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Biology Contributions

Long-Term Recovery of the Adaptive Immune System in Rhesus Macaques After Total Body Irradiation

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Abstract

Purpose: Ionizing radiation causes acute damage to hematopoietic and immune cells, but the long-term immunologic consequences of irradiation are poorly understood. We therefore performed a prospective study of the delayed immune effects of radiation using a rhesus macaque model.

Methods and Materials: Ten macaques received 4 Gy high-energy x-ray total body irradiation (TBI) and 6 control animals received sham irradiation. TBI caused transient lymphopenia that resolved over several weeks. Once white blood cell counts recovered, flow cytometry was used to immunophenotype the circulating adaptive immune cell populations 4, 9, and 21 months after TBI. Data were fit using a mixed-effects model to determine age-dependent, radiation-dependent, and interacting effects. T cell receptor (TCR) sequencing and quantification of TCR Excision Circles were used to determine relative contributions of thymopoiesis and peripheral expansion to T cell repopulation. Two years after TBI, the cohort was vaccinated with a 23-valent pneumococcal polysaccharide vaccine and a tetravalent influenza hemagglutinin vaccine.

Results: Aging, but not TBI, led to significant changes in the frequencies of dendritic cells, CD4 and CD8 T cells, and B cells. However, irradiated animals exhibited increased frequencies of central memory T cells and decreased frequencies of naïve T cells. These consequences of irradiation were time-dependent and more prolonged in the CD8 T cell population. Irradiation led to transient increases in CD8+ T cell TCR Excision Circles and had no significant effect on TCR sequence entropy, indicating T cell recovery was partially mediated by thymopoiesis. Animals that were irradiated and then vaccinated showed normal immunoglobulin G binding and influenza neutralization titers in response to the 4 protein antigens but weaker immunoglobulin G binding titers to 10 of the 23 polysaccharide antigens.

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Conclusions: These findings indicate that TBI causes subtle but long-lasting immune defects that are evident years after recovery from lymphopenia.

Introduction

Total body irradiation (TBI) causes acute, dose-dependent injury to multiple organs.[1] Those who survive acute irradiation are at increased lifetime risk of developing solid tumors[2,3]; however, the other long-term effects are not well understood.[4] This is an important knowledge gap given the use of controlled radiation exposure for clinical purposes, such as bone marrow ablation, and the risk of unforeseen exposure due to clinical error, nuclear accident, or weaponization of nuclear devices. The late effects of TBI on the adaptive immune system are of particular concern given the system's critical role in pathogen defense and vaccine-induced protection.

Analyses of patients with cancer treated with targeted radiation therapy and bone marrow transplant recipients undergoing TBI for pretransplant ablation have established that acute irradiation causes circulating white blood cell (WBC) numbers to drop sharply within days and then gradually recover over months.[5,6] However, recovery of cellularity does not equate to recovery of immune function. Indeed, follow-up of the populations irradiated during the 1945 atomic bombings (A-bomb) and the 1986 Chernobyl nuclear power plant accident indicates that TBI has long-term immunologic consequences. These include a decrease in the ratio of naïve to memory T cells, skewed T cell antigen receptor (TCR) repertoire,[7,8] increased auto-reactive immunoglobulin M (IgM),[9] and elevated IgM, IgA, and IgE.[10] Studies of A-bomb survivors over 50 years after exposure have reported decreased circulating dendritic cells (DC),[11] increased monocytes,[12] increased circulating inflammatory cytokines,[13] and ablated in vitro T cell responses.[14] The net immunologic effect of these changes is unclear. A-bomb survivors appear to be in a state of persistent subclinical inflammation[15] and are less able to clear hepatitis B.[16] In contrast, a study of 2011 to 2013 seasonal influenza vaccinations in A-bomb survivors found no defects in serologic responsiveness compared with age-matched controls.[17] One caveat is that many survivors of the 1945 and 1986 exposures are now of the age where immunosenescence is likely to weaken their adaptive immunity.[18] More generally, studies of adaptive immunity in irradiated humans are limited by survivor bias, differences in ages at time of exposure, pre-existing morbidities, varied exposures and doses, and confounding factors postexposure.

Given these complications, the rhesus macaque nonhuman primate (NHP) model is often used for evaluation of immune function and radiation countermeasures.[19-23] Importantly, the rhesus immune system closely recapitulates that of humans, and NHPs are susceptible to many human pathogens and simian pathogens that are similar to human pathogens. Further, NHPs are inherently susceptible to inflammation-associated morbidities found in humans such as obesity and hypertension.[24,25] Similar to humans,[26] rhesus macaques show an acute loss of circulating granulocytes and B, $CD4^+$, and $CD8^+$ T cells days after TBI, with delays in $CD4^+$ T cell recovery leading to a prolonged shift in the CD4/8 T cell ratio.[19,21-23] To investigate the long-term effect of radiation on immunity, we performed a prospective study of male rhesus macaques exposed to 4 Gy TBI.

Methods and Materials

Animal husbandry

Detailed methods can be found in the Supplementary Material.

Twenty male rhesus macaques aged 5.8 ± 0.5 years were obtained from World Wide Primates Incorporated (Miami, FL). Animals were seronegative for simian retroviruses. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals and conducted in compliance with the Wake Forest University Institutional Animal Care and Use Committee requirements in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Complete blood counts (CBCs) with differentials were conducted weekly.

Irradiation

Animals were randomized to receive 4 Gy TBI (n = 14) or sham irradiation (n = 6). This dose was selected to be below the $LD_{10/30}$ (lethal dose to 10% of the population in 30 days) of 5.5 Gy previously described for male macaques.[21] On Day 0, animals were sedated via intramuscular (IM) injection of 15 mg/kg ketamine and then irradiated on a clinical linear accelerator using 2 lateral fields of 6 MV x-rays (174-cm source-axis-distance, 32 × 40 cm^2 field size @ 100 cm from the source, nominal dose rate of 0.8 Gy/min @ 174 cm source-axis-distance,

2.7 minute irradiation time per field). Sham-irradiated animals underwent all procedural steps except that ionizing radiation was not delivered.

Vaccination

One hundred eighteen weeks after TBI, animals were vaccinated with 0.5 mL IM polyvalent polysaccharide vaccine against 23 *Streptococcus pneumoniae* strains (Pneumovax23; Merk) and 0.5 mL IM quadrivalent northern hemisphere 2018/19 seasonal influenza vaccine (FluLaval tetra; GlaxoSmithKline). A second 0.5 mL IM dose of the influenza vaccine was administered 4 weeks later.

Peripheral blood collection and processing

Animals were sedated by IM injection with 15 mg/kg ketamine and femoral vein blood was drawn into sodium heparin-coated collection tubes (BD Bioscience) then shipped overnight at room temperature. The following day, plasma and peripheral blood mononuclear cells (PBMCs) were separated using System-Histopaque-1077 tubes (Sigma). PBMCs were immunophenotyped and remaining cells and plasma cryopreserved.

