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Transcriptional Profiling of Non-Human Primate Lymphoid Organ Responses to Total-Body Irradiation

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Abstract

The global threat of exposure to radiation and its subsequent outcomes require the development of effective strategies to mitigate immune cell injury. In this study we explored transcriptional and immunophenotypic characteristics of lymphoid organs of a non-human primate model after total-body irradiation (TBI). Fifteen middle-aged adult, ovariectomized, female cynomolgus macaques received a single dose of 0, 2 or 5 Gy gamma radiation. Thymus, spleen and lymph node from three controls and 2 Gy (n = 2) and 5 Gy (n = 2) exposed animals were assessed for molecular responses to TBI through microarray-based transcriptional profiling at day 5 postirradiation, and cellular changes through immunohistochemical (IHC) characterization of markers for B and T lymphocytes and macrophages across all 15 animals at time points up to 6 months postirradiation. Irradiated macaques developed acute hematopoietic syndrome. Analysis of array data at day 5 postirradiation identified transcripts with 2-fold difference from control and a false discovery rate (FDR) of $P_{adj} < 0.05$ in lymph node (n = 666), spleen (n = 493) and thymus (n = 3,014). Increasing stringency of the FDR to $P < 0.001$ reduced the number of genes to 71 for spleen and 379 for thymus. IHC and gene expression data demonstrated that irradiated animals had reduced numbers of T and B lymphocytes along with relative elevations of macrophages. Transcriptional analysis revealed unique patterns in primary and secondary lymphoid organs of cynomolgus macaques. Among the many differentially regulated transcripts, upregulation of noncoding RNAs [*MIR34A* for spleen and thymus and *NEAT1* (*NCRNA00084*) for thymus] showed potential as biomarkers of radiation injury and targets for mitigating the effects of radiation-induced hematopoietic syndrome-impaired lymphoid reconstitution.

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INTRODUCTION

Accidental or malicious exposure to ionizing radiation can result in rapid lymphocyte cell death, inflammation, immune suppression or death. Alterations in lymphocyte subset homeostasis, reduced ratios of naïve-to-memory lymphocytes, decreased lymphocyte function, altered T-cell receptor repertoires and suppressed immunity have all been reported as acute and subacute responses to radiation (1–5) and as late effects in atomic bomb survivors (6–11). Little is known about molecular and cellular changes that occur within the primary and secondary lymphoid organs, including the thymus, spleen and lymph nodes, during acute radiation injury in primates.

Macaques are a proven animal model of radiation injury (4, 12), since that they are genetically closely related to humans, with similar physiology and hormonal fluctuations, allowing high translatability of data generated. The majority of non-human primate (NHP) work in the field of radiation has focused on male rhesus macaques, although some work has also been conducted in cynomolgus macaques (1, 12, 13). Macaques exposed to radiation experience precipitous drops in their peripheral blood lymphocyte counts, and with subsequent lymphoid reconstitution exhibit homeostatic aberrations, similar to the late effects of atomic bomb survivors (14–17). Despite efforts to assess the dynamics in immune cell kinetics, few studies have identified cellular and molecular changes at the tissue level in the immune system of macaques after total-body irradiation (TBI), and there is a notable lack of published studies detailing radiation effects on lymphoid organs in female cynomolgus macaques.

Previously published work by our group evaluating the effects of TBI on female cynomolgus macaques demonstrated marked alterations in targeted molecular and general histologic characteristics of thymus, spleen and lymph node (18). The current study extends that work through the use of transcriptional profiling and immunohistochemistry (IHC) to identify key molecular pathways in these same tissues that were altered in response to TBI, and to explore changes in cell populations, with a long-term goal of generating hypotheses for identifying potential biomarkers and/or therapeutic molecular targets that could be used to develop mitigators of radiation injury. To achieve this goal, we conducted microarray-based transcriptional profiling and IHC-based evaluation of immune cell populations in thymus, spleen and lymph node from these same animals that received 0, 2 or 5 Gy TBI. The results extend previous work in this model and provide a broad profile of the responses of lymphoid organs to single dose TBI.

MATERIALS AND METHODS

Animals and Study Design

The overall design of this study is described elsewhere (18). Briefly, 15 ovariectomized, adult female cynomolgus monkeys (*Macaca fascicularis*) of Indonesian origin with median age of 20 years (ranging 15–24 years) and median weight of 3.03 kg (ranging 2.56–4.59 kg) were housed at the Wake Forest School of Medicine (WFSM; Winston Salem, NC). At baseline, complete blood counts and serum chemistries performed on each NHP were within normal limits and animals tested negative for simian retroviruses. The animals were

randomly assigned to one of three groups receiving either 0 (n = 3), 2 (n = 6) or 5 (n = 6) Gy irradiation. Post-mortem examinations were planned at 5, 60 and 180 days postirradiation with two animals each of the 2 and 5 Gy groups assigned to each of these time points and three animals in the 0 Gy group. All animals survived to day 5, but not all animals survived to the end points scheduled at 60 and 180 days.

In this work, gene expression data are limited to the 0 Gy control (n = 3) animals and irradiated animals [2 Gy (n = 2), 5 Gy (n = 2)], which were necropsied on day 5 postirradiation. Immunohistochemical data are reported for all monkeys (n = 15).

Complete necropsy examination was performed on all animals either at the time of their humane end point or the end of the study. Complete animal demographic information including animal survival data was published previously [see Table 1 and ref. (18)]. The WFSM is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All experimental procedures were approved by the Wake Forest University Institutional Animal Care and Use Committee and the Guide for Care and Use of Laboratory Animals (19).

