Chapter XXV: Bone Marrow Transplantation – Animal Models

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Introduction

Adult hematopoiesis occurs mainly within the bone marrow (BM), wherein a hierarchy of hematopoietic stem cells (HSCs) produce the complete hierarchy of mature blood and immune cells. Adult BM is highly sensitive to radiation injury due to the proliferative nature of the blood forming cells. Total body radiation doses in the range of 2-10 Gy can cause death due to hematologic toxicities, including neutropenia and thrombocytopenia, and their consequences, such as bacterial or fungal infection and hemorrhage. For this reason, it is desirable to develop experimental animal models to understand both the cellular mechanisms induced by radiation injury and to study new mechanisms that regulate hematopoietic regeneration following myelosuppressive irradiation. The mouse is a convenient and cost-effective model organism for studying radiation injury to the hematopoietic system. Murine hematopoiesis closely mimics human hematopoiesis and more than 9,000 transgenic mouse models exist to study specific genes and signaling pathways. In this chapter, we will describe the effects of radiation injury on the hematopoietic system in the most commonly used mouse model, the C57BL/6 mouse. We will focus on the application of this model to interrogate the hematopoietic response to ionizing radiation, to test novel targeted therapeutics that promote HSC regeneration after irradiation and to develop clinically relevant pre-clinical models to evaluate novel therapeutics that accelerate hematologic recovery following radiation – based myeloablative stem cell transplantation.

Acute Radiation Injury in the Mouse Model

Histological Assesment Total Bone Marrow Cellularity

Normal hematopoiesis occurs by self-renewal and differentiation of the quiescent long-term hematopoietic stem cells (LT-HSC) into the more proliferative short-term hematopoietic stem cells (ST-HSC). The ST-HSC can then subdivide to form multipotent progenitor (MPP) cells which give rise to progenitors of either the myeloid or lymphoid lineage (depicted in **Figure 3)**. Ionizing radiation (IR) causes acute toxicity to the proliferating ST-HSCs and progenitor cell populations. The acute loss of BM ST-HSCs and progenitor populations results in a loss of total BM cellularity. BM cellularity in mice is best visualized by longitudinal histological sections of mouse femurs stained with hematoxylin and eosin (H&E). **Figure 1** depicts a typical femur section before and after radiation injury. In the non-irradiated femur at left, the femur is densely cellular with hematoxylin (purple) stained hematopoietic cells. At 24 hrs following 5 Gy total body irradiation (TBI, middle image), a dramatic decrease in BM cellularity and frank hemorrhage can be seen by the infiltration of eosin-staining (pink) erythrocytes. By day 14 (right), BM recovery is observed.

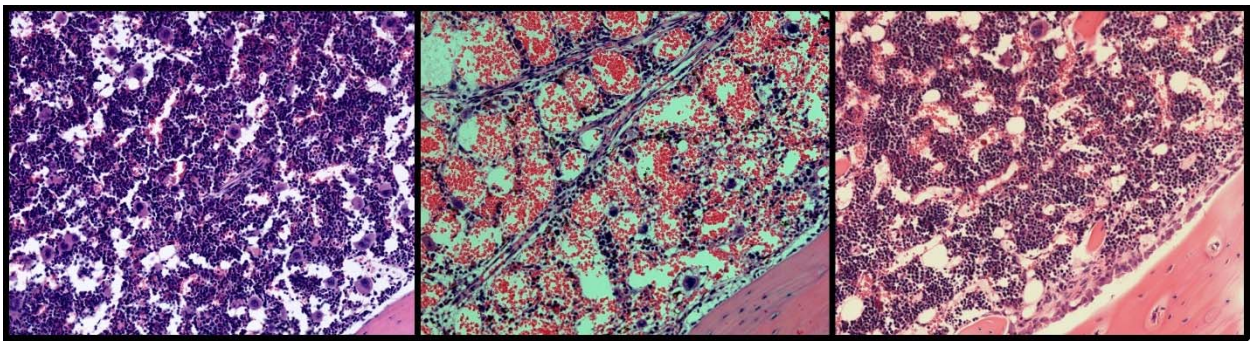

Figure 1. Representative 20x images of mouse femurs sectioned longitudinally and stained H&E. Unpublished data, Chute laboratory, UCLA.