Immunophenotyping

Fresh PBMCs were labeled for 40 minutes with fluorescently conjugated antibodies (targets and clones listed in Table E1) in phosphate buffered saline (PBS), 1% bovine serum albumin (BSA) (Invitrogen), washed, stained for viability with live-dead fix aqua (Invitrogen) in PBS (Sigma) and fixed in 4% paraformaldehyde (ThermoFisher). Fixed cells were analyzed using an LSRII flow cytometer (BD Bioscience) running Diva software (BD Bioscience) and data gated using FlowJo software (BD Bioscience) (Fig. E1). Baseline data were collected 5 months before irradiation.

Influenza microneutralization

Heat-inactivated (1 hour 56°C) sera were serially diluted in virus diluent (Dulbecco's modified Eagle medium, 100 U/mL penicillin-streptomycin, 2 mM L-glutamine, 7.5% BSA, 1 μg/mL L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated Trypsin; ThermoFisher) and mixed 1:2 with virus diluent containing 100 $TCID_{50}$ of each influenza virus (A/Michigan/45/2015 [H1N1]pdm09, A/Singapore/INFIMH-16-0019/2016 [H3N2], B/Colorado/6/2017 [Victoria Lineage], or B/Phuket/3073/2013 [Yamagata Lineage]; all International Reagent Resource). Madin-Darby Canine Kidney (MDCK) cells (London Strain) (International Reagent Resource) were added and plates incubated overnight at 37°C 5% CO_2. Plates were then washed in PBS, fixed in -20°C acetone (VWR) and air-dried. Plates were washed in PBS, 0.1% Tween-20 (Sigma) and incubated for 1 hour with mouse anti-Influenza A nucleoprotein clones A1 and A3 (Millipore) or mouse anti-Influenza B nucleoprotein clones B2 and B4 (Millipore) diluted 1:4000 in PBS, 7.5% BSA, 0.1% Tween-20, and 1% dry nonfat milk (ThermoFisher). After washing, plates were incubated for 1 hour with goat antimouse conjugated to Horseradish peroxidase (HRP), washed, and then incubated with O-phenylenediamine dihydrochloride HRP substrate (Sigma). The reaction was stopped using 0.5 N sulfuric acid (Sigma) and quantified using a Synergy H1 reader (BioTek).

Hemagglutinin-binding enzyme-linked immunosorbent assay

Assay plates were coated with 2 μg/mL hemagglutinin (HA) (B/Phuket/3073/2013 [aa 16-546], [A/Michigan/45/2015] [H1N1]-pd09-like, B/Colorado/6/2017 [aa 16-546], or A/Singapore/ INFIMH-16-0019/2016X [H3N2]; all eEnzyme) washed in PBS 0.1% and Tween-20 (Sigma) and then blocked with 3% nonfat milk (Quality Biological). Sera serially diluted in PBS, 10% fetal bovine serum, 5% goat serum, 0.05% Tween-20 (all Sigma), and 1% nonfat milk, were applied to the plates and then incubated at 4°C overnight. After washing, plates were incubated with antihuman IgG Fc-HRP clone H2 (Sothern Biotech) for 2 hours, washed, and then incubated with tetramethylbenzidine HRP substrate (Sigma). The reaction was stopped using 1 M sulfuric acid (Sigma) and quantified using a Synergy H1 reader.

Signal Joint T Cell Receptor Excision Circle (sj(TREC)) Quantification

$CD4^+$ and $CD8^+$ cells were isolated from cryopreserved PBMC using magnetic-bead positive selection (Miltenyi Biotec). Cells were counted and then lysed in proteinase K (Roche). sjTREC in lysates and serially diluted, quantified NHP sjTREC standard plasmid[27] were amplified using Platinum Taq (Invitrogen) with rhesus-specific sjTREC primers and probes[27] using a CFX Connect or CFX96 RT-PCR instrument (Bio-Rad). Lysate sjTREC content was extrapolated from the sjTREC standard plasmid results.

Pneumococcal IgG multiplex

Human antipneumococcal reference serum 007sp[28] and NHP study sera were precleared using pneumococcal cell wall polysaccharide adsorbents and then assayed using a custom multiplex bead array similar to those described elsewhere.[29,30] Briefly, samples were incubated with fluorescent magnetic microspheres (Luminex Corp) labeled with pneumococcal polysaccharides (FXImmune Diagnostics). After sample binding, microspheres were washed in PBS,

1% BSA (Invitrogen) and incubated for 30 minutes at room temperature with 4 μg/mL biotinylated antihuman/rhesus IgG Fc clone H2 (Southern Biotech). Microspheres were washed and labeled with 8 μg/mL streptavidin-phycoerythrin (Invitrogen). Beads were read on a BioPlex 200 multiplex reader (Bio-Rad) and analyzed using BioPlex Manager software v6.2 (Bio-Rad). NHP test sera IgG-binding of each polysaccharide was then calculated relative to that of 007sp reference serum.

TCR sequencing

RNA was extracted using the RNeasy Mini Kit (Qiagen). TCR α/β libraries were generated with rhesus-specific reverse primers (Takara) in combination with the SMARTer Human TCR Profiling Kit. Libraries were purified using SPRIselect Beads (Beckman Coulter) then sequenced on a MiSeq instrument (Illumina). Sequence annotation and clonal assignments were performed using MiXCR.[31] A threshold of 1000 reads per clone was used, and clones were considered identical if their CDR3 sequence matched within 3 base pairs.

Statistical analyses

Except as noted, data were analyzed using Prism (GraphPad) and plotted as mean ± standard deviation. For results past 100 days postirradiation, data from ± 1 week were grouped together. Unless stated, data were analyzed using a mixed-effects repeated measures model with Greenhouse-Geisser correction for sphericity as implemented in Prism. Significant results were reported as F(degrees of freedom numerator, degrees of freedom denominator) = F statistic followed by the P value for the F statistic. Variables found to change significantly ($P < .05$) with time were interpreted as changing due to aging. Variables found to change significantly with radiation were interpreted as being altered by TBI irrespective of time. Variables found to change significantly in response to the interaction of time and radiation were interpreted as being temporally changed by TBI.

Multiple comparisons were performed by Fisher's least significant difference without correction for repeated measures. Heatmaps were generated using RStudio software (RStudio) with the pheatmap package (Ravio Kolde).

Results

Fourteen animals received 4 Gy TBI, whereas 6 control animals were subject to a sham irradiation. All received supportive care (enteral feeding gavage, subcutaneous fluids, antibiotics, pain medication, blood transfusions) as required. Four animals in the TBI group were euthanized within 3 months due to opportunistic systemic infectious diseases (3 with *Spironucleus* sp. and 1 with *Klebsiella pneumoniae).* As the current study focused on the long-term effect of irradiation, data from these animals were excluded.

TBI causes transient neutropenia and lymphopenia

CBC and differentials were collected from the 16 animals at regular intervals. CBCs 200 days postirradiation were previously reported by Michalson et al.[32] TBI led to an acute decrease in circulating WBC, with mean count dropping to a nadir of 8.8% of baseline after 16 days (Fig 1A). WBC counts recovered over the next 40 days, with no significant difference in total cellularity being detected from 58 days post-TBI onwards. When fit to a mixed-effect model, total WBC count changed significantly with time ($F[4.42,61.7] = 3.02$, $P = .02$) and there was a significant interaction between irradiation and time ($F[22,307] = 3.62$, $P < .0001$), indicating that both aging and time since irradiation affected the total WBC.

