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EXECUTIVE SUMMARY

The immune system provides the first line of defense for exposures to environmental hazards. Protective immunity mechanisms using innate or adaptive responses are employed to mitigate acute challenges or amplify the readiness of the system to respond to future challenges. Some stimuli lead to amplified inflammatory reactions such as delayed hypersensitivity which is required for immunity to parasites and can also lead to adverse consequences such as contact dermatitis. Radiation exposure has the potential to aggravate hypersensitivity reactions as well as to suppress protective immunity. Ionizing radiation at high doses has long been recognized as highly effective in destroying cells of the immune system, particularly lymphocytes. Increased risk of infection follows radiation exposure and potentially reduces immune surveillance of tumor cells. However, in the past decade attention has been turned to the effects of radiation exposure below 1 Gy in the context of risk assessment and evidence has accumulated that doses in the 2 to 50 cGy range may actually be protective rather than deleterious.

In order to examine the effects of low dose ionizing radiation on the immune system we chose to examine an amplified adaptive cellular immunity response. This response is Type IV delayed-type hypersensitivity also called contact hypersensitivity. The agent fluorescein isothiocyanate (FITC) is a low molecular weight, lipophilic, reactive, fluorescent molecule that can be applied to the skin where it (hapten) reacts with proteins (carriers) to become a complete antigen. Exposure to FITC leads to sensitization which is easily measured as a hypersensitivity inflammatory reaction following a subsequent exposure to the ear. Ear swelling, eosinophil infiltration, immunoglobulin E production and cytokine secretion patterns characteristic of a "Th2 polarized" immune response are the components of the reaction. The reaction requires successful implementation of antigen processing and presentation by antigen presenting Langerhans cells, communication with naïve T lymphocytes in draining lymph nodes, expansion of activated T cell clones, migration of activated T cells to the circulation, and recruitment of memory T cells, macrophages and eosinophils to the site of the secondary challenge. Using this model our approach was to quantify system function rather than relying only on indirect biomarkers of cell. We measured the FITC-induced hypersensitivity reaction over a range of doses from 2 cGy to 2 Gy. Irradiations were performed during key events or prior to key events to deplete critical cell populations. In addition to quantifying the final inflammatory response, we assessed cell populations in peripheral blood and spleen, cytokine signatures, IgE levels and expression of genes associated with key processes in sensitization and elicitation/recall. We hypothesized that ionizing radiation would produce a biphasic effect on immune system function resulting in an enhancement at low doses and a depression at higher doses and suggested that this transition would occur in the dose range of 5 to 50 cGy.

We determined that radiation had its greatest impact when administered during periods of critical cell-cell interactions at about 4 days after initial sensitization with hapten and 8 hours after a second exposure (elicitation). When radiation was used to deplete cells to minimum values (4 days post irradiation) during critical periods the effects were smaller. The minimum effective dose for reduction of the overall ear swelling inflammatory response was approximately 25 cGy. Accompanying reductions in the overall responses were reductions in white blood cells and platelets in peripheral blood and spleen. These reductions were most evident at 50 cGy and above but reductions were observed for some conditions at as low as 5 cGy. Some irradiation conditions resulted in increases in blood platelet and eosinophil counts which may be attributable to trafficking of these blood components from other tissue compartments. Immunoglobulin E levels, which are characteristic of contact hypersensitivity to FITC, were highly elevated after FITC treatment but not dramatically affected by irradiation except at 4 days after sensitization when T-cell:B-cell interactions are most robust. Histological analysis of ears showed a high level of cellular infiltrate and measurements of eosinophil peroxidase in ear lysates indicates the presence of eosinophils as reported in the literature. Cytokine profiles in unirradiated animals were consistent with a T_{H2}-polarized immune reaction and irradiation had its greatest differential effect on expression of three molecules (eotaxin/CCL11, IP-10 and MIG/CXCL-9) associated with chemoattraction of monocytes and eosinophils and three molecules (IL-1β, IL-6 and IFN-γ) associated with inflammation and T_{H2}-polarization. Gene expression analysis showed that patterns modulated by irradiation were

consistent with the sensitization reactions expected early after hapten administration with emphasis on attraction of leukocytes to the site of application, establishing conditions for interaction of dendritic cells and T cells, activation of T cells along the T_{H2} pathway and the inflammation events required to facilitate cell chemotaxis and migration to the reaction site. There were modest trends towards more complete stages of sensitization reaction after hapten treatment and vehicle treatment alone trended towards earlier events consistent with an adjuvant role for dibutyl-phthalate. There were stronger trends towards cellular infiltration processes including involvement of adhesion to endothelial cells but weaker MHCII-related antigen presentation activity. Inflammatory cytokine activity showed mixed up and down-regulation. T cell activation events in lymph node were generally resistant to radiation and in untreated animals there was a strong downregulation response (34 of 89 genes) of enzymes involved in managing oxidative stress. Patterns were consistent with a very strong ear inflammatory response which was further stimulated by irradiation at about the same time as the elicitation step.

Taken together these data show that low levels of radiation (≈25 cGy) can inhibit adaptive immunity as measured in the mouse ear swelling test (≈ 30% reduction) especially in the time periods of about 4 days after initial exposure to a lipophilic, reactive hapten or about 8 hours after secondary exposure. The minimum effect was associated with about a 10-15% reduction in white blood cells. At doses between 1 and 2 Gy the levels of inhibition can reach 70% with up to 65% reduction in some white blood cells. Gene and cytokine expression analysis point to chemoattraction of monocytes and eosinophils to reaction sites as sensitive targets as well as antigen presentation events. From a regulatory perspective this suggests that in allergen-sensitive individuals, special care should be taken to avoid chemical exposure during operations when body extremities may be exposed to low dose radiation.

INTRODUCTION

It has long been appreciated that cells of the immune system are radiosensitive and use apoptosis as the primary mechanism of cell death following injury. The hypervariability of the immunoglobulin superfamily of genes expressed in lymphoid cells also led to the appreciation of the nonhomologous end joining mechanism of DNA repair. Clinically, whole body irradiation is used in treatment of some lymphomas and as an immunosuppressive agent for bone marrow transplants. Inflammation at sites of radiotherapy is a common side effect. Many studies with radiation have addressed the changes in cell populations following radiation exposure and have shown a reproducible pattern of relative sensitivities amongst different lymphocyte classes and *in vivo* culture of cells derived from irradiated animals have addressed functional capacity in terms of spontaneous and stimulated mitogenesis and their specific profiles of cytokine production. However, many fewer studies have examined the coordinated responses of an irradiated immune system in terms of responses to challenge by a foreign antigen or organism. This is especially true in the dose regime below 1 Gray. We are trying to understand the functional capacity of the murine immune system in terms of adaptive cellular immunity following low dose whole body exposures to gamma rays using chemical contact hypersensitivity as an adaptive immunity model. In the context of low level radioactive waste handling and contamination remediation, exposure to chemical antigens may interact with low level radiation exposure to determine health risks.

The immune system provides the first line of defense for exposures to environmental hazards. Protective immunity mechanisms using innate or adaptive responses are employed to mitigate acute challenges or amplify the readiness of the system to respond to future challenges. Some stimuli lead to amplified inflammatory reactions such as delayed hypersensitivity which is required for immunity to parasites and can also lead to adverse consequences such as contact dermatitis. Radiation exposure has the potential to aggravate hypersensitivity reactions as well as to suppress protective immunity. Ionizing radiation at high doses has long been recognized as highly effective in destroying cells of the immune system, particularly lymphocytes. Increased risk of infection follows radiation exposure and potentially reduces immune surveillance of tumor cells. However, in the past decade attention has been turned to the effects of radiation exposure below 1 Gy in the context of risk assessment and evidence has accumulated that doses in the 1 to 50 cGy range may actually be protective rather than deleterious.

Selected low dose immune system responses reported in the literature are summarized below for 4 arbitrary dose ranges.

≥1 Gy: Reduction of cell numbers to minimum at 4 days with recovery by 2 weeks. Sensitivity pattern: B> T > Th (CD4+) > Tc (CD8+) > NK. CD8:CD4 ratio 2x ↑. Spleen TNF-α ↑. Increases in mitogen-stimulated cell proliferation.

>0.2 Gy: Hemolytic plaque assays with sheep red blood cells (SRBC) – B-cell mediated but CD4 modulated. D_o's 0.76 – 1.4 Gy if sensitization before irradiation but D_o's > 20 Gy if sensitization ≥ 4 days after. Hemolytic plaques: 25 cGy ↑ (peak), 75 cGy N/C, 1.25 Gy 0.5x ↓, > 1.75 Gy <0.10x ↓

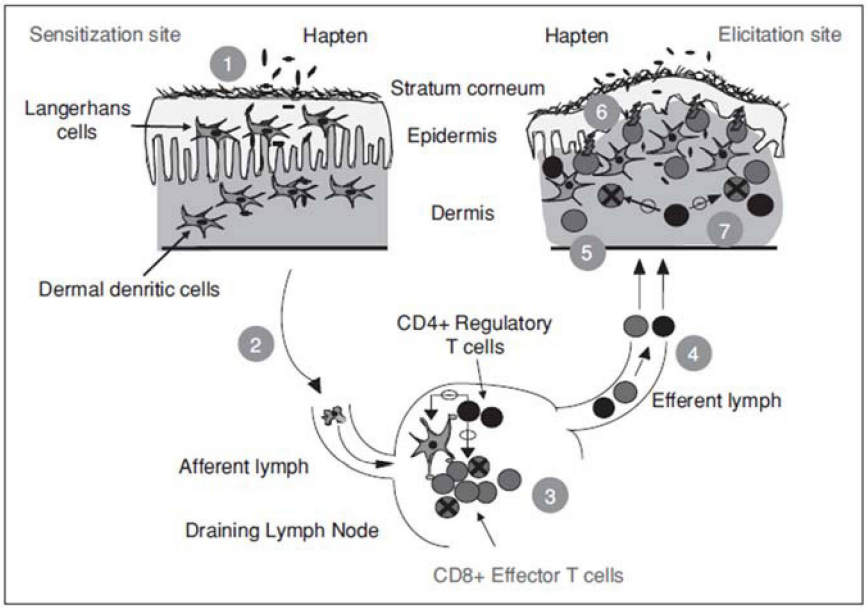
~ 0.2 Gy: IFN-γ ↑ TNF- α ↑ TGF-β ↓ IL-4, IL-6, IL-10 N/C (Ts suppression and T cell proliferation). Glutathione ↑ in splenocytes, NK mediated cytotoxicity 1.4x ↑, SRBC lysis 2x ↑. CHS to DNFB (Th1 response) ear swelling at 3d 0.74x ↓ but Con-A stimulated splenocyte proliferation 1.4 x ↑

Low Doses ≤ 0.25 Gy: Mitogen stimulated splenocyte proliferation: 5cGy ↑, 7.5 cGy ↑ augmented by corticosteroids, > 25 cGy N/C. Hemolytic plaques: 2.5cGy N/C, 7.5 cGy 1.74x ↑, 10 cGy 0.61x ↓ persistent for 2 mo but lost by 4 mo. Reactivity to IL-1β ↑ at 2.5 to 25cGy. T cell proliferation in co-culture with irradiated macrophages (microenvironment effect) 2 – 4 cGy ↓. 2 week ingestion of radon to 0.012 cGy resulted in decreased IgE levels in response to picryl chloride DTH if irradiation is before sensitization but not after. Radioadaptation with 1-2 cGy priming dose before 1.5 – 4 Gy challenge dose after 24 hrs: chromosome breaks, HPRT mutation, apoptosis and tumor induction effects inhibited.

Guided by these published observations we hypothesized that ionizing radiation would produce a biphasic effect on immune system function resulting in an enhancement at low doses and a depression at higher doses. We further hypothesized that this transition would occur in the dose range of 10 to 50 cGy.

The immune system responds to environmental challenges according to two broad mechanisms: innate and adaptive immunity. Innate immunity along with coagulation responses are designed to respond quickly to injuries and potential infection by generic pathogens. Adaptive immunity incorporates innate responses but allows the immune system to generate new specificity and amplify the sensitivity of reactions to foreign substances and organisms. It is the basis for vaccination.

Contact hypersensitivity (formally, Type IV delayed-type hypersensitivity) is an adaptive inflammatory response of the skin following repeated exposure to a chemical agent. Its underlying mechanisms are related to those of normal responses to parasite infection, and inappropriately regulated responses leading to asthma. Contact hypersensitivity (CHS) can be induced by painting the abdominal skin of C57BL/6 mice with fluorescein isothiocyanate (FITC) in a suitable solvent system. The low molecular weight, lipophilic FITC reacts as a hapten with proteins (carriers) of the skin to create complete antigens. These FITC-protein conjugates are processed by antigen presenting cells which migrate to regional lymph nodes and "present" antigens to naïve T lymphocytes. FITC-peptide specific T cells are then activated and differentiate into several T cell subclasses including T memory cells that redistribute to distal skin and lymphoid sites. This is the sensitization (S) phase of the CHS response. After several days or weeks, FITC is applied to the ear of a sensitized mouse where, after 24 to 48 hours, a local inflammatory reaction takes place causing the ear the swell due to infiltration of immune cells (including eosinophils, a unique FITC-induced response). This is the elicitation (E) phase of the CHS response. The magnitude of the ear swelling can be quantified as a measure of the strength of the inflammatory response. A specific pattern of cytokine expression (T_{H2}) along with production of immunoglobulin E serve as biomarkers. The diagram below pictorially illustrates the contact hypersensitivity process that we chose to use to evaluate adaptive immune function.



Pathophysiology of CHS. Sensitization phase: Haptens penetrate the stratum corneum. Hapten loading by skin dendritic cells (step 1) parallels activation and migration of DC through the afferent lymphatic vessels to the draining lymph nodes (step 2). Migrating DC are located in the para-cortical area of the draining LN where they can present haptenated peptides on MHC class I and II molecules to CD8+ and

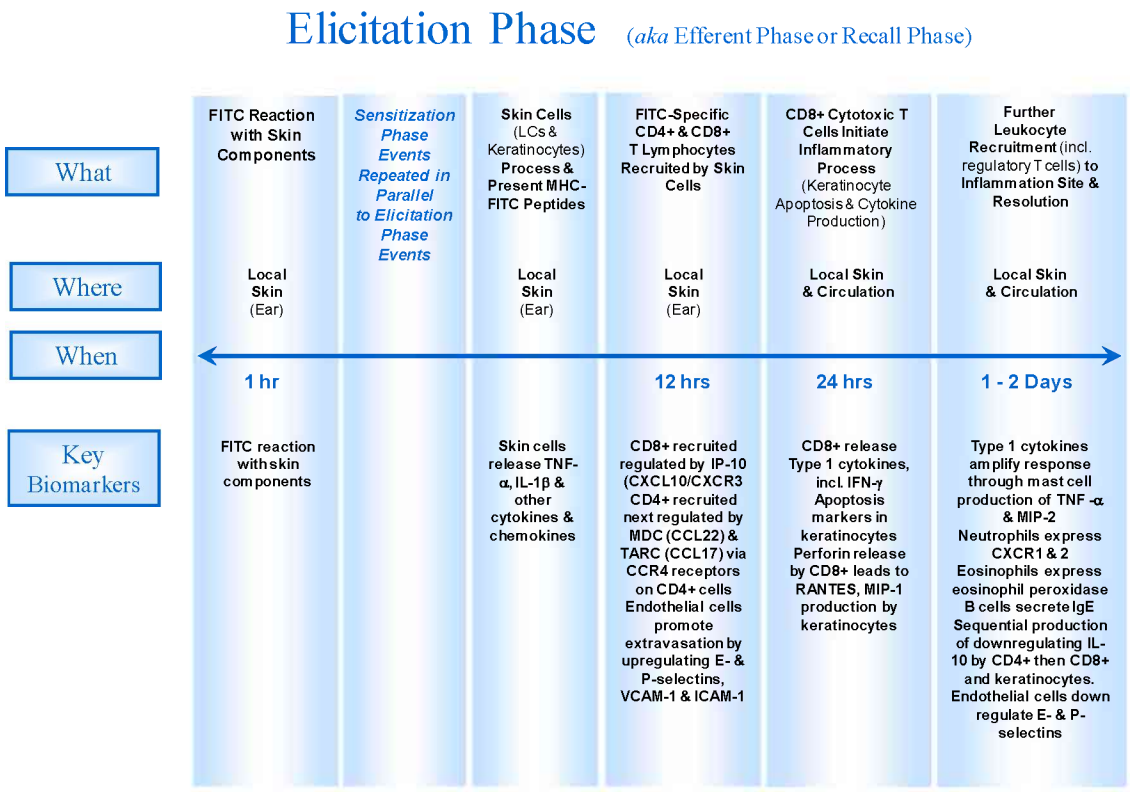
CD4+ T cells, respectively (step 3). Specific T cells precursors expand clonally in the draining LN and diffuse to the bloodstream through the efferent lymphatic vessels and the thoracic duct (step 4). During this process they acquire skin-specific homing antigens (CLA and CCR4) and become memory T cells. Primed T cells preferentially diffuse in the skin after transendothelial migration. At the end of the sensitization step everything is ready for the development of a CHS reaction upon challenge with the relevant hapten. Elicitation phase: When the hapten is painted for a second (and subsequent) time, it diffuses through the epidermis and could be loaded by LC or other skin cells expressing MHC molecules, such as keratinocytes and dermal dendritic cells, which are then able to activate trafficking specific T cells (step 5). CD8+ cytotoxic T cell activation initiates the inflammatory process through keratinocyte apoptosis and cytokine/chemokine production (step 6). This is responsible for the recruitment of leukocytes (including regulatory T cells) from the blood to the skin leading to the development of skin lesions (step 7). From figure 1 of P. Saint-Mezard et al. Eur J Dermatol 2004; 14: 284-95.

