (Supplemental Figure 3B) from untreated and irradiated (20 Gy) human PBMC at 0 and 24 hours show an initial small increase in the percentage of Bcl-2⁺ CD4⁺ T cells (2.16% to 2.49%) with a negligible decrease from 1.21% to 0.12% at 24 hours (Supplemental Figure 3A). Bcl-2⁺ CD8⁺ T cells decreased from 2.11% to 2.92% at 0

hours, with no detectable Bcl-2⁺ CD8⁺ T cells by 24 hours after 20 Gy (Supplemental Figure 3B), and no alteration in the ratio of Bcl-2⁺CD4⁺:Bcl-2⁺CD8⁺. At the 24 hour timepoint there was a significant increase in Bax⁺ CD4⁺ T cells from 3.63% to 26.7% (Supplemental Figure 3A). There was also a large increase in Bax⁺ CD8⁺ T cells from 6.98% to 37.4% at 24 hours (Supplemental Figure 3B). The ratio of Bax⁺CD4⁺:Bax⁺CD8⁺ cells did not change (Figure 3B). In contrast while the percentage of Bcl-2⁺Bax⁺ CD4⁺ T cells changed little (97.2% to 96.6%) immediately following radiation, but markedly decreased from 95.2% to 73.3% at 24 hours (Supplemental Figure 3A). Again, there was a negligible decrease in Bcl-2⁺Bax⁺ CD8⁺ T cells at 0 hours and a significant decrease in Bcl-2⁺Bax⁺ CD8⁺ T cells from 90.3% to 62.6% at the 24 hour timepoint, with the ratio of Bcl-2⁺Bax⁺ CD4⁺: Bcl-2⁺Bax⁺ CD8⁺ cells remaining at 1:1 24 hours following irradiation (Supplemental Figure 3B). Isotype controls at 24 hours after 20 Gy showed no detectable Bax⁺ and/or Bcl-2⁺ CD4⁺ or CD8⁺ T cells.

Overall, there were very small decreases in mean percentages of Bcl-2⁺CD4⁺ and Bcl-2⁺CD8⁺ T cells after irradiation at 24 hours. The mean percentage of Bax⁺ CD8⁺ T cells was greater than Bax⁺CD4⁺ T cells ($p=0.0002$) at 24 hours after irradiation. The mean percentage of Bax⁺ Bcl-2⁺ CD4⁺ T cells was greater than the percentage of Bax⁺ Bcl-2⁺ CD8⁺ T cells at 24 hours after irradiation ($p=0.009$) (Supplemental Figure 10C). The absolute number of Bcl-2⁺ CD4⁺T and Bcl-2⁺ CD8⁺T cells after irradiation decreased compared to unirradiated cells at 24 hours, while the absolute number of Bax⁺ cells (Bax⁺ CD8⁺ T > Bax⁺ CD4⁺ T) cells significantly increased after irradiation at 24 hours ($p=0.001$ and 0.0017) (Supplemental Figure 3D). The absolute number of Bax⁺

Bcl-2⁺ CD4⁺ T and Bax⁺ Bcl-2⁺ CD8⁺ T cells decreased after irradiation at 24 hours($p=<0.05$) (Supplemental Figure 3D). The number of Bax⁺ cells as compared to Bcl-2⁺Bax⁺ cells was greater in CD8⁺ T cells than CD4⁺ T cells, (Supplemental Figure 3D) with a lower absolute number of CD8⁺ T cells than CD4 T cells after irradiation. As shown in Figure 10F, there was a larger fold change in Bax⁺ CD8⁺ T cells (8 fold) than Bax⁺ CD4⁺ T cells (3.6 fold), while the fold change of Bcl-2⁺Bax⁺ CD4 versus Bcl-2⁺Bax⁺ CD4 after irradiation was similar (~2 fold) (Supplemental Figure 3E).

Bax expression as compared to Bax/Bcl-2 expression is significantly less in conventional dendritic cells and monocytes

APC were treated with 0 Gy and 20 Gy (2000cGy) and stained for subset surface makers as well as intracellularly for Bax and Bcl-2 at 0, 1, 4, 8, and 24 hours. Bcl-2⁺ monocytes decreased from 86.6% to 60% at 24 hours following irradiation (Supplemental Figure 4A). Bcl-2⁺ conventional dendritic cells decreased from 89.2% to 60.9% at 24 hours (Supplemental Figure 4B). The ratio of Bcl-2⁺monocytes:Bcl-2⁺conventional dendritic cells (1:1) was unchanged following irradiation. Changes in the percentage of Bax⁺ monocytes and Bax⁺ conventional dendritic cells were very small and statistically insignificant at both time points, (Supplemental Figure 4A and 4B) with no change in the ratio of Bax⁺monocytes:Bax⁺conventional dendritic cells from 1:1 after radiation at 24 hours. The percentage of Bcl-2⁺Bax⁺ monocytes increased from 10.9% to 30.7% at 24 hours (Supplemental Figure 4A). There was a larger increase in Bcl-2⁺Bax⁺ conventional dendritic cells from 8.15% to 29.2% at the 24 hour timepoint (Supplemental Figure 4B). Again, at 24 hours, the ratio of Bcl-2⁺Bax⁺ monocytes: Bcl-2⁺Bax⁺

conventional dendritic cells remained unchanged at 1:1 following irradiation. Isotype controls at 24 hours after 20 Gy showed no detectable Bax⁺ and/or Bcl-2⁺ monocytes or conventional dendritic cells.

Overall, the mean percentage of Bcl-2⁺ monocytes and Bcl-2⁺ conventional dendritic cells decreased 24 hours after irradiation while the mean percentage of Bax⁺ monocytes and Bax⁺ conventional dendritic cells slightly increased at 24 hours (Supplemental Figure 4C). At 24 hours after irradiation, the increase in Bax⁺ monocytes was a little larger than Bax⁺ conventional dendritic cells ($p=<0.0001$). The small shift in the mean percentage of Bax⁺ Bcl-2⁺ monocytes compared to Bax⁺ Bcl-2⁺ conventional dendritic cells was not statistically different ($p=.767$) (Supplemental Figure 4C).

The decreased absolute number of Bcl-2⁺ monocytes after irradiation as compared to unirradiated cells at 24 hours was statistically significant ($p=0.036$) (Supplemental Figure 4D). The increased percentage of Bax⁺ monocytes may be explained by the increase in the absolute number of Bax⁺ monocytes after irradiation as compared to unirradiated cells at 24 hours ($p=0.033$) (Supplemental Figure 4D). There was an increase in the absolute number of Bax⁺ Bcl-2⁺ monocytes after irradiation as compared to unirradiated cells at 24 hours, but this difference was not statistically significant ($p=0.289$) (Supplemental Figure 4D). The absolute number of Bcl-2⁺ conventional dendritic cells decreased ($p=0.05$) and the absolute number of Bax⁺ conventional dendritic cells increased after irradiation as compared to unirradiated cells at 24 hours ($p=0.002$) (Supplemental Figure 4E). There was an increase in the absolute number of Bax⁺ Bcl-2⁺ conventional dendritic cells after irradiation as compared to unirradiated cells at 24 hours but it was not statistically significant ($p=0.1004$) (Supplemental Figure

4E). When unirradiated cells were compared to irradiated cells at 24 hours, the absolute number of Bcl-2⁺ monocytes decreased by about 2 fold and the fold change in the absolute number of Bcl-2⁺ conventional dendritic cells decreased by about 1.5 fold ($p=0.242$) (Supplemental Figure 4F). Bax⁺ monocytes increased by approximately 2 fold and the Bax⁺ conventional dendritic cells increased by about 1.5 fold ($p=0.0245$) (Supplemental Figure 4F). The absolute number of Bcl-2⁺Bax⁺ monocytes and conventional dendritic cells increased by about 2 fold ($p=0.1053$). Overall, there is a larger fold change in Bax⁺ monocytes than Bax⁺ conventional dendritic cells, while the fold change of Bcl-2⁺Bax⁺ CD4 versus Bcl-2⁺Bax⁺ CD4 after irradiation was similar (Supplemental Figure 4F). The higher absolute number and fold change of Bax⁺ monocytes as compared to Bax⁺ conventional dendritic cells versus Bax⁺Bcl-2⁺ APC may help to explain why monocytes are less radioresistant than dendritic cells.

Discussion

Although radiation is widely used as a component of preparatory regimens for hematopoietic stem cell transplantation, the associated immunomodulatory effects are poorly understood. The experiments described here were designed to further elucidate the effects of radiation on the balance of T cell and APC subsets during the critical window, within 2-4 days, after radiation when immune modulation is likely to optimally modify GVT or graft versus leukemia (GVL) effects.

Following irradiation, with 20 Gy, there was a selective loss (CD8 T > CD4 T) of T cell subsets from healthy donor human PBMC. Radiation changed the balance according to a hierarchy of radiation sensitivity with the naive T cells being the most sensitive, followed by the CD4-CD8- T cells, and memory T cells, being the least radiosensitive. There was also a selective loss of APC (monocytes > total and conventional dendritic cells), although the magnitude of the APC depletion was substantially less than that observed in T cells. The loss of T cells was influenced by the expression of intracellular levels of Bax, which is expressed early in the caspase-mediated apoptotic pathway and increases within the first few hours after radiation (Murphy et al. 2002). This does not induce cell death alone, but elevated levels of Bax accelerate apoptosis following a death signal (Oltvai, Milliman, and Korsmeyer 1993). The selective loss of T cells was influenced by the intracellular level of Bax. Overall, CD8 T cells that expressed higher levels of Bax than CD4 T cells were more sensitive to radiation with associated cell loss. Monocytes expressed higher levels of Bax than dendritic cells, but overall APC expressed less Bax than T cells and were more radioresistant (underwent less apoptosis) than T cells. These results demonstrate that Bax

plays an important role in the sensitivity of T cells and APC to radiation-induced apoptosis. Bax and Bcl-2 compete with each other to tip the balance to promote or inhibit apoptosis, respectively. As a result, it was vital to examine Bcl-2 expression as well. Bcl-2 plays a major role in regulating the cellular response to apoptotic signals. Bcl-2 is considered to be a novel proto-oncogene because it blocks apoptosis in many cell types and is thought to provide selective survival advantage for cells by blocking apoptosis (Boise et al. 1995; Hockenberry et al. 1990; Krajewski et al. 1993; Williams 1991; Yang et al. 1997). Contrary to studies in mice where higher levels of Bcl-2 expression conferred a survival advantage for certain T cell subsets (Seino, Harada, and Taniguchi 2004; Tamada et al. 1998; Yao et al. 2009), high levels of Bcl-2 expression in human T cells from PBMC did not protect T cells from radiation-induced cell death. The level of Bcl-2 in irradiated cells did not substantially change in CD4 or CD8 T cells after irradiation, while the level of Bax expression significantly increased. Intrinsically APC expressed higher levels of Bcl-2 which decreased slightly after radiation. Overall, this data suggests that radiation-induced Bax expression played a role in selective depletion of T and APC cell subsets. Though other anti-apoptotic proteins including Bcl-XL (Boise et al. 1995), and their impact on T cell subset survival is the subject of continuing investigation, studies have not been performed on the role of these anti-apoptotic proteins in APC survival.

In exploring the downstream target PARP, we found that the loss of T cells correlated with the level of cleaved PARP. CD8 T cells that expressed higher levels of cleaved PARP than CD4 T cells after irradiation underwent more radiation-induced death. In contrast, cleaved PARP could not be detected in monocytes or conventional

dendritic cells following irradiation. Since previous studies in Chapter 2 showed that the NF κ B pathway is activated in APC and is known to inhibit apoptosis through transcription of apoptotic inhibitor proteins, there could be an interaction between NF κ B activity and PARP activity after radiation. In these experiments, I κ B α was used as a marker of NF κ B activation, since I κ B α activity is necessary for NF κ B translocation to the nucleus. However, based upon these results, there does not seem to be an interaction between I κ B α and PARP, though I κ B α (via NF κ B) could be interacting with more upstream proteins in the apoptotic pathway.

One of the later steps in apoptosis is DNA fragmentation which is commonly detected by "end-labeling" or "TUNEL" (terminal deoxynucleotidyltransferase dUTP nick end labeling) (Darzynkiewicz et al. 1997; Enari et al. 1998; Luznik et al. 2003; Walker et al. 1993). T cell and APC subsets also expressed TUNEL after radiation, further demonstrating that these cells were dying by apoptosis after radiation. As expected, there was greater TUNEL expression in T cell subsets than APC, and the level of TUNEL expression in both T cell subsets and APC corresponded with their sensitivity to radiation. Though APC are known to die by different mechanisms, such as mitotic catastrophe (Brown and Attardi 2005), these results demonstrate that apoptosis plays an important role in radiation-induced APC apoptosis and that the monocytes and dendritic cells have different sensitivities to radiation.

In conclusion, radiation causes a selective loss of both T cell and APC subsets following irradiation of fresh healthy donor human PBMC, with T cells being significantly more radiosensitive than APC. This results in an altered balance of T and APC cell subsets in a hierarchical fashion based on the relative sensitivity of these

cellular subsets to radiation induced apoptosis, which correlates with the level of Bax expression after irradiation, and not Bcl-2 in the p53 mitochondrial mediated apoptotic pathway.

The changes described here are likely to have important functional effects on the immune function, since radiation shifts the balance toward host memory T cells. The enhanced effects of host memory T cells could increase the risk of allograft ransplant rejection in hosts. In addition, the radioresistance of APC and selective loss of monocytes as opposed to conventional dendritic cells results in an enhanced ratio of APC: T cells and APC type (dendritic cells). Understanding that radiation the radiation-induced shifts in the balance toward dendritic cells after radiation is important because there may be increased priming capacity by DC of alloreactive T cells leading to enhanced induction of cytotoxic T lymphocyte responses. Furthermore, we have demonstrated in previous studies that the NFkB pathway is activated in dendritic cells and enhances function of dendritic cells after radiation, which could lead to increased risk of allograft rejection by the host or GVHD in transplant patients receiving radiation therapy prior to hematopoietic stem cell transplantation, since radioresistant APC are present within the early the post-transplant period. In contrast, where the radioresistance of memory T cells and APC could be detrimental, supplemental methods (i.e. other treatments), in addition to radiation, may be required to cytoreduce the radioresistant subsets further. In addition, since Bax expression is lower in APC subsets, therapeutic approaches to increase Bax expression to enhance radiation sensitivity and depletion of APC subsets might also be clinically beneficial.

Elucidation of the underlying etiology of APC is critical for optimizing the use of radiation for purposes of modulating APC function in order to improve clinical outcomes. The mechanisms of cell death, in which Bax plays an important role, could be useful for determining outcomes after radiation as it relates to good GVT effects versus bad (tumor recurrence and/or progression) GVT effects.

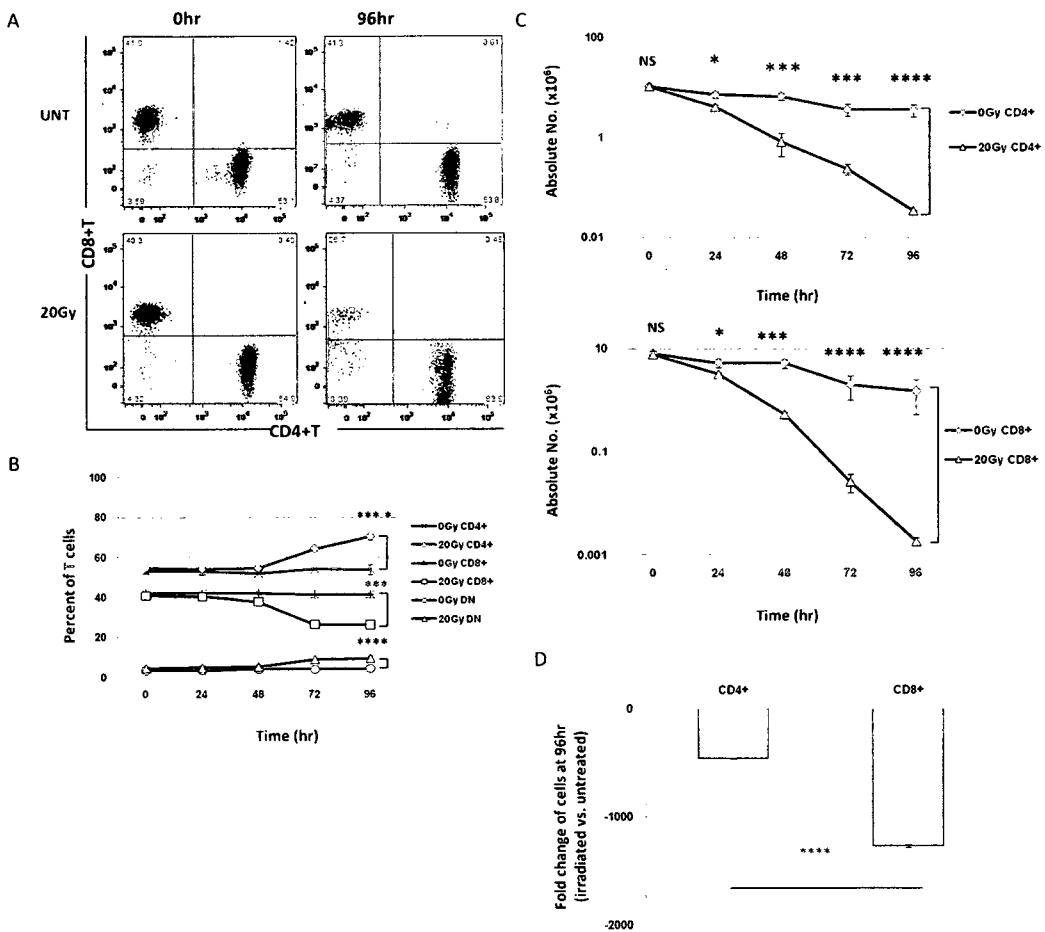


Figure 1. Effect of irradiation on CD4⁺ and CD8⁺ T cell subsets.