Diet

Prior to study initiation, the animals had consumed diets formulated at WFSM, which contained milk and soy proteins with varying soy isoflavone content (20, 21). Animals were transitioned initially to a laboratory chow diet (Purina LabDiet® Monkey Diet 5037, Richmond, IN) for three months and then were switched to a diet mimicking the nutritional intake of a typical Western diet for a minimum of two weeks prior to irradiation. The Western diet was produced in-house and contained 34% of calories as fat, 18% of calories as protein and 48% of calories as carbohydrates, and a moderately atherogenic cholesterol content of 0.20 mg/calorie. Protein was animal-sourced (casein, lactalbumin). Fats were 40% saturated, 34% monounsaturated and 26% polyunsaturated fatty acids. The animals had unlimited access to water and for environmental enrichment they were supplemented with fresh fruits and vegetables.

Irradiation

Non-human primates were sedated with ketamine (10 mg/kg) (Vedco Inc., St. Joseph, MO) and sequentially irradiated with two opposed lateral fields of 6-MV X rays at a dose rate of 60 cGy/min using a Varian 2100C linear accelerator (Varian Medical Systems, Inc., Palo Alto, CA) for single TBI doses of 0 (sham treatment), 2 or 5 Gy to the mid-plane. The radiation source was 120 cm from the skin.

Clinical Monitoring and Husbandry

Beginning on the day of irradiation, animals were administered prophylactic antibiotics including both enrofloxacin (5 mg/kg every 12 h; Bayer HealthCare LLC, Shawnee Mission, KS) and ceftazidime (30 mg/kg every 12 h; Patterson Veterinary, Devens, MA). Antibiotics were continued daily in all animals for up to 52 days after TBI or sham TBI. All monkeys were monitored daily for signs of radiation-induced illness including vomiting, diarrhea, weight loss, hemorrhage and alopecia. Using the methods of Uckun *et al.* (22) as modified

from the Children's Cancer Group Clinical Toxicity Criteria, monkeys were evaluated for signs of hematologic failure or infection. The animals received supportive care as needed including parenteral fluids and supplemental nutrition. If animals became moribund or reached grade 4 toxicity, as previously defined in primate trials at WFSM (23, 24), they were humanely euthanized by intravenous pentobarbital overdose.

Transcriptional Profiling of Lymphoid Tissue

At necropsy, sections of spleen, thymus and inguinal lymph node tissue samples were preserved in RNA_{later} (Applied Biosystems, Foster City, CA) followed by storage at -80°C . RNA was extracted from tissues using TRI Reagent[®] (Applied Biosystems[®], Foster City, CA), RNA content and purity was determined using a NanoDrop[™] 2000 (Thermo Scientific[™], Waltham, MA) and RNA quality was determined by measuring the RNA integrity number (RIN) with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA with RIN > 8.0 was used for downstream applications. Microarray procedures were performed by Cogenics (Morrisville, NC). Briefly, 1 μg of total RNA was assayed using GeneChip[™] Rhesus Macaque Genome Arrays (Affymetrix[®] Inc., Waltham, MA) and the GeneChip One-Cycle Target Labeling Kit (Affymetrix), according to the manufacturer's recommended protocol. This microarray included 52,024 probe sets for approximately 47,000 transcripts. The labeled RNA was fragmented, hybridized to arrays for 17 h, washed and scanned. Scanned images were analyzed using GeneChip Operating Software (Affymetrix) to produce transcript intensity data.

Immunohistochemistry

Sections of spleen, thymus and axillary lymph nodes were collected at the time of necropsy and fixed in 4% paraformaldehyde. After fixation for a minimum of 24 h, tissues underwent routine processing, were embedded in paraffin, sectioned at 4 μm , stained with either hematoxylin and eosin (H&E) or primary antibodies for IHC and evaluated by light microscopy by one of two veterinary pathologists (JMC and DLC) (25). To evaluate the effects of radiation on the immune system, tissues from affected and clinically healthy control animals were stained for T cells (anti-CD3; Dako Inc., Carpinteria, CA, A0452, clone F7.2.38, 1:300 dilution), B cells (anti-CD20cy; Dako Inc., M0755, clone L26, 1:1500) and macrophages (Sigma-Aldrich[®] LLC, St. Louis, MO, 279M-16, clone HAM56, 1:50). All IHC was performed using a Leica Bond RX System (Leica Biosystems Inc., Buffalo Grove, IL). Vector[®] Red (Burlingame, CA) was the chromogen and tissues were counterstained with Mayer's hematoxylin. Slides were imaged using a BX61VS Olympus microscope (Olympus[®] Inc., Waltham, MA) and IHC was quantified using the custom programmable Visiopharm Integrator System (VIS) version 6.5.0.2303 (Hoersholm, Denmark) image analysis software.

Analyses

RNA data analysis. The transcript intensity data from the microarrays were analyzed using GeneSifter[®] Analysis Edition software (PerkinElmer[®] Inc., Waltham, MA). The expression profiling data described in this article are limited to the 0 Gy control and irradiated animals that were necropsied day 5 postirradiation [see Table 1 and ref. (18)]. Day 5 postirradiation transcript intensity data were RMA-normalized, converted to log₂ scale and screened for

heterogeneity among samples and groups. Sixty- and 180 day postirradiation data were not collected as planned due to unexpected animal deaths [see Table 1 and ref. (18)]. For a transcript to be considered differentially expressed between treatment groups, we set the cutoff filter at a fold change greater than two. We tested the data with supervised analysis of variance (ANOVA). *P* values were corrected for multiple comparisons using the Benjamini-Hochberg method (26, 27) to control the false discovery rate (FDR) to $P < 0.05$. Principal components analysis (PCA), pattern navigation, cluster analysis, heat mapping and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed on filtered data subsets and shared genes between all tissues using Genesifter and the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 (28, 29). Euclidean distances representing the numeric difference between treatment vectors were calculated as part of hierarchical clustering dendrograms using average linkage (Supplementary Fig. S1; <http://dx.doi.org/10.1667/RR15100.LS1>).