A breakdown of events in the sensitization and elicitation phases which includes timing and biomarkers of events is shown in the two diagrams below.

Sensitization Phase (*aka* Induction or Afferent Phase)

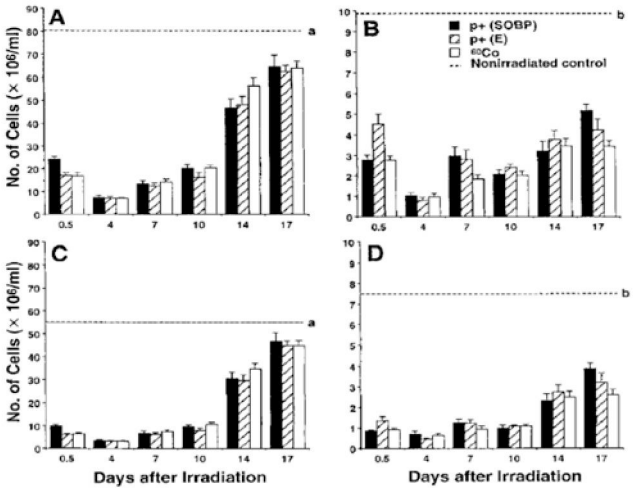
What	FITC reaction with skin components	LCs detect & ingest FITC conjugates Keratinocytes activated	LC maturation & migration to draining lymph node. LCs process & present MHC-II + FITC peptides	LCs activate naïve CD4+ & CD8+ T lymphocytes	T cell clonal expansion & differentiation	T cell migration to periphery Immune memory established
Where	Local skin (Back or abdomen)	Local skin	Skin, afferent lymphatic vessels & lymph node	Lymph node	Lymph node & efferent lymphatic vessels	Peripheral circulation & skin
When	Rapid	15 min	Peaks at 24 hrs	5 - 7 Days / Ongoing processes 4 - 7 days		Stable for months
Key Biomarkers	FITC reaction with skin components	LCs Release IL-1β Keratinocytes release TNF-α & GM-CSF LC & keratin-ocyes tightly bind via E cadherins	TNF- α & IL-1β → Cadherins E ↓ facilitating LC migration & LC CCRs ↓ LCs secrete MMP 3,9 CCR7 ↑ → LC binding to CCL21 TNF- α → CCL21 ↑ on lymphatic vessels MHC II & co-stimulatory molecules ↑	T cell Receptor + LC MHC II - peptide complex interaction (Recognized by CD4+ T Cells) LC → CD80, & CD 86 ↑ & ligate CD28 on T cells IL-2 secretion NF-kβ & AP-1 ↑ Cytokine production "polarized" to Th2 pattern: IL-4, IL-5, IL-10, IL-13 ↑	T cell division Changes in chemokine & addressin receptors to emigrate from node. T cells entering in skin express CCR4, α4β1 integrin & CLA which interact with endothelial cell ligands: E-, P-selectin, CCL17	T cell CCR7 - subset can't migrate to nodes: *peripheral memory cells.* CCR7 + subset migrates to nodes but not skin: *central memory cells.*

Abbreviations:
FITC = Fluorescein Isothiocyanate
LC = Langerhans Cells



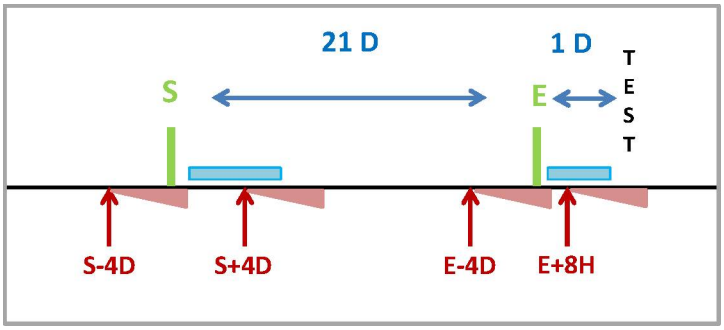
APPROACH

Use chose to use contact hypersensitivity (CHS) to the hapten FITC to generate an adaptive immune response characterized by a rapid and robust inflammatory reaction in the mouse ear swelling test. Then we scheduled irradiation and antigen exposures to match key events (depicted above) in development of adaptive immunity and probe effects of (1) maximum cell depletion and (2) maximum cell-cell interaction activity in lymph nodes and skin during sensitization and elicitation phases. We found previously that maximum cell depletion after irradiation is at 4 days post irradiation with gradual recovery over 14 days. This is illustrated below



The panel illustrates these responses for cells in the spleen and peripheral blood following gamma ray and proton exposures. The figure is from "Effects of Whole-Body Proton Irradiation on the Immune System" by Kajioka, Andres, Mao, Moyers, Nelson, Slater & Gridley, Radiation Research 153: 587 (2000). The panels are as follows: Number of leukocytes in the spleen (panel A) and blood (panel B), and number of lymphocytes in the spleen (panel C) and blood (panel D) with time after whole-body irradiation. The dotted horizontal lines represent the means for nonirradiated controls.

Guided by the previously-reported dose-time response we irradiated 4 days before sensitization (S-4D) or elicitation (E-4D) to probe cell number effects. Dendritic cell (and keratinocyte) activation and migration followed by interactions with naïve T cells (e.g. antigen presentation) in the lymph nodes and the beginning of T cell clonal expansion occur at 4 days after sensitization. Maximum cell chemotaxis, vascular adhesion changes and other innate immunity changes initiating inflammation following elicitation are maximum at 6 – 12 hours after elicitation. Therefore, to probe effects on critical periods of cell-cell interactions used S+4D and E+8H regimens. To assess the magnitude and features of the adaptive immune response methods were employed to: quantify ear swelling, characterize cell populations in blood and spleen, characterize the inflammatory response and biomarkers after 0, 2, 5, 10, 20, 50, 100, 200 cGy ^{60}Co-γ in the S-4D, S+4D, E-4D and E+8H schedules. This is summarized below.



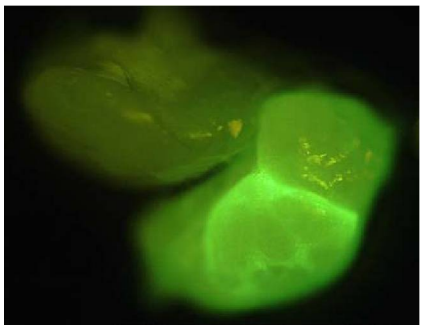
Protocol timelines. Sensitization (S) and Elicitation (E) phase hapten exposures (green) and four irradiation schemes (red) at $T_{irradiate}$ +/-4 days or +8 hours showing periods of maximum peripheral blood cell depletion (pink wedges) and periods of critical cell interaction (blue bars).

RESULTS

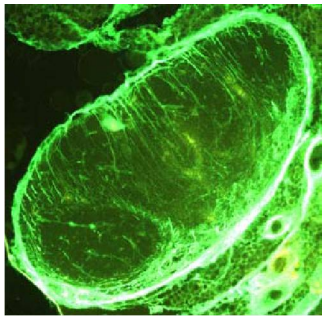
Protocol Development

Our initial efforts sought to establish the best concentrations and administration techniques for the immune sensitizer FITC (fluorescein isothiocyanate). FITC reacts with proteins in the skin to stimulate antigen presenting cells (Langerhans cells and dendritic cells) to migrate to draining lymph nodes where they interact (antigen presentation) with naïve T lymphocytes to generate populations of activated lymphocytes specific for FITC-peptide conjugates. FITC also reacts with small molecules containing –SH and –$NH3^+$ groups and these small conjugates are drained by the lymphatic structures. However, T cells only respond to FITC-peptide conjugates that are "presented" by antigen presenting cells (APC) in the context of major histocompatibility (MHC) antigens. The figure below illustrates the distribution of FITC conjugates in lymph nodes that was primarily associated with lymphatic vessels and excluded from follicles.

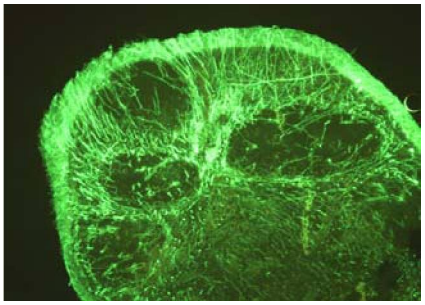
Histological experiments investigated the events occurring in and around the draining lymph nodes where antigen presenting cells of the skin migrate to interact with T cells. Lymph nodes were observed to be highly labeled with fluorescein conjugates with nodes closest to the site of FITC application showing the greatest labeling. The label appears to be correlated to lymphatic and regular vessels as well as structural elements of the tissue. The fluorescein conjugate labeling extends even to the more distant spleen.



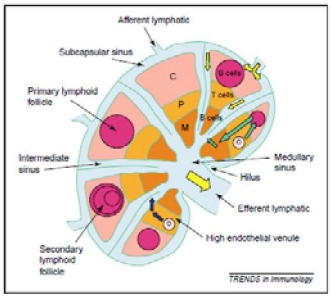
Nodes adjacent to untreated and treated ears



Labeling of vessels and cortex.

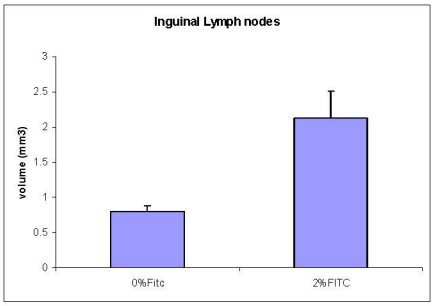


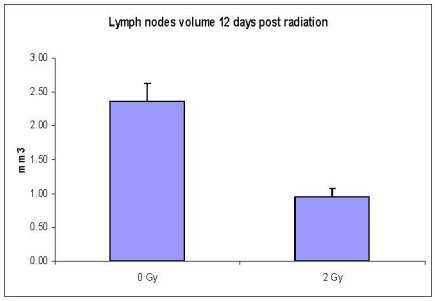
Labeling of vessels and cortex but exclusion from follicles.



The general structure of a lymph node. C, P and M indicate the cortical, paracortical and medullary regions, respectively. E. Crivellato et al. TRENDS Immun. 25 (2004)

We found that lymph node sizes are: 1) significantly increased after immune stimulation, 2) reduced moderately in volume by the radiation exposure at 21 days, but 3) are significantly reduced 12 days following radiation exposure. This indicates that infiltration is occurring as expected due to the immune stimulation but that cell populations have recovered well by 21 days after the radiation exposure as is also observed for circulating leukocytes and the spleen. Below left are results for 2% FITC (no irradiation) applied once only on the back and evaluated at 1 month – volumes were calculated from length, width, height measurements from photographs. Below right are results from untreated ICR mice evaluated 12 days after whole body irradiation with 2 Gray showing transient cell-depletion related shrinkage.



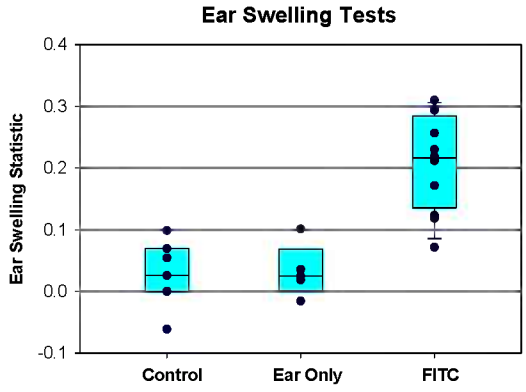


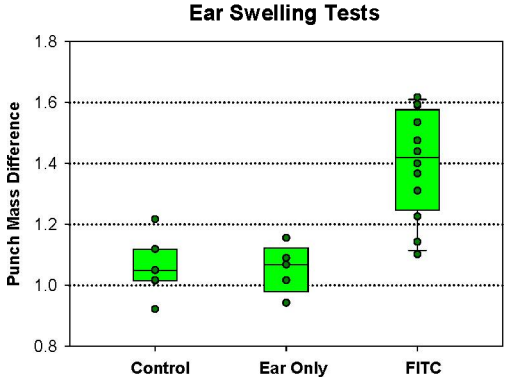
Swelling elicited by hapten.

Shrinkage and cell loss after irradiation.

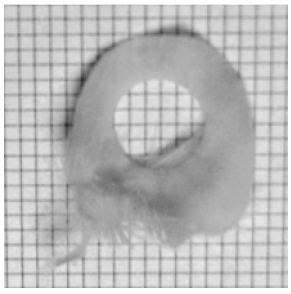
Initially we chose hapten administration on back skin to avoid irritation from bedding on shaved skin. We began with 0.5% FITC in the solvent system 50:50 acetone:dibutyl phthalate (DBP) applied once to shaved back skin (sensitization) followed by administration of the same solution to the right ear at the time of interest (elicitation) and measurements of the CHS reaction 24 hours later. This is a standard method used for contact hypersensitivity testing after 4 – 7 days. Since our goal was to measure longer term adaptive immunity we conducted our ear swelling tests at 1 month post sensitization. While some ear swelling was observed the response did not show adequate statistical significance. Tests with lower concentrations also failed to show adequate statistical significance. A reassessment of the literature suggested two improvements. First, administration of FITC to abdominal or thoracic skin gave stronger responses under some circumstances and many investigators have chosen to sensitize with multiple applications. Second, a recent publication determined that the DBP solvent component actually acts as an immune adjuvant in stimulating antigen presentation. However, the optimum concentrations for the adjuvant effects were closer to 10% rather than 50%. We therefore ultimately settled on two daily sensitizations to belly skin using 2% FITC in 90:10 acetone:DBP and measured responses at 21 and 35 days. This procedure yields reproducible, robust hypersensitivity reactions

The two figures below illustrate the very good response obtained at 21 days using either a micrometer-base thickness measure or the tissue punch mass as indices of the reaction. In this experiment 2% FITC was applied twice to belly skin (sensitization) and once to the back of the right ear (elicitation). "Control" is solvent only (90:10 acetone:DBP) and "ear only" indicates application to the back of the right ear without prior sensitization. The ear swelling statistic (left panel) used relative thickness changes normalized to ear size [(right ear final - right ear pre-elicitation)/right ear pre-elicitation - (left ear final - left ear pre-elicitation)/left ear pre-elicitation or $(R_f - R_o)/R_o - (L_f - L_o)/L_o)]$ while the punch mass difference was simply right ear (treated) punch mass – left ear (control) punch mass ($R_m - L_m$). Both measures are now used. A Mitotoyu non-rotating thickness gauge with a 1.4 Newton force was tested and modified with a 6 mm tip to give reliable thickness measurements in the "ear swelling" test. Typical baseline thicknesses were of the order 210 microns.