The loss in BM cellularity can be quantified by counting the cells isolated from each femur using a hemocytometer or cell counter. The time course of BM cell loss and recovery following 5 Gy TBI is shown in **Figure 2.** BM from one femur is depleted of mature red blood cells using a red blood cell lysis, stained with the viability stain Trypan blue to exclude dead cells, and enumerated using a Bio-Rad TC-20 automated cell counter.

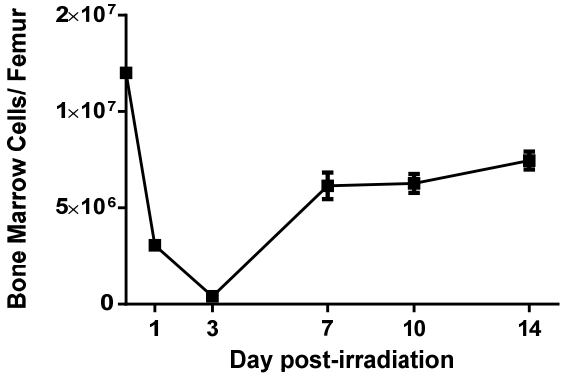

Figure 2. Bone marrow cellularity following 5 Gy TBI. Unpublished data, Chute laboratory, UCLA.

Hematopoietic Stem and Progenitor Flow Cytometry Analysis

Flow cytometry is a fast and reliable means to assess radiation effects on HSC and progenitor cell populations. **Figure 3** shows the cell surface markers which are utilized to define the LT-HSC, ST-HSC, MPP, and other progenitor cell populations present in the adult mouse (Challen et al., 2009; Heiser et al., 2014). Murine LT-HSC can be defined as CD34-Flt3-ckit+sca-1+lin- cells or CD150+CD48-ckit+sca-1+lin- cells (Kiel M, et al. *Cell* 121, 1109-1121, 2005).