IHC data analysis. IHC data from all animals (5, 60 and 180 days, as well as unscheduled) were included in the analyses. Customized algorithms were developed using VIS[®] software for quantification of the total percentage area of positive IHC signal in each lymphoid tissue section. Data were pooled from animals across all time points for each tissue in the different irradiation groups and analyzed using ANOVA with Bonferroni correction for multiple comparisons. Due to limitations in the quantity of thymic tissue available at the time of necropsy, CD3 was not quantified for thymus in one monkey that received a 5 Gy dose of radiation, CD20 was not quantified for thymus in one monkey that received a 2 Gy dose of radiation and one that received a 5 Gy dose of radiation, and HAM56 was not quantified for thymus in one animal receiving a 2 Gy dose and two animals receiving a 5 Gy dose of radiation. $P < 0.05$ was considered significant and all data were analyzed using GraphPad Prism version 7 software (La Jolla, CA).

RESULTS

Effects of TBI on Transcriptional Profiles

Rhesus-specific gene microarrays included 52,024 probe sets for approximately 47,000 transcripts were used to evaluate gene expression signatures in the lymph node, spleen and thymus at days 0, 2 and 5 post-TBI. A total of 666 differentially expressed transcripts were identified in lymph node tissue (>2 -fold change, $P_{\text{adj}} < 0.05$). The PCA of these 666 transcripts showed high clustering within treatment groups (Fig. 1A). To determine the transcripts with the greatest difference between groups, the filter was adjusted to >6 -fold change (Table 1). The 666 lymph node transcripts with >2 -fold change were analyzed for KEGG pathways with DAVID and ranked by fold enrichment (Table 2). The top four KEGG pathways identified were primary immunodeficiency, African trypanosomiasis and B-cell receptor signaling pathway and hematopoietic lineage.

A total of 493 differentially expressed transcripts were identified in spleen (>2 -fold change, $P_{\text{adj}} < 0.05$). The PCA showed significant clustering of transcripts by exposure groups (Fig. 2B). To determine the transcripts with the greatest difference between groups the filter was adjusted to >6 -fold change (Table 3). The following upregulated transcripts were identified after irradiation: *MIR34A*, *TYMS* and *DDB2*. The following downregulated transcripts were

identified after irradiation: *HBB*, *MAMU-DQA1*, *TAC3*, *FCRL3*, *MS4A1*, *BCL11A*, *CD19*, *FCMR*, *IGHG1*, *AFF3*, *CXCR5*, *TLR10*, *FCRLA*, *TACSTD2*, *RFLNB*, *CD79A*, *LTB* and *WFDC21P*. The 493 splenic transcripts with >2-fold change were analyzed for KEGG pathways using DAVID and ranked by fold enrichment (Table 4). The top four KEGG pathways identified were primary immunodeficiency, intestinal immune network for IgA production, hematopoietic cell lineage and B-cell receptor signaling pathway.

A total of 3,014 differentially expressed transcripts were identified in the thymus (>2-fold change, $P_{adj} < 0.02$). Similar to spleen and lymph node, PCA showed high clustering of transcripts by exposure group (Fig. 1C). To determine the transcripts with the greatest difference between groups the filter was adjusted to > 6-fold change (Table 5). The following upregulated transcripts were identified after irradiation: *KRT4*, *MIR34A*, *NEAT1*, *CEA-CAM5*, *SAA2*, *RNASE1*, *MGP*, *S100P*, *PTG1*, *HSPB1*, *APOD*, *FST*, *HOPX*, *TAGLN*, *RERGL*, *EPHA3*, *ANXA1*, *GSN*, *EFEMP1*, *IGHG1*, *PRELP*, *CP*, *COCH* and *KRT76*. The following downregulated transcripts were identified after irradiation: *UHRF1*, *TOP2A*, *NUSAP1*, *TMSB15A*, *CCNB2*, *E2F2*, *ARPP21*, *KIF14*, *DEPDC1B*, *COX8C*, *KIF15*, *CDK1*, *BUB1*, *CD1A*, *CD1B*, *KIF11*, *ST3GAL6*, *CKAP2*, *RAG1*, *SH2D1A*, *SLIT1*, *SCGB3A1*, *FOXM1*, *RAB11FIP4*, *CYP2U1*, *SPOCD1*, *CD1E*, *MNS1*, *ELOVL4*, *CD8A*, *CD8B*, *CHRNA3*, *CDCA7*, *HIST1H2AD*, *CYP2U1*, *RORC*, *KCNJ10*, *GCSAM*, *KIF23*, *CCR9*, *ANLN*, *CD1C*, *MOB1B*, *TFDP2*, *TMEM171*, *CAMK4*, *CDK6*, *UBE2T*, *ATAD2*, *BCL2L1*, *LEF1*, *TFDP2*, *TCRB*, *BCL11B*, *DLEU2*, *PITPNM2*, *MPHOSPH9*, *ZNF711*, *SLC14A1*, *CD3G*, *THEMIS*, *AFF3* and *SMC3*. The 3,214 thymic transcripts with >2-fold change were analyzed for KEGG pathways with DAVID and ranked by fold enrichment (Table 6). The top four KEGG pathways identified were DNA replication, complement and coagulation cascades, ECM-receptor interaction and cell cycle.