Neutrophils and lymphocytes make up the bulk of WBCs in NHP, and both populations dropped within days of irradiation (Fig 1B,C). Neutrophil numbers recovered to those of controls within 4 weeks. Lymphocyte counts in the control animals were marginally higher than the irradiated group before irradiation. Regardless, it took 8 weeks after irradiation for lymphocyte numbers to return to baseline or match control animals. There was a transient over-recovery of lymphocytes before numbers stabilized. Both neutrophil and lymphocyte counts changed significantly over time (neutrophils: $F[4.42,61.7] = 3.02$, $P = .02$; lymphocytes: $F[2.67,37.3] = 11.1$, $P < .0001$) and showed significant interaction between irradiation and time (neutrophils: $F[22,307] = 3.62$, $P < .0001$; lymphocytes: $F[22,307] = 8.62$, $P < .0001$). Together, these results indicated that absolute numbers of circulating neutrophils and lymphocytes were affected by both TBI and aging and that the effect of radiation decreased over time.

In addition to neutrophils, the numbers of 2 other populations pivotal to innate immunity, eosinophils and monocytes, dropped dramatically after irradiation and then rapidly recovered (Fig 1D,E). Both populations exhibited time-dependent decreases (monocytes: $F[4.25,59.3] = 3.44$, $P = .012$; eosinophils $F[4.57,63.6] = 2.48$, $P = .045$) although only monocytes exhibited an interaction between irradiation and time ($F[22,307] = 3.47$, $P < .0001$).

Overall, these results are consistent with previous studies in mice, NHPs, and humans showing that high-dose TBI leads to an acute loss of circulating WBCs followed by recovery over several weeks. They also reaffirm previous findings that the major WBC populations decrease in number with age. The prospective

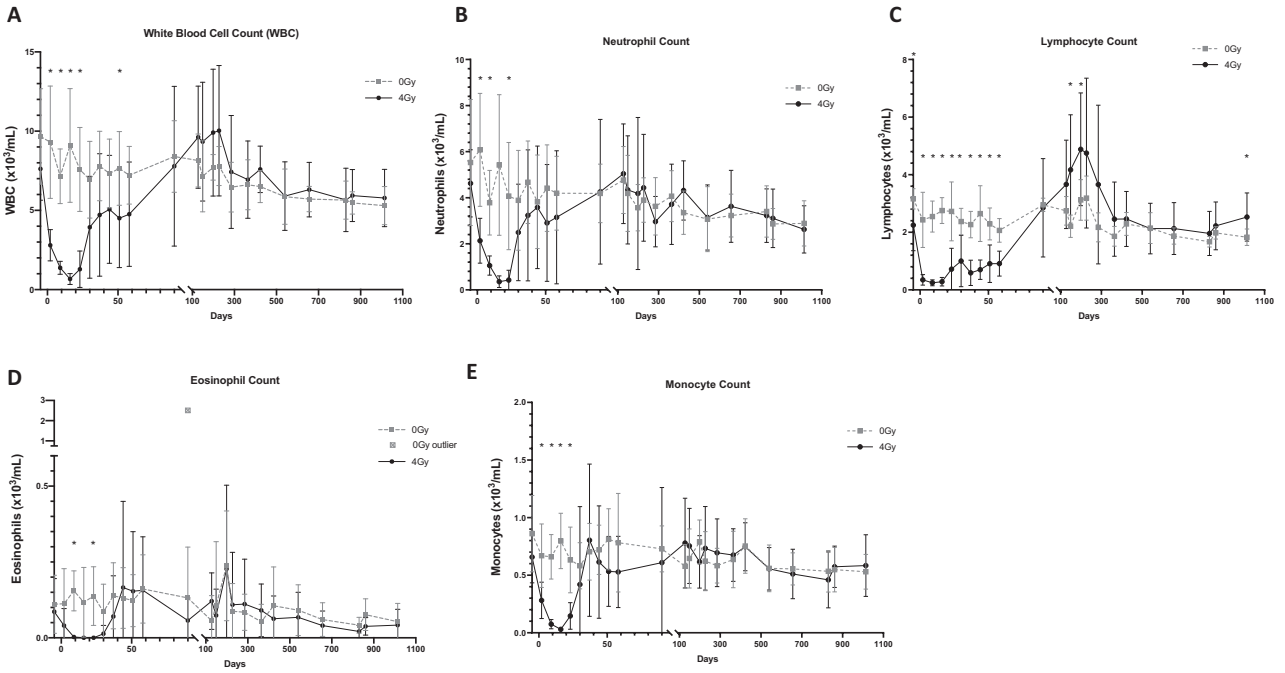

Figure 1 Acute loss and gradual recovery of nonhuman primate (NHP) white blood cell populations after total body irradiation (TBI). Counts of (A) white blood cells, (B) neutrophils, (C) lymphocytes, (D) eosinophils, and (E) monocytes in rhesus macaques sham-irradiated or irradiated at day 0. One outlier eosinophil count in the sham group was excluded. $^*P < .05$ by Fisher's least significant difference test.

model reported here is thus consistent with published data and represents a relevant model for investigation of the effects of TBI on immunity.

TBI perturbs the long-term balance between naïve and memory T cells

Circulating lymphocytes are an equilibrium of naïve, effector, and memory B and T cells. We therefore immunophenotyped PBMC to characterize the balance of these populations plus the frequency (%) DC, in the 21 months after TBI. Importantly, the first postradiation timepoint for this analysis was approximately 3.5 months after exposure, when absolute numbers of WBC had recovered (Fig 1A).

To evaluate changes in DC, we measured the percentage of myeloid (mDC) and plasmacytoid DCs (pDC)[33] within PBMC. Although the percentage of cells classified as mDC and pDC decreased over time (mDC: $F[1.046,14.64] = 8.124$, $P = .0117$; pDC $F[1.048,22.00] = 14.77$, $P = .0008$) (Fig 2A,B), there was no significant effect of irradiation or interaction between time and irradiation. This suggested that although aging caused a decrease in frequency of circulating DC, TBI did not have any long-term effect.

The frequency of circulating B cells fell significantly as time passed ($F[2.284, 31.98] = 12.41$, $P < .0001$) (Fig 2C). Radiation exposure did not have a significant effect on this frequency. To further evaluate the long-term effect of radiation on B cells, we measured the percentage of B cells expressing CD80 at baseline and after irradiation and CD27 after irradiation only. CD80 provides costimulatory signals from T cells and is a marker of B cell activation. CD27 expression correlates with B cell somatic hypermutation and is a marker of memory B cells.[34] The percentage of $CD80^{high}$ B cells decreased significantly with time ($F[1.229,22.94] = 9.203$, $P = .0039$) (Fig 2D). The percentage of $CD27^+$ B cells changed significantly with time ($F[1.428,19.99] = 56.06$, $P < .0001$) (Fig. 2E), but irradiation had no significant effect on the expression of either marker. Collectively, these results imply that although aging led to a drop in the percentage of circulating B cells, radiation did not have a long-term effect on B cell frequency or naïve/memory balance.