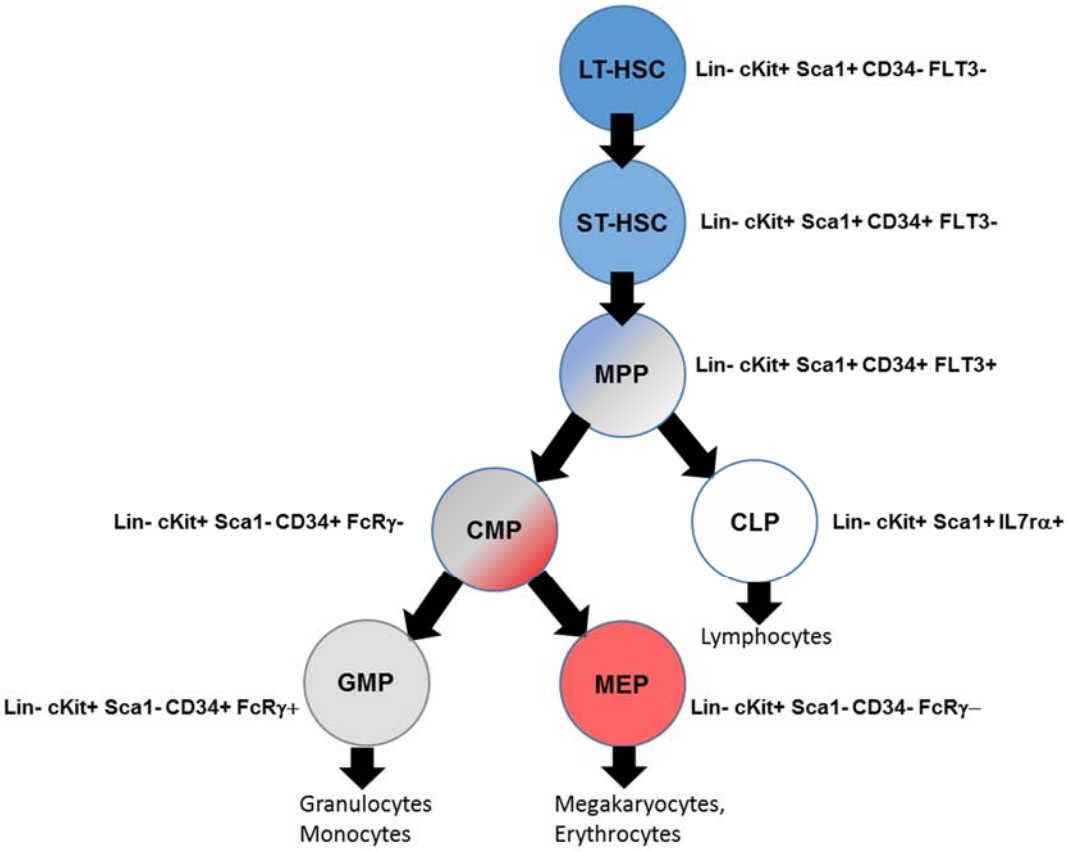

Figure 3: Cell surface markers for hematopoietic stem and progenitor populations. (LT-HSC = Long-term hematopoietic stem cell, ST-HSC= short-term HSC, MPP = Multipotent Progenitor cell, CLP = Common Lymphoid Progenitor, CMP = Common Myeloid Progenitor, GMP = Granulocyte-Monocyte Progenitor, MEP = Megakaryocyte-Erythroid Progenitor)

The acute effects of radiation toxicity to the ST-HSC and myeloid progenitor pools are depicted in the representative flow analysis shown in **Figure 4**. BM was isolated from the femurs of non-irradiated control and 5 Gy - irradiated adult C57BL/6 mice at 24 hours following TBI. BM cells were then depleted of mature red blood cells using a red blood cell lysis. The remaining cells were then analyzed by flow cytometry to assess response to radiation injury. In the gating strategy shown in **Figure 4**, we first excluded cellular debris by drawing a P1 gate based on the known forward (FSC) and side scatter (SSC) properties of hematopoietic cells (Doan et al., *Nat Med* 19:295-304, 2013). When performing FACs analysis on irradiated samples, it is necessary to include a viability stain such as 7AAD to exclude dead cells which have a high degree of autofluorescence. For this reason, in our next gate we excluded both 7AAD+ dead cells as well as cells that express mature lineage markers (lin+ cells). Exclusion of 7AAD+ and lin+ cells leaves us with the lineage negative (lin-) subset of the BM. At 24 hours post radiation injury, the lin- cell subset is severely depleted (**Figure 4**). Within the lin- population, we then looked at expression of the cell surface markers c-kit and sca-1. As can be seen in **Table 1**, the double positive c-kit+sca-1+ lin- (KSL) population encompasses a mix of LT-HSCs, ST-HSCs, MPPs, and CLPs. Whereas, the c-kit+sca-1- lin- (c-kit+lin-) designates cells of the

myeloid lineage and includes the CMP, GMP, and MEP populations. At 24 hours, both the c-kit+lin- myeloid progenitor cells and KSL population were significantly depleted by 5 Gy TBI.