A total of 77 differentially expressed transcripts were shared among the spleen, lymph node and thymus (Fig. 2). These transcripts were analyzed for KEGG pathways and ranked by fold enrichment. The three KEGG pathways identified were the p53 signaling pathway (fold enrichment: 16.2; genes: *BBC3*, *CDKN1A* and *ZMAT3*), HIF-1 signaling pathway (fold enrichment: 12.0; genes: *CDKN1A*, *ENO2* and *PRKCB*) and measles (fold enrichment: 9.8; genes: *BBC3*, *TNFRSF10B* and *PRKCQ*).

Immunohistochemistry

The T- and B-lymphocyte and macrophage patterns in thymus, spleen and lymph node were evaluated by IHC. As expected, CD3 IHC resulted in staining of T cells in the periarteriolar lymphatic sheath in spleen, paracortex of lymph node and cortex and medulla of thymus. Likewise, CD20 IHC stained B cells in follicles of spleen and lymph node, and scattered throughout the medulla of thymus. Finally, HAM56 IHC staining was less intense in these tissues, but did stain scattered macrophages in follicles of spleen and lymph node and cortical sinus and medulla of lymph node. Control monkey thymus had little to no HAM56 staining.

In lymph nodes from animals that received 2 and 5 Gy TBI, a significant reduction ($P < 0.05$) of CD3⁺ T cells was observed (Figs. 3A–C and 4B). Similarly, there was significant ($P < 0.05$) reduction in CD20⁺ B cells in spleens from monkeys that received 2 and 5 Gy

exposure and in lymph nodes of monkeys that received a 5 Gy dose (Figs. 3D–I and 4D and E). The limited number of sections available for HAM56⁺ macrophage immunoreactivity did not allow for a useful statistical assessment of differences (Fig. 4G–I). No significant differences between groups were identified in thymus for any of the three immunomarkers (Fig. 4C, F and I), although there was a trend toward lower CD3⁺ staining in the spleen of monkeys exposed to 5 Gy compared to 0 Gy controls (Fig. 4A, $P = 0.07$). As an alternative method of comparison, total mass of T cells within the spleen of each animal was estimated by multiplying spleen weight obtained at necropsy by percentage CD3⁺ IHC area obtained using quantitative IHC. These estimated masses were compared using ANOVA with Bonferroni correction for multiple comparisons. There was significantly less CD3⁺ mass in the spleen from animals that received either 2 or 5 Gy vs. 0 Gy dose ($P = 0.01$, data not shown). Similar analyses were done for CD20 and HAM56 and results were not different from data shown in Fig. 4.

Given the marked reduction in both T and B cells in secondary immune organs, we sought to determine if one population of lymphocytes was preferentially decreased over the other within the lymph node and spleen. To make this determination, the ratio of CD3⁺ to CD20⁺ IHC area was calculated for these tissues in each animal and compared using ANOVA with Bonferroni correction for multiple comparisons. As shown in Fig. 5A, there was a significant increase ($P < 0.05$) of the CD3⁺:CD20⁺ ratio in lymph node at 5 days after 5 Gy irradiation compared to 0 Gy control. This finding suggests greater loss of B cells compared to T cells at this early time point. The CD3⁺:CD20⁺ ratio was not significantly different from 0 Gy control animals at later times postirradiation.

DISCUSSION

The thymus, spleen and lymph nodes are primary and secondary lymphoid organs that are essential to the formation and maturation of lymphocytes necessary for acquired immunity. The effects of radiation on these organs can be both acute and long-term in individuals, manifesting as either life-threatening sepsis or impaired immunity as a result of inadequate lymphoid reconstitution.

Our study suggested a greater decrease in B cells than T cells in lymph node at 5 days postirradiation, consistent with the kinetics of relative loss of circulating CD20⁺ B cells to CD3⁺ T cells in male macaques after TBI reported elsewhere (14). Variable sensitivity of lymphocytes across immune organs was also identified. Although B- and T-cell populations had not recovered in the lymph node by 20 days after TBI (data not shown), the ratio of B to T cells at 20 days had returned to that seen in lymph node of 0 Gy control monkeys. It is possible that T-cell death was slower than B-cell death with both populations reaching approximately the same decline. Alternatively, B-cell populations may start recovering by 20 days due to repopulation of B cells in the lymph node. Regardless, these findings are consistent with several other studies in mice that demonstrated a higher sensitivity of B cells compared to T cells (30–33).

The highest ratio of CD3⁺ T cells to CD20⁺ B cells occurred at approximately 5 days in the lymph node. In general, a germinal center response to a particular antigen occurs over a

seven-day period. This is a well-orchestrated process between T-follicular helper cells and B cells. Total-body irradiation is followed by a period in which the host is susceptible to death from sepsis from opportunistic infections due to bone marrow suppression and acute gastroenteritis. Our data suggest that at these doses TBI has the greatest effect on T and B cells in the lymph node during a time of great risk when early; and late initiation phases of germinal center formation would be helpful in battling radiation-induced gastrointestinal bacterial translocation. Our findings suggest that the cellular dynamics in lymph nodes are severely compromised at one of the most critical times necessary for mounting an immune response to pathogens.

Several transcripts from each tissue were associated with KEGG pathways related to hematopoietic cell lineage and primary immunodeficiency, most likely due to radiation-induced loss of lymphocyte populations. A number of the transcripts were dramatically downregulated, including those related to B cells including IgHG1 and MS4A1 in both spleen and lymph node, which was not surprising given the loss of B cells in these tissues, as shown by IHC.

MIR34A was one of the most highly upregulated transcripts observed in lymphoid tissues. In monkeys irradiated with 5 Gy, *MIR34a* was 22-fold higher in spleen, 8-fold higher in lymph node and 10-fold higher in thymus compared to controls. *MIR34a* is a highly conserved member of the mir34 family of noncoding microRNAs. In addition to its role as a tumor suppression miRNA, recently published studies have shown this miRNA represses Foxp1 activity, thus disrupting B-lymphocyte development (34). Furthermore, published studies of irradiated mice have shown that p53 targets *MIR34A* leading to apoptosis (35–38). The very high levels observed in TBI-associated lymphoid tissues here may be associated with lymphocytic p53-mediated apoptosis. Therefore, this particular miRNA may serve as a marker of dosimetry (39, 40) or possibly a target for mitigating the effects of radiation injury.