Although the percentage of circulating B cells fell as the animals aged, the percentage of T cells increased ($F[2.18,30.5] = 22.2$, $P < .001$) (Fig 2F). Peripheral T cells in primates are primarily $CD4^+$ or $CD8^+$ plus a small percentage of $CD4^+$ $CD8^+$ cells of unclear function.[35] Although the ratio of $CD4^+$ to $CD8^+$ T cells changed over time ($F[1.213,16.99] = 50.67$, $P < .0001$) (Fig 2G), there was no significant effect of irradiation or interaction between time and irradiation on either the percentage of T cells or the CD4/CD8 ratio. There was no significant difference in $CD4^+$ $CD8^+$ frequency between the control and irradiated groups (Fig 2H). When considered in combination with the lymphocyte absolute

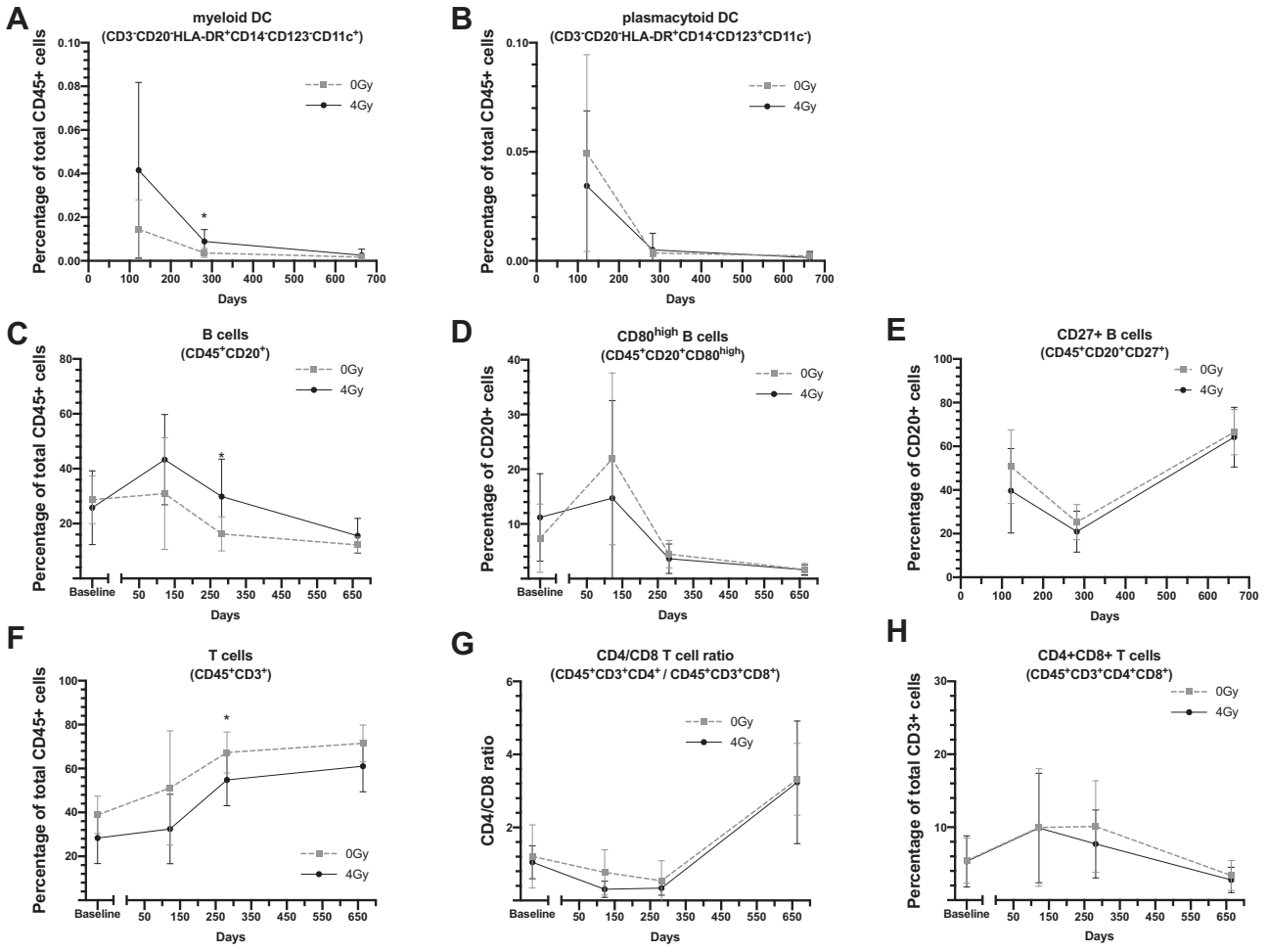

Figure 2 Homeostatic rebalancing of B, T, and dendritic cells in the peripheral blood of rhesus macaques after total body irradiation (TBI). Peripheral blood mononuclear cells (PBMCs) were collected at baseline and/or 122, 282, 664 days (4, 9, 21 months) after irradiation. PBMCs were stained for viability and CD45 plus (A, B) CD3, CD20, HLA-DR, CD14, CD123, and CD11c (dendritic cells), (C-E) CD20, CD27, and CD80 (B cells), or (F-H) CD3, CD4, and CD8 (T cells) and analyzed by flow cytometry. Data are expressed as percentages of the parent population noted on the y-axis. $*P < .05$ by Fisher's least significant difference test.

counts over time (Fig 1C), these data indicated that lymphocyte recovery post-TBI did not lead to long-term changes in T cell numbers beyond those caused by aging.

To appraise the long-term effect of TBI on T cell subpopulations we evaluated CD28 and CD95 expression for identification of naïve ($CD28^{mid}CD95^{low}$), effector memory (T_{EM}: $CD28^{low/-}CD95^{mid/high}$), and central memory ($T_{CM}$: $CD28^{high}CD95^{high}$) populations.[33] The relative proportion of $CD4^+$ and $CD8^+$ naïve cells changed with time ($CD4^+$: $F[1.912,26.76] = 7.155$, $P = .0036$; $CD8^+$: $F[11.891,26.47] = 15.55$, $P < .0001$) and with radiation exposure ($CD4^+$: $F[1,14] = 8.898$, $P = .0099$; $CD8^+$: $F[1,14] = 6.389$, $P = .0241$) (Fig 3A,D). The percentage of $CD4^+$ naïve T cells, but not $CD8^+$ naïve T cells, also showed a significant interaction between time and irradiation ($F[2,28] = 12.74$, $P < .0001$). The proportions of naïve T cells in irradiated animals were significantly lower than in age-matched controls at the first post-TBI timepoint tested ($CD4^+$: $P = .0002$; $CD8^+$: $P = .0083$). Thus, aging led to a drop in the frequency of naïve $CD4^+$ T cells and an increase in the frequency of naïve $CD8^+$ T cells. Further, irradiation led to significant decreases in the frequencies of both $CD4^+$ and $CD8^+$ naïve T cells. The effect of radiation on naïve $CD4^+$, but not naïve $CD8^+$, T cells decreased over time.