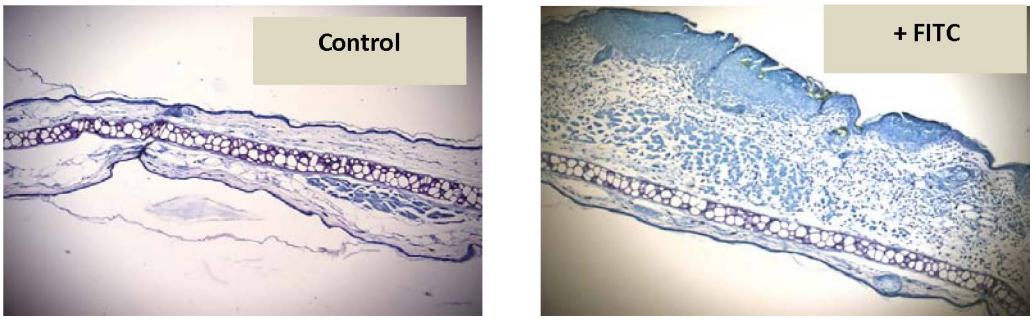




The adjacent figure illustrates an ear from an ICR mouse from which a tissue punch has been used to isolate a 6 mm diameter sample. Several punch sizes were tested before settling on a 6 mm diameter which allows the most reproducible portion of the pinna (external ear) to be sampled. Whereas in this example the punch was centered on the ear, the optimum position is approximately 1 mm from the outer edge to avoid excess cartilage and folds. The grid size is 1 mm.



The ear swelling reaction was associated with a cellular infiltrate that included eosinophils and was quite firm as opposed to a more compliant edema dominated by fluids. The figure below illustrates the basic ear swelling response when abdominal application of 2% FITC in 9:1 acetone : dibutyl phthalate is followed after 21 days by application of FITC to the right ear causing an inflammatory reaction 24 hrs later



In addition to the swelling reaction, ear tissues were also used to establish an extraction protocol for an eosinophil peroxidase assay (biochemical estimate of the number of eosinophils infiltrating the ear) and to estimate protein yields from 6 and 8 mm tissue punches of ears. Ear tissue extracts were prepared with a microcentrifuge homogenizer and high speed centrifugation to minimize particulate debris and yields of protein per 6 mm ear punch were determined. Such punches have a wet weight of the order of 6.5 milligrams. Purified human eosinophil oxidase was used as a reference standard.

Blood was isolated by cardiac puncture and used for blood smears and for preparation of plasma. Blood plasma was assayed for IgE concentration using an ELISA kit (BD Pharmigen BDOptEIA Set #555248) and showed about a 4.5-fold increase in FITC-treated animals over controls indicating production of IgE by B lymphocytes in response to T-lymphocyte stimulation. Elevated levels of IgE are specific for hypersensitivity reactions as IgE normally represents only 1/2000 of the immunoglobin concentration in blood.

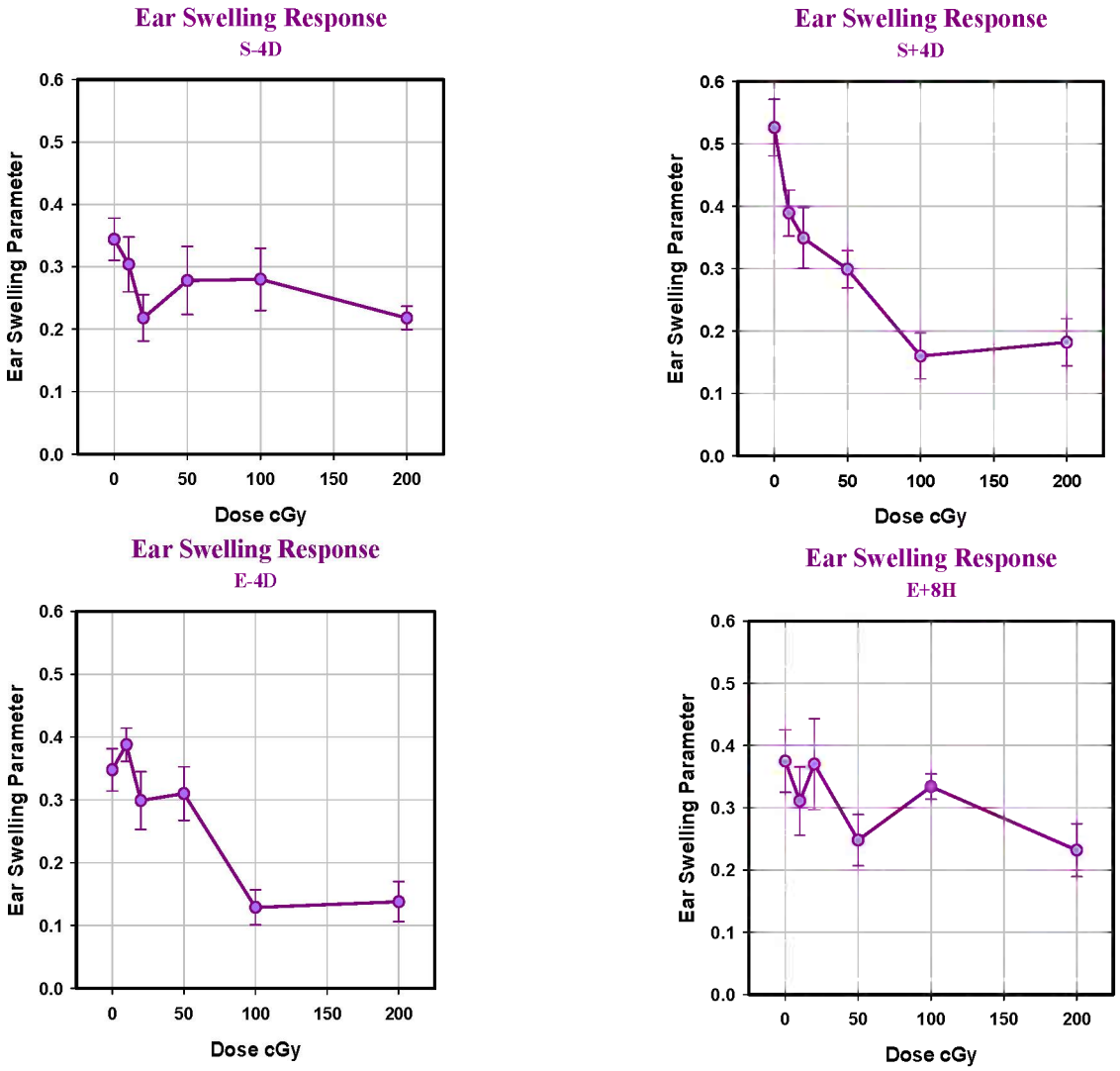
Blood smears were prepared for Wright-Giemsa staining to cross-check automated blood counts and hematology parameters (e.g. hematocrit, mean red cell volume, etc.) from a Scil Vet ABC blood analyzer). Eosinophil and other white cell counts were obtained and compared to automated threepart differential cell counts. There were several parameters for which the two methods gave inconsistent values. The Scil system has subsequently been serviced and recalibrated to improve performance.

A 13 cytokine Millipore immunobead assay (Milliplex Map Mouse Cytokine Kit) was performed on plasma samples to verify changes in response to FITC sensitization. We anticipate a “Th2” type immune response in which major changes are increases in: IL-4 (interleukin 4), IL-5, IL-6 but not “Th1” type increases in IL-2, GM-CSF and interferon gamma. Several histological samples were prepared to characterize anatomical sites important to the overall contact hypersensitivity response.

10 cGy to 2 Gy Dose Responses

Ear Swelling

Below, the ear swelling dose response in the range 10 cGy to 2 Gy is quantified for the 4 irradiations regimens: S-4D, S+4D, E-4D and E+8H. S+4D and E-4D schedules were the most effective in reducing the ear swelling response.

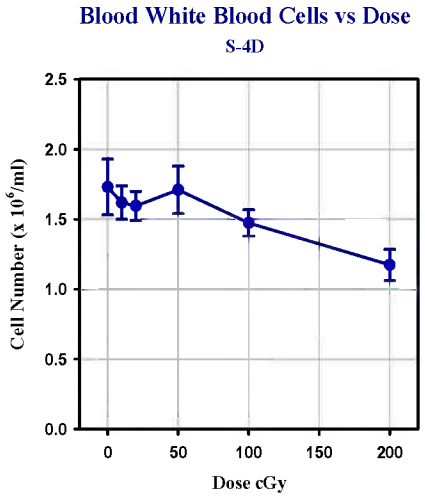


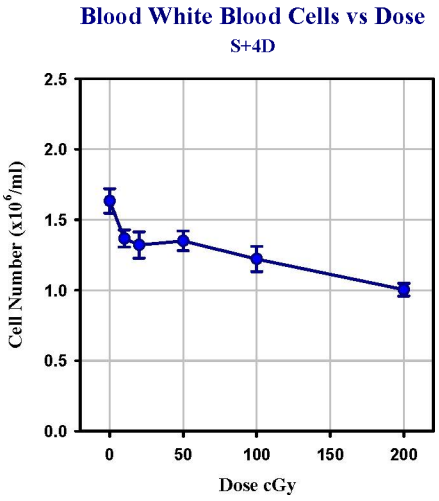
It can be seen that for the S+4D protocol that ear swelling is inhibited at doses at and above 10 cGy whereas in other protocols a dose of 25 cGy to 50 cGy is required. The data suggests that disruption of dendritic:T cell interactions and T activation are the sensitive processes for sensitization while cell numbers are critical to the elicitation step.

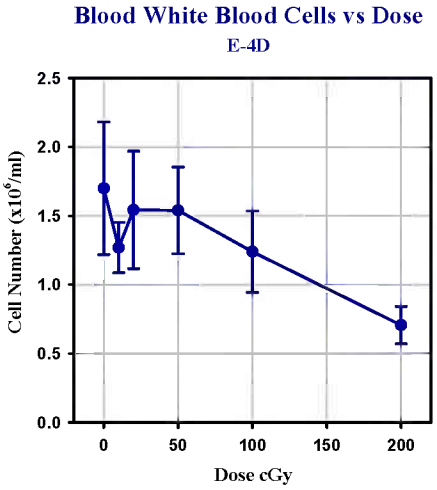
White Blood Cell Populations in Blood and Spleen

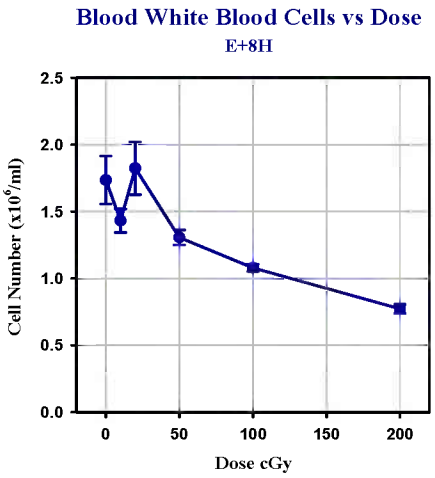
The set of panels on the next page illustrate the populations of total white blood cells in peripheral blood or in spleens measured one day after the elicitation phase application of FITC. Cell

numbers are in (10^6/ml) and are quantified for the 4 irradiations regimens: S-4D, S+4D, E-4D and E+8H. Spleen cell numbers represent total spleen cells diluted into 2 ml F10 medium. Spleen cell values for the S-4D and S+4D regimens were not significantly changed after irradiation and are not shown (Spleen at S-4D and S+4D).

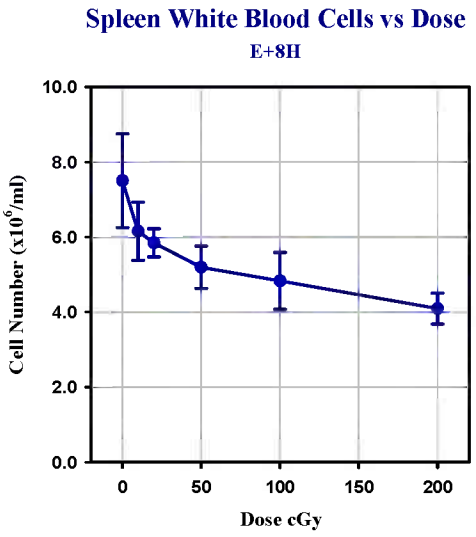


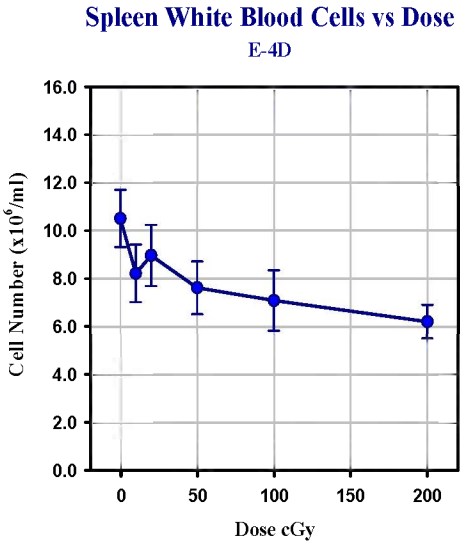






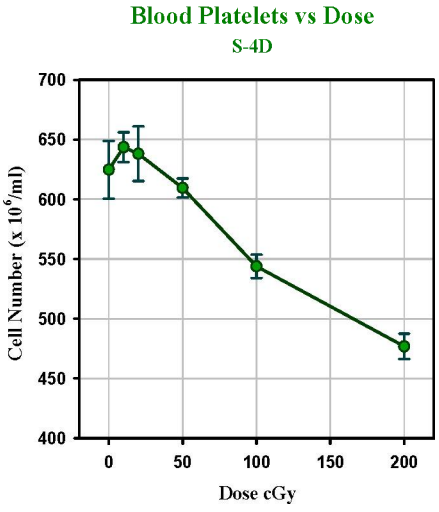
Peripheral blood leukocyte numbers at 1 day after elicitation were reduced after ≥50 cGy except in the S+4D protocol which was sensitive at the 10 cGy paralleling the ear swelling response. For leukocytes in the spleen, sensitization phase irradiations were ineffective but during the elicitation phase, cell numbers were reduced at doses ≥ 25 cGy. as shown below.

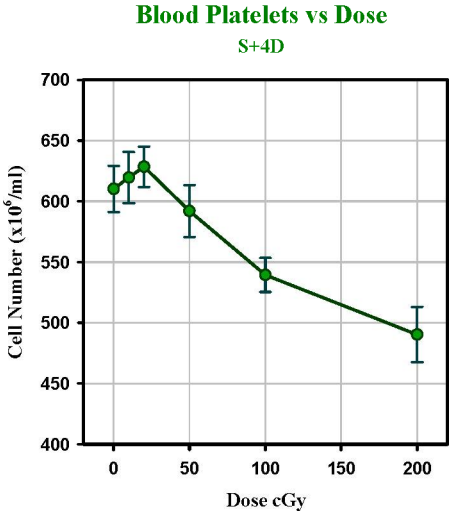




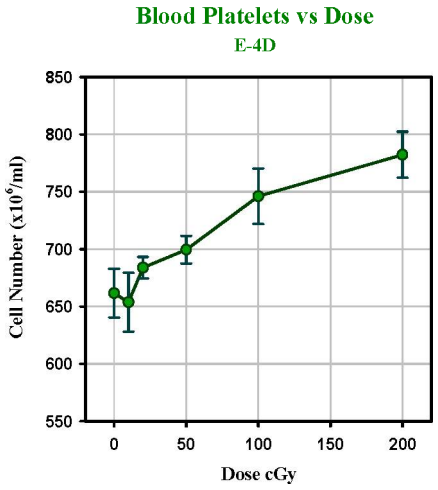
Platelet Populations in Blood and Spleens

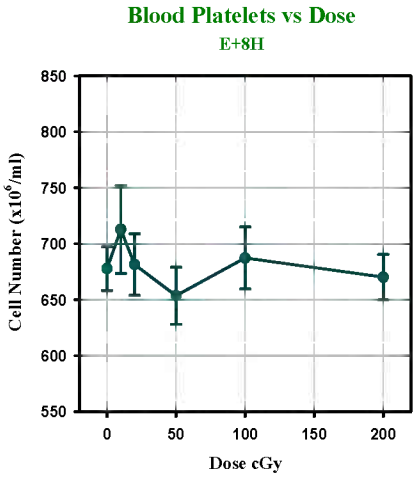
The next series of panels illustrates the populations of platelets in peripheral blood or in spleens measured one day after the elicitation phase application of FITC. Cell numbers are in (10^6/ml) and are quantified for the 4 irradiations regimens: S-4D, S+4D, E-4D and E+8H. Spleen cell numbers represent total spleen cells diluted into 2 ml F10 medium. Spleen cell values for the E-4D and E+8H regimens were not significantly changed after irradiation and are not shown.