NEAT1, (aka Nuclear Paraspeckle Assembly Transcript 1, noncoding RNA 00084, *NCRNA00084*) is a 3.2-kb-long noncoding RNA that is a critical component of paraspeckle nuclear bodies. Thymic NEAT1 was upregulated up to 8-fold due to irradiation. It appears to be induced by DNA damage and genotoxic stress, is upregulated by p53, and may act as a transcriptional regulator of several genes associated with cell survival and cancer progression, including lymphocytic leukemias (41, 42).

Thymidylate synthetase (*TYMS* or TS), one of the most upregulated transcripts from spleen and lymph node, catalyzes the methylation of deoxyuridylate to deoxythymidylate and maintains the thymidine-5-prime monophosphate pool critical for DNA replication and repair (43). Increases in *TYMS* in response to radiation suggest that cells present within these tissues were actively repairing radiation-induced DNA damage.

Toll-like receptor 10 (*TLR10*) was one of the most differentially decreased transcripts in irradiated spleen and lymph node. Studies have shown that *TLR10* may play a role in the pathogenesis of lymphoma (44) and is expressed by IgM⁺IgD⁺CD27⁺ B cells (45) suggesting a role in development and maintenance of this subpopulation of B cells in

humans. Marginal zone B cells are generated in the spleen and are important for early defense against bacterial pathogens such as *Streptococcus pneumoniae*, by responding to capsular polysaccharides (46). Published studies of mice have shown enhanced radiosensitivity of marginal zone B cells relative to other B-cell populations (47, 48). The TLR10 reductions in lymph node and spleen may reflect greater radiosensitivity of marginal zone B cells. Loss of this subset of B cells would place individuals exposed to radiation at increased risk of invasive infection by encapsulated bacteria. Interestingly, one 5 Gy irradiated monkey in this study succumbed to sepsis and had bacterial colonies in the lungs despite administration of prophylactic antibiotics (18).

Hemoglobin subunit beta (*HBB*) was the most downregulated transcript among differentially expressed genes in the spleen (by 62-fold) and lymph node (by 51-fold) in monkeys exposed to 5 Gy TBI. *HBB* encodes the beta hemoglobin subunit, which interacts with the *HBA* alpha subunit to form the hemoglobin molecule in erythrocytes. It is possible that the diminution of *HBB* expression is reflective of reduced tissue content of viable erythrocytes, since *HBB* has been used as a biomarker for blood (49). The potential function of this transcript in normal spleen and lymph node tissue is not clear, although expression of *HBB* in the absence of *HBA* expression occurs, and reductions in *HBB* expression in tumor cells have been associated with increased apoptotic responses to reactive oxygen species (50). *BCL11A*, another transcript, which was significantly decreased in spleen from irradiated monkeys, has been linked to *HBB* expression. *BCL11A* encodes a zinc finger transcription factor, which interacts with the HMG-box-containing transcription factor *SOX6* to facilitate switching of expression of fetal hemoglobin subunit gamma to *HBB* in adult human erythroid cells (51). Yu and colleagues describe *BCL11A* as having a binding site 85 kb upstream of the *HBB* gene in the mouse (52). They also demonstrated that *BCL11A* is essential for early B cells and common lymphoid progenitor cells to survive and proliferate. The reduced expression of *HBB* might be due to the decreased *BCL11A*, which might be downregulated due to apoptotic signaling triggered by radiation-induced DNA damage. Alternatively, reduced *HBB* and *BCL11A* may be related to diminished viable erythrocytes in irradiated tissues (53). Further investigation of the origin of decreases in these transcripts is warranted. It is possible that therapies aimed at enhancing *BCL11A* activity may mitigate radiation-induced lympho-depletion, particularly in B cells.

In our previously published study (18), we showed a decrease in thymopoiesis in irradiated monkeys as evidenced by decreased T-cell receptor excision circle (TREC) output. In this study transcriptional profiling revealed that the transcription factor *E2F2*, a target of retinoblastoma protein responsible for controlling the G₁-S phase of the cell cycle was one of the most differentially decreased transcripts in thymus (21-fold lower in the 5 Gy irradiated group). In contrast, Murga *et al.* (54) reported that *E2F2*^{-/-} knockout mice had enhanced T-cell proliferation and accumulated autoreactive effector/memory T lymphocytes, suggesting that *E2F2* represses transcriptional activity necessary for normal S-phase entry. A potential explanation for decreased *E2F2* levels in thymus of irradiated monkeys is that lymphocyte progenitor cells downregulate expression of this gene as part of a proliferative response. However, the response is not enough to overcome massive loss of progenitor cells; therefore, TREC output is decreased overall despite the upregulation of proliferation by the few surviving progenitor cells.

We previously demonstrated reductions in thymus weight, thymopoiesis and bone marrow cellularity in these animals, and qRT-PCR data demonstrated alterations in T-cell markers (reduced CD3 in thymus and spleen) and macrophage markers (increased CD68), suggestive of increased macrophage/T-cell ratios in lymphoid tissues from irradiated animals (18). IHC immunoreactivity for the macrophage marker HAM56 also tended to be elevated in these tissues in the current study, although not significantly different from controls, perhaps due to low numbers of animals, and undersampling of tissues by IHC (single sections of tissues) versus PCR data, which represent a larger tissue sample. In addition, the exact epitope and gene product responsible for HAM56 immunoreactivity has yet to be identified.