(A) Gated CD3⁺ T cells from unirradiated human PBMC (top row) or after 20Gy (bottom row) at 0 and 96hrs, were analyzed by flow cytometry for CD4 versus CD8. (B) The mean percentage (\pm standard error) of CD4⁺, CD8⁺, CD4⁺CD8⁻ (DN) at 0, 24, 48, 72, and 96hrs before and after 20Gy irradiation. (C) Mean absolute numbers (\pm standard error) for CD4⁺ T cells (upper panel) and CD8⁺ T cells (bottom panel) at 0, 24, 48, 72, and 96 hrs after 0Gy versus 20Gy. (D) Mean fold change (ratio of irradiated (20Gy) versus unirradiated) (\pm standard error) of different CD4⁺ and CD8⁺ at 96hrs. P=<0.0001 for CD4⁺ versus CD8⁺ (B-D). NS= not significant, *p=<0.05 and ***p=<0.001, and

****P<0.0001. Unless otherwise stated, the data shown here are representative of five independent experiments.

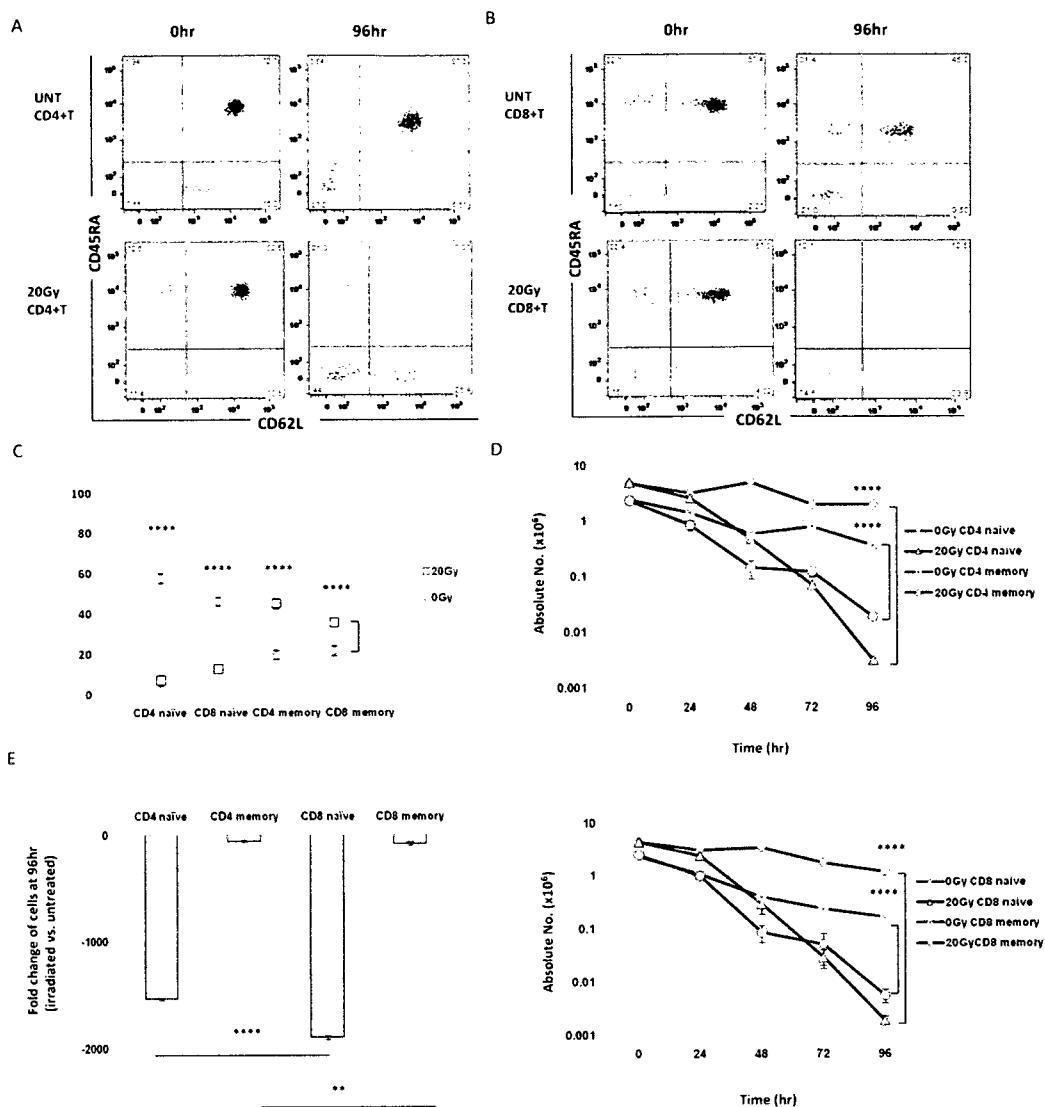


Figure 2. Effect of irradiation on naïve ($CD45RA^+$ $CD62L^+$) and effector memory ($CD45RA^-CD62L^-$) $CD4^+$ and $CD8^+$ T cells.

(A) Gated $CD3^+$ $CD4^+$ T cells from unirradiated human PBMC (top row) or after 20Gy (bottom row) at 0 and 96hrs, were analyzed by flow cytometry for $CD45RA$ versus $CD62L$. (B) Gated $CD3^+$ $CD8^+$ T cells from unirradiated human PBMC (top row) or after 20Gy (bottom row) at 0 and 96hrs, were analyzed by flow cytometry for $CD45RA$ versus $CD62L$. (C) The mean percentage (\pm standard error) of naïve and memory $CD4^+$ and $CD8^+$ T cells.

and CD8⁺ are shown at 96hrs after 0 and 20Gy irradiation. (D) Mean absolute numbers (\pm standard error) for naïve and memory CD4⁺ T cells (top row) and naïve and memory CD8⁺ T cells (bottom row) at 0, 24, 48, 72, and 96 hrs after 0Gy versus 20Gy. (E) Mean fold change (ratio of irradiated (20Gy) versus unirradiated) (\pm standard error) of different CD4 and CD8 subsets at 96hrs. (C-E) NS= not significant, *p=<0.05, **p=<0.01, and ****P=<0.0001.

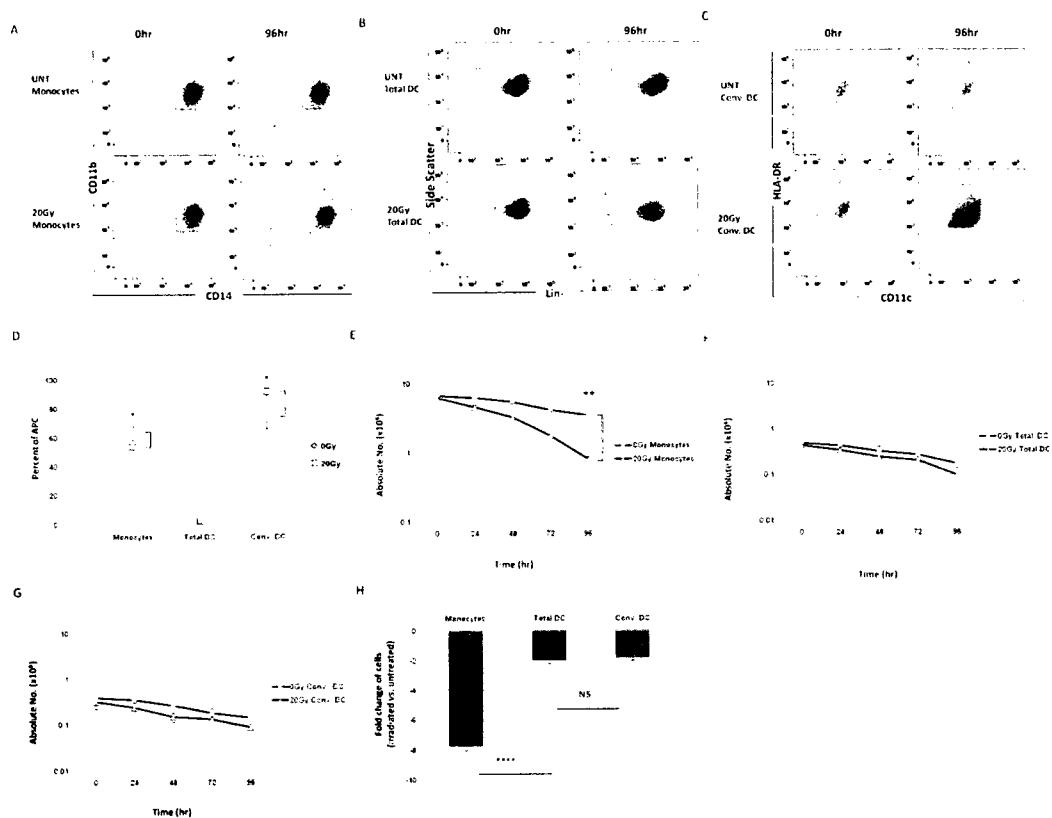


Figure 3. Effect of irradiation on APC subsets.

(A) Gated HLA-DR⁺ cells from unirradiated human PBMC (top row) or after 20Gy (bottom row) at 0 and 96hrs, were analyzed by flow cytometry for CD11b versus CD14 monocytes. (B) Unirradiated human PBMC (top row) or after 20Gy (bottom row) at 0

and 96hrs, were analyzed by flow cytometry for Side Scatter versus Lin- total dendritic cells. (C) Gated Lin⁻ dendritic cells from human PBMC (top row) or after 20Gy (bottom row) at 0 and 96hrs, were analyzed by flow cytometry for HLA-DR versus CD11c conventional dendritic cells. (D) The mean percentage (\pm standard error) of monocytes, total dendritic cells, and conventional dendritic cells are shown at 96hrs before and after 20Gy irradiation. (E) Mean absolute numbers (\pm standard error) for monocytes at 0, 24, 48, 72, and 96 hrs after 0Gy versus 20Gy. (F) Mean absolute numbers (\pm standard error) for total dendritic cells at 0, 24, 48, 72, and 96 hrs after 0Gy versus 20Gy. (G) Mean absolute numbers (\pm standard error) for conventional dendritic cells at 0, 24, 48, 72, and 96 hrs after 0Gy versus 20Gy. (H) Mean fold change (ratio of irradiated (20Gy) versus unirradiated) (\pm standard error) of different APC subsets at 96hrs. (B-H) NS= not significant, * p = <0.05 , **p= <0.01 , and ****P= <0.0001 .

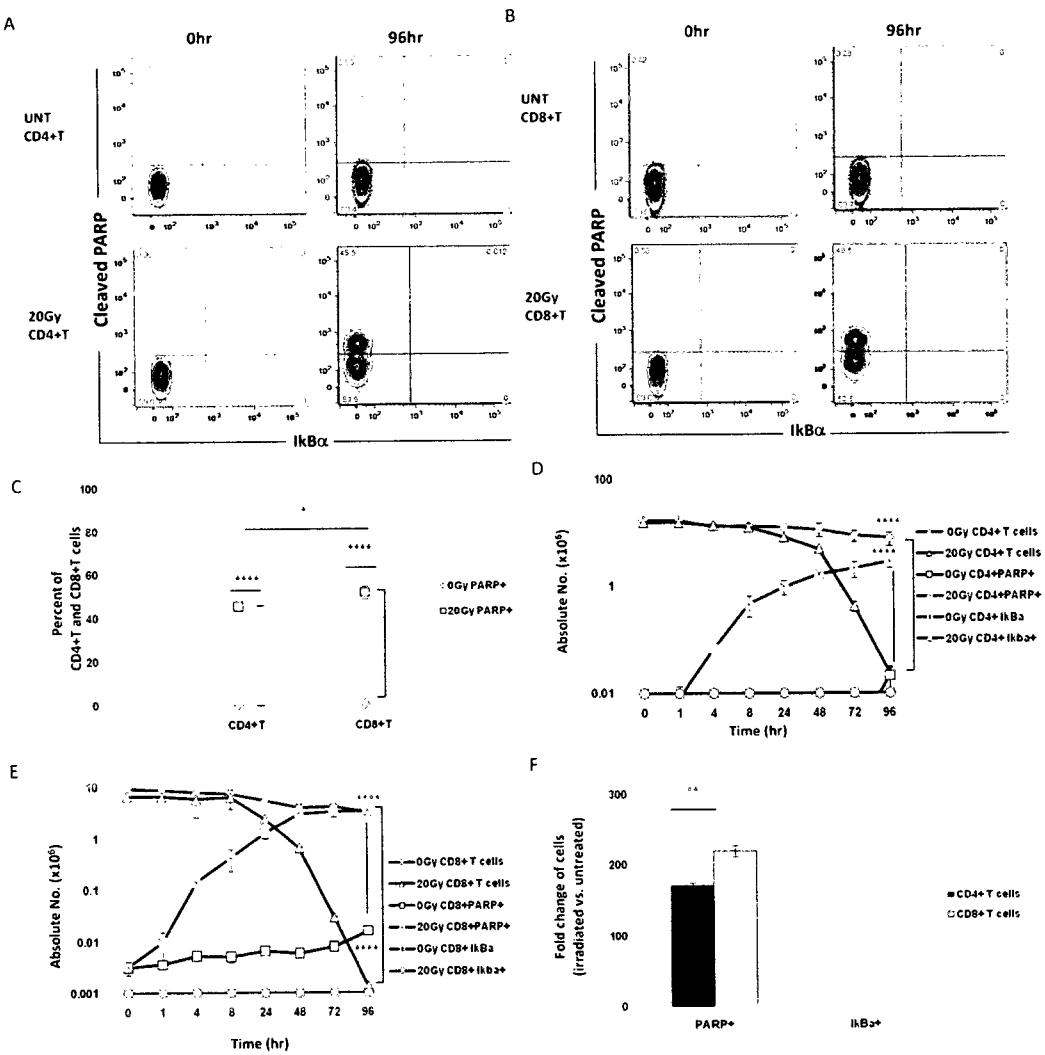


Figure 4. Radiation does not induce IκBα in T cells and CD8⁺ T cells display greater sensitivity to radiation (greater cleaved PARP) than CD4⁺ T cells to radiation.

- (A) Gated CD4⁺ cells from unirradiated human PBMC (top row) or after 20Gy (bottom row) at 0 and 96hrs, were analyzed by flow cytometry for cleaved PARP versus IκBα.
- (B) Gated CD8⁺ cells from unirradiated human PBMC (top row) or after 20Gy (bottom

row) at 0 and 96hrs, were analyzed by flow cytometry for cleaved PARP versus IkB α . (C) The mean percentage (\pm standard error) of CD4 $^+$ or CD8 $^+$ PARP+ cells are shown at 96hrs after 0 and 20Gy irradiation. (D) Mean absolute numbers (\pm standard error) for CD4, CD4 PARP $^+$ and CD4 IkB α $^+$ cells at 0, 24, 48, 72, and 96 hrs after 0Gy versus 20Gy. (E) Mean absolute numbers (\pm standard error) for CD8, CD8 PARP $^+$ and CD8 IkB α $^+$ cells at 0, 24, 48, 72, and 96 hrs after 0Gy versus 20Gy. (F) Mean fold change (ratio of irradiated (20Gy) versus unirradiated) (\pm standard error) of CD4 or CD8 PARP $^+$ or IkB α $^+$ cells at 96hrs. (C-F) ** (p=<0.01) and ****(p=<0.0001).

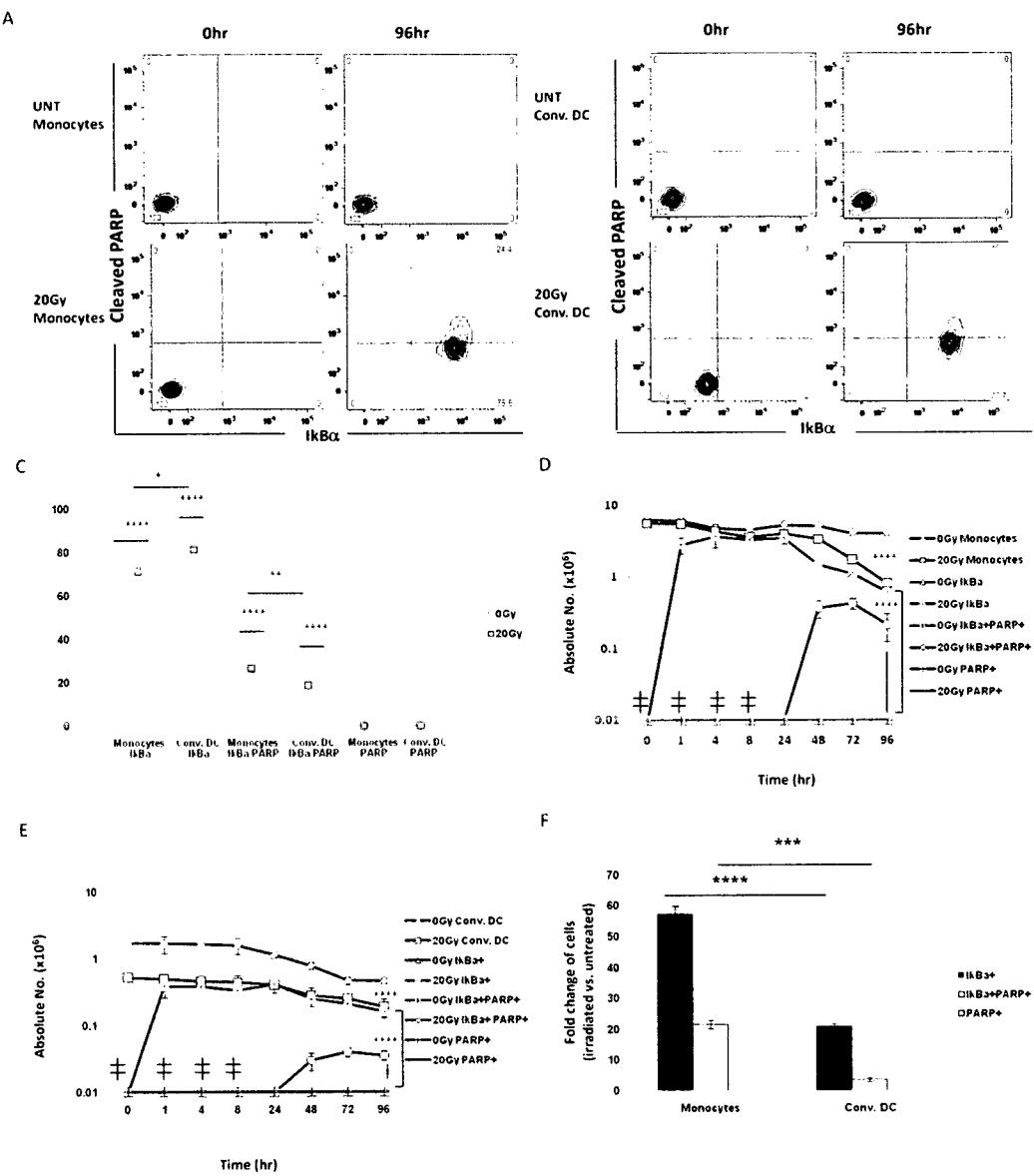


Figure 5. APC are more radioresistant (decreased cleaved PARP) than T cell subsets and their radioresistance could be due to increased IkB α activity.