The frequencies of both T_{EM} populations were significantly affected by time ($CD4^+$: $F[1.701,23.81] = 6.021$, $P = .0102$; $CD8^+$: $F[1.968,27.55] = 10.45$, $P = .0004$). The frequency of $CD8^+$, but not $CD4^+$, T_{EM} was also significantly disturbed by irradiation ($CD8^+$: $F[1,14] = 25.66$, $P = .0002$) (Fig 3B,E). These data indicated that aging led to an increase in T_{EM} frequency, with radiation causing a sustained increase in the frequency of $CD8^+$ T_{EM}.

$CD8^+$, but not $CD4^+$, T_{CM} were subject to significant time-dependent effects ($CD8^+$: $F[1.828,25.60] = 4.277$, $P = .0278$) and were sensitive to irradiation ($CD8^+$: $F[1,14] = 17.31$, $P = .001$) (Fig 3C,F). In both $CD4^+$ and

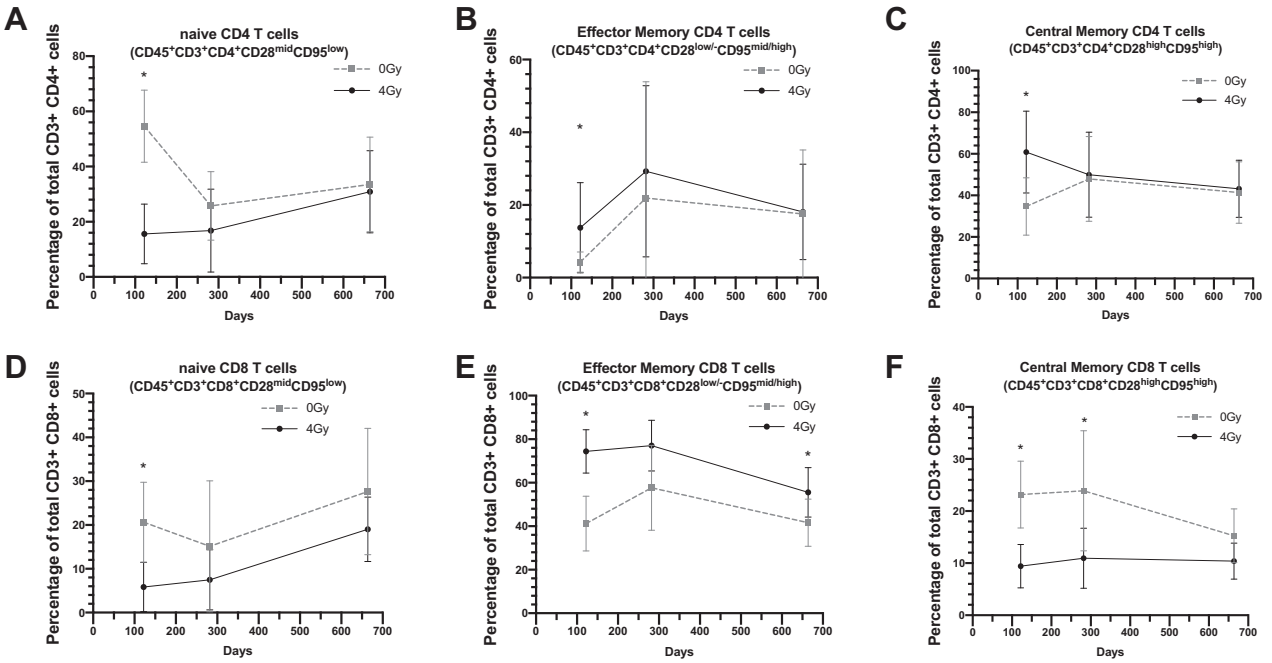

Figure 3 Homeostatic rebalancing of $CD4^+$ but not $CD8^+$ T cell populations in the peripheral blood of rhesus macaques after total body irradiation (TBI). Peripheral blood mononuclear cells (PBMCs) were collected at baseline and/or 122, 282, 664 days post-irradiation. PBMCs were stained for viability and CD45 plus CD3, CD4, CD8, CD45RA, CD95, and CD28 and gated on (A-C) CD4 or (D-F) CD8 plus, (A, D) naïve ($CD28^{mid}CD95^{low}$), (B, E) effector memory ($CD28^{low/-}CD95^{mid/high}$), and (C, F) central memory ($CD28^{high}CD95^{high}$) populations. Data are expressed as percentages of the parent population noted on the y-axis. $*P < .05$ by Fisher's least significant difference test.

$CD8^+$ T_{CM} populations there was significant interaction between time and radiation exposure ($CD4^+$: F[2,28] = 4.538, $P = .0196$; $CD8^+$: F[2,28] = 4.490, $P = .0204$). This indicated that aging did not affect the frequency of $CD4^+$ T_{CM} over the 21-month study, but that the frequencies of both $CD4^+$ and $CD8^+$ T_{CM} were affected by radiation exposure, with the effect on $CD8^+$ T_{CM} being more prolonged.

Thymopoiesis and peripheral expansion reconstitute the peripheral T cell compartment after TBI

The number of circulating T cells dropped dramatically a few days after TBI and recovered over several months (Fig 1C). This reconstitution could be mediated by increased thymic output of naïve cells (thymopoiesis) and/or by peripheral expansion of naïve or memory cells. This is an important distinction, as thymic radiation damage may corrupt TCR selection,[36] while peripheral expansion cannot generate TCR diversity.

During thymopoiesis, excised TCR DNA forms TCR rearrangement excision circles (TRECs). TRECs are not replicated during proliferation, and so TRECs become diluted with subsequent cell divisions. TREC numbers are thus highest in populations of recent thymic emigrants and lowest in cell populations that have undergone extensive peripheral expansion.[27] To determine whether TBI increased thymic output, we quantified TREC in $CD4^+$ and $CD8^+$ T cells isolated from the control and irradiated animals (Fig 4A,B). We did not observe a statistically significant change in CD4+ or CD8+ TREC numbers with respect to either time or irradiation. However, CD4+ T cells in 1 irradiated animal exhibited a transient 70-fold increase in sjTREC at the first timepoint after irradiation (122 days; outlier shown in plot), and several of the irradiated animals showed a transient spike in CD8+ TREC numbers after irradiation, as evidenced by the larger standard deviations of the irradiated samples 9 months (~282 days) after irradiation. Interpreting these data is complicated by the animals in the irradiated group having higher and more variable CD4+ and CD8+ sjTREC counts than the control group preirradiation. However, they suggest that if the thymus contributes to CD4+ T cell repopulation after irradiation then it primarily does so acutely (earlier than 3 months after exposure) and that the contribution to CD8+ T cell repopulation is transient and variable.