Strengths of the current study include the use of a NHP model, the simultaneous exploration of gene expression and cellular marker IHC in multiple lymphoid organs, the use of two different radiation doses, which were carefully monitored and administered and a well-established protocol for monitoring animal health. Limitations of the study relate primarily to the small number of animals and individual cells, although the strong responses of lymphoid tissues to radiation exposure allowed us to evaluate global responses for hypothesis generation. The 2 and 5 Gy radiation doses were determined to be sublethal based on previous work in male rhesus monkeys and were selected as a starting point. Assessing a broader range of doses which would be likely to occur in a malicious event was beyond the scope of this project. The use of ovariectomized female cynomolgus monkeys, as a model, is both a strength and a weakness in the current study. This paradigm has been useful in modeling surgical menopause in women, and many studies have demonstrated that monkeys respond to ovariectomy, and to exogenous hormone regimens, with remarkable similarity to that observed in women. Nevertheless, they represent a somewhat restricted population, and since we did not simultaneously study males or premenopausal females, we cannot make gender-based comparisons or draw conclusions relating to relative susceptibilities to radiation exposure.

In summary, cellular changes and unique gene expression profiles in primary and secondary lymphoid organs were identified in female cynomolgus macaques after TBI. Many of the targets identified correlated with loss of immune cells during a critical time of immune function after TBI and potential pathogen exposure. These data are useful for generating hypotheses for acute responses to radiation in lymphoid organs and for potential identification of targets for pharmacologic preservation of immunity after radiation exposure, developing strategic vaccine regimens and identifying mitigating agents for treatment after radiation exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

PCA analysis of differentially expressed transcripts, obtained 5 days after 0, 2 and 5 Gy irradiation, in lymph node (panel A), spleen (panel B) and thymus (panel C). Filter was set at fold change > 2 , $P_{\text{adj}} < 0.05$. These data show distinct differences between irradiated and control groups. For all tissues, the control and irradiated groups clustered separately from each other, and the vectors of the 2 and 5 Gy irradiated groups differed from each other, indicative of a dose response.

FIG. 2.

Venn diagram showing shared differentially expressed transcripts among lymph node, spleen and thymus tissues. Filter was set at fold change > 2 , $P_{\text{adj}} < 0.05$. A total of 77 differentially expressed transcripts were shared among the three tissue types.

FIG. 3.

Photomicrographs of IHC stains of lymphoid tissues from control and irradiated animals. Panels A–C: IHC staining for CD3 in lymph node from 0, 2 and 5 Gy irradiated animals, respectively. Panels D–F: IHC staining for CD20 in lymph node from 0, 2 and 5 Gy irradiated animals, respectively. Panels G–I: IHC staining for C20 in spleen from 0, 2 and 5 irradiated Gy animals.

FIG. 4.

Percentage area positive by IHC in tissues at 5 days after 0, 2 and 5 Gy irradiation. Panels A-C: Comparison of CD3⁺ positive staining in spleen, lymph node and thymus, respectively. Panels D-F: Comparison of CD20 staining in spleen, lymph node and thymus, respectively. Panel G-I: Comparison of HAM56 staining in spleen, lymph node and thymus, respectively. Data were analyzed using ANOVA with Bonferroni correction for multiple comparisons. Groups with differing letters (a or b) are significantly different from one another ($P < 0.05$). Data are mean $\pm$ standard deviation (SD).

FIG. 5.

Ratio of CD3 to CD20 percentage area positive by IHC in tissues at 5, 15–20 or 53–165 days after 0, 2 and 5 Gy. Panel A: Comparison of CD3:CD20 ratio in lymph node. Panel B: Comparison of CD3:CD20 ratio in spleen. Data were analyzed using ANOVA with Bonferroni correction for multiple comparisons. Groups with differing letters (a or b) are significantly different from one another ($P < 0.05$). Data are the mean $\pm$ SD.

TABLE 1

The Top Differentially Expressed Transcripts in Lymph Nodes of Irradiated Monkeys

Gene	0 Gy	2 Gy ^a	5 Gy ^a
Increased			
CFB	1	4.69	14.20
FOS	1	3.86	15.32
MIR34A	1	6.38	8.25
TYMS	1	4.71	7.48
SFRP4	1	3.74	6.07
Decreased			
HBB	1	-35.81	-51.71
XCL1	1	-11.00	-5.04
CNR1	1	-6.16	-8.85
FCRL3	1	-4.13	-7.94
IGHG1	1	-4.09	-7.86
FCRLA	1	-3.59	-6.04
MS4A1	1	-3.01	-6.17
TLR10	1	-2.69	-6.87
SMC3	1	-9.71	-1.27

^aFold changes are shown relative to 0 Gy control group. The threshold was fold change > 6 and $P_{\text{adj}} < 0.05$.

TABLE 2
Top Four KEGG Pathways of Differentially Expressed Transcripts in Lymph Nodes of Irradiated Monkeys

Pathway	Fold enrichment	P	Genes	Fold change 2 Gy ^a	Fold change 5 Gy
Primary immunodeficiency	10.6	0.0031	BLNK	-2.38	-2.23
			BTX	-1.69	-2.08
			CD19	-2.91	-5.34
			CD8A	-2.13	-2.81
			CD8B	-2.78	-3.28
African trypanosomiasis	7.5	0.015	FAS	2.01	2.47
			HBB	-35.84	-51.71
			IDO1	3.49	3.00
			PRKCB	-1.65	-2.28
			BLNK	-2.38	-2.23
B cell receptor signaling pathway	5.9	0.0011	BTX	-1.69	-2.08
			CD19	-2.91	-5.34
			CD72	-2.30	-2.75
			CR2	1.01	-2.04
			FOS	3.86	15.32
Hematopoietic cell lineage	5.7	0.00047	RAC2	-1.69	-2.24
			CD14	1.69	2.04
			CD19	-2.91	-5.34
			CD37	-2.35	-3.74
			CD8A	-2.13	-2.81
			CD8B	-2.78	-3.28
			CR2	1.01	-2.04
			FCGR1A	1.32	2.15
			MS4A1	-2.48	-5.83

^aFold changes relative 0 Gy control group.