(A) Gated HLA-DR $^+$ CD11b $^+$ CD14 $^+$ cells from unirradiated human PBMC (top row) or after 20Gy (bottom row) at 0 and 96hrs, were analyzed by flow cytometry for cleaved PARP versus IkB α . (B) Gated Lin $^-$ HLA-DR $^+$ CD11c $^+$ cells from unirradiated human

PBMC (top row) or after 20Gy (bottom row) at 0 and 96hrs, were analyzed by flow cytometry for cleaved PARP versus I κ B α . (C) The mean percentage (\pm standard error) of monocytes or conventional DC I κ B α ⁺, I κ b α ⁺PARP⁺, or PARP⁺ cells are shown at 96hrs after 0 and 20Gy irradiation. (D) Mean absolute numbers (\pm standard error) for monocytes, monocytes I κ B α ⁺, monocytes I κ B α ⁺ PARP⁺, and monocytes PARP⁺ at 0, 24, 48, 72, and 96 hrs after 0Gy versus 20Gy. (E) Mean absolute numbers (\pm standard error) for conventional dendritic cells, conventional dendritic cells I κ B α ⁺, conventional dendritic cells I κ B α ⁺ PARP⁺, and conventional dendritic cells PARP⁺ at 0, 24, 48, 72, and 96 hrs after 0Gy versus 20Gy. (F) Mean fold change (ratio of irradiated (20Gy) versus unirradiated) (\pm standard error) of monocytes or conventional dendritic cells that are I κ B α ⁺, I κ B α ⁺PARP⁺, or PARP⁺ cells at 96hrs. (C-F) * (p=<0.05), **(p=<0.01), *** (p=<0.001) and ****(p=<0.0001); ≠ (data not displayed that are =<0.01).

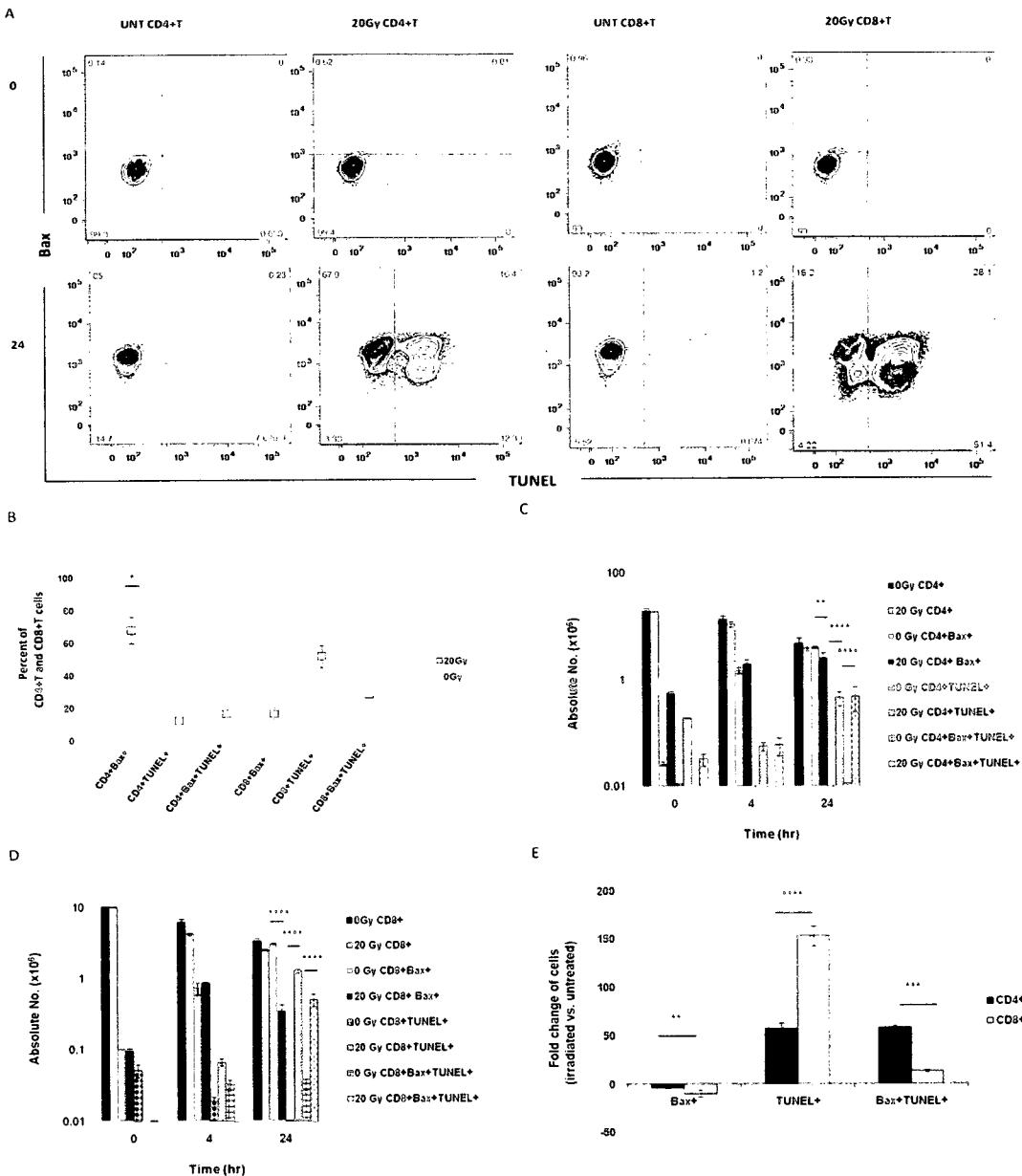


Figure 6. CD8⁺ T cells are more radiosensitive than CD4⁺ T cells as indicated by greater activation of Bax and TUNEL.

(A) Gated CD4⁺ and CD8⁺ cells from unirradiated or after 20Gy human PBMC at 0hrs (top row) or at 24hrs (bottom row), were analyzed by flow cytometry for Bax versus TUNEL. (B) The mean percentage (\pm standard error) of CD4⁺ or CD8⁺ Bax⁺, TUNEL⁺,

or Bax⁺TUNEL⁺ cells are shown at 24hrs after 0 and 20Gy irradiation. All p values are ($=<0.05$) (C) Mean absolute numbers (\pm standard error) for CD4⁺, CD4⁺Bax⁺, CD4⁺TUNEL⁺, CD4⁺ Bax⁺TUNEL⁺ cells at 0, 1, 4, 8, and 24 hrs after 0Gy versus 20Gy. (D) Mean absolute numbers (\pm standard error) for CD8⁺, CD8⁺Bax⁺, CD8⁺TUNEL⁺, CD8⁺ Bax⁺TUNEL⁺ cells at 0, 1, 4, 8, and 24 hrs after 0Gy versus 20Gy. (E) *(p=<0.05).

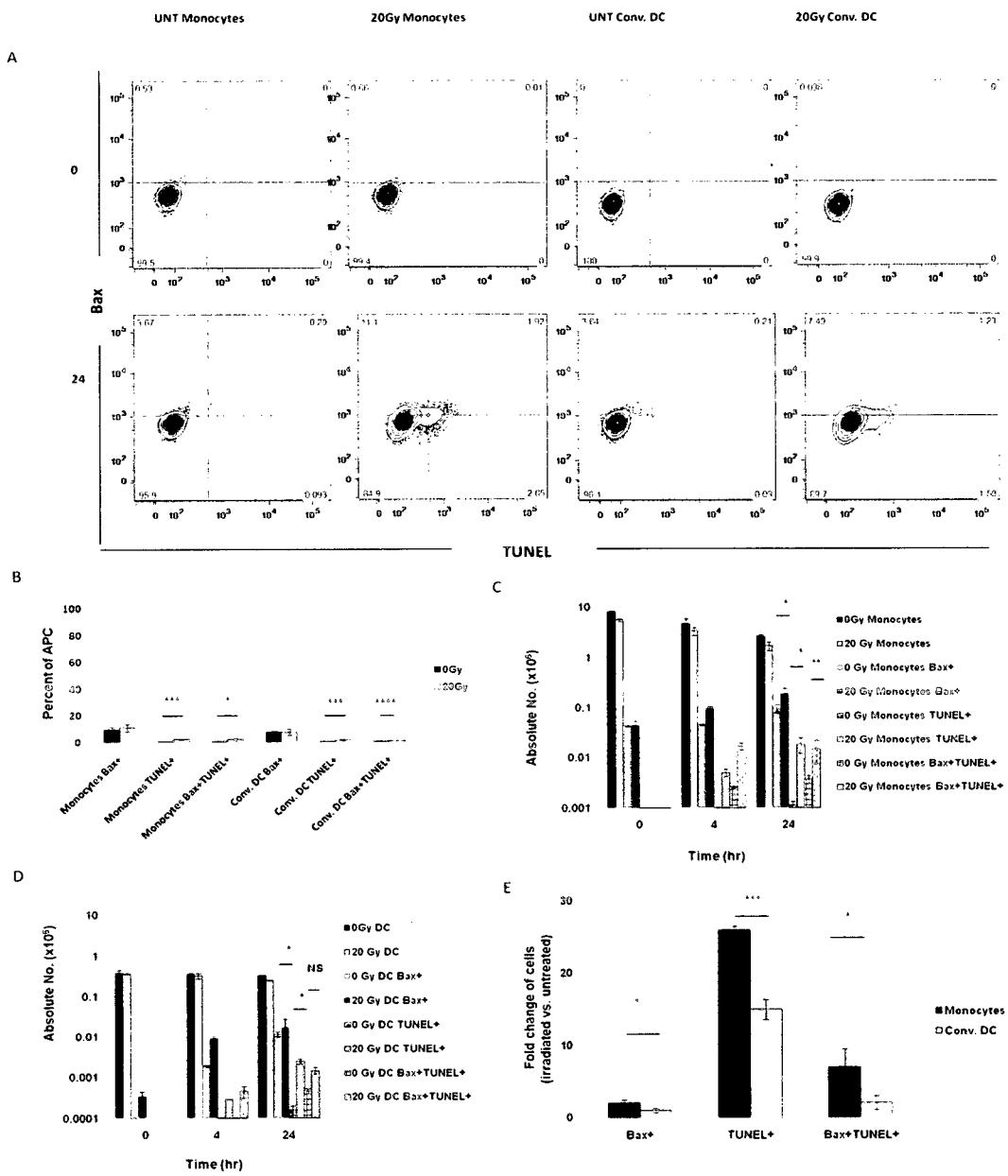


Figure 7. Conventional Dendritic Cells are slightly more radioresistant than Monocytes as indicated by greater activation of Bax and TUNEL.

(A) Gated HLA-DR⁺CD11b⁺ CD14⁺ and Lin-HLA-DR⁺ CD11c⁺ cells from unirradiated or after 20Gy human PBMC at 0hrs (top row) or at 24hrs (bottom row), were analyzed by flow cytometry for Bax versus TUNEL. (B) The mean percentage (\pm standard error) of

monocytes or conventional dendritic cells Bax⁺, TUNEL⁺, or Bax⁺TUNEL⁺ cells are shown at 24hrs after 0 and 20Gy irradiation. (C) Mean absolute numbers (\pm standard error) for monocytes, monocytes Bax⁺, monocytes TUNEL⁺, and monocytes Bax⁺TUNEL⁺ cells at 0, 1, 4, 8, and 24 hrs after 0Gy versus 20Gy. (D) Mean absolute numbers (\pm standard error) for conventional dendritic cells, conventional dendritic cells Bax⁺, conventional dendritic cells TUNEL⁺, conventional dendritic cells Bax⁺TUNEL⁺ at 0, 1, 4, 8, and 24 hrs after 0Gy versus 20Gy(D) Mean fold change (ratio of irradiated (20Gy) versus unirradiated) (\pm standard error) of monocytes or conventional dendritic cells Bax⁺, TUNEL⁺, or Bax⁺TUNEL⁺ at 24 hrs. (B-E) NS= not significant, *(p=<0.05), ** (p=<0.01), ***(p=<0.001), and ****(p=0.0001).

References

- Ame, J. C., C. Spenlehauer, and G. de Murcia. 2004. The parp superfamily. *Bioessays* 26, no. 8: 882-93. *J*
- Anderson, B. E., J. M. McNiff, C. Matte, I. Athanasiadis, W. D. Shlomchik, and M. J. Shlomchik. 2004. Recipient cd4+ t cells that survive irradiation regulate chronic graft-versus-host disease. *Blood* 104, no. 5: 1565-73. *J*
- Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 1995. Cd28 costimulation can promote t cell survival by enhancing the expression of bcl-xL. *Immunity* 3, no. 1: 87-98. *J*
- Brown, J. M. and L. D. Attardi. 2005. The role of apoptosis in cancer development and treatment response. *Nat Rev Cancer* 5, no. 3: 231-7. *J*
- Darzynkiewicz, Z., G. Juan, X. Li, W. Gorczyca, T. Murakami, and F. Traganos. 1997. Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 27, no. 1: 1-20. *J*
- Demaria, S., N. Bhardwaj, W. H. McBride, and S. C. Formenti. 2005. Combining radiotherapy and immunotherapy: A revived partnership. *Int J Radiat Oncol Biol Phys* 63, no. 3: 655-66. *J*
- Domen, J., K. L. Gandy, and I. L. Weissman. 1998. Systemic overexpression of bcl-2 in the hematopoietic system protects transgenic mice from the consequences of lethal irradiation. *Blood* 91, no. 7: 2272-82. *J*
- Edinger, M., P. Hoffmann, J. Ermann, K. Drago, C. G. Fathman, S. Strober, and R. S. Negrin. 2003. Cd4+cd25+ regulatory t cells preserve graft-versus-tumor activity

- while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* 9, no. 9: 1144-50. *J*
- Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata. 1998. A caspase-activated dnase that degrades DNA during apoptosis, and its inhibitor icad. *Nature* 391, no. 6662: 43-50. *J*
- Field, E. H. and S. Strober. 2001. Tolerance, mixed chimerism and protection against graft-versus-host disease after total lymphoid irradiation. *Philos Trans R Soc Lond B Biol Sci* 356, no. 1409: 739-48. *J*
- Friedman, E. J. 2002. Immune modulation by ionizing radiation and its implications for cancer immunotherapy. *Curr Pharm Des* 8, no. 19: 1765-80. *J*
- Fuks, Z. and S. Slavin. 1981. The use of total lymphoid irradiation (tli) as immunosuppressive therapy for organ allotransplantation and autoimmune diseases. *Int J Radiat Oncol Biol Phys* 7, no. 1: 79-82. *J*
- Hallahan, D. E., D. R. Spriggs, M. A. Beckett, D. W. Kufe, and R. R. Weichselbaum. 1989. Increased tumor necrosis factor alpha mrna after cellular exposure to ionizing radiation. *Proc Natl Acad Sci U S A* 86, no. 24: 10104-7. *J*
- Higuchi, M., D. Zeng, J. Shizuru, J. Gworek, S. Dejbakhsh-Jones, M. Taniguchi, and S. Strober. 2002. Immune tolerance to combined organ and bone marrow transplants after fractionated lymphoid irradiation involves regulatory nk t cells and clonal deletion. *J Immunol* 169, no. 10: 5564-70. *J*
- Hockenberry, D., G. Nunez, C. Milliman, R. D. Schreiber, and S. J. Korsmeyer. 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348, no. 6299: 334-6. *J*

Hong, J. H., C. S. Chiang, C. Y. Tsao, P. Y. Lin, W. H. McBride, and C. J. Wu. 1999.

Rapid induction of cytokine gene expression in the lung after single and
fractionated doses of radiation. *Int J Radiat Biol* 75, no. 11: 1421-7. J

Ishihara, H., K. Tsuneoka, A. B. Dimchev, and M. Shikita. 1993. Induction of the

expression of the interleukin-1 beta gene in mouse spleen by ionizing radiation. J
Radiat Res 133, no. 3: 321-6.