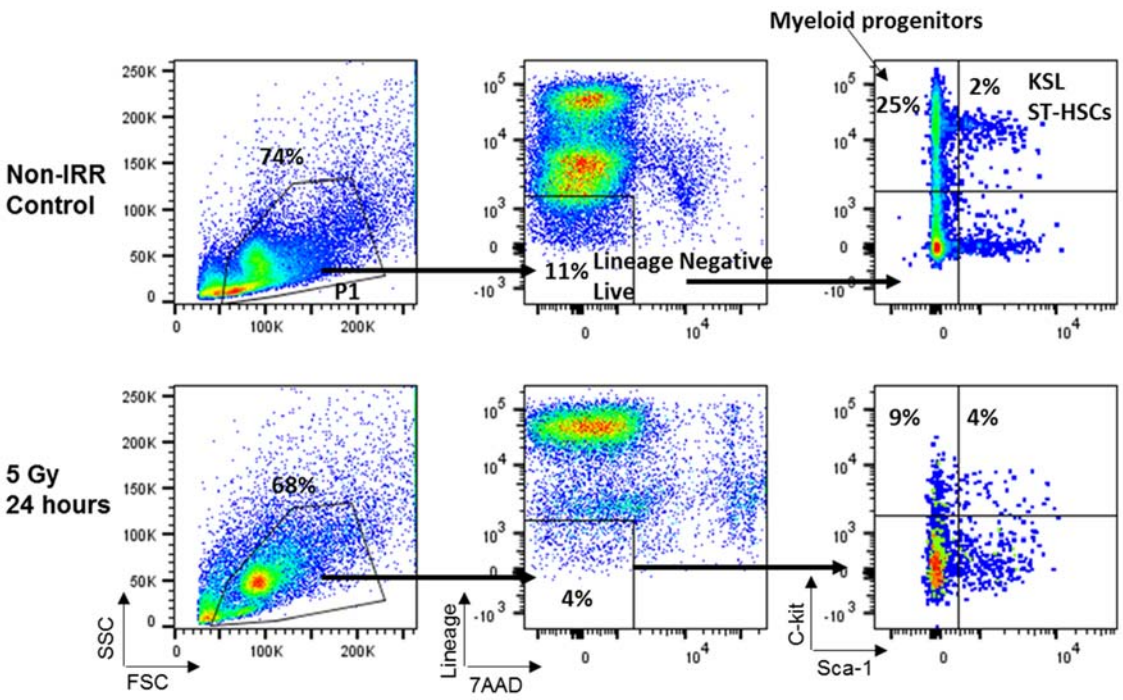

Figure 4. Representative flow cytometric analysis of the BM in steady state and following 5 Gy TBI.

Recovery of BM HSC and progenitor populations following injury is a good indicator of functional hematopoietic regeneration. However, true functional assessment of BM recovery after injury is measured by the ability of the adult BM to produce mature blood elements of all lineages. For this reason, the following sections will evaluate ways to functionally measure hematopoietic recovery after radiation injury.

Peripheral Blood Complete Cell Count (CBC) Analysis

In the acute phase of radiation injury (day 0 to day +28), patients are susceptible to death due to infection or bleeding from a lack of mature circulating blood cells and platelets. This is particularly true in the case of multi-organ radiation injury models where the barrier function of the skin or gastrointestinal tract is compromised allowing pathogens into circulation. (Mettler and Voelz, 2002; Waselenko et al., 2004). Therefore, recovery of mature circulating neutrophils, lymphocytes, and platelets are a critical measure of BM function after radiation injury.

Although there is an immediate decline in BM progenitor cell populations, there is a delay in observing an effect in the peripheral blood (PB) since the mature blood cells are relatively radioresistant. This is evident in a standard time course measurement of complete blood cell counts (CBC) after injury. Representative CBC data for total peripheral blood white blood cells (WBCs), neutrophils, and lymphocytes following 5 Gy TBI in a C57BL/6 mouse are shown in **Figure 5**. Following 5 Gy TBI, PB WBCs drop precipitously. The nadir in PB WBCs after 5 Gy

occurs typically at day +10. Note, at this same radiation dose the nadir in total bone marrow counts occurs at day +3 **(Figure 2).** If the TBI dose is increased to 7 Gy, PB counts fall eveb more rapidly due to toxicity to both progenitor and mature blood cell populations. At 7 Gy, the nadir is reached by day +4 and the recovery of mature PB cells is delayed compared to the 5 Gy TBI dose. By day 20, CBC counts are recovered at 5 Gy but remain depressed following 7 Gy TBI.

Typically, doses less than 7 Gy TBI are sub-lethal in the C57BL/6 mouse model. TBI doses between 5-7 Gy are useful for evaluating the effects of therapeutic mitigators on short-term recovery of PB mature elements and BM HSC and progenitor populations. It is important to note here that the act of sampling blood from an irradiated mouse is stressful to the animal and has been demonstrated to increase mortality in LD50/30 studies (Plett et al., 2012). For this reason, we recommend using a separate cohort of mice for each analysis time point following $\geq$ 5 Gy TBI.

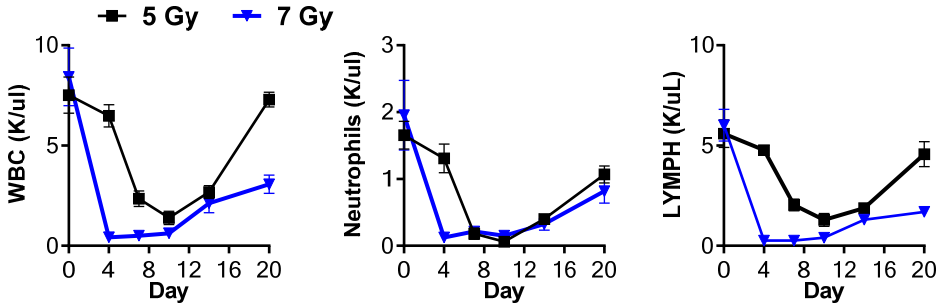

Figure 5. Time course of peripheral blood WBCs, neutrophils, and lympocytes following 5 Gy (black) and 7 Gy (blue) TBI. Unpublished data, Chute laboratory.