To determine whether TBI caused long-term changes to TCR diversity, we sequenced TCRα and β mRNA from PBMC collected at baseline and 9 months post-irradiation. The 9-month timepoint was selected as T cell numbers had recovered to near baseline (Figs 1C and 2F). Cryopreserved PBMC from 5 control and 6 irradiated animals yielded sufficient high-quality RNA for baseline

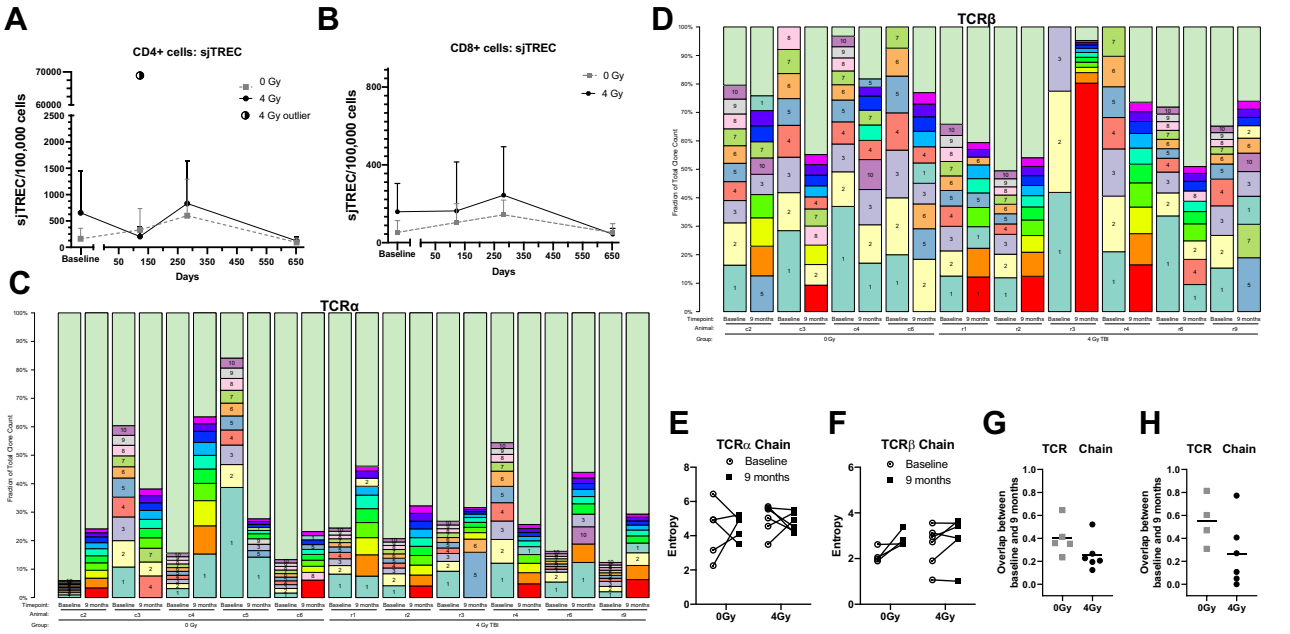

Figure 4 Thymopoiesis contributes to T cell repopulation after total body irradiation (TBI). (A, B) $CD4^+$ and $CD8^+$ T cells were isolated by positive selection from peripheral blood mononuclear cells (PBMCs) collected at baseline, 122, 282, and 664 days post-irradiation and their T cell receptor (TCR) rearrangement excision circle (TREC) contents quantified by quantitative polymerase chain reaction (qPCR). (C-H) TCRα and TCRβ chains were sequenced from PBMC at baseline and 9 months postirradiation. Panels (C, D) show cumulative frequency distribution of top 10 clones in each animal at each timepoint. Each color represents a distinct clone, and clones found in the same animal at both timepoints are numbered. Panels (E, F) show entropy for each chain in each animal at baseline and 9 months postexposure. Lines link samples from the same animal. Panels (G, H) show the mean and per-animal cumulative frequencies of overlapping clones between baseline and 9 months postirradiation. (A color version of this figure is available at https://doi.org/10.1016/j.adro.2021.100677.)

and postexposure TCRα and/or TCRβ sequencing. We first evaluated the top 10 α and β clones in each animal at baseline and 9 months post-TBI. The distribution and identity of the immunodominant clones in each of the nonirradiated animals changed considerably between timepoints, revealing the dynamic nature of the TCR repertoire. We observed similar dynamism in immunodominant clones in the irradiated group (Fig 4C,D). To evaluate global TCR diversity beyond the top 10 clones, we calculated the TCRα and TCRβ Shannon entropy index for each animal at both timepoints. This index incorporates both the number of clones and frequency of each clone, and the higher the index, the greater the diversity of T cell clones in an animal.[37] Although entropies in each animal changed over time, there were no consistent trends (Fig 4E,F), and mixed-effects modeling did not indicate any effect of time or radiation on the entropies. Thus, radiation did not significantly constrain or enhance overall T cell repertoire diversity. Next, we determined the overlap in clones between baseline and 9 months post-TBI for each animal. The cumulative frequencies of overlapping TCRα and TCRβ clones over time were less in irradiated animals, although these differences did not reach statistical significance (Fig 4G,H). There was, therefore, a trend for irradiated animals to have a higher proportion of clones at 9 months that were not present at baseline.

Collectively, these data revealed that although dominant TCR clones changed with time, single high-dose irradiation did not lead to a significant long-term loss of TCR diversity or sustained domination of the repertoire through expansion of a small number of clones. New TCR clones only arise during thymopoiesis, and so these findings further support the model that long-term repopulation and homeostasis of circulating T cells after TBI were partially driven by thymic output rather than solely by peripheral expansion.

TBI leads to long-term deficiencies in response to select antigens

These findings do not mean that the adaptive immune systems of irradiated animals fully recovered. We therefore evaluated in this cohort the immune response to 2 multivalent vaccines: a 23-serotype pneumococcal polysaccharide vaccine (23vP) and a tetravalent 2018/2019 seasonal influenza vaccine containing 4 influenza HA proteins. The pneumococcal vaccine and the first dose of the influenza vaccine were administered approximately