Joffre, O., T. Santolaria, D. Calise, T. Al Saati, D. Hudrisier, P. Romagnoli, and J. P. van
Meerwijk. 2008. Prevention of acute and chronic allograft rejection with
cd4+cd25+foxp3+ regulatory t lymphocytes. *Nat Med* 14, no. 1: 88-92. J

Kohrt, H. E., B. B. Turnbull, K. Heydari, J. A. Shizuru, G. G. Laport, D. B. Miklos, L. J.
Johnston, S. Arai, W. K. Weng, R. T. Hoppe, P. W. Lavori, K. G. Blume, R. S.
Negrin, S. Strober, and R. Lowsky. 2009. Tli and atg conditioning with low risk
of graft-versus-host disease retains antitumor reactions after allogeneic
hematopoietic cell transplantation from related and unrelated donors. *Blood* 114,
no. 5: 1099-109. J

Krajewski, S., S. Tanaka, S. Takayama, M. J. Schibler, W. Fenton, and J. C. Reed. 1993.
Investigation of the subcellular distribution of the bcl-2 oncprotein: Residence in
the nuclear envelope, endoplasmic reticulum, and outer mitochondrial
membranes. *Cancer Res* 53, no. 19: 4701-14. J

Lan, F., D. Zeng, M. Higuchi, J. P. Higgins, and S. Strober. 2003. Host conditioning with
total lymphoid irradiation and antithymocyte globulin prevents graft-versus-host
disease: The role of cd1-reactive natural killer t cells. *Biol Blood Marrow
Transplant* 9, no. 6: 355-63. J

- Lan, F., D. Zeng, M. Higuchi, P. Huie, J. P. Higgins, and S. Strober. 2001. Predominance of nk1.1+tcr alpha beta+ or dx5+tcr alpha beta+ t cells in mice conditioned with fractionated lymphoid irradiation protects against graft-versus-host disease: "Natural suppressor" Cells. *J Immunol* 167, no. 4: 2087-96.
- Luznik, L., J. E. Slansky, S. Jalla, I. Borrello, H. I. Levitsky, D. M. Pardoll, and E. J. Fuchs. 2003. Successful therapy of metastatic cancer using tumor vaccines in mixed allogeneic bone marrow chimeras. *Blood* 101, no. 4: 1645-52.
- Maris, M. B., B. M. Sandmaier, B. E. Storer, T. Chauncey, M. J. Stuart, R. T. Maziarz, E. Agura, A. A. Langston, M. Pulsipher, R. Storb, and D. G. Maloney. 2004. Allogeneic hematopoietic cell transplantation after fludarabine and 2 gy total body irradiation for relapsed and refractory mantle cell lymphoma. *Blood* 104, no. 12: 3535-42.
- Mielcarek, M., P. J. Martin, W. Leisenring, M. E. Flowers, D. G. Maloney, B. M. Sandmaier, M. B. Maris, and R. Storb. 2003. Graft-versus-host disease after nonmyeloablative versus conventional hematopoietic stem cell transplantation. *Blood* 102, no. 2: 756-62.
- Murphy, M., M. J. Mabruk, P. Lenane, A. Liew, P. McCann, A. Buckley, P. Billet, M. Leader, E. Kay, and G. M. Murphy. 2002. The expression of p53, p21, bax and induction of apoptosis in normal volunteers in response to different doses of ultraviolet radiation. *Br J Dermatol* 147, no. 1: 110-7.
- Oltvai, Z. N., C. L. Milliman, and S. J. Korsmeyer. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, bax, that accelerates programmed cell death. *Cell* 74, no. 4: 609-19.

Paulos, C. M., C. Wrzesinski, A. Kaiser, C. S. Hinrichs, M. Chieppa, L. Cassard, D. C.

Palmer, A. Boni, P. Muranski, Z. Yu, L. Gattinoni, P. A. Antony, S. A.

Rosenberg, and N. P. Restifo. 2007. Microbial translocation augments the function of adoptively transferred self/tumor-specific cd8+ t cells via tlr4 signaling. *J Clin Invest* 117, no. 8: 2197-204.

Pillai, A. B., T. I. George, S. Dutt, P. Teo, and S. Strober. 2007. Host nkt cells can

prevent graft-versus-host disease and permit graft antitumor activity after bone marrow transplantation. *J Immunol* 178, no. 10: 6242-51.

Ren, H., J. Shen, C. Tomiyama-Miyaji, M. Watanabe, E. Kainuma, M. Inoue, Y.

Kuwano, and T. Abo. 2006. Augmentation of innate immunity by low-dose irradiation. *Cell Immunol* 244, no. 1: 50-6.

Rieser, C., G. Bock, H. Klocker, G. Bartsch, and M. Thurnher. 1997. Prostaglandin e2 and tumor necrosis factor alpha cooperate to activate human dendritic cells:

Synergistic activation of interleukin 12 production. *J Exp Med* 186, no. 9: 1603-8.

Seino, K., M. Harada, and M. Taniguchi. 2004. Nkt cells are relatively resistant to

apoptosis. *Trends Immunol* 25, no. 5: 219-21.

Steinauer, K. K., I. Gibbs, S. Ning, J. N. French, J. Armstrong, and S. J. Knox. 2000.

Radiation induces upregulation of cyclooxygenase-2 (cox-2) protein in pc-3 cells.

Int J Radiat Oncol Biol Phys 48, no. 2: 325-8.

Strober, S. 1987. Total lymphoid irradiation in alloimmunity and autoimmunity. *J*

Pediatr 111, no. 6 Pt 2: 1051-5.

Tamada, K., M. Harada, K. Abe, T. Li, and K. Nomoto. 1998. IL-4-producing NK1.1⁺ t cells are resistant to glucocorticoid-induced apoptosis: Implications for the Th1/Th2 balance. *J Immunol* 161, no. 3: 1239-47.

Teitz-Tennenbaum, S., Q. Li, R. Okuyama, M. A. Davis, R. Sun, J. Whitfield, R. N. Knibbs, L. M. Stoolman, and A. E. Chang. 2008. Mechanisms involved in radiation enhancement of intratumoral dendritic cell therapy. *J Immunother* 31, no. 4: 345-58.

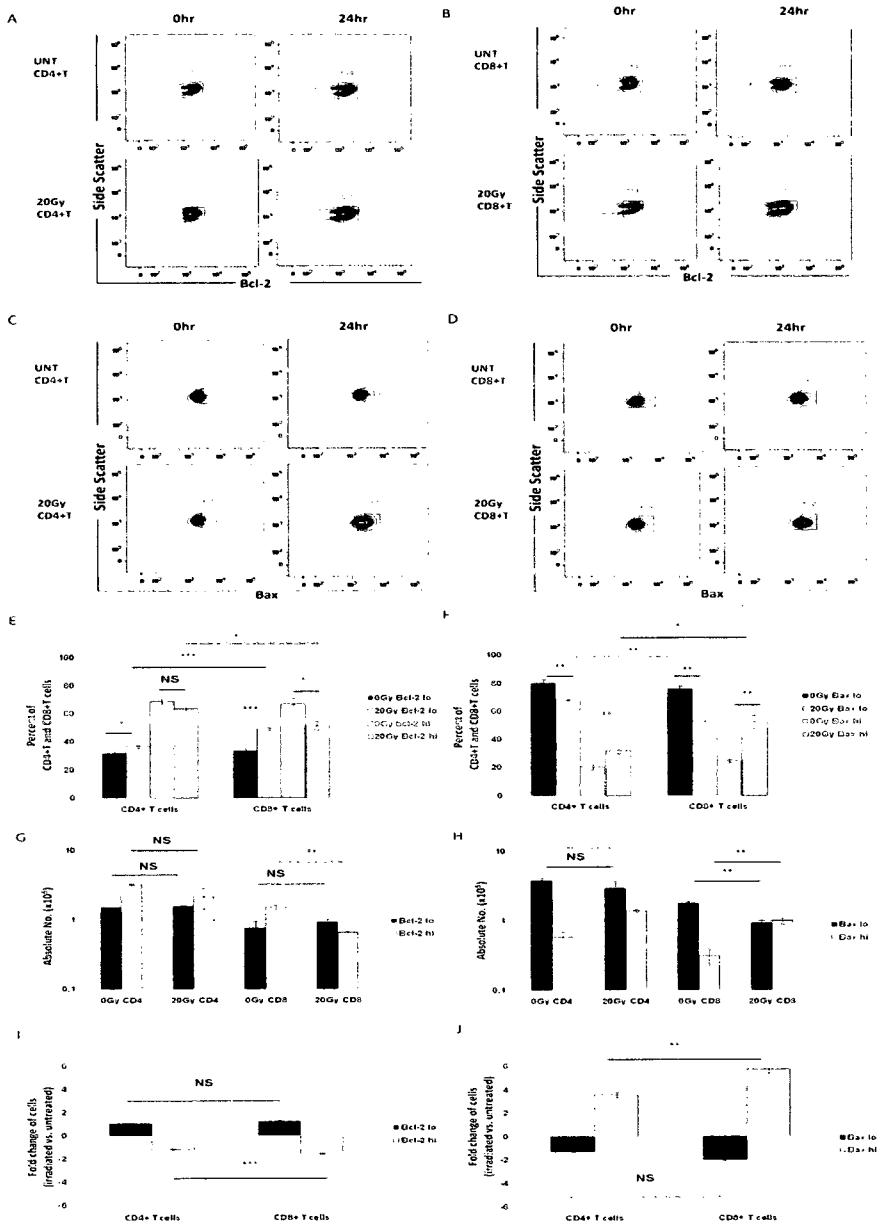
Veis, D. J., C. M. Sorenson, J. R. Shutter, and S. J. Korsmeyer. 1993. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* 75, no. 2: 229-40.

Walker, P. R., L. Kokileva, J. LeBlanc, and M. Sikorska. 1993. Detection of the initial stages of DNA fragmentation in apoptosis. *Biotechniques* 15, no. 6: 1032-40.

Williams, G. T. 1991. Programmed cell death: Apoptosis and oncogenesis. *Cell* 65, no. 7: 1097-8.

Yang, J., X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. I. Peng, D. P. Jones, and X. Wang. 1997. Prevention of apoptosis by bcl-2: Release of cytochrome c from mitochondria blocked. *Science* 275, no. 5303: 1129-32.

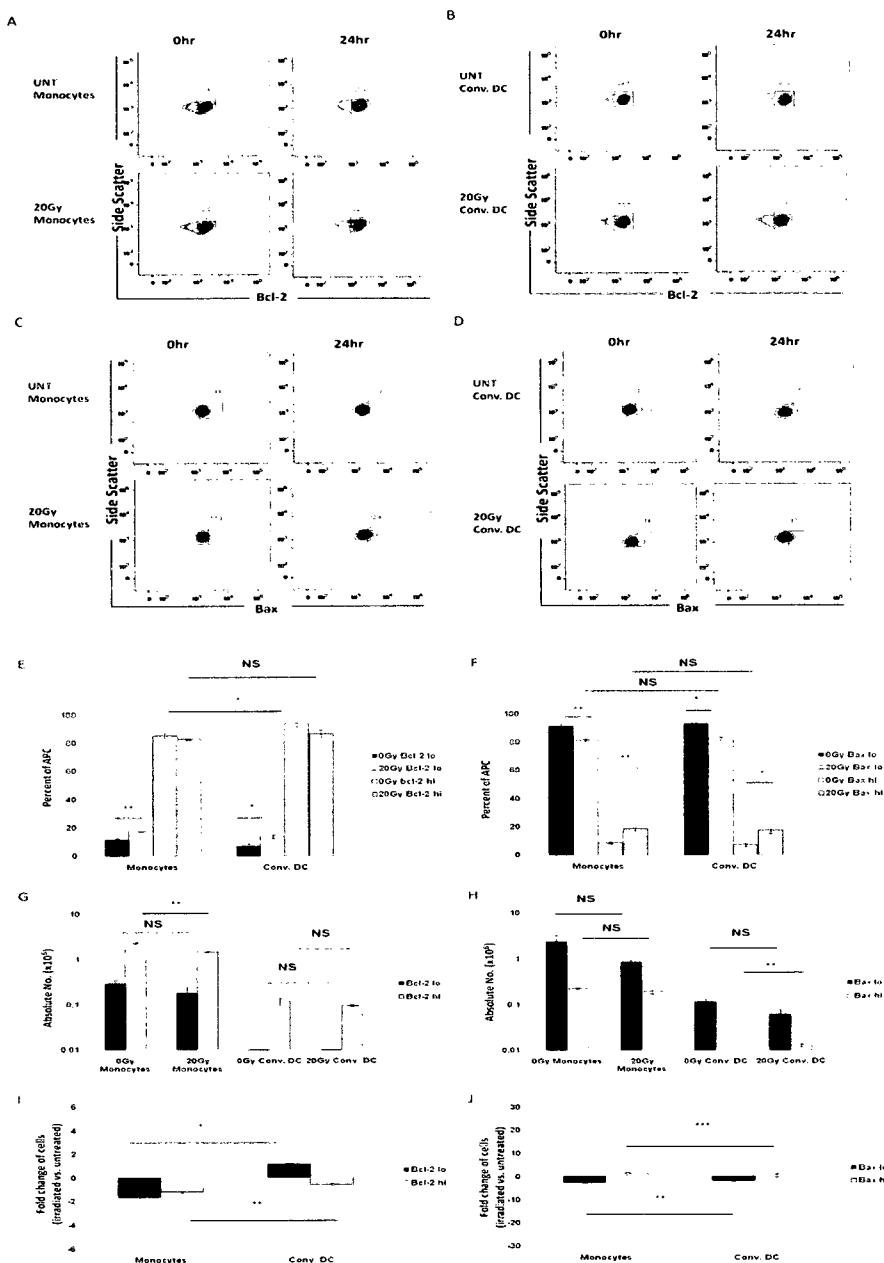
Yao, Z., Y. Liu, J. Jones, and S. Strober. 2009. Differences in bcl-2 expression by t-cell subsets alter their balance after in vivo irradiation to favor CD4⁺bcl-2hi NKT cells. *Eur J Immunol* 39, no. 3: 763-75.



Supplemental Figure 1. Irradiation increased expression of Bax in T cell subsets while Bcl-2 expression remained relatively constant.

(A) Gated CD3⁺CD4⁺ cells from unirradiated (top row) or after 20Gy (bottom row) at 0 and 24hrs, were analyzed by flow cytometry for Side Scatter versus Bcl-2. (B) Gated CD3⁺CD8⁺ cells from unirradiated (top row) or after 20Gy (bottom row) at 0 and 24hrs,

were analyzed by flow cytometry for Side Scatter versus Bcl-2. (C) Gated CD3⁺CD4⁺ cells from unirradiated (top row) or after 20Gy (bottom row) at 0 and 24hrs, were analyzed by flow cytometry for Side Scatter versus Bax. (D) Gated CD3⁺CD8⁺ cells from unirradiated (top row) or after 20Gy (bottom row) at 0 and 24hrs, were analyzed by flow cytometry for Side Scatter versus Bax. (E) The mean percentage (\pm standard error) of CD4⁺ and CD8⁺ Bcl-2 lo and Bcl-2 hi cells at 24hrs after 0 and 20Gy irradiation. (F) The mean percentage (\pm standard error) of CD4⁺ and CD8⁺ Bax lo and Bax hi cells at 24hrs after 0 and 20Gy irradiation. (G) Mean absolute numbers (\pm standard error) for CD4⁺ or CD8⁺ Bcl-2 lo and hi cells at 24 hrs after 0Gy versus 20Gy. (H) Mean absolute numbers (\pm standard error) for CD4⁺ or CD8⁺ Bax lo and hi cells at 24 hrs after 0Gy versus 20Gy. (I) Mean fold change (ratio of irradiated (20Gy) versus unirradiated) (\pm standard error) of CD4⁺, CD8⁺ Bcl-2 lo or hi cells at 24hrs. (J) Mean fold change (ratio of irradiated (20Gy) versus unirradiated) (\pm standard error) of CD4⁺, CD8⁺ Bax lo or hi at 24hrs. (E-I) *($p=<0.05$), **($p=<0.01$), and ***($p=<0.001$).

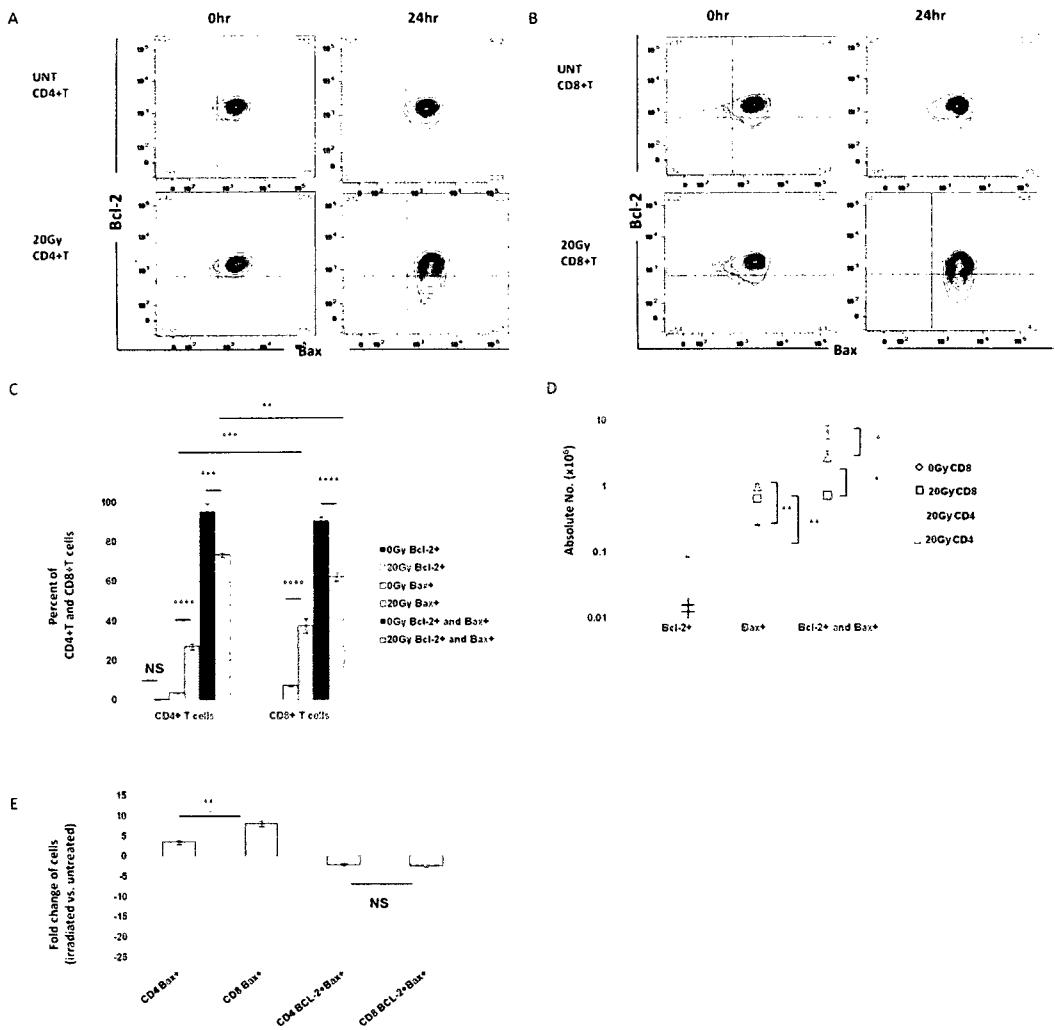


Supplemental Figure 2. Irradiation slightly increased expression of Bax in APC cell subsets while Bcl-2 expression remained relatively constant.