LD50/30 Survival Analysis

The ultimate goal for a novel hematopoietic regenerative therapy is the ability to rescue an exposed individual from death due to acute radiation sickness. For this reason, it is useful to evaluate therapeutics in a murine LD50/30 survival model. At UCLA, TBI doses around 7.5 – 8.5 Gy, delivered by a Cs137 source, cause death in approximately 50% of adult C57BL/6 mice by day 30 (LD50/30). A representative survival curve is shown for a cohort of C57BL/6 mice irradiated with 7 and 8 Gy TBI on a Cs137 irradiator (**Figure 6**). Mice are more susceptible to death due to hematopoietic failure from days 10-24 following radiation injury. The LD50/30 dose varies based on age, sex, and weight of the C57BL/6 mouse. Plett et al. also demonstrated that antibiotic administration in the drinking water and subcutaneous saline injection can promote survival in LD50/30 studies in the C57BL/6 mouse model (Plett et al., 2012). In the C57Bl/6 mouse, TBI doses greater than 9 Gy are 100% lethal without a rescue BM transplant. Radiation sensitivity is also known to vary highly by strain with Balb/c mice being highly radiosensitive (Williams et al., 2010). John Storer of the Jackson laboratory

documented the differences in LD50/30 in ten common mouse strains (Roscoe B. Jackson Memorial Laboratory. and Green, 1975).

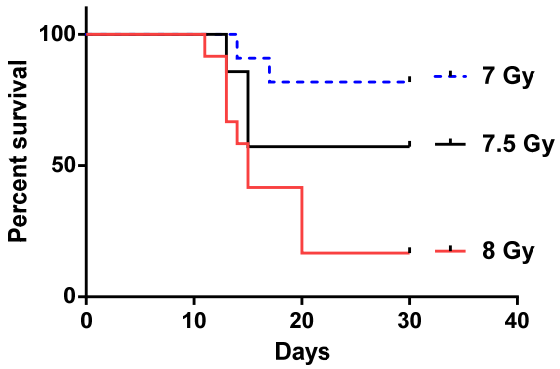

Figure 6. Percent survival in the C57/Bl6 mouse following 7 (blue), 7.5 (black), and 8 (red) Gy TBI in a Cesium-137 irradiator. Unpublished data, Chute laboratory, UCLA.

Colony Forming Cell (CFC) Assays

An easy, commercially-available (StemCell Technologies) assay for recovery of myeloid progenitors after radiation injury is the methycellulose-based colony forming cell (CFC) assay. In this assay, whole BM or KSL cells are introduced into methycellulose containing a standard set of cytokines for which promote the growth of primitive erythroid progenitor cells (BFU-E), granulocyte-macrophage progenitor cells (CFU-GM, CFU-G and CFU-M), and multi-potential granulocyte, erythroid, macrophage, megakaryocyte progenitor cells (CFU-GEMM)(Doan et al. *Nat Med* 19:295-304, 2013; Himburg et al. *Nat Med* 16:475-482, 2010). While the CFC assay is a good measure of myeloid progenitor recovery, it does not provide any information on HSC or lymphoid cell recovery.

Long-Term Hematopoietic Stem Cell Transplant Assays

Since the long-term hematopoietic stem cell (LT-HSC) has the ability to self-renew or differentiate to form all the blood cell lineages, it is the ideal target for a regenerative therapy to promote the long-term reconstitution of the hematopoietic system. Current therapeutics for hematopoietic regeneration target specific blood lineages (e.g. granulocyte colony stimulating factor, GCSF, Neupogen, and Peg-GCSF, Neulasta, Amgen, Inc.). For example, Neupogen promotes neutrophil recovery by promoting expansion of myeloid progenitors. Likewise, erythropoietin (Amgen) promotes erythrocyte recovery. However, no cytokine capable of stimulating HSC growth has yet to be successfully translated into clinical practice. Our laboratory has recently identified two candidate growth factors that are secreted by BM vascular niche cells, pleiotrophin (PTN) and epidermal growth factor (EGF), that promote HSC regeneration and hematopoietic reconstitution following TBI (Doan et al., 2013; Himburg et al., 2012; Himburg et al., 2010; Himburg et al., 2014). To validate the ability of these factors to promote expansion of a true LT-HSC stem cell, we used “the gold-standard” congenic competitive bone marrow transplantation assay. This is the standard assay for assessing the