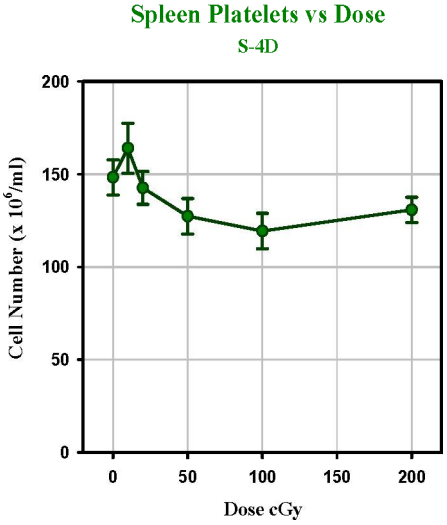


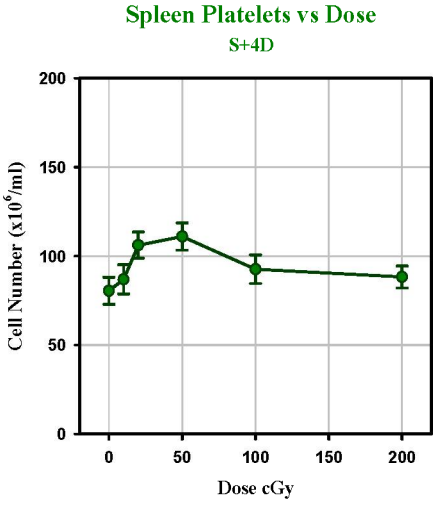
Platelet numbers showed minor increases (not statistically significant) after 10 and 25 cGy during the sensitization phase but dropped below control levels above 50 cGy (above). However. when irradiation was associated with the elicitation phase, the numbers were significantly elevated at ≥ 25 cGy for the E-4D protocol but not significantly altered for the E+8H protocol. Elevation could be from trafficking from other tissue sites or altered megakaryocyte differentiation in the bone marrow.





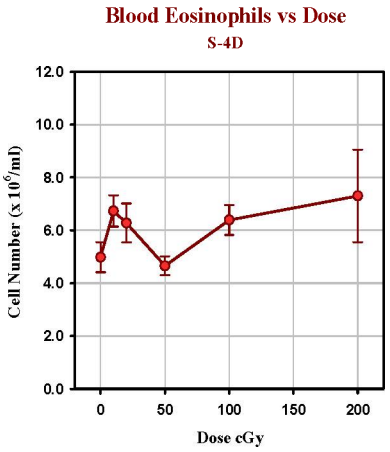
In the spleen, no significant differences were seen for E-4D or E+8H protocols while ~ 10-15% decrease or increases were observed in the S-4D and S+4D protocols at ≥ 50 cGy.

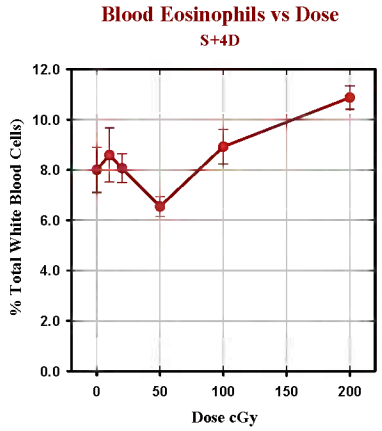


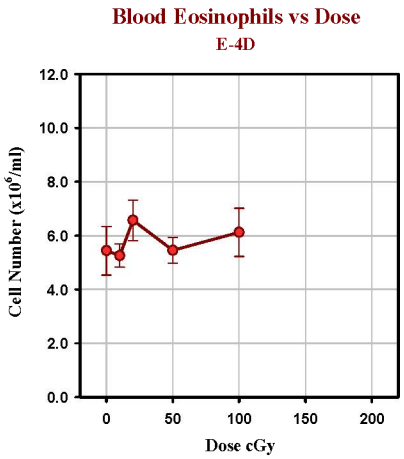


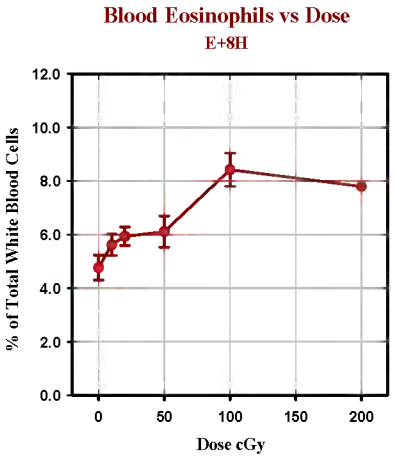
Eosinophil Populations in Blood and Spleens

Normally, eosinophils are a minor blood component but the CHS reaction to FITC is a Type 2 polarized reaction that involves eosinophil activity and is associated with elevated percentages. The next series of panels illustrates the proportion of eosinophils in peripheral blood or spleens as % total leukocytes measured one day after the E phase application of FITC.

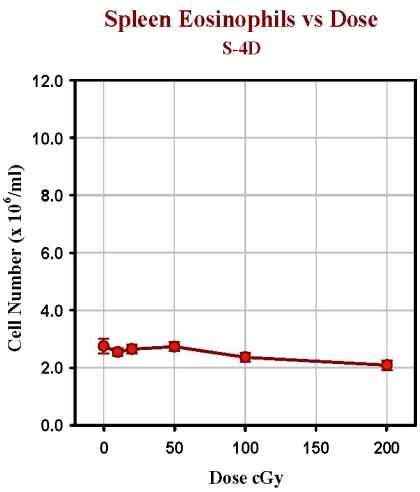


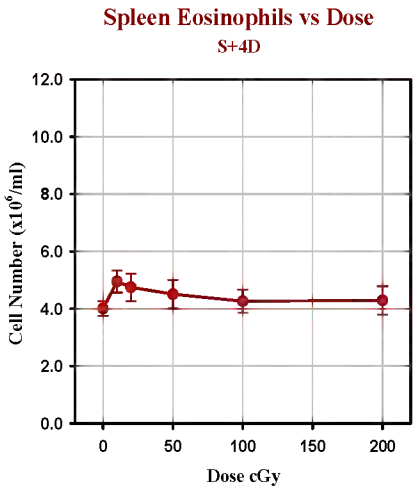


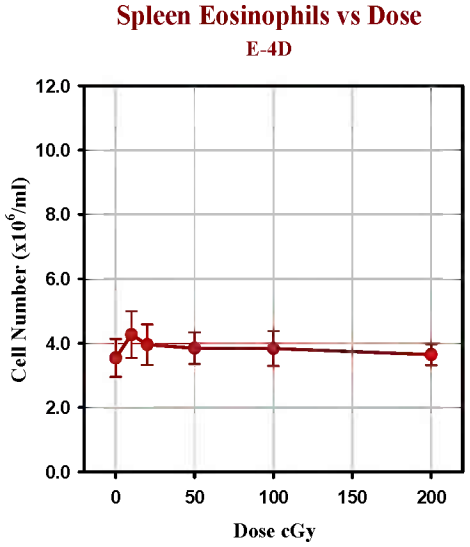


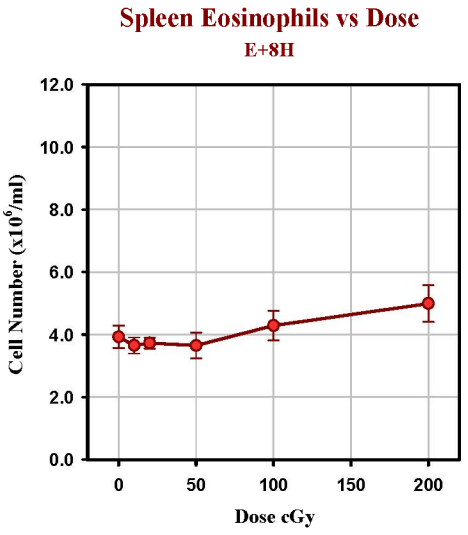


In peripheral blood, eosinophil levels show elevation after 1 Gy but there is considerable scatter in the response. For eosinophils in the spleen there are no substantial changes elicited by irradiation in any of the four standard treatment regimens.



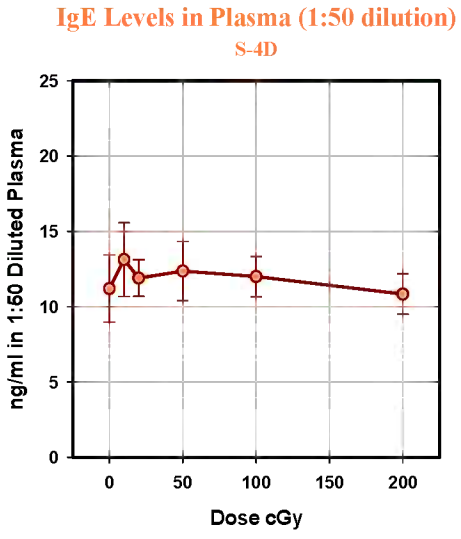


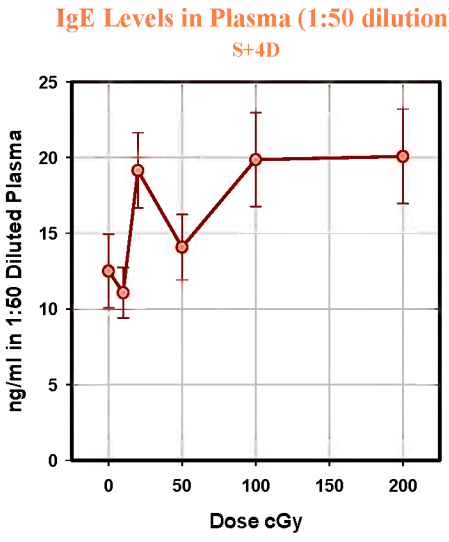


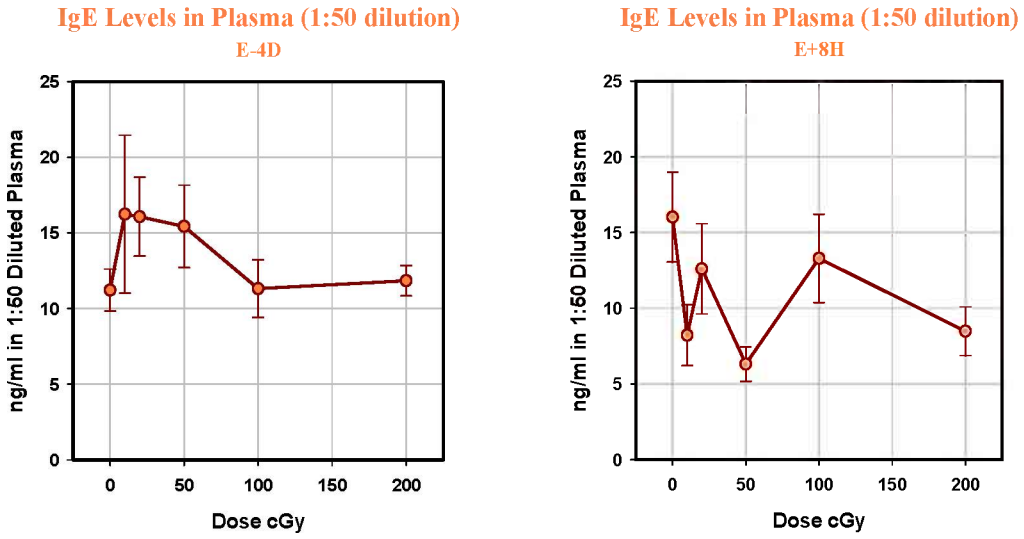


Immunoglobulin E Levels in Plasma

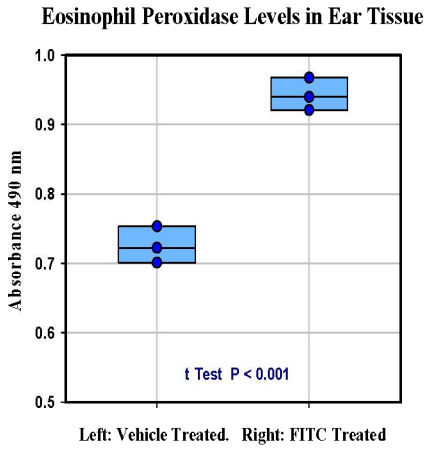
Complementing the eosinophil cell counts are serum IgE levels and a surrogate biochemical measure of eosinophils in ear tissue. IgE is typically the least abundant immunoglobulin isotype representing only 0.05% of the Ig concentration, compared to 75% for IgGs however it is is capable of triggering the most powerful inflammatory reactions. IgE can upregulate the expression of Fc receptors. on mast cells, basophils, and dendritic cells. Crosslinking of antigens to IgE on mast cells causes degranulation and release of many mediators from the mast cells and granulocytes including IL-4 and IL-13. IgE levels were very stable to the S-4D but elevated above 25 cGy in the S+4D protocol (with significant variability). Data suggest an elevation after 10 - 50 cGy in the E-4D and a highly variable decrease in the E+8H regimen.







Eosinophil peroxidase is an eosinophil-specific enzyme whose levels were measured (no radiation in example) in ear punch lysates to estimate eosinophil infiltration into the ear. Example shows eosinophil infiltration elicited during inflammatory response.



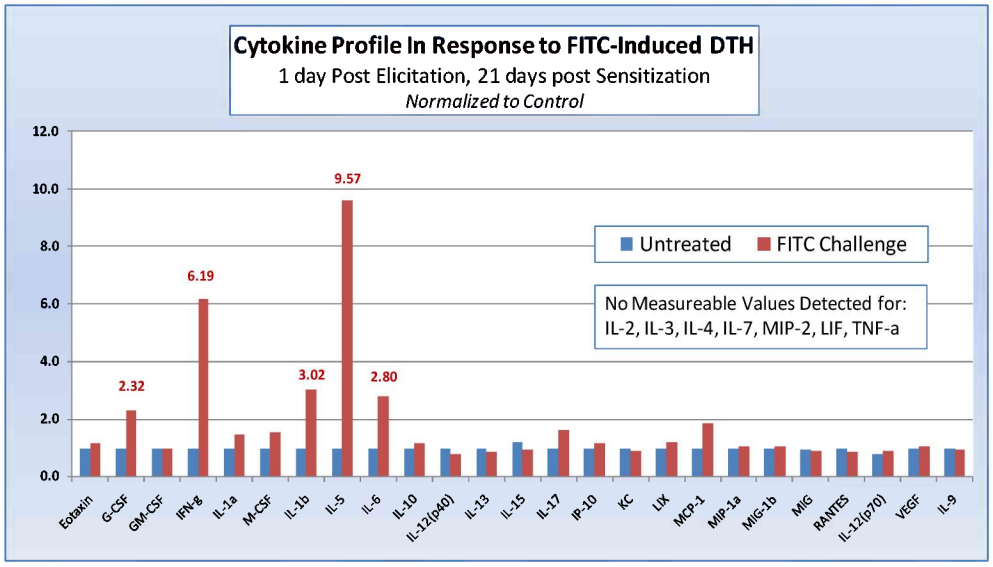
Cytokine Levels in Plasma Measured by Multiplex Immunobead Assays.