(A) Gated HLA-DR⁺CD11b⁺ CD14⁺ from unirradiated (top row) or after 20Gy (bottom row) at 0 and 24hrs, were analyzed by flow cytometry for Side Scatter versus Bcl-2. (B)

Gated Lin⁻HLA-DR⁺ CD11c⁺ cells from unirradiated or after 20Gy human PBMC at 0hrs (top row) or at 24hrs (bottom row), were analyzed by flow cytometry for Side Scatter versus Bcl-2. (C) Gated HLA-DR⁺CD11b⁺ CD14⁺ from unirradiated (top row) or after 20Gy (bottom row) at 0 and 24hrs, were analyzed by flow cytometry for Side Scatter versus Bax. (D) Gated Lin⁻HLA-DR⁺ CD11c⁺ cells from unirradiated or after 20Gy human PBMC at 0hrs (top row) or at 24hrs (bottom row), were analyzed by flow cytometry for Side Scatter versus Bax. (E) The mean percentage (\pm standard error) of monocytes and conventional dendritic cells Bcl-2 lo and Bcl-2 hi cells at 24 hrs after 0 and 20Gy irradiation. (F) The mean percentage (\pm standard error) of monocytes and conventional dendritic cells Bax lo and Bax hi cells at 24 hrs after 0 and 20Gy irradiation. (G) Mean absolute numbers (\pm standard error) for monocytes or conventional dendritic cells Bcl-2 lo and hi cells at 24 hrs after 0Gy versus 20Gy. (H) Mean absolute numbers (\pm standard error) for monocytes or conventional dendritic cells Bax lo and hi cells at 24 hrs after 0Gy versus 20Gy. (I) Mean fold change (ratio of irradiated (20Gy) versus unirradiated) (\pm standard error) of monocytes or conventional dendritic cells Bcl-2 lo or hi at 24 hrs. (J) Mean fold change (ratio of irradiated (20Gy) versus unirradiated) (\pm standard error) of monocytes or conventional dendritic cells Bax lo or hi at 24 hrs.

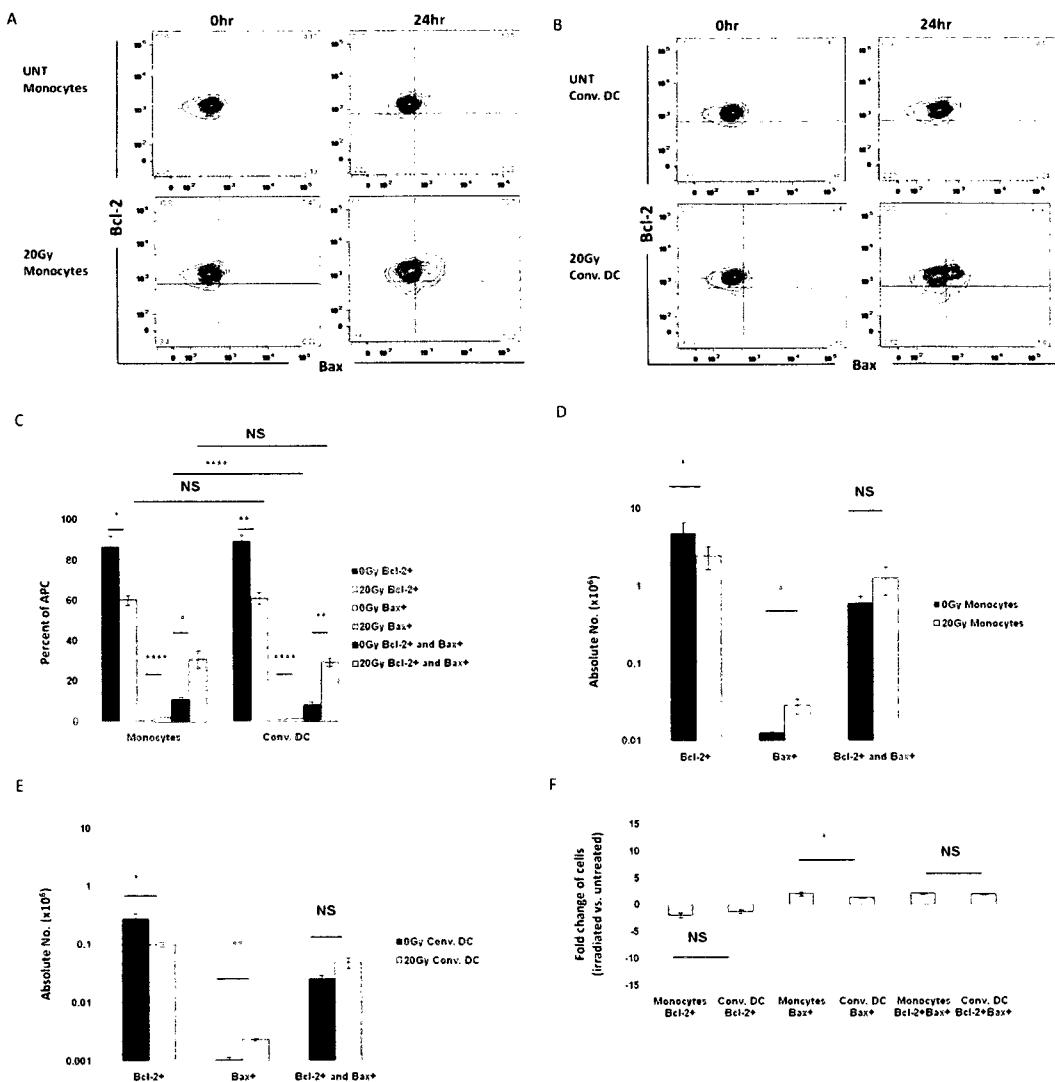
NS=not significant, *($p=<0.05$), ** ($p=<0.01$), ***($p=<0.001$), and ****($p=<0.0001$).



Supplemental Figure 3. Greater Bax⁺ CD8⁺ T cells (than CD4⁺ T cells) compared to T cell Bax⁺Bcl-2⁺ expression could explain greater radiosensitivity of CD8⁺ T cells.

(A) Gated CD3⁺CD4⁺ cells from unirradiated (top row) or after 20Gy (bottom row) at 0 and 24hrs, were analyzed by flow cytometry for Bcl-2 versus Bax. (B) Gated CD3⁺CD8⁺ cells from unirradiated (top row) or after 20Gy (bottom row) at 0 and 24hrs, were analyzed by flow cytometry for Bcl-2 versus Bax. (C) The mean percentage (\pm standard error) of CD4⁺ and CD8⁺ Bcl-2⁺, Bax⁺, or Bcl-2⁺Bax⁺ cells at 24 hrs after 0Gy

versus 20Gy. (D) Mean absolute numbers (\pm standard error) for CD4 $^{+}$ and CD8 $^{+}$ Bcl-2 $^{+}$, Bax $^{+}$, or Bcl-2 $^{+}$ Bax $^{+}$ cells at 24 hrs after 0Gy versus 20Gy. (E) Mean fold change (ratio of irradiated (20Gy) versus unirradiated) (\pm standard error) of CD4 $^{+}$ or CD8 $^{+}$ Bax $^{+}$, or Bax $^{+}$ Bcl-2 $^{+}$ cells at 24 hrs. (C-E) **(p=<0.01), ***(p=<0.001), and ****(p=<0.0001); (data displayed are =<0.01).



Supplemental Figure 4. Greater Bax⁺ Monocyte expression (than Conventional Dendritic Cells) compared to APC Bax⁺Bcl-2⁺ expression after irradiation could explain greater radiosensitivity of Monocytes.

(A) Gated HLA-DR⁺CD11b⁺ CD14⁺ from unirradiated (top row) or after 20Gy human PBMC (bottom row) at 0 and 24 hrs, were analyzed by flow cytometry for Bcl-2 versus Bax. (B) Gated Lin⁻HLA-DR⁺ CD11c⁺ cells from unirradiated or after 20Gy human PBMC at 0hrs (top row) or at 24hrs (bottom row), were analyzed by flow cytometry for

Bcl-2 versus Bax. (C) The mean percentage (\pm standard error) of monocytes and conventional dendritic cells Bcl-2 $^+$, Bax $^+$, or Bcl-2 $^+$ Bax $^+$ cells at 24 hrs after 0Gy versus 20Gy. (D) Mean absolute numbers (\pm standard error) for monocytes Bcl-2 $^+$, Bax $^+$, or Bcl-2 $^+$ Bax $^+$ cells at 24 hrs after 0Gy versus 20Gy. (E) Mean absolute numbers (\pm standard error) for conventional dendritic cells Bcl-2 $^+$, Bax $^+$, or Bcl-2 $^+$ Bax $^+$ cells at 24 hrs after 0Gy versus 20Gy. (F) Mean fold change (ratio of irradiated (20Gy) versus unirradiated) (\pm standard error) of monocytes or conventional dendritic cells Bcl-2 $^+$, Bax $^+$, or Bax $^+$ Bcl-2 $^+$ at 24hrs. (C-F) NS=not significant, *($p=<0.05$), **($p=<0.01$), and ****($p=<0.0001$).

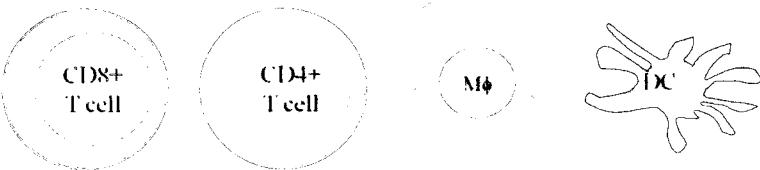
CHAPTER 4

SUMMARY AND FUTURE RESEARCH

Summary

By characterizing the relevant functional radiation-induced intracellular signaling changes in APC and identifying factors associated with the relative radioresistance of certain APC subsets, this thesis supports the theory that radiation directly affects immune cells in a functionally relevant manner. These results also show that enhanced APC function/responsiveness and radioresistance are interrelated.

Because radioresistant APC subsets express lower levels of Bax (compared to T cells), radiation induces lower levels of cleaved PARP and lower rates of apoptosis in radioresistant APC subsets. The table below represents a summary of radiation's effects on the mitochondrial-caspase mediated apoptotic pathway and the increase in TUNEL (readout of apoptosis).



Bax	↑	↑	↑	↑
Bcl-2	↓	↓	↓	↓
Cleaved PARP	↑	↑	↑	↑
TUNEL	↑	↑	↑	↑

Table 1. Radiation induced changes in mitochondrial mediated caspase apoptotic pathway in immune cell subsets.

It is conceivable that the NFκB pathway should also be activated in these cells since NFκB is necessary for immune cell survival, promotes transcription of anti-apoptotic genes (Ghosh and Karin 2002), and is activated by radiation in tumor cells (Magne et al. 2006). The studies presented here did not examine the role of NEMO in the increase in Bax⁺ APC after radiation . If Bax⁺ APC increase after radiation while NEMO activation is inhibited, this would suggest that radiation-induced activation of Bax is NFκB pathway dependent.

In addition to not knowing which and how APC survive, it was not known whether NFκB is activated by radiation in APC or if radiation-induced activation of the NFκB pathway is necessary and/or sufficient for APC responsiveness. The studies in chapter 2, demonstrate that radiation does induce activation of the NFκB pathway in human monocytic cell line and human APC from PBMC, and these studies show that

ATM radiation-induced phosphorylation of NEMO, an upstream target of the pathway, was necessary and sufficient for enhanced APC responsiveness (as indicated by increased T cell proliferation, co-stimulatory molecule expression, and cytokine production). If radiation-induced activation of Bax in APC also requires ATM mediated activation of the NF κ B pathway this would further link the two pathways. Overall, APC that survive radiation, due to low Bax which could result from the NF κ B pathway inhibiting an increase in Bax levels, would have enhanced function after radiation due to NF κ B pathway activation and inhibiting this pathway would decrease APC survival in addition to inhibiting APC function.

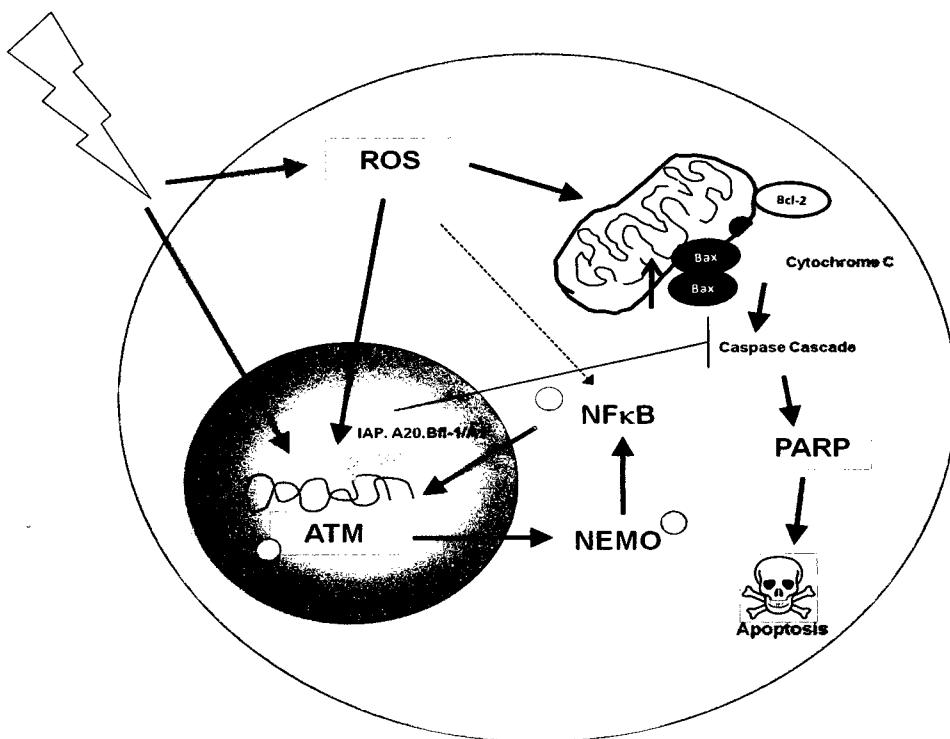


Figure 1. Overview of radiation-induced signaling changes in APC.

In this model, radiation results in DNA damage (direct and indirect via ROS production) that activates the NF κ B pathway via phosphorylation of NEMO which is ATM mediated, and is not due to direct activation of NF κ B by ROS. This activation of NF κ B leads to transcription of genes that promote maturation and activation of APC, which leads to enhanced responsiveness of APC after radiation, as well as, anti-apoptotic proteins that block caspase function or inhibit cytochrome c release (Chen et al. 2002; Magne et al. 2006). A direct interaction between activation of NF κ B and inhibition or activation of pro-apoptotic factors such as Bax is not known and therefore no direct link is shown between NF κ B and Bax in the model. ROS produced by radiation also activates the

mitochondrial caspase mediated pathway in which the relationship between pro-apoptotic and anti-apoptotic factors determines the susceptibility to apoptosis. If more pro-apoptotic factors are present, mitochondria release Cytochrome c, which activates the caspases, and finally cleaved PARP that leads to apoptosis. In this case, varying Bax expression among APC subsets correlated with the differential susceptibility to apoptosis (as indicated by PARP expression). Furthermore, characterizing radiation-induced changes in APC and the relative radioresistance of the different subsets (monocytes and dendritic cells) along with T cell subsets (CD4 and CD8 naïve and memory) was important because of the clinical relevance of understanding how radiation modulates the immune system. Potential examples of this relevance are demonstrated in the following scenarios:

- 1) Radioresistant APC are present within the early post-transplant period after HSCT. If these cells are surviving, their function after radiation will be enhanced and thereby, can increase GVT effects (i.e. enhanced host dendritic cell ratio after radiation could lead increase priming by activated host dendritic cells of cytotoxic T lymphocytes and increase their cytolytic activity). On the other hand, the survival and activation of host APC after HSCT may be deleterious and promote acute GVHD.
- 2) Radiation also selectively depletes T cell subsets with memory T cells being the most radioresistant (chapter 3). The depletion of host T cells enhances engraftment and reduces solid organ rejection. In addition, there would be residual resistant host memory T cells that could increase the risk of allograft transplant rejection in hosts. The effects of

radiation on APC and T cell subsets along with the associated predicated clinical outcomes are summarized in table 1 below.

Radiation-induced Δ's	GVT	GVHD	Solid Organ Allograft Tolerance
Host APC activation	↑	↑	↓
↑Host DC:Monocytes	↑	↑	↓
Host CD4 T cell depletion			↑
Host residual memory T cells			↓

Table 2. Radiation induced PMBC subset changes and predicted clinical outcomes.

Since there is no way to know whether or not patients will have improved GVT effects or suffer from GHVD, characterizing radiation induced changes in APC has provided potentially useful markers (NFκB pathway molecules, Bax and other pro-apoptotic proteins) that could be used to optimize radiation treatment protocols for anti-tumor therapies as well preparatory radiation regimens for successful HSCT (i.e. no GVHD).

Future Research

The findings reported here demonstrate the importance of radiation as an independent immune modulatory agent, with the potential to suppress or enhance the immune response, and point to several promising directions for future research. Such studies are timely, since the use of radiation as a component of tumor vaccine strategies is an area of active investigation. Future studies should seek to elucidate methods by which NEMO, I κ B α and NF κ B activation after radiation may directly enhance tumor immunity. Described below are examples of studies that could further elucidate the role of radiation as a potentiator of tumor immunity.