TABLE 3

The Top Differentially Expressed Transcripts in Spleen of Irradiated Monkeys

Gene	0 Gy	2 Gy ^a	5 Gy ^a
Increased			
MIR34A	1	10.68	22.90
TYMS	1	3.05	8.04
DDB2	1	2.89	6.14
Decreased			
HBB	1	-37.55	-62.23
MAMU-DQA1	1	-17.51	-108.59
TAC3	1	-9.81	-10.46
FCRL3	1	-6.17	-14.13
MS4A1	1	-4.99	-17.23
BCL11A	1	-5.19	-14.77
CD19	1	-4.77	-13.34
FCMR	1	-4.76	-12.55
IGHG1	1	-4.23	-8.86
AFF3	1	-4.13	-8.86
CXCR5	1	-4.48	-7.46
TLR10	1	-3.19	-9.01
FCRLA	1	-4.41	-6.49
TACSTD2	1	-4.12	-6.35
RFLNB	1	-3.61	-6.23
CD79A	1	-3.57	-6.14
LTB	1	-3.11	-6.00
WFDC21P	1	-2.17	-6.43

^aFold changes are shown relative to 0 Gy control group. The threshold was fold change > 6 and $P_{\text{adj}} < 0.05$.

TABLE 4
Top Four KEGG Pathways of Differentially Expressed Transcripts in Spleens of Irradiated Monkeys

Pathway	Fold enrichment	P	Genes	Fold change 2 Gy ^a	Fold change 5 Gy
Primary immunodeficiency	10.6	0.0011	AICDA	-2.46	-3.79
			CD19	-4.77	-13.34
			IL2RG	-1.52	-2.14
			IL7R		
Intestinal immune network for IgA production	9.5	0.00036	PTPRC	-1.63	-2.61
			AICDA	-2.46	-3.79
			CXCR4	-1.93	-3.10
			ITGB7	-1.51	-2.54
			IL6	-3.72	-5.40
			HLA-DOB	-4.14	-4.95
Hematopoietic cell lineage	8.4	9.6E-6	HLA-DPA1	-2.10	-1.35
			CD19	-4.77	-13.34
			CD37	-2.40	-5.56
			CD5	-1.38	-2.23
			CD7	-1.87	-2.89
			CR1	-3.11	-4.12
			CR2	-6.43	-16.19
			IL6	-3.72	-5.40
			IL7R	-1.81	-3.35
			MS4A1	-4.27	-12.34
B cell receptor signaling pathway	7.7	0.00025	CARD11	-1.81	-2.22
			CD19	-4.77	-13.34
			CR2	-6.43	-16.19
			NFATC2	-1.45	-2.58
			RAC2	-1.70	-2.27
			SYK	-1.74	-2.85
			VAV2	-2.49	-2.41

^aFold changes relative to 0 Gy control group.

TABLE 5

The Top Differentially Expressed Transcripts in Thymus of Irradiated Monkeys

Gene	Increased			Decreased							
	0 Gy	2 Gy ^a	5 Gy ^a	Gene	0 Gy	2 Gy ^a	5 Gy ^a	Gene	0 Gy	2 Gy ^a	5 Gy ^a
KRT4	1	26.92	16.08	UHRF1	1	-9.28	-53.07	CDCA7	1	-3.25	-11.22
MIR34A	1	7.26	10.32	TOP2A	1	-9.11	-36.81	HIST1H2AD	1	-6.54	-5.73
NEAT1	1	8.01	7.14	NUSAP1	1	-9.59	-27.20	CYP2U1	1	-4.09	-8.80
CEACAM5	1	8.20	6.78	TMSB15A	1	-9.52	-25.91	RORC	1	-4.34	-8.13
SAA2	1	8.20	5.97	CCNB2	1	-7.67	-24.53	KCNJ10	1	-5.29	-6.33
RNASE1	1	5.18	8.65	E2F2	1	-7.96	-21.63	GCSAM	1	-3.77	-8.71
MGP	1	5.38	8.00	ARPP21	1	-7.70	-20.51	KIF23	1	-4.41	-7.43
S100P	1	6.77	5.25	KIF14	1	-7.31	-20.62	CCR9	1	-3.33	-9.80
PTGE1	1	6.12	5.81	DEPDC1B	1	-7.25	-17.74	ANLN	1	-4.30	-7.46
HSPB1	1	5.56	6.20	COX8C	1	-8.12	-13.50	CD1C	1	-3.77	-8.31
APOD	1	4.46	7.44	KIF15	1	-7.00	-15.56	MOB1B	1	-3.71	-8.26
FST	1	6.54	4.95	CDK1	1	-6.20	-16.09	TFDP2	1	-3.84	-7.86
HOPX	1	6.47	5.00	BUB1	1	-4.78	-18.55	TMEM171	1	-4.08	-6.95
TAGLN	1	5.98	6.00	CD1A	1	-5.92	-14.48	CAMK4	1	-3.47	-8.06
RERGL	1	4.06	7.88	CD1B	1	-6.00	-12.26	CDK6	1	-3.51	-7.89
EPHA3	1	3.79	8.44	KIF11	1	-6.12	-10.75	UBE2T	1	-3.97	-6.92
ANXA1	1	6.23	5.10	ST3GAL6	1	-5.90	-10.52	ATAD2	1	-3.73	-7.27
GSN	1	4.77	6.55	CKAP2	1	-4.68	-13.11	BCL2L1	1	-3.60	-7.40
EFEMP1	1	4.86	6.24	RAG1	1	-6.75	-8.56	LEF1	1	-3.06	-8.60
IGHG1	1	3.20	8.75	SH2D1A	1	-4.87	-11.24	TFDP2	1	-3.76	-6.87
PRELP	1	4.01	6.83	SLIT1	1	-6.43	-8.51	TCRB	1	-3.61	-6.97
CP	1	2.10	6.83	SCGB3A1	1	-6.97	-7.50	BCL11B	1	-2.40	-10.43
COCH	1	1.35	8.18	FOXM1	1	-5.25	-9.63	DLEU2	1	-3.79	-6.55
KRT76	1	6.07	1.62	RAB11FIP4	1	-4.80	-10.12	PITPNM2	1	-3.80	-6.46
				CYP2U1	1	-4.75	-10.16	MPHOSPH9	1	-3.40	-6.54
				SPOCD1	1	-4.81	-9.62	ZNF711	1	-3.14	-6.26
				CD1E	1	-5.31	-8.64	SLC14A1	1	-2.47	-7.88