Contact hypersensitivity in response to FITC has been shown to be associated with the T_{H2} polarization differentiation pathway and MHC II restriction. This is distinct from other haptens such as fluorodinitrophenol. The cytokines associated with these processes organize into distinct patterns which can be used as signatures for determining which pathway is employed for a particular set of circumstances. We expected from the literature to see a T_{H2} process. The table below outlines the signatures for the two alternate pathways.

Cytokine signatures associated with the T_{H1} vs T_{H2} polarization differentiation pathways.

Cytokine	Function(s)	Th 1	Th 2
IL-1 β	Th2 response, CD4+ cell proliferation	-	↑
IL-2	T cell growth factor, CD8+ Tc cell activation, Macrophage activation	↑↑	-
IL-4	B cell activation, IgE production, Inhibits Th1 response	↓	↑↑
IL-5	B cell activation, IgG to IgE switch, Eosinophil growth and recruitment	-	↑↑
IL-6	Stimulates IL-2 production and B cell IgG production	-	↑↑
IL-7	Pre-T & B cell growth factor		
IL-10	Inhibits Th1 reaction, Inhibits macrophages	↓	↑
IL-12(p70)	Activates NK cells and Th1 response, Inhibits Th2 response.	↑	↓
IL-13	B cell growth factor, IgG to IgE switch	-	↑
GM-CSF	Granulocyte, dendritic cell and macrophage growth factor	↑↑	↑
Interferon-γ	Activates CD8+ Tc cells, Inhibits Th2 reaction, Activates macrophages	↑↑	↓
MCP-1	Chemokine		
TNF-α	Endothelial cell activation, Pro-inflammation, Apoptosis	↑	↑

We found that five cytokines were elevated more than 2-fold in a pattern consistent with a T_H2 polarized response characteristic of FITC as hapten. The cytokines were measured by Milliplex™ Map immunobead array on blood plasma samples.



When evaluated according to the four irradiation schedules, four cytokines (*, eotaxin/CCL-11, IL-5, IP-10 and MIG/CXCL-9) showed the most robust changes after one or more radiation regimen as compared to unirradiated controls. The patterns suggest potential effects on the elicitation or recall phase due to alterations in cell homing and migration to the site of inflammation. They also point to eosinophils as a key cell type in the contact hypersensitivity response. This confirms the expectations from the literature. The reduction in activity of IL-1β, IL-6 and IFN-γ following irradiation suggest a diminished inflammation reaction and a trend away from a T_{H1} polarization.

Cytokine Profiles in Response to Irradiation in the Four Schedules

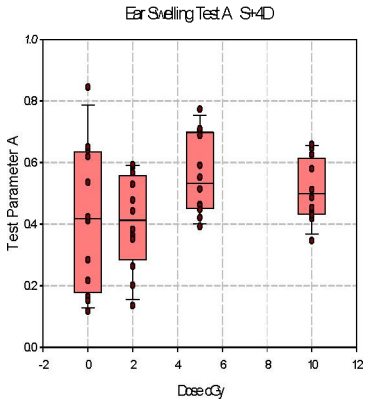
	Cytokine	CH Baseline vs Control	Radiation Effects vs CH Baseline				Producing Cell(s)	Normal Function of Cytokine
			S-4D	S+4D	E-4D	E+8H		
	G-CSF	↑↑	↑	≈	↓	↑	macrophages, endothelium	granulopoiesis in bone marrow
*	Eotaxin	↑	↑↑	↑	↑	≈	endothelium	eosinophil chemotaxis
	GM-CSF	≈	≈	≈	≈	≈	macrophages, T, NK, mast cells	granulopoiesis and mast cell proliferation
	IFN-γ	↑↑	≈	≈	↓	≈	NK cells	stimutation of T_H1 and T_H2 effector cells
	IL-1α	↑	≈	≈	≈	≈	macrophages, B, dendritic cells	T cell co-stimulation, B maturation
	IL-1β	↑↑	≈	↓	≈	≈	macrophages, B, dendritic cells	T cell co-stimulation, B maturation
	IL-2	≈	≈	≈	↓	≈	T_H1 cells	growth and differentiation of T cells
	IL-3	≈	≈	≈	≈	≈	activated T_H, mast cells, NK	myelopoiesis to RBC and granulocytes, mast cell histamine release
	IL-4	≈	≈	≈	≈	≈	T_H2, newly activated CD4+	T proliferation, B differentiation, IgE synthesis
*	IL-5	↑↑	↓	↓	↓↓	↓↓	T_H2, mast cells, eosinophils	eosinophil production, B differentiation
	IL-6	↑↑	↓	≈	≈	≈	macrophages, T_H2, B, endothelium	B activation, plasma cell antibody secretion
	IL-7	≈	≈	≈	≈	≈	bone marrow stroma	differentiation of lymphoid lineage
	IL-9	≈	≈	≈	≈	≈	CD4+ T_H2	potentiates IgM, IgE, IgE and mast cell stimulation
	IL-10	≈	↓	≈	≈	≈	CD8+ T cells, macrophages	macrophage cytokine production, T_H1 cytokines up, T_H2 stimulation
	IL-12(p40)	↓	≈	↓	≈	≈	dendritic cells, T, macrophage	differentiation into T cytotoxic, NK production of IFN-γ and TNF-α
	IL-12(p70)	≈	≈	≈	≈	≈	dendritic cells, T, macrophage	differentiation into T cytotoxic, NK production of IFN-γ and TNF-α
	LIF	≈	≈	≈	≈	≈	T cells	myeloid cell differentiation
	IL-13	≈	↑	↑	≈	↓	activated T_H2, mast, NK	B growth and differentiation - IgE production, T_H1 cell inhibition
	LIX	↑	↓	↓	≈	≈	eosinophils	neutrophil activation and chemotaxis
	IL-15	↓	≈	≈	↓	≈	macrophages	NK cell production
	IL-17	↑	≈	≈	≈	≈	T_H17 cells	angiogenesis and inflammatory cytokines
*	IP-10	≈	↓	≈	↓↓	↑↑	monocytes, endothelium, fibroblasts	monocyte chemotaxis, T cell adhesion to endothelium
	KC	≈	↑	≈	≈	≈	macrophages, epithelium, neutrophils	neutrophil chemotaxis
	MCP-1	↑	↓	≈	↓	↑	macrophages	memory T cell, monocytes, dendritic cell recruitment to inflammatory sites
	MIP-1a	≈	≈	↓	↓	≈	macrophages	granulocyte activation and pro-inflammatory cytokine release
	MIP-1b	≈	≈	≈	≈	≈	macrophages	granulocyte activation and pro-inflammatory cytokine release
	M-CSF	↑	≈	≈	↓	≈	monocytes, granulocytes, fibroblasts	monocyte and granulocyte proliferation
	MIP-2	≈	≈	≈	≈	≈	macrophages, epithelium, neutrophils	neutrophil chemotaxis
*	MIG	≈	≈	≈	↓↓	≈	macrophages, monocyte, endothelium	T_H1 chemoattractant
	RANTES	≈	≈	≈	↓	≈	T cells	chemotaxis & adhesion of of eosinophils, basophils, T cells
	TNF-α	≈	≈	≈	↓	≈	activated macrophages and NK	proinflammatory through NFkB, MAPK and apoptosis

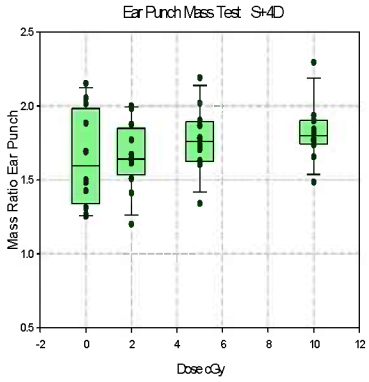
Extension of Measurements to Lower Dose Range: 0, 2, 5, and 10 cGy

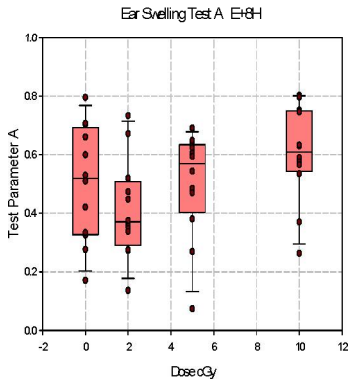
During the no cost extension period, the experimental regimens used for the 10 cGy to 2 Gy dose range were extended to lower doses in consultation with DOE Low Dose Program Manager Noelle Metting, ScD.. Sample sizes of 12 animals were used in the two most sensitive of the four time sequences employed before (S+4D and E+8H) and doses of 2, 5 and 10 cGy were administered.

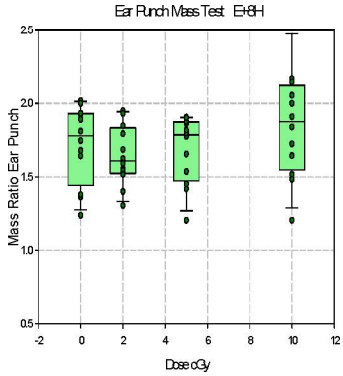
Ear swelling Test and Spleen Mass

Ear swelling test and spleen mass were assessed using the S+4D and E+8H protocols that probe the effects of irradiation at the time of major T-cell activation and proliferation in the lymph nodes after sensitization of belly skin and onset of inflammatory/cell infiltration response shortly after immune recall is elicited in ears. The ear swelling reaction was assessed both by micrometer for changes in thickness normalized to before-elicitation control (vehicle only) ear or by the mass of a 6 mm tissue punch. While there was a slight trend towards enhanced swelling at 5 and 10 cGy in the S+4D protocol and at 10 cGy in the E+8H protocol none of the changes reached statistical significance.

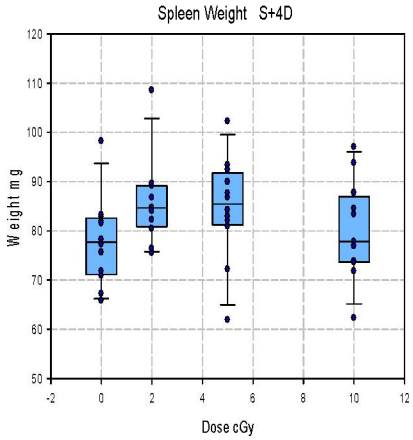


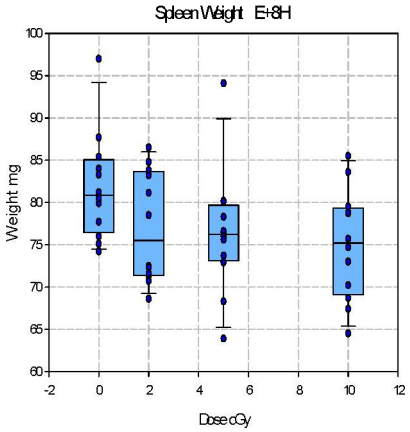






Spleen mass exhibited a slight enhancement after 2 and 5 cGy in the S+4D protocol and a slight decrease in the E+8 H but neither response was significantly significant.



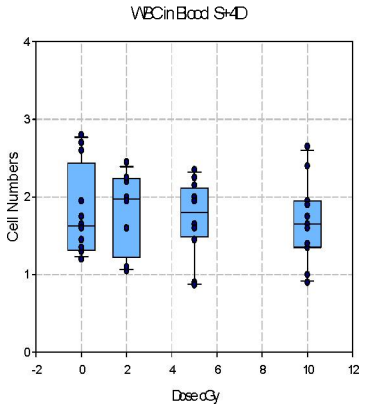


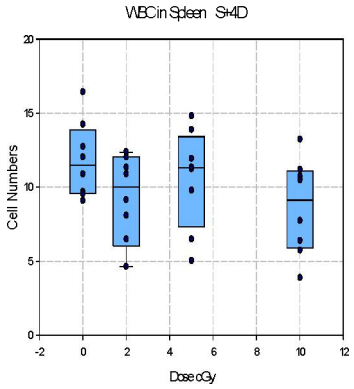
Cell Populations in Peripheral Blood and Spleen

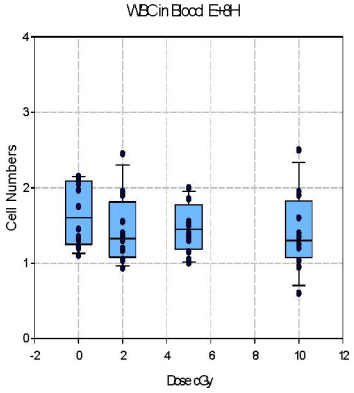
Blood cell counts in peripheral blood and spleen were obtained using a Scil™ Heska Vet ABC cell veterinary blood counter as for the higher dose range experiments and were measured for the S+4D and E+8H protocols.

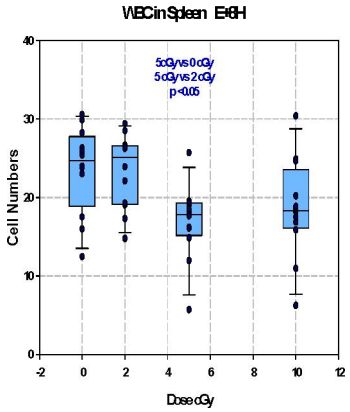
White Blood Cell Populations in Blood and Spleen

Total white blood cell counts were generally unchanged in either peripheral blood or spleen after either protocol. However WBC in spleen were depleted at $p < 0.05$ at 5 cGy relative to either 0 or 2 cGy. Differences in spleen number parameter for S+4D vs 8+8H were due to different dilution volumes for the splenocytes. Caution should be used in interpreting differences in the spleen which could be due to cell trafficking differences rather than absolute depletion.



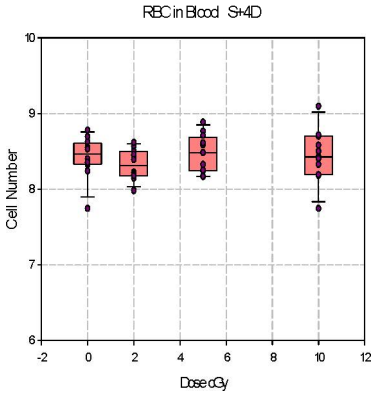


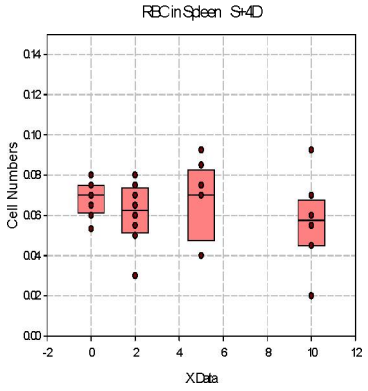


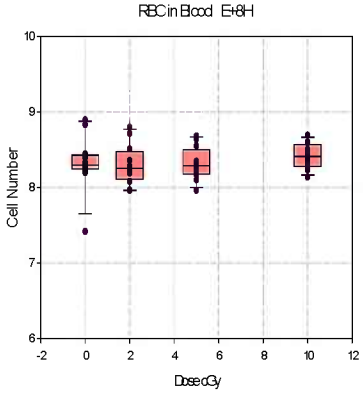


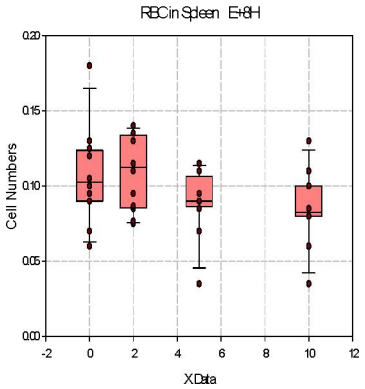
Red Blood Cell Populations in Blood and Spleen

Total red blood cell counts were statistically unchanged in either peripheral blood or spleen after either protocol.