1: Isolate APC from mouse tumor models of common human cancers (i.e. breast, lung, or colon) after localized radiation treatment to assess activation of NF κ B and its upstream targets.

Rationale: Results presented within this thesis demonstrate that NF κ B pathway activation after radiation in human APC is necessary for enhanced APC responsiveness/function. Confirmation of NF κ B pathway activation in APC from mouse model tumors would provide in vivo validation of results to date and facilitate further exploration of how this activation can be used to directly enhance tumor immunity.

Experimental Design and Methods: For these experiments, syngeneic or spontaneous tumor models should be used. Tumors will be harvested at d 0, 2, 5, 10 after localized

radiation, digested to obtain single-cell suspensions and used for flow-cytometric analysis of APC and T cell subsets for NEMO, I κ B α , and NF κ B activation.

Expected Results: It is expected that these experiments will demonstrate radiation-induced phosphorylation of NEMO, I κ B α , and NF κ B in APC, but not T cells isolated from tumors.

2: To correlate radiation-induced changes in specific aim 1 with function in tumor models.

Rationale: Results from this thesis have demonstrated that radiation-induced NF κ B pathway activation enhances APC responsiveness. In vivo validation is needed to confirm activation of this pathway in correlating has useful correlates to clinical outcomes.

Experimental Design and Methods: Normal mice with OVA expression tumors would be locally irradiated and injected with OT 1 specific CD8 $^{+}$ T cells that are fluorescently labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE). T cells would be isolated from the tumors and analyzed by flow cytometry for cell proliferation (Lugade et al. 2008; Sancho et al. 2008). In addition, OT 1 specific CD8 $^{+}$ T cells would be cocultured with APC, both from the unirradiated and irradiated OVA expressing tumors of different mice to assess cytokine production in vitro. As T cell proliferation and cytokine production are indicative of APC responsiveness, correlates could then be drawn between the extent to which NEMO, I κ B α , or NF κ B is phosphorylated after radiation and the probable enhanced responsiveness of APC from irradiated tumors in these mice.

Expected Results: It is expected that radiation will enhance tumor responsiveness as a result of enhanced APC responsiveness after radiation. However, responsiveness may vary with cancer type.

In vivo experiments introduce complexities not seen in vitro. The major difference is that the effect of radiation on the tumor cells could be influenced by the tumor microenvironment in which they are abnormal interactions between tumor and host cells and is often characterized by hypoxia, nutrient deprivation, acidosis, and aberrant stroma (Mueller and Fusenig, Nature Reviews Cancer 4, 839-849, 2004; Strausberg, tumor microenvironments, the immune system, and cancer survival, 2005). In a in vivo system, immune cells within the microenvironment can receive signals (i.e. release and activation of chemokines and leukocyte adhesion molecules by radiation) that can result in homing of APC and T cells to the tumor site to promote an anti-tumor response and immune cells in the tumor environment can be dysfunctional which could effect how immune cells respond to tumor antigens. In addition, T cells and APC experience less cell-cell contact among themselves in vivo than in vitro which could affect the functional results obtained from studies on T and APC interactions. Also, APC's are often expanded in vitro for functional experiments and increasing the numbers of APC could result in enhanced functional outcomes (ex: APC responsiveness after radiation) that may not been seen in vivo. Therefore, the complexities of an in vivo tumor model system must be taken into account when interpreting results of radiation-induced activation of signaling pathways and subsequent functional changes.

The clinical applicability of this research is vital as every day in the United States an estimated 6,000 people need bone marrow transplants for hereditary and malignant

diseases. Many of these patients will be treated using preparatory regimens that include radiation. Therefore, there is a need to better understand how radiation affects immune cell subsets and the significance of relevant changes that occur in order to optimize these treatment regimens. Thousands of new cases of hematopoietic malignancies such as non-Hodgkins lymphoma and leukemia are diagnosed every day. As shown in Chapter 2, the NF κ B pathway is vital for APC responsiveness after radiation. Therefore, NF κ B activation or activation of an upstream target could be used as a systemic biomarker in patients who have undergone radiation therapy for cancer treatment in preparation of bone marrow transplantation or HSCT. Future experiments should examine the following:

3A: Utilization of mouse GVHD models for correlative studies and functional studies in vivo.

Rationale: APC can increase the risk for GVHD as a result of their ability to present host antigen to donor T cells, and this ability depends on APC responsiveness. Since NF κ B determines APC responsiveness, correlation between NF κ B pathway activation and GVHD would provide compelling evidence to study inhibition of NEMO in mouse transplantation models, and possibly in the future, in human patients.

Experimental Design: For correlative studies, the level of NF κ B pathway activation in mouse models of GVHD would be determined and compared to levels in mice without GVHD. Higher levels of NEMO, I κ B α , or NF κ B activation in GHVD mouse models would indicate that future studies should examine inhibition of this pathway (i.e. by NBD

peptide) in vivo. The NBD peptide would be administered systemically to mice throughout the radiation preparatory regimen for HSCT transplantation and immediately after transplantation to determine if direct NEMO inhibition reduces the risk for GVHD as compared to control mice.

3B: Correlate levels of NF κ B pathway activation in blood samples from patients with or without GVHD that have undergone preparatory allogeneic TBI/TLI radiation regimens for transplantation.

Rationale: APC responsiveness is necessary in processes such as engraftment of donor marrow/cells and chimerism. Correlating NF κ B pathway activity to patient markers of successful transplantation could be useful in optimizing preparatory regimens for treatment. Simultaneous analysis of NF κ B pathway proteins and other intracellular proteins that may be activated by radiation and are involved in the regulation and development of the immune system could be studied using multiplex cytometric arrays with fluorescent nanoparticles that allow for measurement of many different analytes in a few microliters of sample.

Experimental Design: Patient blood samples obtained before and after the preparatory regimens using radiation for BMT or HSCT would be assessed for NF κ B pathway activation and levels of activation would be correlated to engraftment of donor cells, chimerism and the extent of GVHD.

3C: Correlate levels of NF κ B pathway activation in blood samples from patients with GVT versus tumor recurrence and/or tumor persistence that will undergo preparatory allogeneic TBI/TLI radiation regimens for transplantation.

Rationale: APC responsiveness is necessary for initiation and enhancement of GVT responses after radiation. Correlating NF κ B pathway activity to patient outcomes could be useful for optimizing the use of radiation in promoting anti-tumor responses.

Experimental Design: Patient blood samples obtained before and after the preparatory regimens using radiation for BMT or HSCT would be assessed for NF κ B pathway activation and levels of activation would be correlated to GVT rates. This information could then be used to design protocols to improve GVT by NF κ B pathway modification in specific patients.

Similar studies as presented in specific aim 3 could also be used to examine the use of NF κ B as a systemic biomarker of APC responsiveness in clinical trials where radiation is combined with immunotherapies to enhance anti-tumor responses.

It would also be important to further study the significance of the relative radioresistance of APC compared to T cell subsets as residual APC are likely to have an important impact on immune function and thereby clinical outcomes. Though most T cells are extremely radiosensitive, previous studies by others have demonstrated that there are also more radioresistant T cells such as CD4 $^{+}$ CD25 $^{+}$ T regulatory cells that will also have an impact on immune function as well (Joffre et al. 2008). Thus, additional studies are warranted for mechanisms of cell survival after radiation in human T

regulatory cells. Similar studies should be performed with Natural Killer T cells, as they have been shown to be radioresistant and to expand after preparatory radiation regimen for HSCT in humans and could be affecting immune function after radiation (Lowsky et al. 2005). Irradiated host APC are present within the early post-transplant period and could increase the risk of allograft transplant rejection in hosts by presenting donor alloantigens to residual memory T cells in the host. On the other hand, the radioresistance of APC could benefit graft versus tumor effects.

The results presented in chapter 3 have demonstrated that varying expression of Bax contributes to radiation-induced sensitivity of T cells and resistance of APC subsets. Simultaneous analysis of Bax, Bcl-2, other anti and pro-apoptotic proteins like Bcl-XL, and p53 upregulated modulator of apoptosis (PUMA)(Nakano and Vousden 2001), along with readouts of apoptotic death and mitotic catastrophe induced by radiation could be correlated to GVT rates as above.

Overall, the work presented in this thesis has laid a foundation for continued exploration of how radiation enhances the immune response as well as how to approach the characterization of other intracellular signaling markers of APC responsiveness that can potentially be used as clinical correlates for outcomes of radiation therapy.

CHAPTER 5

REFERENCES

- Abdulkarim, B., S. Sabri, E. Deutsch, S. Vaganay, E. Marangoni, W. Vainchenker, P. Bongrand, P. Busson, and J. Bourhis. 2000. Radiation-induced expression of functional fas ligand in ebv-positive human nasopharyngeal carcinoma cells. *Int J Cancer* 86, no. 2: 229-37.
- Ahn, K. S. and B. B. Aggarwal. 2005. Transcription factor nf-kappab: A sensor for smoke and stress signals. *Ann N Y Acad Sci* 1056: 218-33.
- Albanese, J. and N. Dainiak. 2000. Ionizing radiation alters fas antigen ligand at the cell surface and on exfoliated plasma membrane-derived vesicles: Implications for apoptosis and intercellular signaling. *Radiat Res* 153, no. 1: 49-61.
- Ame, J. C., C. Spenlehauer, and G. de Murcia. 2004. The parp superfamily. *Bioessays* 26, no. 8: 882-93.
- Anderson, B. E., J. M. McNiff, C. Matte, I. Athanasiadis, W. D. Shlomchik, and M. J. Shlomchik. 2004. Recipient cd4+ t cells that survive irradiation regulate chronic graft-versus-host disease. *Blood* 104, no. 5: 1565-73.
- Antonia, S., J. J. Mule, and J. S. Weber. 2004. Current developments of immunotherapy in the clinic. *Curr Opin Immunol* 16, no. 2: 130-6.
- Baldwin, A. S., Jr. 1996. The nf-kappa b and i kappa b proteins: New discoveries and insights. *Annu Rev Immunol* 14: 649-83.

- Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y. J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu Rev Immunol* 18: 767-811.
- Belka, C., P. Marini, W. Budach, K. Schulze-Osthoff, F. Lang, E. Gulbins, and M. Bamberg. 1998. Radiation-induced apoptosis in human lymphocytes and lymphoma cells critically relies on the up-regulation of cd95/fas/apo-1 ligand. *Radiat Res* 149, no. 6: 588-95.
- Bellas, R. E., J. S. Lee, and G. E. Sonenshein. 1995. Expression of a constitutive nf-kappa b-like activity is essential for proliferation of cultured bovine vascular smooth muscle cells. *J Clin Invest* 96, no. 5: 2521-7.
- Bentires-Alj, M., E. Dejardin, P. Viatour, C. Van Lint, B. Froesch, J. C. Reed, M. P. Merville, and V. Bours. 2001. Inhibition of the nf-kappa b transcription factor increases bax expression in cancer cell lines. *Oncogene* 20, no. 22: 2805-13.
- Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 1995. Cd28 costimulation can promote t cell survival by enhancing the expression of bcl-xl. *Immunity* 3, no. 1: 87-98.
- Bottero, V., V. Busuttil, A. Loubat, N. Magne, J. L. Fischel, G. Milano, and J. F. Peyron. 2001. Activation of nuclear factor kappab through the ikk complex by the topoisomerase poisons sn38 and doxorubicin: A brake to apoptosis in hela human carcinoma cells. *Cancer Res* 61, no. 21: 7785-91.
- Brightbill, H. D., D. H. Libratty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, P. J. Brennan, B. R. Bloom, P. J. Godowski, and R. L. Modlin. 1999. Host defense mechanisms

- triggered by microbial lipoproteins through toll-like receptors. *Science* 285, no. 5428: 732-6.
- Brody, J. D., M. J. Goldstein, D. K. Czerwinski, and R. Levy. 2009. Immunotransplantation preferentially expands t-effector cells over t-regulatory cells and cures large lymphoma tumors. *Blood* 113, no. 1: 85-94. J
- Brown, J. M. and L. D. Attardi. 2005. The role of apoptosis in cancer development and treatment response. *Nat Rev Cancer* 5, no. 3: 231-7. J
- Buggins, A. G., N. Lea, J. Gaken, D. Darling, F. Farzaneh, G. J. Mufti, and W. J. Hirst. 1999. Effect of costimulation and the microenvironment on antigen presentation by leukemic cells. *Blood* 94, no. 10: 3479-90. J
- Burns, T. F., E. J. Bernhard, and W. S. El-Deiry. 2001. Tissue specific expression of p53 target genes suggests a key role for killer/dr5 in p53-dependent apoptosis in vivo. *Oncogene* 20, no. 34: 4601-12. J
- Cameron, R. B., P. J. Spiess, and S. A. Rosenberg. 1990. Synergistic antitumor activity of tumor-infiltrating lymphocytes, interleukin 2, and local tumor irradiation. Studies on the mechanism of action. *J Exp Med* 171, no. 1: 249-63. J
- Chakravarty, P. K., A. Alfieri, E. K. Thomas, V. Beri, K. E. Tanaka, B. Vikram, and C. Guha. 1999. Flt3-ligand administration after radiation therapy prolongs survival in a murine model of metastatic lung cancer. *Cancer Res* 59, no. 24: 6028-32. J
- Chen, X., B. Shen, L. Xia, A. Khaletzkiy, D. Chu, J. Y. Wong, and J. J. Li. 2002. Activation of nuclear factor kappaB in radioresistance of tp53-inactive human keratinocytes. *Cancer Res* 62, no. 4: 1213-21. J

- Chen, Y. R., C. F. Meyer, and T. H. Tan. 1996. Persistent activation of c-jun n-terminal kinase 1 (jnk1) in gamma radiation-induced apoptosis. *J Biol Chem* 271, no. 2: 631-4. J
- Chen, Y. R., X. Wang, D. Templeton, R. J. Davis, and T. H. Tan. 1996. The role of c-jun n-terminal kinase (jnk) in apoptosis induced by ultraviolet c and gamma radiation. Duration of jnk activation may determine cell death and proliferation. *J Biol Chem* 271, no. 50: 31929-36. J
- Chen, Y., J. Zhang, S. A. Moore, Z. K. Ballas, J. P. Portanova, A. M. Krieg, and D. J. Berg. 2001. Cpg DNA induces cyclooxygenase-2 expression and prostaglandin production. *Int Immunol* 13, no. 8: 1013-20. J
- Chiang, C. S., J. H. Hong, Y. C. Wu, W. H. McBride, and G. J. Dougherty. 2000. Combining radiation therapy with interleukin-3 gene immunotherapy. *Cancer Gene Ther* 7, no. 8: 1172-8. J
- Chiang, C. S., R. G. Syljuasen, J. H. Hong, A. Wallis, G. J. Dougherty, and W. H. McBride. 1997. Effects of il-3 gene expression on tumor response to irradiation in vitro and in vivo. *Cancer Res* 57, no. 18: 3899-903. J
- Clark, G. J., N. Angel, M. Kato, J. A. Lopez, K. MacDonald, S. Vuckovic, and D. N. Hart. 2000. The role of dendritic cells in the innate immune system. *Microbes Infect* 2, no. 3: 257-72. J
- Cock, J. G., A. D. Tepper, E. de Vries, W. J. van Blitterswijk, and J. Borst. 1998. Cd95 (fas/apo-1) induces ceramide formation and apoptosis in the absence of a functional acid sphingomyelinase. *J Biol Chem* 273, no. 13: 7560-5. J

Cremesti, A., F. Paris, H. Grassme, N. Holler, J. Tschopp, Z. Fuks, E. Gulbins, and R.