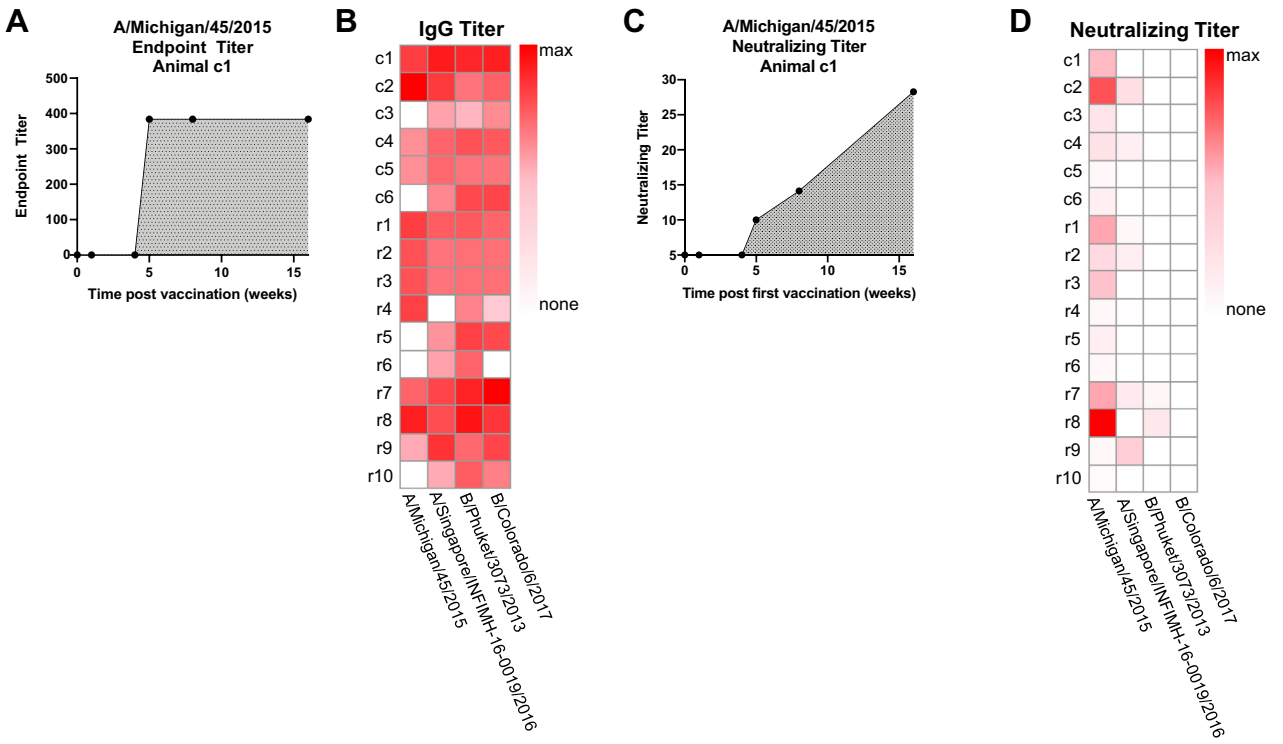

Figure 5 Two years after total body irradiation (TBI), irradiated animals show normal antibody responses to seasonal influenza vaccination. One hundred eighteen and 122 weeks after irradiation, macaques were vaccinated with a tetravalent seasonal influenza vaccine. Sera were assayed for (A, B) hemagglutinin-binding immunoglobulin G (IgG) and (C,D) neutralizing antibodies against the influenza strains included in the vaccine. (A) Baseline-normalized hemagglutinin (HA)-binding IgG for each animal and HA were plotted as shown and area under the curve (AUC, shaded) calculated. (B) Heatmap shows log(AUC + 1) for the above-baseline response to each HA over time for all animals and HAs. (C) Neutralization titers for each influenza strain and animal were plotted as shown and the AUC calculated. (D) Heatmap shows the AUC for the neutralization response over time for all animals and influenza strains.

118 weeks (826 days) after TBI or sham irradiation. A booster influenza vaccine dose was administered 4 weeks later. Serum was collected from each animal immediately before vaccination (baseline) and 1-, 4-, 5-, 8-, and 16-weeks post.

HA-specific IgG titers for each animal were normalized to baseline and plotted against time. The area under each curve (AUC) was calculated as shown in Figure 5A for a representative animal/HA. The IgG responses for all animals and all HAs are summarized in Figure 5B. With the exception of irradiated animal r6, all vaccinated animals had detectable IgG responses to at least 3 of the vaccine HAs. The majority of control (n = 4 of 6) and irradiated (n = 6 of 10) animals had IgG responses to all 4 HAs, and there were no significant differences between the control and irradiated groups in the AUC of the IgG responses for any of the HAs. In addition, there were no significant differences in IgG responses between the control and irradiated groups after the animals had received only the prime dose (Fig E3). Thus, irradiation did not affect the amount of anti-HA IgG produced in response to vaccine strain HA proteins.

Sera were then tested for their ability to neutralize the 4 influenza virus strains represented in the vaccine. Neutralizing titers for each virus were plotted against time and AUC calculated as shown in Figure 5C. The AUCs for all 4 viruses and 16 animals are shown in Figure 5D. Baseline normalization was not required as there were no detectable neutralizing responses before the boost.

The responses to the 2 influenza B vaccine strains were weak in all animals, with no animals generating neutralizing antibodies to B/Colorado/6/2017 and only 2 generating neutralizing antibodies to B/Phuket/3073/2013. Influenza B does not naturally infect NHPs.[38] The vaccine doses were therefore likely these animals' first exposure to type B HA, and so the poor neutralization titers were unsurprising.

In contrast, influenza A is zoonotic, and the animals in the study were likely to have experienced similar viruses previously.[38] This was borne out by the neutralizing responses to A/Michigan/45/2015 and A/Singapore/INFIMH-16-0019/2016. There were no significant differences in influenza A responses between the irradiated and sham-irradiated animals (Fig 5D).

The humoral response to 23vP was quantified by measuring serum IgG for each of the 23 pneumococcal polysaccharides using a multiplex bead array. IgG titers for each polysaccharide were normalized, plotted against

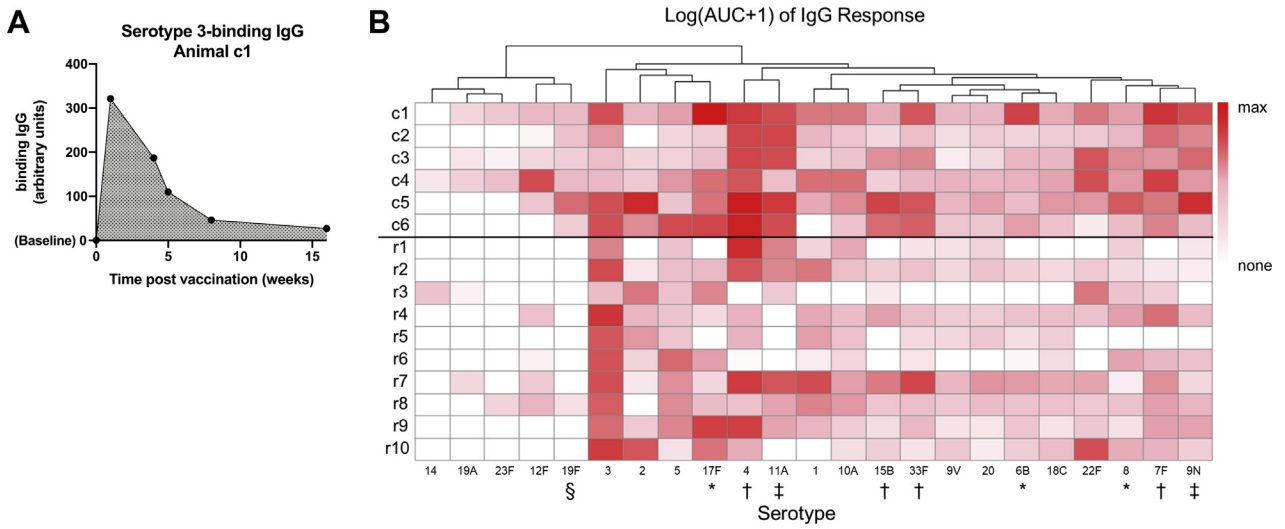

Figure 6 Selectively deficient immunoglobulin G (IgG) responses to 23-valent polysaccharide vaccine 2 years after irradiation. One hundred eighteen weeks after irradiation, macaques were vaccinated with a 23-valent pneumococcus polysaccharide vaccine and then serotype-specific IgG antibodies were assayed. (A) Data were plotted as shown and area under the curve (AUC, shaded) calculated. (B) Heatmap shows log(AUC + 1) for the above-baseline response to each serotype over time. $^{*}P < .05$, $^{\dagger}P < .01$, $^{\ddagger}P < .001$, $^{\S}P < .0001$ by *t* test.