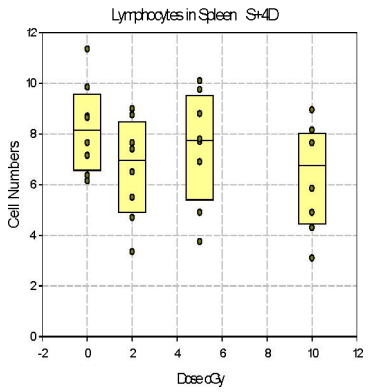


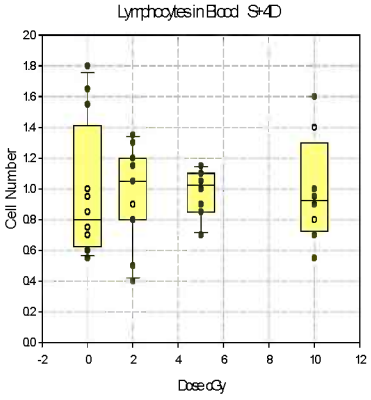


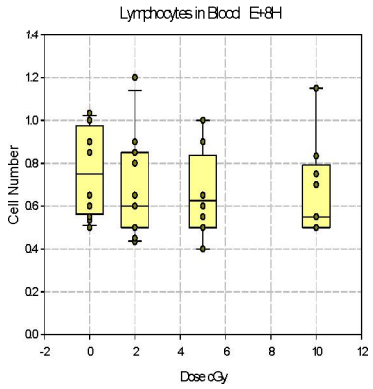


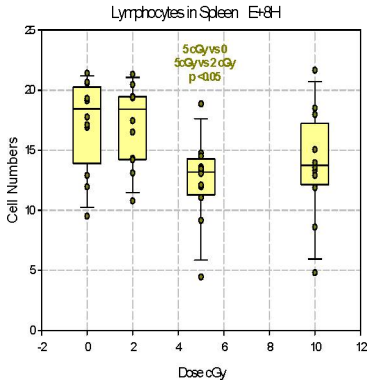
Lymphocyte Populations in Blood and Spleen

Lymphocyte numbers were generally unchanged with the exception of a relative depletion in the spleen after 5 cGy irradiation versus either 0 or 2 cGy in the E+8H protocol. Some caution should be used in interpreting spleen numbers which could be due to altered cell trafficking as well as overall depletion.



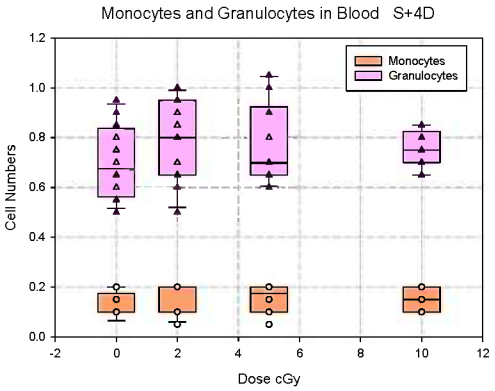


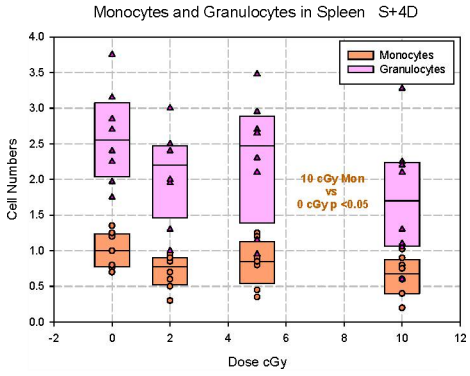


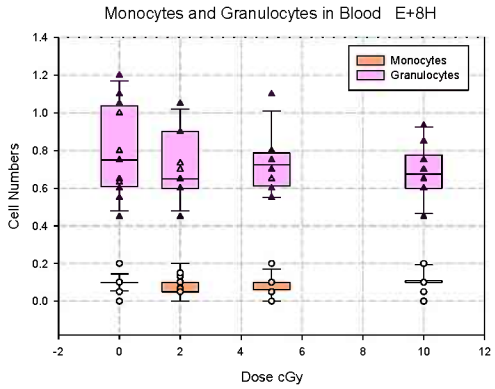


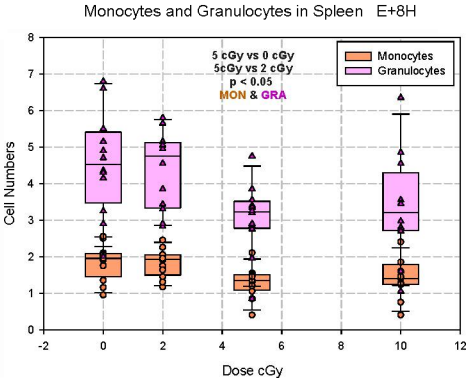
Monocyte and Granulocyte Populations in Blood and Spleen

Monocytes and Granulocyte numbers were stable in the blood under the treatment regimens but showed small relative depletion in the spleen at 5 and 10 cGy versus 0 and 2 cGy. Some caution should be used in interpreting spleen numbers which could be due to altered cell trafficking as well as overall depletion.



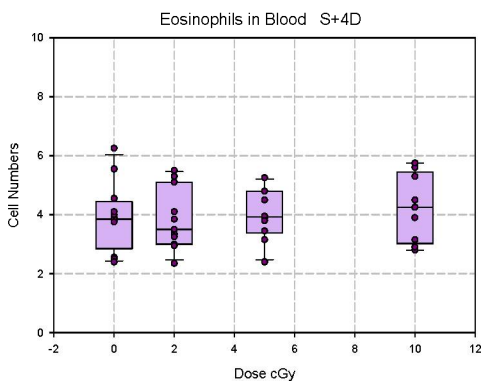


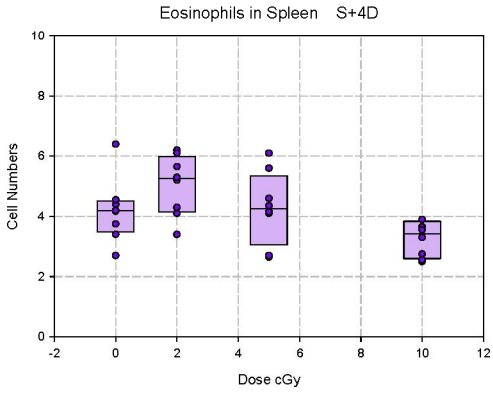


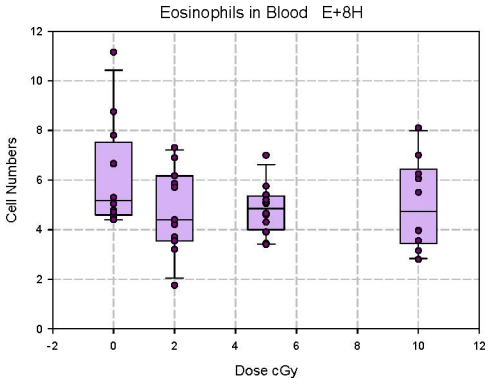


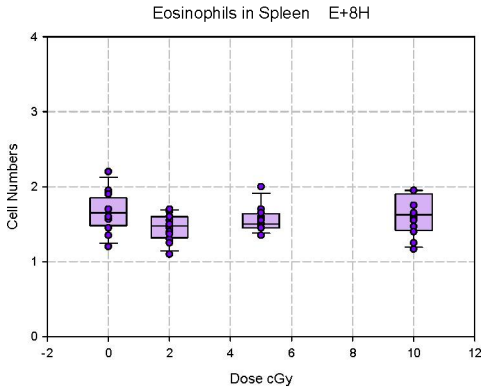
Eosinophill Populations in Blood and Spleen

Eosinophil numbers were unchanged under all treatment conditions.



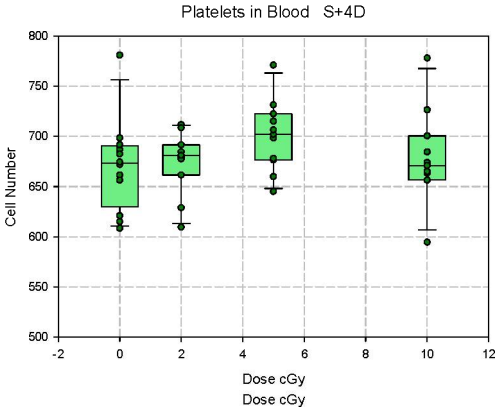


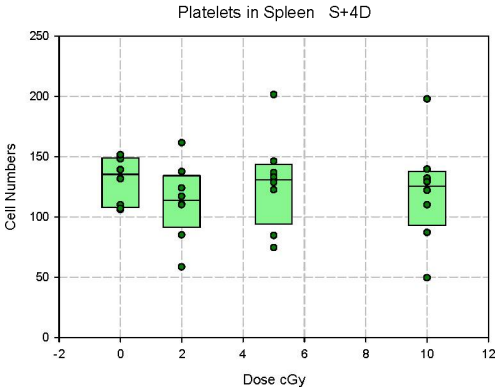


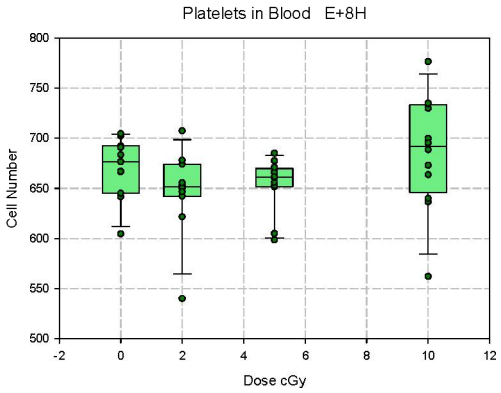


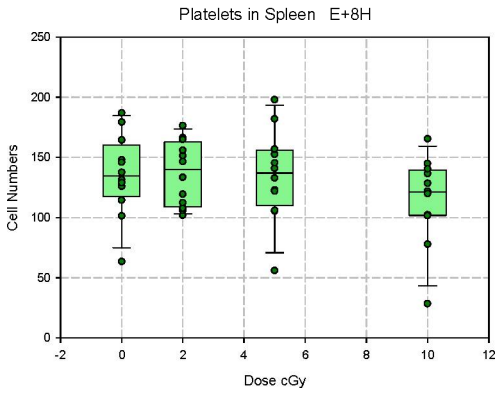
Platelet Populations in Blood and Spleen

Platelets showed a trend (but not statistically significant)towards elevation at 5 cGy in blood in the S+4D protocol but









Overall, quantification of the ear swelling responses and associated blood and spleen cell populations exhibited little or no changes in the lower dose range. The exceptions were minor cell number differences at the 5 cGy level in the spleen which may reflect cell trafficking differences rather than overall cell losses or gains.

Transcriptomic Analysis of Critical Events and Time Periods in the Adaptive Immune Response in Response to Irradiation

During the final no-cost extension period we turned our attention to determining the effects of radiation on individual steps in formation of the immune memory and mounting of the recall response that occur during the sensitive periods. Our original plan was to utilize flow cytometry methods for characterizing lymph node cell composition, dendritic and T cell differentiation status as well as quantification of regulatory molecules involved in cell adhesion and recruitment to the site of inflammation. Reductions in available staff time and other resources during laboratory and office moves mitigated against this approach. So we determined that the greatest return on investment would be to isolate tissues from animals treated according to the four protocols and outsource analysis of targeted gene arrays associated with the cells and processes of interest.

Targeted Qiagen Arrays

The purpose of these experiments was to identify gene expression patterns associated with key steps in establishing and recalling adaptive immunity and the effects of gamma ray exposure at critical times relative to these events. Most gamma ray exposures were at 1 Gy but both 10 cGy and 1 Gy were used for T-cell and dendritic cell activation endpoints. Tissue samples were skin from belly locations associated with sensitization phase hapten exposure, ear punches associated with elicitation phase and inflammation of reaction, and pooled lymph nodes at the time of dendritic cell interaction with naïve T lymphocytes. Treatments were control, acetone + dibutylphthalate (also has adjuvant activity), and fluorescein isothiocyanate in vehicle. Nine data sets were generated using targeted (89 test genes plus 7 contols) PCR arrays from SA Biosciences/Qiagen. The nine data sets were as follows.

DATA SET 1. Skin activation events according to protocol 1. Irradiate at sensitization (S) – 4 days (S-4D, DAY 1). Sensitize belly skin twice (S1, DAY 4, S2, DAY5). Euthanize and harvest belly skin tissue on DAY 6 (S1+2D = S2+1D). Responses should reflect keratinocyte + dendritic cell activities in skin at time point of minimum circulating blood cells (4 day nadir after radiation). Conditions 0 Gy or 1 Gy vs Untreated, DBP or FITC. Array # PAMM 052Z – Innate Immunity Genes.

DATA SET 2. Skin activation events according to protocol 2. Sensitize belly skin twice (S1, DAY 1, S2, DAY2). Irradiate on DAY2 close to time of S2. Euthanize and harvest belly skin tissue on DAY 3 (S1+2D = S2+1D). Responses should reflect keratinocyte + dendritic cell activities in skin acutely after sensitization and co-irradiation. Conditions 0 Gy or 1 Gy vs Untreated, DBP or FITC. Array # PAMM 052Z – Innate Immunity Genes.

DATA SET 3. Ear tissue activation events according to protocol 5. Sensitize belly skin twice (S1, DAY 1, S2, DAY2). Allow adaptive immunity to FITC develop for 14 days after S1. Irradiate at Day 13 (Elicitation (E) – 1 day, E-1D) Elicit inflammation with ear treatment on day 14. Euthanize and harvest ear tissue punches on DAY 15(E+1D). Responses should reflect inflammatory events and cellular infiltration in ear at time point where irradiation should inhibit resident APC's, T memory cells responding to elicitation treatment. Conditions 0 Gy or 1 Gy vs Untreated, DBP or FITC. Array # PAMM 052Z – Innate Immunity Genes.

DATA SET 4. Lymph node events at time of dendritic cell: naïve T interaction/activation events according to protocol 3. Irradiate at sensitization – 4 days (S-4D, DAY 1). Sensitize belly skin twice (S1, DAY 4, S2, DAY5). Euthanize and harvest draining lymph nodes at DAY 8 (4 days after sensitization). Responses should reflect dendritic cell activation, migration, presentation and T cell activation & clonal expansion events in the node at time point of maximum interaction. Conditions 0 Gy, 10 cGy or 1 Gy vs DBP or FITC. Array # PAMM 053Z – T Cell Activation Genes.

DATA SET 5. Skin oxidative stress & activation events according to protocol 2. Sensitize belly skin twice (S1, DAY 1, S2, DAY2). Irradiate on DAY2 close to time of S2. Euthanize and harvest belly skin tissue on DAY 3 (S1+2D = S2+1D). Responses should reflect oxidative stress and compensation activities in skin acutely after sensitization and co-irradiation. Conditions 0 Gy or 1 Gy vs Untreated, DBP or FITC. Array # PAMM 065Z Oxidative Stress Genes.

DATA SET 6. Ear tissue activation events according to protocol 5. Sensitize belly skin twice (S1, DAY 1, S2, DAY2). Allow adaptive immunity to FITC develop for 14 days after S1. Irradiate at Day 13 (Elicitation – 1 day, E-1D) Elicit inflammation with ear treatment on day 14. Euthanize and harvest ear tissue punches on DAY 15 (E+1D). Responses should reflect inflammatory events and cellular infiltration in ear at time point where irradiation should inhibit resident APC's, T memory cells responding to elicitation treatment. Conditions 0 Gy or 1 Gy vs Untreated, DBP or FITC. Array # PAMM 097Z – Inflammasome Genes.

DATA SET 7. Activation events for dendritic cells in skin according to protocol 1. Irradiate at sensitization – 4 days (S-4D, DAY 1). Sensitize belly skin twice (S1, DAY 4, S2, DAY5). Euthanize and harvest belly skin tissue on DAY 6 (S1+2D = S2+1D). Responses should reflect early activation of dendritic cells due to oxidative stress and compensation activities in skin acutely after sensitization under conditions when immune cell populations are maximally depleted by irradiation. Conditions 0 Gy or 1 Gy vs Untreated, DBP or FITC. Array # PAMM 406Z Dendritic Cell Genes.