Kolesnick. 2001. Ceramide enables fas to cap and kill. *J Biol Chem* 276, no. 26: 23954-61. J

D'Amico, A. and L. Wu. 2003. The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells are within the bone marrow hemopoietic precursors expressing flt3. *J Exp Med* 198, no. 2: 293-303. J

Dale, D. C., L. Boxer, and W. C. Liles. 2008. The phagocytes: Neutrophils and monocytes. *Blood* 112, no. 4: 935-45. J

Darzynkiewicz, Z., G. Juan, X. Li, W. Gorczyca, T. Murakami, and F. Traganos. 1997. Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 27, no. 1: 1-20. J

Delamarre, L., H. Holcombe, and I. Mellman. 2003. Presentation of exogenous antigens on major histocompatibility complex (mhc) class i and mhc class ii molecules is differentially regulated during dendritic cell maturation. *J Exp Med* 198, no. 1: 111-22. J

Delves, P. J. and I. M. Roitt. 2000. The immune system. First of two parts. *N Engl J Med* 343, no. 1: 37-49. J

_____. 2000. The immune system. Second of two parts. *N Engl J Med* 343, no. 2: 108-17. X

Demaria, S., N. Bhardwaj, W. H. McBride, and S. C. Formenti. 2005. Combining radiotherapy and immunotherapy: A revived partnership. *Int J Radiat Oncol Biol Phys* 63, no. 3: 655-66. J

Demaria, S. and S. C. Formenti. 2007. Sensors of ionizing radiation effects on the immunological microenvironment of cancer. *Int J Radiat Biol* 83, no. 11-12: 819-
25. J

Demaria, S., N. Kawashima, A. M. Yang, M. L. Devitt, J. S. Babb, J. P. Allison, and S. C. Formenti. 2005. Immune-mediated inhibition of metastases after treatment with local radiation and ctla-4 blockade in a mouse model of breast cancer. *Clin Cancer Res* 11, no. 2 Pt 1: 728-34. J

Deveraux, Q. L. and J. C. Reed. 1999. Iap family proteins--suppressors of apoptosis. *Genes Dev* 13, no. 3: 239-52. J

Didelot, C., M. Barberi-Heyob, A. Bianchi, P. Becuwe, J. F. Mirjolet, M. Dauca, and J. L. Merlin. 2001. Constitutive nf-kappab activity influences basal apoptosis and radiosensitivity of head-and-neck carcinoma cell lines. *Int J Radiat Oncol Biol Phys* 51, no. 5: 1354-60. J

Domen, J., K. L. Gandy, and I. L. Weissman. 1998. Systemic overexpression of bcl-2 in the hematopoietic system protects transgenic mice from the consequences of lethal irradiation. *Blood* 91, no. 7: 2272-82. J

Eder, M., G. Geissler, and A. Ganser. 1997. Il-3 in the clinic. *Stem Cells* 15, no. 5: 327-33. J

Edinger, M., P. Hoffmann, J. Ermann, K. Drago, C. G. Fathman, S. Strober, and R. S. Negrin. 2003. Cd4+cd25+ regulatory t cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med* 9, no. 9: 1144-50. J

- Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata. 1998. A caspase-activated dnase that degrades DNA during apoptosis, and its inhibitor icad. *Nature* 391, no. 6662: 43-50. J
- Feng, H., Y. Zeng, L. Whitesell, and E. Katsanis. 2001. Stressed apoptotic tumor cells express heat shock proteins and elicit tumor-specific immunity. *Blood* 97, no. 11: 3505-12. J
- Field, E. H. and S. Strober. 2001. Tolerance, mixed chimerism and protection against graft-versus-host disease after total lymphoid irradiation. *Philos Trans R Soc Lond B Biol Sci* 356, no. 1409: 739-48. J
- Fonteneau, J. F., M. Larsson, A. S. Beignon, K. McKenna, I. Dasilva, A. Amara, Y. J. Liu, J. D. Lifson, D. R. Littman, and N. Bhardwaj. 2004. Human immunodeficiency virus type 1 activates plasmacytoid dendritic cells and concomitantly induces the bystander maturation of myeloid dendritic cells. *J Virol* 78, no. 10: 5223-32. J
- Fonteneau, J. F., M. Larsson, and N. Bhardwaj. 2002. Interactions between dead cells and dendritic cells in the induction of antiviral CTL responses. *Curr Opin Immunol* 14, no. 4: 471-7. J
- Friedman, E. J. 2002. Immune modulation by ionizing radiation and its implications for cancer immunotherapy. *Curr Pharm Des* 8, no. 19: 1765-80. J
- Fuks, Z. and S. Slavin. 1981. The use of total lymphoid irradiation (TLI) as immunosuppressive therapy for organ allotransplantation and autoimmune diseases. *Int J Radiat Oncol Biol Phys* 7, no. 1: 79-82. J

- Gallucci, S. and P. Matzinger. 2001. Danger signals: Sos to the immune system. *Curr Opin Immunol* 13, no. 1: 114-9. J
- Ganss, R., E. Ryschich, E. Klar, B. Arnold, and G. J. Hammerling. 2002. Combination of t-cell therapy and trigger of inflammation induces remodeling of the vasculature and tumor eradication. *Cancer Res* 62, no. 5: 1462-70. J
- Ghosh, S. and M. Karin. 2002. Missing pieces in the nf-kappab puzzle. *Cell* 109 Suppl: S81-96. J
- Guan, B., P. Yue, G. L. Clayman, and S. Y. Sun. 2001. Evidence that the death receptor dr4 is a DNA damage-inducible, p53-regulated gene. *J Cell Physiol* 188, no. 1: 98-105. J
- Guermonprez, P., L. Saveanu, M. Kleijmeer, J. Davoust, P. Van Endert, and S. Amigorena. 2003. Er-phagosome fusion defines an mhc class i cross-presentation compartment in dendritic cells. *Nature* 425, no. 6956: 397-402. J
- Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery, and S. Amigorena. 2002. Antigen presentation and t cell stimulation by dendritic cells. *Annu Rev Immunol* 20: 621-67. J
- Gulley, J. L., P. M. Arlen, A. Bastian, S. Morin, J. Marte, P. Beetham, K. Y. Tsang, J. Yokokawa, J. W. Hodge, C. Menard, K. Camphausen, C. N. Coleman, F. Sullivan, S. M. Steinberg, J. Schlom, and W. Dahut. 2005. Combining a recombinant cancer vaccine with standard definitive radiotherapy in patients with localized prostate cancer. *Clin Cancer Res* 11, no. 9: 3353-62. J
- Gulley, J. L., R. A. Madan, and P. M. Arlen. 2007. Enhancing efficacy of therapeutic vaccinations by combination with other modalities. *Vaccine* 25 Suppl 2: B89-96. J

Hallahan, D. E., D. R. Spriggs, M. A. Beckett, D. W. Kufe, and R. R. Weichselbaum.

1989. Increased tumor necrosis factor alpha mRNA after cellular exposure to ionizing radiation. *Proc Natl Acad Sci U S A* 86, no. 24: 10104-7. J

Harris, P. and P. Ralph. 1985. Human leukemic models of myelomonocytic development: A review of the HL-60 and U937 cell lines. *J Leukoc Biol* 37, no. 4: 407-22. J

Hartgers, F. C., C. G. Figdor, and G. J. Adema. 2000. Towards a molecular understanding of dendritic cell immunobiology. *Immunol Today* 21, no. 11: 542-5. J

Hayden, M. S., A. P. West, and S. Ghosh. 2006. NF-κB and the immune response. *Oncogene* 25, no. 51: 6758-80. J

Herr, I., D. Wilhelm, T. Bohler, P. Angel, and K. M. Debatin. 1997. Activation of CD95 (apo-1/fas) signaling by ceramide mediates cancer therapy-induced apoptosis. *EMBO J* 16, no. 20: 6200-8. J

Higuchi, M., D. Zeng, J. Shizuru, J. Gworek, S. Dejbakhsh-Jones, M. Taniguchi, and S. Strober. 2002. Immune tolerance to combined organ and bone marrow transplants after fractionated lymphoid irradiation involves regulatory NK T cells and clonal deletion. *J Immunol* 169, no. 10: 5564-70. J

Ho, E., G. Chen, and T. M. Bray. 1999. Supplementation of N-acetylcysteine inhibits NF-κB activation and protects against alloxan-induced diabetes in CD-1 mice. *FASEB J* 13, no. 13: 1845-54. J

Hockenberry, D., G. Nunez, C. Milliman, R. D. Schreiber, and S. J. Korsmeyer. 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348, no. 6299: 334-6. J

- Hong, J. H., C. S. Chiang, C. Y. Tsao, P. Y. Lin, W. H. McBride, and C. J. Wu. 1999. Rapid induction of cytokine gene expression in the lung after single and fractionated doses of radiation. *Int J Radiat Biol* 75, no. 11: 1421-7. J
- Hoppe, R. T. 1987. The non-hodgkin's lymphomas: Pathology, staging, treatment. *Curr Probl Cancer* 11, no. 6: 363-447. J
- Ishihara, H., K. Tsuneoka, A. B. Dimchev, and M. Shikita. 1993. Induction of the expression of the interleukin-1 beta gene in mouse spleen by ionizing radiation. *Radiat Res* 133, no. 3: 321-6. J
- Iwasaki, A. and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 5, no. 10: 987-95. J
- Joffre, O., T. Santolaria, D. Calise, T. Al Saati, D. Hudrisier, P. Romagnoli, and J. P. van Meerwijk. 2008. Prevention of acute and chronic allograft rejection with cd4+cd25+foxp3+ regulatory t lymphocytes. *Nat Med* 14, no. 1: 88-92. J
- Jones, D. T., K. Ganeshaguru, A. E. Virchis, N. I. Folarin, M. W. Lowdell, A. B. Mehta, H. G. Prentice, A. V. Hoffbrand, and R. G. Wickremasinghe. 2001. Caspase 8 activation independent of fas (cd95/apo-1) signaling may mediate killing of b-chronic lymphocytic leukemia cells by cytotoxic drugs or gamma radiation. *Blood* 98, no. 9: 2800-7. J
- Kaneko, Y. S., K. Ikeda, and M. Nakanishi. 1999. Phorbol ester inhibits DNA damage-induced apoptosis in u937 cells through activation of protein kinase c. *Life Sci* 65, no. 21: 2251-8. J

- Karsunky, H., M. Merad, A. Cozzio, I. L. Weissman, and M. G. Manz. 2003. Flt3 ligand regulates dendritic cell development from flt3+ lymphoid and myeloid-committed progenitors to flt3+ dendritic cells in vivo. *J Exp Med* 198, no. 2: 305-13.
- Kim, K. W., S. H. Kim, J. G. Shin, G. S. Kim, Y. O. Son, S. W. Park, B. H. Kwon, D. W. Kim, C. H. Lee, M. Y. Sol, M. H. Jeong, B. S. Chung, and C. D. Kang. 2004. Direct injection of immature dendritic cells into irradiated tumor induces efficient antitumor immunity. *Int J Cancer* 109, no. 5: 685-90.
- Kitagawa, R. and M. B. Kastan. 2005. The atm-dependent DNA damage signaling pathway. *Cold Spring Harb Symp Quant Biol* 70: 99-109.
- Kohrt, H. E., B. B. Turnbull, K. Heydari, J. A. Shizuru, G. G. Laport, D. B. Miklos, L. J. Johnston, S. Arai, W. K. Weng, R. T. Hoppe, P. W. Lavori, K. G. Blume, R. S. Negrin, S. Strober, and R. Lowsky. 2009. Tli and atg conditioning with low risk of graft-versus-host disease retains antitumor reactions after allogeneic hematopoietic cell transplantation from related and unrelated donors. *Blood* 114, no. 5: 1099-109.
- Koren, H. S., S. J. Anderson, and J. W. Lerrick. 1979. In vitro activation of a human macrophage-like cell line. *Nature* 279, no. 5711: 328-31.
- Krajewski, S., S. Tanaka, S. Takayama, M. J. Schibler, W. Fenton, and J. C. Reed. 1993. Investigation of the subcellular distribution of the bcl-2 oncprotein: Residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res* 53, no. 19: 4701-14.
- Krieg, A. M. 2004. Antitumor applications of stimulating toll-like receptor 9 with cpg oligodeoxynucleotides. *Curr Oncol Rep* 6, no. 2: 88-95.

- Krutzik, P. O., J. M. Irish, G. P. Nolan, and O. D. Perez. 2004. Analysis of protein phosphorylation and cellular signaling events by flow cytometry: Techniques and clinical applications. *Clin Immunol* 110, no. 3: 206-21. J
- Lan, F., D. Zeng, M. Higuchi, J. P. Higgins, and S. Strober. 2003. Host conditioning with total lymphoid irradiation and antithymocyte globulin prevents graft-versus-host disease: The role of cd1-reactive natural killer t cells. *Biol Blood Marrow Transplant* 9, no. 6: 355-63. J
- Lan, F., D. Zeng, M. Higuchi, P. Huie, J. P. Higgins, and S. Strober. 2001. Predominance of nk1.1+tcr alpha beta+ or dx5+tcr alpha beta+ t cells in mice conditioned with fractionated lymphoid irradiation protects against graft-versus-host disease: "Natural suppressor" Cells. *J Immunol* 167, no. 4: 2087-96. J
- Larsson, M., J. F. Fonteneau, and N. Bhardwaj. 2001. Dendritic cells resurrect antigens from dead cells. *Trends Immunol* 22, no. 3: 141-8. J
- Liu, S. Z., S. Z. Jin, X. D. Liu, and Y. M. Sun. 2001. Role of cd28/b7 costimulation and il-12/il-10 interaction in the radiation-induced immune changes. *BMC Immunol* 2: 8. J
- Lowsky, R., T. Takahashi, Y. P. Liu, S. Dejbakhsh-Jones, F. C. Grumet, J. A. Shizuru, G. G. Laport, K. E. Stockerl-Goldstein, L. J. Johnston, R. T. Hoppe, D. A. Bloch, K. G. Blume, R. S. Negrin, and S. Strober. 2005. Protective conditioning for acute graft-versus-host disease. *N Engl J Med* 353, no. 13: 1321-31. J
- Lugade, A. A., E. W. Sorensen, S. A. Gerber, J. P. Moran, J. G. Frelinger, and E. M. Lord. 2008. Radiation-induced ifn-gamma production within the tumor microenvironment influences antitumor immunity. *J Immunol* 180, no. 5: 3132-9. J

Luznik, L., J. E. Slansky, S. Jalla, I. Borrello, H. I. Levitsky, D. M. Pardoll, and E. J.

Fuchs. 2003. Successful therapy of metastatic cancer using tumor vaccines in
mixed allogeneic bone marrow chimeras. *Blood* 101, no. 4: 1645-52. J

Lynch, D. H., A. Andreasen, E. Maraskovsky, J. Whitmore, R. E. Miller, and J. C. Schuh.
1997. Flt3 ligand induces tumor regression and antitumor immune responses in
vivo. *Nat Med* 3, no. 6: 625-31. J

Magne, N., R. A. Toillon, V. Bottero, C. Didelot, P. V. Houtte, J. P. Gerard, and J. F.

Peyron. 2006. Nf-kappab modulation and ionizing radiation: Mechanisms and
future directions for cancer treatment. *Cancer Lett* 231, no. 2: 158-68. J

Main, J. M. and R. T. Prehn. 1955. Successful skin homografts after the administration of
high dosage x radiation and homologous bone marrow. *J Natl Cancer Inst* 15, no.
4: 1023-9. J

Maraskovsky, E., K. Brasel, M. Teepe, E. R. Roux, S. D. Lyman, K. Shortman, and H. J.

McKenna. 1996. Dramatic increase in the numbers of functionally mature
dendritic cells in flt3 ligand-treated mice: Multiple dendritic cell subpopulations
identified. *J Exp Med* 184, no. 5: 1953-62. J

Maris, M. B., B. M. Sandmaier, B. E. Storer, T. Chauncey, M. J. Stuart, R. T. Maziarz, E.

Agura, A. A. Langston, M. Pulsipher, R. Storb, and D. G. Maloney. 2004.

Allogeneic hematopoietic cell transplantation after fludarabine and 2 gy total
body irradiation for relapsed and refractory mantle cell lymphoma. *Blood* 104, no.
12: 3535-42. J

Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol* 12:

991-1045. J

- _____. 2002. The danger model: A renewed sense of self. *Science* 296, no. 5566: 301-5. *X*
- Mayo, M. W., C. Y. Wang, P. C. Cogswell, K. S. Rogers-Graham, S. W. Lowe, C. J. Der, and A. S. Baldwin, Jr. 1997. Requirement of nf-kappab activation to suppress p53-independent apoptosis induced by oncogenic ras. *Science* 278, no. 5344: 1812-5. *J*
- McBride, W. H., C. S. Chiang, J. L. Olson, C. C. Wang, J. H. Hong, F. Pajonk, G. J. Dougherty, K. S. Iwamoto, M. Pervan, and Y. P. Liao. 2004. A sense of danger from radiation. *Radiat Res* 162, no. 1: 1-19. *J*
- McLellan, A. D., E. B. Brocker, and E. Kampgen. 2000. Dendritic cell activation by danger and antigen-specific t-cell signalling. *Exp Dermatol* 9, no. 5: 313-22. *J*
- Medzhitov, R. and C. A. Janeway, Jr. 1997. Innate immunity: Impact on the adaptive immune response. *Curr Opin Immunol* 9, no. 1: 4-9. *J*
- Medzhitov, R. and C. Janeway, Jr. 2000. Innate immune recognition: Mechanisms and pathways. *Immunol Rev* 173: 89-97. *J*
- Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh, and C. A. Janeway, Jr. 1998. Myd88 is an adaptor protein in the htoll/il-1 receptor family signaling pathways. *Mol Cell* 2, no. 2: 253-8. *J*
- Melkonyan, H. S., T. E. Ushakova, and S. R. Umansky. 1995. Hsp70 gene expression in mouse lung cells upon chronic gamma-irradiation. *Int J Radiat Biol* 68, no. 3: 277-80. *J*

- Meyn, R. E., L. C. Stephens, K. A. Mason, and D. Medina. 1996. Radiation-induced apoptosis in normal and pre-neoplastic mammary glands in vivo: Significance of gland differentiation and p53 status. *Int J Cancer* 65, no. 4: 466-72. *J*
- Mielcarek, M., P. J. Martin, W. Leisenring, M. E. Flowers, D. G. Maloney, B. M. Sandmaier, M. B. Maris, and R. Storb. 2003. Graft-versus-host disease after nonmyeloablative versus conventional hematopoietic stem cell transplantation. *Blood* 102, no. 2: 756-62. *J*
- Morel, A., N. Fernandez, A. de La Coste, H. Haddada, M. Viguier, B. S. Polla, B. Antoine, and A. Kahn. 1998. Gamma-ray irradiation induces b7.1 costimulatory molecule neoexpression in various murine tumor cells. *Cancer Immunol Immunother* 46, no. 5: 277-82. *J*
- Murphy, M., M. J. Mabruk, P. Lenane, A. Liew, P. McCann, A. Buckley, P. Billet, M. Leader, E. Kay, and G. M. Murphy. 2002. The expression of p53, p21, bax and induction of apoptosis in normal volunteers in response to different doses of ultraviolet radiation. *Br J Dermatol* 147, no. 1: 110-7. *J*
- Muul, L. M., C. Silvin, S. P. James, and F. Candotti. 2008. Measurement of proliferative responses of cultured lymphocytes. *Curr Protoc Immunol* Chapter 7: Unit 7 10 1-*J* 7 10 24.
- Nakano, K. and K. H. Vousden. 2001. Puma, a novel proapoptotic gene, is induced by p53. *Mol Cell* 7, no. 3: 683-94. *J*
- Nikitina, E. Y. and D. I. Gabrilovich. 2001. Combination of gamma-irradiation and dendritic cell administration induces a potent antitumor response in tumor-bearing