time, and then the above-baseline AUC was calculated. An example plot for 1 animal and serotype is shown in Figure 6A, and the AUCs for all serotypes and animals are shown in Figure 6B. Some serotypes, for example, 3, 2, 10A, and 9V, elicited similar IgG responses in control and TBI animals. However, the irradiated animals showed significantly weaker IgG responses to 10 of the 23 serotypes in the vaccine. The difference in response to serotype 19F was particularly striking, with 100% (n = 6 of 6) of control animals exhibiting a response and only 10% (n = 1 of 10) of the TBI animals responding ($P < .00001$). In addition, while all 6 of the control animals showed some IgG response to a minimum of 17 serotypes, 3 of the 10 irradiated animals showed responses to 12 or fewer serotypes. Therefore, despite the total WBC counts and frequencies of DC, B, and T cell populations all returning to their expected setpoints, irradiated animals mounted significantly weaker humoral responses to select bacterial polysaccharide antigens over 2 years after radiation exposure.

Discussion

Most previous studies of the long-term effect of radiation on immunity have been observational, retrospective analyses years after the initial radiation exposure or have been performed in human patients with underlying diseases. Here, we report a multiyear prospective study of immunologic recovery in rhesus macaques after 4 Gy TBI. We found that after a period of neutro- and lymphopenia, the numbers and ratios of the key cellular mediators of immune responses returned to those of age-matched sham-irradiated controls. However, radiation exposure led to subtle, long-term differences in the ability of animals to mount a humoral response to polysaccharide antigens in the setting of vaccination.

The animals in the current study recovered from lymphopenia approximately 3 months after TBI, but both $CD4^{+}$ and $CD8^{+}$ T cells were initially skewed toward memory populations, and the memory:naïve balance gradually recovered over time. This suggests T cell repopulation was initially driven by peripheral expansion of surviving memory cells. This is consistent with prior observations from lymphopenic mice, humans, and irradiated NHPs.[19,39] Although peripheral expansion can lead to constriction of TCR diversity,[39] we did not observe a significant change in TCR sequence entropy once the memory:naïve cell balance returned to baseline, suggesting any TCR skewing was transient.

Although we did not observe a statistically significant difference in TREC numbers 4 to 9 months after radiation exposure, T cells from irradiated animals had greater variability in CD8 TREC numbers in comparison to the nonirradiated controls. This implies that thymic output contributed to the peripheral repopulation of CD8 cells but that the degree or kinetics of thymopoietic recovery varied between animals. Notably, this phenomenon has also been reported in other NHP TBI studies.[19] The transient decrease in the CD4/8 T cell ratio following irradiation indicated that CD8 T cells recovered faster than CD4 cells, as previously observed in patients recovering from lymphopenia.[40,41]

Recently, Hale et al.[30] vaccinated 5.1- to 6.4-year-old macaques with 23vP 2 years after irradiation at 7.5 to 8.5 Gy using linear accelerator-derived photons. Comparison

of IgG responses to 11 polysaccharide antigens at baseline and 16 weeks after vaccination suggested irradiated animals may be unable to generate a sustained response to select polysaccharides 2 years after TBI. The current prospective study reinforces this finding, as the IgG response to 10 of the 23 pneumococcal polysaccharide antigens tested was either weakened or completely ablated 2 years after TBI. This indicates that despite a quantitative recovery of a TBI-ablated immune system, there are qualitative deficiencies in adaptive immunity 2 years postirradiation. Interestingly, the responses to specific serotypes differed between the 2 studies, with Hale and colleagues observing compromised responses to serotype 5, a serotype for which we found no difference in response postirradiation. Conversely, Hale et al reported only a nonsignificant reduction in the number of animals responding to serotype 19F (75% of controls vs 44% of TBI animals). These distinctions may reflect radiation dose-dependent differences in response, the use of a binary classifier for response in Hale et al. (above-baseline IgG titers at 16 weeks after vaccination) versus a quantitative metric (area under the time-response curve from 0-16 weeks) in the current study, or the preclearance of nonspecific homologous antibodies from sera in the current work but not in the assays reported by Hale et al.

Although irradiated animals had deficiencies in their ability to generate IgG responses to several polysaccharide antigens, we did not observe any defect in IgG responses to any of the 4 influenza protein antigens administered in parallel to the polysaccharides. We tested more polysaccharides than proteins. However, our previous study of NHPs irradiated at a mixture of ages and doses also found no significant difference in the ability of the irradiated animals to mount an IgG response to tetanus toxoid protein.[30] Further, our study of A-bomb survivors indicated that irradiation did not cause a deficit in the ability of people to mount an antibody response to seasonal trivalent influenza vaccines.[17] Our cumulative findings in multiple cohorts and species therefore suggest that high-dose TBI causes long-term defects in the ability to mount an adaptive immune response to polysaccharide but not protein antigens.

Although humoral responses to protein vaccines arise from follicular B cell differentiation to memory B cells, a process that requires T follicular helper cell help (T-dependent), responses to plain polysaccharide vaccines such as 23vP are T-independent and don't generate memory B cells. Instead, polysaccharide vaccines likely act by cross-linking B cell antigen receptors on splenic marginal zone B (MZ B) cells to drive an extrafollicular plasma cell response. Notably, children under 2 years old have few MZ B cells and do not mount effective responses to 23vP but can create humoral immunity in response to protein vaccines.[42-44] Similarly, 23vP is only weakly efficacious in the elderly,[45] and mouse studies indicate aging reduces the ability of MZ B cells to capture antigens and migrate to chemokines.[46] Although the effect of radiation on splenic MZ B cells in NHP has not been reported, we previously observed that TBI of NHP causes an acute contraction of splenic B cell counts and disrupts the splenic architecture.[22,47] The poor response to polysaccharide antigens observed in irradiated animals may, therefore, be a consequence of MZ B cell loss or malfunction.

Regardless of the mechanisms, our finding is of importance as it suggests irradiated individuals may be less able to mount a response to some infections or vaccines even after the WBC stabilizes. This is of particular concern with regard to *S. pneumoniae* as pneumonia is the most common serious infection found in patients with cancer.[48,49] Further, human infections by *S. pneumoniae* serotypes 19F, 11A, and 9N, the 3 serotypes with the most significant radiation-induced deficiencies in IgG response, have each been associated with increased mortality.[50]

Collectively, the study reported here adds to the growing body of mouse, NHP, and human data indicating that in addition to the dramatic acute effect of radiation exposure, TBI leads to subtle long-term immunologic defects.

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Supplementary Materials

Supplementary material for this article can be found at https://doi.org/10.1016/j.adro.2021.100677.

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