DATA SET 8. Activation events for dendritic cells in skin according to protocol 2. Sensitize belly skin twice (S1, DAY 1, S2, DAY2). Irradiate on DAY2 close to time of S2. Euthanize and harvest belly skin tissue on DAY 3 (S1+2D = S2+1D). Euthanize and harvest belly skin tissue on DAY 6 (S1+2D = S2+1D). Responses should reflect activation of dendritic cells due to oxidative stress and compensation activities in skin acutely after sensitization and co-irradiation. Conditions 0 Gy or 1 Gy vs Untreated, DBP or FITC. Array # PAMM 406Z Dendritic Cell Genes.

DATA SET 9. Activation events for dendritic cells in skin according to protocol 3. Irradiate at sensitization – 4 days (S-4D, DAY 1). Sensitize belly skin twice (S1, DAY 4, S2, DAY5). Euthanize and harvest belly skin tissue at DAY 8 (4 days after sensitization). Responses should reflect later dendritic cell activation, migration, and antigen presentation events in the skin when sensitization occurred under conditions when immune cell populations are maximally depleted by irradiation. Conditions 0 Gy, 10 cGy or 1 Gy vs DBP or FITC. Array # PAMM 406Z Dendritic Cell Genes.

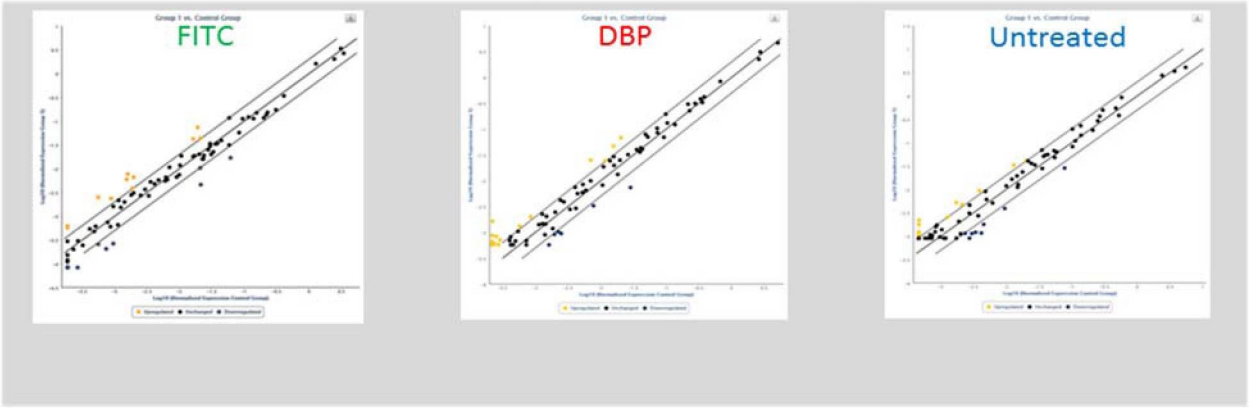
Results generally demonstrate that the effects of radiation were dependent on the sensitization & elicitation event schedule and there were distinct responses for the untreated, acetone + dibutyl phthalate vehicle control (with adjuvant activity) and FITC hapten treatment in vehicle. Depending on the specifics, as few as 1 and as many as 70 out of 89 genes were 2-fold up or down regulated by the radiation exposure. The table below summarizes results from 6 selected conditions from the 9 data sets.

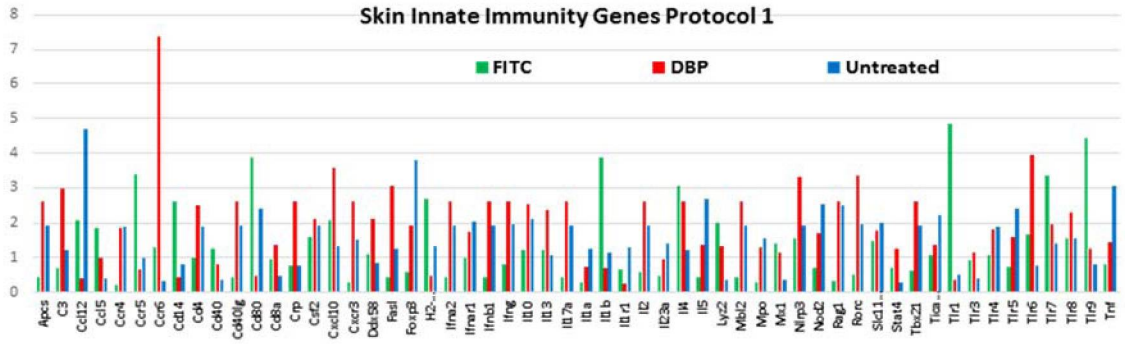
Target Tissue	Gene Set	Treatment Protocol	Number of Genes Upregulated > 2-fold			Number of Genes Downregulated > 2-fold			Number of Genes Unchanged (<2 fold up or down)		
			FITC	Veh	Ctrl	FITC	Veh	Ctrl	FITC	Veh	Ctrl
Skin	Innate Immunity	1 @ 1 Gy	10	26	12	15	6	8	64	57	69
Skin	Oxidative Stress	2 @ 1 Gy	4	5	15	2	2	34	83	82	40
Skin	Dendritic Cell Activation	1 @ 1 Gy	5	16	20	12	2	9	72	71	60
Ears	Innate Immunity	5 @ 1 Gy	4	13	5	10	28	8	75	58	76
Ears	Inflammasome	5 @ 1 Gy	56	3	3	14	5	12	19	81	74
Lymph Nodes	T cell Activation	3 @ 1 Gy	2	3	--	2	1	--	85	85	--
	T cell Activation	3 @ 0.1 Gy	0	4	--	1	1	--	88	84	--

Examples of Gene Expression Patterns for Three Selected Protocols

A breakdown of the responses for Skin Innate Immunity responses is illustrated below as scatterplots of 1 Gy vs 0 Gy fold-regulation correlations and a bar chart detailing the magnitude of responses by gene where 12 – 26 genes were significantly changed for FITC vs vehicle vs untreated conditions. The complexity of this set of responses was typical for the nine data sets.

Data Set 1: Skin, Innate Immunity Genes, Protocol 1

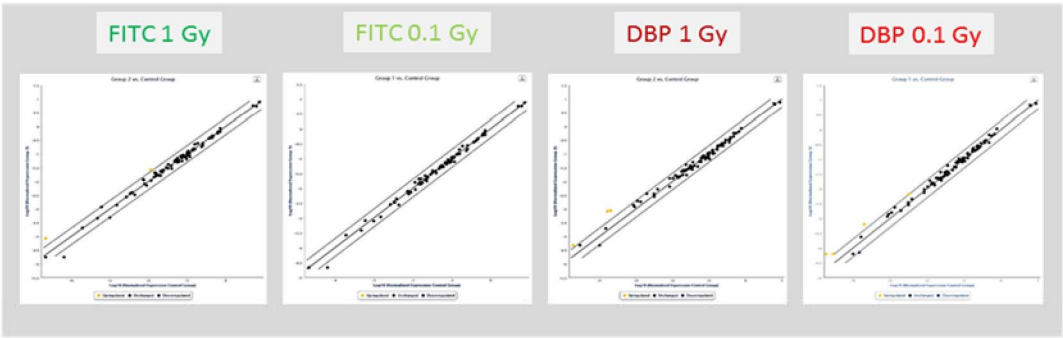


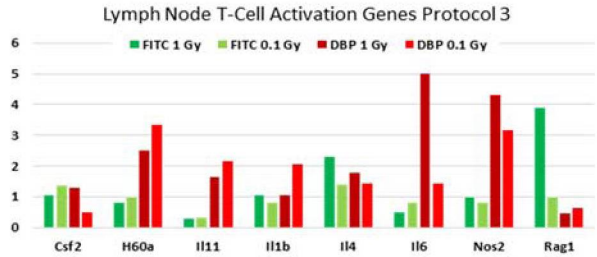


In the next illustration for T-cell activation genes in lymph nodes, only a tiny fraction of genes were modulated but showed dose-dependent patterns of response.

Data Set 4: Lymph Node T-Cell Activation Genes Protocol 3

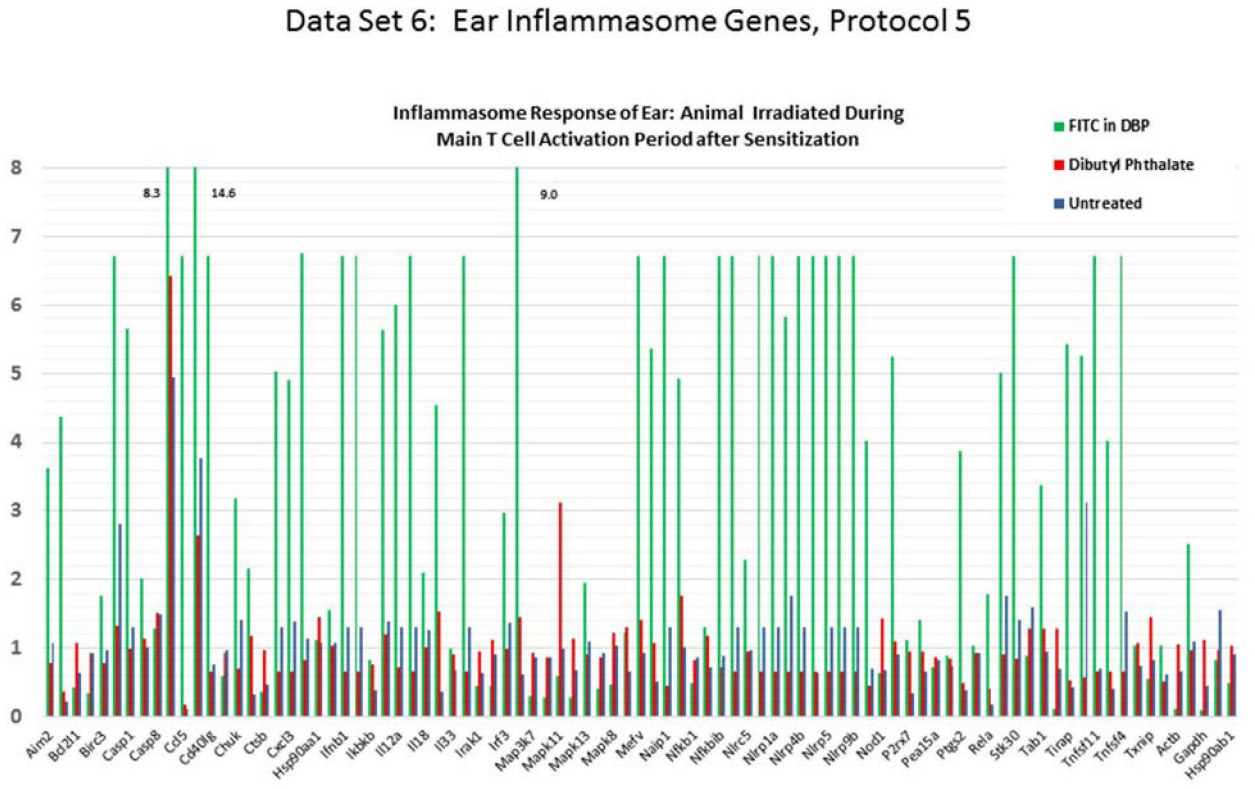
Treated vs Untreated at 2 Doses





The most responsive gene set was for inflammasome-related gees in ear tissue during the major inflammation reaction. Under conditions of hapten treatment there was a very robust response that was augmented by radiation exposure. Much more muted effects were observed for vehicle treatment alone or no treatment. In the figure, every other gene is labeled for legibility. The complete gene list is as follows.

Aim2,Bcl2, Bcl2l1, Birc2, Birc3, Card6, Casp1, Casp12, Casp8, Ccl12, Ccl5, Ccl7, Cd40l, Cflar, Chuk, Ciita, Ctsb, Cxcl1, Cxcl3, Fadd, Hsp90aa1, Hsp901, Ifnb1, Ifng, Ikbkb, Ikbkg, Il12a, Il12b, Il18, Il1b, Il33, Il6, Irak1, Irf1, Irf3, Irf4, Map3k7, Mapk1, Mapk11, Mapk12, Mapk13, Mapk3, Mapk8, Mapk9, Mefv, Myd88, Naip1, Naip5, Nfkb1, Nfkbia, Nfkbib, Nlrc4, Nlrc5, Nlrp12, Nlrp1a, Nlrp3, Nlrp4b, Nlrp4e, Nlrp5, Nlrp6, Nlrp9b, Nlrx1, Nod1, Nod2, P2rx7, Panx1, Pea15a, Pstpip1, Ptgs2, Pycard, Rela, Ripk2, Stk30, Sugt1, Tab1, Tab2, Tirap, Tnf, Tnfsf11, Tnfsf14, Tnfsf4, Traf6, Txnip, Xiap, Actb, B2m, Gapdh, Gusb, Hsp90ab1



What pathways and processes are recognized in these responses? For the six selected data sets shown in the table above the following highlights are noted.

DATA SET 1. *Skin innate immunity activation events according to protocol 1.* Responses should reflect keratinocyte + dendritic cell activities in skin at the time point of minimum circulating blood cells (4 day nadir after radiation). *After FITC treatment:* 1 Gy γ elicited significant upregulation of: CCL12 and CXCL10 which attract eosinophils, monocytes and lymphocytes, CD80 which is costimulatory for T cell activation, IL-1β which is a general proinflammatory cytokine, IL-4 which stimulate T_H cell differentiation towards the T_{H2} polarization and three toll-like receptor genes which mediate interferon production by macrophages. Significant down regulation occurred for: CCR4 and CXCR3 involved in

chemotaxis, CD40 which mediates activated T cell interactions with antigen presenting cells (APC), IL-1α and IL-23α which promote inflammation and myeloperoxidase which is a marker of monocytes and eosinophils. *After dibutyl-phthalate + acetone vehicle treatment:* 1 Gy γ elicited significant upregulation of: CCR6 and CD40 which promote T cell:dendritic cell interactions, IL-2, IL-4 and interferon γ which indicate T cell and MHC II activation with T_{H2} polarization, two toll-like receptors which promote IL-1β processing and GMCSF which promotes proliferation of monocytes and granulocytes. Significant down regulation occurred for: CCL12 a chemoattractant for eosinophils and monocytes, toll like receptor-1 which mediates cytokine production and ILR1 which mediates inflammation. *With no treatment*, 1 Gy γ elicited significant upregulation of: CCL12 a chemoattractant for eosinophils and monocytes, CD80 a costimulatory molecule for T cell activation, IFNα,βR which mediates stimulation of macrophages and B cells, IL-5 and IL-10 which stimulate mast cells and eosinophil differentiation, and TNF-α a general proinflammatory molecule. Significant down regulation occurred for: T cell activating CCL5, CD40 which mediated dendritic cell:T cell interactions, STAT 4 which mediates cytokine signaling and TLR3 which promotes IL-1β processing. Taken together, these patterns are consistent with the sensitization reactions expected early after hapten administration with emphasis on attraction of leukocytes to the site of application, establishing conditions for interaction of dendritic cells and T cells, activation of T cells along the T_{H2} pathway and the inflammation events required to facilitate cell chemotaxis and migration to the reaction site. There were modest trends towards more complete stages of sensitization reaction after hapten treatment and vehicle treatment alone trended towards earlier events consistent with an adjuvant role for dibutyl-phthalate.

DATA SET 3. *Ear tissue innate immunity activation events according to protocol 5.* Responses should reflect radiation effects on inflammatory events and cellular infiltration in the ear shortly after the elicitation step at the time point where irradiation may be expected to inhibit resident APC's and T memory cells responding to elicitation treatment two weeks after sensitization at the distal belly skin site. *After FITC treatment:* 1 Gy γ elicited significant upregulation of: ICAM-1 which increases adhesion of leukocytes to endothelial cells and promotes extravasation, very strong upregulation of CD4 which promotes MHCII antigen presentation interactions, as well as CCR8 and ILR1 which promote inflammation. Significant down regulation occurred for: proinflammatory IL-1β, IL-1α, IL-6. *After dibutyl-phthalate + acetone vehicle treatment:* 1 Gy γ elicited significant upregulation of: endothelial adhesion molecule ICAM-1, CCL12 a chemoattractant for eosinophils and monocytes, IL-1α and interferon γ which are proinflammatory and promote T_{H2} polarized reactions. Significant down regulation occurred for: CD4, CD40, CD40L which promote antigen presentation via MHCII, IL-4 and IL-5 which promote T_{H2} polarized reactions, and IL-1β and IL-6. *With no treatment*, 1 Gy γ elicited significant upregulation of: CCL12 and integrin α_M which attract and promote adhesion for eosinophils, monocytes, granulocytes and macrophages, proinflammatory IL-1α and TLR4 which promotes IL-1 processing. Significant down regulation occurred for: CCL5 which is chemotactic for T cells, CD80 the co-stimulatory molecule for activation of T cells, and IL-4 & interferon γ which regulate MHCII-dependent antigen presentation and macrophage activation. Taken together, these patterns reflect a different set of events than during early sensitization. There are stronger trends towards cellular infiltration processes including involvement of adhesion to endothelial cells but weaker MHCII-related antigen presentation activity. Inflammatory cytokine activity showed mixed up and down-regulation.