- mice: Approach to treatment of advanced stage cancer. *Int J Cancer* 94, no. 6: 825-33.
- Nishioka, A., Y. Ogawa, I. Kubonishi, S. Kataoka, N. Hamada, M. Terashima, T. Inomata, and S. Yoshida. 1999. An augmentation of fas (cd95/apo-1) antigen induced by radiation: Flow cytometry analysis of lymphoma and leukemia cell lines. *Int J Mol Med* 3, no. 3: 275-8. J
- Nordberg, J. and E. S. Arner. 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* 31, no. 11: 1287-312. J
- O'Neill, D. W., S. Adams, and N. Bhardwaj. 2004. Manipulating dendritic cell biology for the active immunotherapy of cancer. *Blood* 104, no. 8: 2235-46. J
- Ogawa, N. and H. Ohashi. 1997. [study of apoptosis in sjogren's syndrome]. *Rinsho Byori* 45, no. 7: 643-8. J
- Oh, Y. T., D. W. Chen, G. J. Dougherty, and W. H. McBride. 2004. Adenoviral interleukin-3 gene-radiation therapy for prostate cancer in mouse model. *Int J Radiat Oncol Biol Phys* 59, no. 2: 579-83. J
- Olive, P. L. and J. P. Banath. 2004. Phosphorylation of histone h2ax as a measure of radiosensitivity. *Int J Radiat Oncol Biol Phys* 58, no. 2: 331-5. J
- Olsson, I., U. Gullberg, I. Ivhed, and K. Nilsson. 1983. Induction of differentiation of the human histiocytic lymphoma cell line u-937 by 1 alpha,25-dihydroxycholecalciferol. *Cancer Res* 43, no. 12 Pt 1: 5862-7. J
- Oltvai, Z. N., C. L. Milliman, and S. J. Korsmeyer. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, bax, that accelerates programmed cell death. *Cell* 74, no. 4: 609-19. J

Pajonk, F., K. Pajonk, and W. H. McBride. 1999. Inhibition of nf-kappab, clonogenicity, and radiosensitivity of human cancer cells. *J Natl Cancer Inst* 91, no. 22: 1956-60. J

Palucka, K. A., N. Taquet, F. Sanchez-Chapuis, and J. C. Gluckman. 1998. Dendritic cells as the terminal stage of monocyte differentiation. *J Immunol* 160, no. 9: 4587-95. J

Passmore, J. S., P. T. Lukey, and S. R. Ress. 2001. The human macrophage cell line u937 as an in vitro model for selective evaluation of mycobacterial antigen-specific cytotoxic t-cell function. *Immunology* 102, no. 2: 146-56. J

Paulos, C. M., C. Wrzesinski, A. Kaiser, C. S. Hinrichs, M. Chieppa, L. Cassard, D. C. Palmer, A. Boni, P. Muranski, Z. Yu, L. Gattinoni, P. A. Antony, S. A. Rosenberg, and N. P. Restifo. 2007. Microbial translocation augments the function of adoptively transferred self/tumor-specific cd8+ t cells via tlr4 signaling. *J Clin Invest* 117, no. 8: 2197-204. J

Pillai, A. B., T. I. George, S. Dutt, P. Teo, and S. Strober. 2007. Host nkt cells can prevent graft-versus-host disease and permit graft antitumor activity after bone marrow transplantation. *J Immunol* 178, no. 10: 6242-51. J

Radford, I. R. 1999. Initiation of ionizing radiation-induced apoptosis: DNA damage-mediated or does ceramide have a role? *Int J Radiat Biol* 75, no. 5: 521-8. J

Rahman, I., A. Kode, and S. K. Biswas. 2006. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat Protoc* 1, no. 6: 3159-65. J

- Ravi, R., A. Bedi, and E. J. Fuchs. 1998. Cd95 (fas)-induced caspase-mediated proteolysis of nf-kappab. *Cancer Res* 58, no. 5: 882-6. J
- Ren, H., J. Shen, C. Tomiyama-Miyaji, M. Watanabe, E. Kainuma, M. Inoue, Y. Kuwano, and T. Abo. 2006. Augmentation of innate immunity by low-dose J irradiation. *Cell Immunol* 244, no. 1: 50-6.
- Rieser, C., G. Bock, H. Klocker, G. Bartsch, and M. Thurnher. 1997. Prostaglandin e2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: J synergistic activation of interleukin 12 production. *J Exp Med* 186, no. 9: 1603-8.
- Robles, A. I., N. A. Bemmels, A. B. Foraker, and C. C. Harris. 2001. Apaf-1 is a J transcriptional target of p53 in DNA damage-induced apoptosis. *Cancer Res* 61, no. 18: 6660-4.
- Rovere, P., C. Vallinoto, A. Bondanza, M. C. Crosti, M. Rescigno, P. Ricciardi-Castagnoli, C. Rugarli, and A. A. Manfredi. 1998. Bystander apoptosis triggers dendritic cell maturation and antigen-presenting function. *J Immunol* 161, no. 9: 4467-71. J
- Russell, J. S., U. Raju, G. J. Gumin, F. F. Lang, D. R. Wilson, T. Huet, and P. J. Tofilon. 2002. Inhibition of radiation-induced nuclear factor-kappab activation by an anti-ras single-chain antibody fragment: Lack of involvement in radiosensitization. *Cancer Res* 62, no. 8: 2318-26. J
- Ryan, K. M., M. K. Ernst, N. R. Rice, and K. H. Vousden. 2000. Role of nf-kappab in p53-mediated programmed cell death. *Nature* 404, no. 6780: 892-7. J

- Salio, M., N. Dulphy, J. Renneson, M. Herbert, A. McMichael, A. Marchant, and V. Cerundolo. 2003. Efficient priming of antigen-specific cytotoxic t lymphocytes by human cord blood dendritic cells. *Int Immunol* 15, no. 10: 1265-73. J
- Sancho, D., D. Mourao-Sa, O. P. Joffre, O. Schulz, N. C. Rogers, D. J. Pennington, J. R. Carlyle, and C. Reis e Sousa. 2008. Tumor therapy in mice via antigen targeting to a novel, dc-restricted c-type lectin. *J Clin Invest* 118, no. 6: 2098-110. J
- Scandling, J. D., S. Busque, S. Dejbakhsh-Jones, C. Benike, M. T. Millan, J. A. Shizuru, R. T. Hoppe, R. Lowsky, E. G. Engleman, and S. Strober. 2008. Tolerance and chimerism after renal and hematopoietic-cell transplantation. *N Engl J Med* 358, no. 4: 362-8. J
- Scheidereit, C. 2006. Ikappab kinase complexes: Gateways to nf-kappab activation and transcription. *Oncogene* 25, no. 51: 6685-705. J
- Schmitz, M. L., S. Bacher, and O. Dienz. 2003. Nf-kappab activation pathways induced by t cell costimulation. *FASEB J* 17, no. 15: 2187-93. J
- Seino, K., M. Harada, and M. Taniguchi. 2004. Nkt cells are relatively resistant to apoptosis. *Trends Immunol* 25, no. 5: 219-21. J
- Sheard, M. A. 2001. Ionizing radiation as a response-enhancing agent for cd95-mediated apoptosis. *Int J Cancer* 96, no. 4: 213-20.
- Sheard, M. A., B. Vojtesek, L. Janakova, J. Kovarik, and J. Zaloudik. 1997. Up-regulation of fas (cd95) in human p53wild-type cancer cells treated with ionizing radiation. *Int J Cancer* 73, no. 5: 757-62. J
- Shortman, K. and Y. J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat Rev Immunol* 2, no. 3: 151-61. J

- Siebenlist, U., G. Franzoso, and K. Brown. 1994. Structure, regulation and function of nf-kappa b. *Annu Rev Cell Biol* 10: 405-55. *J*
- Slavin, S., Z. Fuks, H. S. Kaplan, and S. Strober. 1978. Transplantation of allogeneic bone marrow without graft-versus-host disease using total lymphoid irradiation. *J Exp Med* 147, no. 4: 963-72. *J*
- Slavin, S., Z. Fuks, S. Strober, H. Kaplan, R. J. Howard, and D. E. Sutherland. 1979. Transplantation tolerance across major histocompatibility barriers after total lymphoid irradiation. *Transplantation* 28, no. 5: 359-61. *J*
- Sojka, D. K., M. Donepudi, J. A. Bluestone, and M. B. Mokyr. 2000. Melphalan and other anticancer modalities up-regulate b7-1 gene expression in tumor cells. *J Immunol* 164, no. 12: 6230-6. *J*
- Somersan, S., M. Larsson, J. F. Fonteneau, S. Basu, P. Srivastava, and N. Bhardwaj. 2001. Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells. *J Immunol* 167, no. 9: 4844-52. *J*
- Stammler, G. and M. Volm. 1996. Expression of heat shock proteins, glutathione peroxidase and catalase in childhood acute lymphoblastic leukemia and nephroblastoma. *Cancer Lett* 99, no. 1: 35-42. *J*
- Steinauer, K. K., I. Gibbs, S. Ning, J. N. French, J. Armstrong, and S. J. Knox. 2000. Radiation induces upregulation of cyclooxygenase-2 (cox-2) protein in pc-3 cells. *Int J Radiat Oncol Biol Phys* 48, no. 2: 325-8. *J*
- Strickland, I. and S. Ghosh. 2006. Use of cell permeable nbd peptides for suppression of inflammation. *Ann Rheum Dis* 65 Suppl 3: iii75-82. *J*

Strober, S. 1987. Total lymphoid irradiation in alloimmunity and autoimmunity. *J*

Pediatr 111, no. 6 Pt 2: 1051-5. *J*

Strober, S., S. Slavin, Z. Fuks, H. S. Kaplan, M. Gottlieb, C. Bieber, R. T. Hoppe, and F.

C. Grumet. 1979. Transplantation tolerance after total lymphoid irradiation.

Transplant Proc 11, no. 1: 1032-8. *J*

Strober, S., S. Slavin, M. Gottlieb, I. Zan-Bar, D. P. King, R. T. Hoppe, Z. Fuks, F. C.

Grumet, and H. S. Kaplan. 1979. Allograft tolerance after total lymphoid

irradiation (tli). *Immunol Rev* 46: 87-112. *J*

Sundstrom, C. and K. Nilsson. 1976. Establishment and characterization of a human

histiocytic lymphoma cell line (u-937). *Int J Cancer* 17, no. 5: 565-77. *J*

Tamada, K., M. Harada, K. Abe, T. Li, and K. Nomoto. 1998. IL-4-producing NK1.1+ t

cells are resistant to glucocorticoid-induced apoptosis: Implications for the Th1/Th2

balance. *J Immunol* 161, no. 3: 1239-47. *J*

Teitz-Tennenbaum, S., Q. Li, R. Okuyama, M. A. Davis, R. Sun, J. Whitfield, R. N.

Knibbs, L. M. Stoolman, and A. E. Chang. 2008. Mechanisms involved in

radiation enhancement of intratumoral dendritic cell therapy. *J Immunother* 31,

no. 4: 345-58. *J*

Teitz-Tennenbaum, S., Q. Li, S. Rynkiewicz, F. Ito, M. A. Davis, C. J. McGinn, and A.

E. Chang. 2003. Radiotherapy potentiates the therapeutic efficacy of intratumoral *J*

dendritic cell administration. *Cancer Res* 63, no. 23: 8466-75. *J*

Tepper, A. D., E. de Vries, W. J. van Blitterswijk, and J. Borst. 1999. Ordering of

ceramide formation, caspase activation, and mitochondrial changes during CD95-

and DNA damage-induced apoptosis. *J Clin Invest* 103, no. 7: 971-8. *J*

- Thery, C. and S. Amigorena. 2001. The cell biology of antigen presentation in dendritic cells. *Curr Opin Immunol* 13, no. 1: 45-51. J
- Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 3, no. 2: 133-46. J
- Trinchieri, G. and A. Sher. 2007. Cooperation of toll-like receptor signals in innate immune defence. *Nat Rev Immunol* 7, no. 3: 179-90. J
- Unanue, E. R. 1980. Cooperation between mononuclear phagocytes and lymphocytes in immunity. *N Engl J Med* 303, no. 17: 977-85. J
- Van Antwerp, D. J., S. J. Martin, T. Kafri, D. R. Green, and I. M. Verma. 1996. Suppression of tnf-alpha-induced apoptosis by nf-kappab. *Science* 274, no. 5288: 787-9. J
- Veis, D. J., C. M. Sorenson, J. R. Shutter, and S. J. Korsmeyer. 1993. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* 75, no. 2: 229-40. J
- Vereecke, R., G. Buffenoir, R. Gonzalez, N. Cambier, D. Hetuin, F. Bauters, P. Fenaux, and B. Quesnel. 2000. Gamma-ray irradiation induces b7.1 expression in myeloid leukaemic cells. *Br J Haematol* 108, no. 4: 825-31. J
- Verhasselt, V., W. Vanden Berghe, N. Vanderheyde, F. Willems, G. Haegeman, and M. Goldman. 1999. N-acetyl-l-cysteine inhibits primary human t cell responses at the dendritic cell level: Association with nf-kappab inhibition. *J Immunol* 162, no. 5: 2569-74. J
- Verheij, M. and H. Bartelink. 2000. Radiation-induced apoptosis. *Cell Tissue Res* 301, no. 1: 133-42. J

- Verheij, M., G. A. Ruiter, S. F. Zerp, W. J. van Blitterswijk, Z. Fuks, A. Haimovitz-Friedman, and H. Bartelink. 1998. The role of the stress-activated protein kinase (sapk/jnk) signaling pathway in radiation-induced apoptosis. *Radiother Oncol* 47, no. 3: 225-32. *J*
- Wadgaonkar, R., K. M. Phelps, Z. Haque, A. J. Williams, E. S. Silverman, and T. Collins. 1999. Creb-binding protein is a nuclear integrator of nuclear factor-kappab and p53 signaling. *J Biol Chem* 274, no. 4: 1879-82. *J*
- Waer, M., V. Palathumpat, H. Sobis, and M. Vandeputte. 1990. Induction of transplantation tolerance in mice across major histocompatibility barrier by using allogeneic thymus transplantation and total lymphoid irradiation. *J Immunol* 145, no. 2: 499-504. *J*
- Walker, P. R., L. Kokileva, J. LeBlanc, and M. Sikorska. 1993. Detection of the initial stages of DNA fragmentation in apoptosis. *Biotechniques* 15, no. 6: 1032-40. *J*
- Wang, C. Y., M. W. Mayo, and A. S. Baldwin, Jr. 1996. Tnf- and cancer therapy-induced apoptosis: Potentiation by inhibition of nf-kappab. *Science* 274, no. 5288: 784-7. *J*
- Wang, H. J., Z. Y. Wu, P. Fan, and J. M. Bian. 2005. [the nuclear factor kappa b activation: The key step of cell proliferation in estrogen receptor-negative breast cancer cells]. *Zhonghua Wai Ke Za Zhi* 43, no. 15: 1014-6. *J*
- Wang, T., Y. C. Hu, S. Dong, M. Fan, D. Tamae, M. Ozeki, Q. Gao, D. Gius, and J. J. Li. 2005. Co-activation of erk, nf-kappab, and gadd45beta in response to ionizing radiation. *J Biol Chem* 280, no. 13: 12593-601. *J*
- Wang, Y., A. Meng, H. Lang, S. A. Brown, J. L. Konopa, M. S. Kindy, R. A. Schmiedt, J. S. Thompson, and D. Zhou. 2004. Activation of nuclear factor kappab in vivo *J*

- selectively protects the murine small intestine against ionizing radiation-induced damage. *Cancer Res* 64, no. 17: 6240-6.
- Watters, D. 1999. Molecular mechanisms of ionizing radiation-induced apoptosis. *Immunol Cell Biol* 77, no. 3: 263-71. *J*
- Watts, C. and S. Amigorena. 2000. Antigen traffic pathways in dendritic cells. *Traffic* 1, no. 4: 312-7. *J*
- Williams, G. T. 1991. Programmed cell death: Apoptosis and oncogenesis. *Cell* 65, no. 7: 1097-8. *J*
- Xu, Y. 2006. DNA damage: A trigger of innate immunity but a requirement for adaptive immune homeostasis. *Nat Rev Immunol* 6, no. 4: 261-70. *J*
- Yang, C. R., C. Wilson-Van Patten, S. M. Planchon, S. M. Wuerzberger-Davis, T. W. Davis, S. Cuthill, S. Miyamoto, and D. A. Boothman. 2000. Coordinate modulation of sp1, nf-kappa b, and p53 in confluent human malignant melanoma cells after ionizing radiation. *FASEB J* 14, no. 2: 379-90.
- Yang, J., X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. I. Peng, D. P. Jones, and X. Wang. 1997. Prevention of apoptosis by bcl-2: Release of cytochrome c from mitochondria blocked. *Science* 275, no. 5303: 1129-32. *J*
- Yao, Z., Y. Liu, J. Jones, and S. Strober. 2009. Differences in bcl-2 expression by t-cell subsets alter their balance after in vivo irradiation to favor cd4+bcl-2hi nkt cells. *Eur J Immunol* 39, no. 3: 763-75. *J*
- Zanke, B. W., K. Boudreau, E. Rubie, E. Winnett, L. A. Tibbles, L. Zon, J. Kyriakis, F. F. Liu, and J. R. Woodgett. 1996. The stress-activated protein kinase pathway *J*

- mediates cell death following injury induced by cis-platinum, uv irradiation or heat. *Curr Biol* 6, no. 5: 606-13.
- Zhang, G. and S. Ghosh. 2000. Molecular mechanisms of nf-kappab activation induced by bacterial lipopolysaccharide through toll-like receptors. *J Endotoxin Res* 6, no. 6: 453-7. J
- Zhou, B. B. and S. J. Elledge. 2000. The DNA damage response: Putting checkpoints in perspective. *Nature* 408, no. 6811: 433-9. J
- Zong, Z. W., T. M. Cheng, Y. P. Su, X. Z. Ran, N. Li, G. P. Ai, and H. Xu. 2006. Crucial role of sdf-1/cxcr4 interaction in the recruitment of transplanted dermal multipotent cells to sublethally irradiated bone marrow. *J Radiat Res (Tokyo)* 47, no. 3-4: 287-93. J