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Increased						Decreased					
Gene	0 Gy	2 Gy	5 Gy ^a	Gene	0 Gy	2 Gy	5 Gy ^a	Gene	0 Gy	2 Gy	5 Gy ^a
				MNS1	1	-6.03	-7.54	CD3G	1	-2.86	-6.79
				ELOVL4	1	-4.50	-9.20	THEMIS	1	-3.10	-6.18
				CD8A	1	-4.54	-8.68	AFF3	1	-2.81	-6.81
				CD8B	1	-4.27	-9.23	SMC3	1	-8.58	-1.90
				CHRNA3	1	-4.58	-8.09				

^aFold changes are shown relative to 0 Gy control group. The threshold was fold change > 6 and $P_{adj} < 0.05$.

TABLE 6

Top Four KEGG Pathways of Differentially Expressed Transcripts in Thymuses of Irradiated Monkeys

Fold enrichment	p	Genes	Fold change		Fold enrichment	P	Genes	Fold change	
			2 Gy ^a	5 Gy				2 Gy ^a	5 Gy
DNA replication									
3.1	0.004	FEN1	-2.50	-3.61	Cell cycle	9.7E-8	BUB1	-1.37	-18.55
		MCM2	-2.61	-3.72			BUB3	-1.07	-2.20
		MCM3	-3.04	-6.79			CCNA2	-1.23	-4.32
		MCM7	-2.72	-4.94			CCNB2	-1.41	-24.53
		POLA2	-1.75	-2.03			CCND1	2.73	2.42
		POLD1	-1.50	-2.10			CDC20	-1.36	-9.99
		POLD3	-1.57	-3.34			CDC25B	-2.04	-3.42
		RFC5	-2.60	-2.83			CDC25C	-2.71	-4.56
		RNASEH2B	-2.98	-3.20			CDC6	-1.42	-10.26
		RPA3	-2.39	-2.95			CDK1	-1.42	-16.09
Complement and coagulation cascades									
3.0	9.9E-5	C1R	3.24	3.40			CDK2	-1.16	-2.88
		C1S	3.30	4.91			CDK6	-1.19	-7.89
		C2	2.24	2.52			CDKN1A	3.08	4.23
		C3AR1	2.35	4.65			CDKN1C	3.03	4.39
		C4B	2.16	2.67			CHEK1	-1.40	-8.23
		C5a	2.76	3.04			ESPL1	-1.22	-3.21
		C7	2.74	5.42			HDAC1	-1.74	-2.28
		CFD	4.10	8.17			MAD1L1	-1.18	-2.24
		CFH	2.83	3.16			MAD2L1	-1.31	-6.97
		CR1	-2.18	-1.67			MCM2	-2.61	-3.72
		F3	4.49	2.89			MCM3	-1.19	-6.79
		PLAU	2.43	2.22			MCM7	-1.15	-4.94
		PROS1	2.62	2.72			MYC	-2.02	-3.14
		SERPINA1	1.94	2.71			ORC3	-1.11	-2.31
		SERPING1	3.53	4.46			PTTG1	-1.18	-6.86
							SMAD3	1.45	2.04

Fold enrichment	<i>p</i>	Genes	Fold change		<i>P</i>	Genes	Fold change	
			2 Gy ^a	5 Gy			2 Gy ^a	5 Gy
ECM-receptor interaction	1.7E-5	TFPI	1.73	2.88		STAG2	-1.08	-2.11
		VWF	4.23	4.95		TFDP2	-1.21	-7.70
						TGFB3	2.64	2.50
						TTK	-5.86	-13.26
2.9						WEE1	-1.27	-3.83
		CD36	4.43	5.45				
		COL4A1	3.70	4.07				
		COL4A5	3.67	4.31				
		COL4A6	3.78	5.63				
		COL5A2	2.75	2.70				
		COL6A1	4.04	4.02				
		COL6A3	4.74	4.99				
		FN1	4.83	3.95				
		ITGA3	2.63	3.60				
		ITGA4	-2.34	-3.18				
		ITGA6	-2.63	-1.75				
		ITGA8	2.31	4.53				
		ITGB5	3.27	4.26				
		LAMA2	2.70	4.18				
		LAMA5	2.99	3.19				
		LAMB1	1.96	2.48				
		LAMB2	2.69	3.15				
		LAMC1	3.25	4.52				
		SDC1	3.22	4.47				
		THBS1	3.15	3.35				
		VWF	4.23	4.95				

^aFold changes relative to 0 Gy controls.