DATA SET 4. *Lymph node events at time of dendritic cell: naïve T interaction/activation events according to protocol 3.* Responses should reflect dendritic cell activation, migration, presentation and T cell activation & clonal expansion events in the node at time point of maximum interaction. T cell activation events in lymph nodes were surprisingly unresponsive to irradiation. Only 1 – 5 genes out of 89 responded under two radiation dose conditions following FITC or vehicle treatments. *After FITC treatment:* 1 Gy γ elicited significant upregulation of: Rag1 associated with VDJ recombination and IL-4 associated with isotype switching to IgE. Significant down regulation occurred for: IL-11associated wit JAK signaling. *After dibutyl-phthalate + acetone vehicle treatment:* 1 Gy γ elicited significant

upregulation of: inducible nitric oxide synthetase, IL-4, proinflammatory IL-6 and H60a component of MCH complex indicating enhanced oxidative stress, inflammation and antigen presentation. Significant down regulation occurred for: Rag-1 and CSF-2 indicating reduced VDJ recombination and proliferation of granulocytes and monocytes. Taken together, these patterns are consistent with a modest enhancement by irradiation of oxidative stress and perhaps induction of non-homologous end joining DNA repair associated with B cell maturation to an IgE producing phenotype.

DATA SET 5. *Skin oxidative stress & activation events according to protocol 2.* Responses should reflect oxidative stress and compensation activities in skin acutely after sensitization and co-irradiation. Surprisingly, very few genes showed radiation-elicited changes after vehicle or FITC hapten treatment (only 4 or 9 out of 89) suggesting little or no additive effect of antigen-induced and radiation-induced oxidative stress. In untreated animals there was a strong downregulation response (34 of 89 genes) suggesting a rapid compensatory anti-oxidant response to radiation-induced reactions in the absence of immune stimulation. There was no clear pattern to the sets of enzymes up or down-regulated that might suggest a bias towards mitochondrial versus cytoplasm & membrane based sources of free radicals.

DATA SET 6. *Ear inflammasome-related tissue activation events according to protocol 5.* Responses should reflect inflammatory events and cellular infiltration in ear at time point where irradiation should inhibit resident APC's, T memory cells responding to elicitation treatment. *After FITC treatment:* 1 Gy γ elicited the most robust response of all conditions tested with 70 out of 89 genes exhibiting significant up- or down-regulation. Up-regulated responses included: four CCL and CXCL chemoattractants for leukocytes, six proinflammatory interleukins (including IL-1β, IL-6, IL-2), eleven NLR-family inflammasome components which promote Il-1β & IL-18 maturation and NFκB transcription factor activation, TNF-α and 3 related cytokines and a prostaglandin endoperoxidase. *After dibutyl-phthalate + acetone vehicle treatment* there were only modest changes*:* 1 Gy γ elicited significant upregulation of: CCL-7 and CCL-12 chemoattractants and MAP Kinase 1 mediating cytokine signaling. Significant down regulation occurred for: apoptosis promoting Bcl-2, NLR_{X1} which would promote IL-1β and IL-18 maturation. *With no treatment*, 1 Gy γ elicited significant upregulation of: CARD6, CCL-7, CCL-12 and TNF-α. This would be consistent with a general proinflammatory response, maturation of IL-1β and IL-18 and chemoattraction of monocytes and eosinophils but overall a modest response as compared to FITC treatment. Significant down regulation occurred for: IL-1β and TNF-α – similar ligands associated with general inflammation, cathepsin B and Bcl-2 associated with proteolytic processing and apoptosis. Taken together, these patterns are consistent with a very strong inflammatory response as observed in the ear swelling test which is further stimulated by irradiation at about the same time as the elicitation step.

DATA SET 7. *Activation events for dendritic cells in skin according to protocol 1.* Responses should reflect early activation of dendritic cells due to oxidative stress and compensation activities in skin acutely after sensitization under conditions when immune cell populations are maximally depleted by irradiation. Radiation elicited a complex pattern of positive and negative cell migration and chemoattractant activities under all three treatment conditions. *After FITC treatment:* 1 Gy γ elicited significant upregulation of: CCL-11 (strong responses), IL-16 and TLR-9 associated with chemoattraction of monocytes and eosinophils and proinflammatory regulation. Significant down regulation occurred for: chemoattractant CCL-3, -4 and -20 chemokines and C-lectin activities that would promote IL-1β and IL-18 maturation. *After dibutyl-phthalate + acetone vehicle treatment:* 1 Gy γ elicited significant upregulation of: CXCL2 (strong response), CCL2, -4, -17 and CXCL-1 associated with chemoattraction of polymorphonuclear leukocytes and monocytes as well as IL-10 which is anti-inflammatory and IL-12A made by dendritic cells following antigen stimulation and promotes T_{H1} pattern maturation of T_H cells. *With no treatment*, 1 Gy γ elicited significant upregulation of: CCL-12 (strong response) for chemoattraction of eosinophils and monocytes, CD2 (strong response) adhesion molecule on NK and T cells, and CD40 + CD40L which promote cell interactions between antigen presenting cells and T cells. Significant down regulation occurred for: CXCL-10 and -12 which are chemoattractant for eosinophils,

monocytes and natural killer cells and CCR9 which mediates T cell migration. Taken together, these patterns are consistent with complex regulation of cell migration to sites of antigen presentation with trends towards T_{H2} responses.

ADMINISTRATIVE ISSUES

We experienced many substantive administrative issues that delayed progress on this project and we are appreciative of the Department of Energy's patience as we adapted to these issues. They have been documented in detail in no-cost extension requests but are briefly re-iterated here. (1) Co-investigator and immunologist Lora Green, Ph.D. passed away unexpectedly early in the project which permanently impeded planning, experimental design and data interpretation. Some compensatory support came from Daila Gridley, Ph.D. who served as a consultant immunologist. (2) Our faculty and staff were transferred to a new department as part of a university department reorganization. Our primary association became with the Department of Basic Sciences rather than the Department of Radiation Medicine. This affected staff availability on the project and significantly altered our access to various equipment items and impacted their proper maintenance. Of particular concern were a flow cytometry system and ^{60}cobalt irradiator. We no longer had adequate access to a Becton Dickenson FACSCalibur flow cytometry system and made arrangements to occasionally outsource measurements which necessitated training of staff on the new system and validation of new reagents and protocols. Further, we had to schedule use of (and pay for) the shared equipment maintained by the other department causing a several month delay. (3) The Eldorado Model 6 ^{60}cobalt irradiator (required for all irradiation experiments) experienced a hydraulic system failure and needed repair. The Department of Radiation Medicine owned the equipment and we had to wait for its decisions regarding the repair. Accordingly, we suspended all non-essential activities on the project for several months until such repairs and recalibration were completed. (4) The Loma Linda University School of Dentistry was given the authority to take over half of our laboratory space and offices. This required our team to abruptly stop laboratory and office-based activities to oversee relocation to temporary office space, moving lab equipment, etc. in anticipation of School of Dentistry staff moving in to our current (School of Medicine) laboratory suite. We had to relocate, repair and recalibrate several critical pieces of equipment (e.g. Luminex multichannel bead immunoassay system) which were taken off-line for several months. A second move from temporary to permanent facilities was also required. Cumulatively, these issues led to nearly two years of delays and required continual revision and re-planning of the project's activities.

PRESENTATIONS AND PUBLICATIONS

Presentations

1) Poster. Low Dose Ionizing Radiation Modulates Immune Function: New Project Overview
Gregory Nelson, Cecile Favre, Tamako Jones, Mary Campbell-Beachler.
DOE Low Dose Program Investigators' Workshop
April 12-14, 2010, Washington, DC

2) Poster # 778. **Effects of Radiation on Adaptive Immunity: Contact Hypersensitivity Model**
Gregory Nelson, Tamako Jones, Mary Campbell-Beachler, Cecile Favre and Gregory Nelson
DOE Low Dose Program Investigators' Workshop
May 9, 2011, Bethesda, MD

3) Poster. Low dose ionizing radiation modulates adaptive immunity: contact hypersensitivity model
Tamako A. Jones, Mary Campbell-Beachler, Cecile J. Favre, Gregory A. Nelson
Radiation Research Society
Sept 30 - Oct 3, 2012, San Juan Puerto Rico

4) Invited talk, Symposium S1.2. Heavy Ion Radiobiology: Central Nervous System and Immune System.
Gregory A. Nelson
29th Annual meeting of the American Society for Gravitational and Space Research
Nov 5, 2013, Lake Buena Vista, Florida

5) Invited Lecture. Radiation & Immunology
Gregory Nelson, Ph.D
11th NASA Space Radiation Summer
June 16, 2014
Brookhaven National Laboratory, Upton, N.Y.

6) Invited Lecture. Radiation & Immunology
Gregory Nelson, Ph.D
2015 NASA Space Radiation Summer School
June 23, 2015
Brookhaven National Laboratory, Upton, N.Y.

Publications

Two manuscripts are in preparation for submission to Radiation Research
1) Effects of Low Dose Gamma Radiation on Adaptive Immunity.
I. Sensitization Phase of Contact Hypersensitivity
This paper will describe the methods used for evaluation of gamma irradiation on the sensitization phase of adaptive immunity and include data from S-4D and S+4D protocols.

2) Effects of Low Dose Gamma Radiation on Adaptive Immunity.
II. Elicitation/Recall Phase of Contact Hypersensitivity
This paper will describe gamma irradiation on the elicitation phase of adaptive immunity and include data from E-4D and E+8H with discussion of targeted microarray data.

Continuation Funding

Data acquired under this project has led to continuation funding for related investigations from the National Space Biomedical Research Institute as project #RE03701 in support of NASA cooperative agreement NCC9-5884. The project is entitled "Center for Research on Cardiac, Vascular and Acute Effects of Space Radiation". It will extend the work based on young female mice to older male animals and will compare proton radiation to gamma rays. It will also probe the role of the microvasculature in the adaptive immunity response.

SUMMARY

Contact Hypersensitivity (CHS) to the hapten FITC was used as a model for assessment of the effects of radiation on adaptive immunity. CHS is a system level immune response whose sensitization phase requires cell migration, activation, antigen presentation to naïve T cells in a permissive lymph node environment, T cell clonal expansion and redistribution to the periphery followed later by a second hapten exposure (elicitation) that triggers innate immunity events to result in an inflammatory response accompanied by infiltration of T cells, neutrophils and eosinophils. Any step in the highly orchestrated sequence of events could be more or less sensitive to irradiation. We attempted to identify the sensitive steps and assess the doses of radiation can effectively modify those steps in the dose range 2 cGy to 2Gy.

We established the necessary protocols for generating a robust delayed type hypersensitivity reaction and measuring its magnitude and associated biomarkers. This served as a model of cellular adaptive immunity that reports the efficacy of the immune system in responding to a foreign antigen and maintaining the potential to mount an amplified future response to challenge by the same immunogen. We found that the immune system is extremely robust in terms of post-radiation recovery at relatively early time points. This is reflected in the restitution of cell populations, stability of lymphoid organ sizes, restitution of the cytokine environment, etc.

Using the CHS model, adaptive immunity is found to be a robust process that is substantially resistant to the effects of radiation at doses below 50 cGy when investigated at 3 weeks after sensitization to hapten. We did not observe a hypothesized potentiation of immune activity at 20 cGy and below as reported for some other systems assessed at shorter post irradiation time points.

Overall, we determined that radiation had its greatest impact when administered during periods of critical cell-cell interactions at about 4 days after initial sensitization (S+4D) with hapten and 8 hours after (E+8H) a second exposure (elicitation). When radiation was used to deplete cells to minimum values (4 days post irradiation) during critical periods the effects were smaller (S-4D and E-4D). The minimum effective dose for reduction of the overall ear swelling inflammatory response was approximately 25 cGy.

Accompanying reductions in the overall responses were reductions in white blood cells and platelets in peripheral blood and spleen. These reductions were most evident at 50 cGy and above but reductions were observed for some conditions at as low as 5 cGy. Some irradiation conditions resulted in increases in blood platelet and eosinophil counts. Platelet numbers followed a complex dose response depending on the phase of the immune reaction: S-4D (down), S+4D (down), E-4D (up) and E+8H (no change). These responses suggest stimulated trafficking of platelets between compartments rather than just megakaryocyte depletion. Platelets are known to regulate CHS via P-selectins at the level of T cell extravasation at the elicitation site.

Mechanisms involving eosinophil activity *per se* or its regulation are implicated in the response. IgE levels and changes in eosinophil-related cytokines support this view. Immunoglobulin E levels, which are characteristic of contact hypersensitivity to FITC, were highly elevated after FITC treatment but not dramatically affected by irradiation except at 4 days after sensitization when T-cell:B-cell interactions are most robust. The marker enzyme eosinophil peroxidase was highly elevated in ear inflammatory infiltrates indicating eosinophil infiltration. Involvement of eosinophils is consistent with a T_{H2} polarized T-cell response and our results suggests that other T_{H2} responses, for example asthma, may become exacerbated after radiation exposure.

Histological analysis of ears showed a high level of cellular infiltrate and measurements of eosinophil peroxidase in ear lysates indicates the presence of eosinophils as reported in the literature. Cytokine profiles in unirradiated animals were consistent with a T_{H2}-polarized immune reaction and irradiation had its greatest differential effect on expression of three molecules (eotaxin/CCL11, IP-10 and MIG/CXCL-9) associated with chemoattraction of monocytes and eosinophils and three molecules (IL-1β, IL-6 and IFN-γ) associated with inflammation and T_{H2}-polarization.

Gene expression analysis showed that patterns modulated by irradiation were consistent with the sensitization reactions expected early after hapten administration with emphasis on attraction of

leukocytes to the site of application, establishing conditions for interaction of dendritic cells and T cells, activation of T cells along the T_{H2} pathway and the inflammation events required to facilitate cell chemotaxis and migration to the reaction site. There were modest trends towards more complete stages of sensitization reaction after hapten treatment and vehicle treatment alone trended towards earlier events consistent with an adjuvant role for dibutyl-phthalate. There were stronger trends towards cellular infiltration processes including involvement of adhesion to endothelial cells but weaker MHCII-related antigen presentation activity. Inflammatory cytokine activity showed mixed up and down-regulation. T cell activation events in lymph node were generally resistant to radiation and in untreated animals there was a strong downregulation response (34 of 89 genes) of enzymes involved in managing oxidative stress. Patterns were consistent with a very strong ear inflammatory response which was further stimulated by irradiation at about the same time as the elicitation step.

Taken together these data show that low levels of radiation (≈25 cGy) can inhibit adaptive immunity as measured in the mouse ear swelling test (≈ 30% reduction) especially in the time periods of about 4 days after initial exposure to a lipophilic, reactive hapten or about 8 hours after secondary exposure. The minimum effect was associated with about a 10-15% reduction in white blood cells. At doses between 1 and 2 Gy the levels of inhibition can reach 70% with up to 65% reduction in some white blood cells. Gene and cytokine expression analysis point to chemoattraction of monocytes and eosinophils to reaction sites as sensitive targets as well as antigen presentation events. From a regulatory perspective this suggests that in allergen-sensitive individuals, special care should be taken to avoid chemical exposure during operations when body extremities may be exposed to low dose radiation.