ability of a donor cell population to engraft and reconstitute the blood system of a lethally irradiated congenic recipient mouse. This assay can be performed in different ways, best described in a review by Louise Purton and David Scadden (Purton and Scadden, 2007). In brief, BM from C57BL/6 mice expressing the CD45.2 allele can be transplanted into a lethally irradiated (9.5 Gy) B6.SJL mice which are congenic to C57BL/6 mice and express the CD45.1 allele (Himburg et al. *Nat Med* 16:475-482, 2010. Typically, a test cell dose of donor CD45.2 C57BL/6 cells is given with a "competitor" dose of non-irradiated B6.SJL BM (CD45.1+) cells. One to one dilutions of donor and competitor BM cells (e.g. 2 x 10^5 donor cells: 2 x 10^5 competitor BM cells) are typically utilized, but higher dilutions (e.g. 5:1 donor:competitor BM cells) may be necessary if donor mice have been irradiated prior to collection. Donor CD45.2+ cells can be distinguished from host CD45.1+ cells in the PB and BM of recipient using flow cytometry. If host and donor BM cells have equal long-term HSC content and function, there should be approximately 50% donor cell engraftment in this mouse model.

In the case of radiation injury to the LT-HSC, it has been observed that radiation injury to the LT-HSC induces a premature aging phenotype where the LT-HSC undergoes senescence and loses ability to repopulate upon transplantation (Wang et al., 2006)(Shao et al., 2014). For this reason, transplantation of a given dose of irradiated whole BM cells results in significantly lower long term engraftment in recipient mice than the same cell dose of non-irradiated BM cells. For these reasons, we have adapted the standard congenic described above to assess bone marrow from irradiated donor mice. The schematic for this assay is depicted in **Figure 7.** Here, mice are irradiated and treated per study protocol to evaluate a candidate mitigator. At some time period following irradiation and treatment, donor mice are euthanized and BM collected. Our laboratory typically assays for HSC recovery around 7-10 days following TBI so that several doses of the candidate mitigator can be administered. We have found that 5 x 10^6 BM cells collected from 5 Gy - irradiated C57Bl/6 mice at day +10 results provides approximately 5-10% donor cell engraftment in the PB of congenic recipient mice by 16 weeks when a competitor BM cell dose of 1 x 10^5 BM cells is used (5:1 dilution). Therefore, when evaluating candidate mitigators of radiation – induced HSC injury, we perform sufficient replicates to observe differences of > 30% between engraftment of donor CD45.2+ cells over time (4-20 weeks) when comparing control, irradiated donor mice versus irradiated, mitigator – treated donor mice.

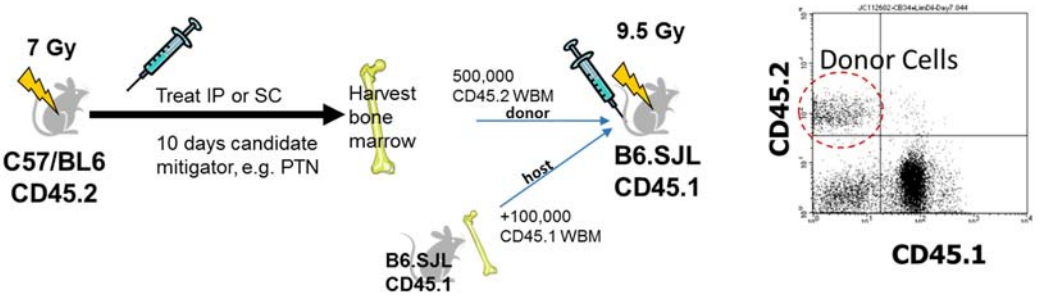

Figure 7. Schematic of Competitive Congenic Bone Marrow Transplant Assay Adapted for Measuring a test dose of irradiated whole bone marrow cells.

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