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blood advances

Autophagy prevents graft failure during murine graft-versus-host disease

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Key Points

- HSC increase autophagy in response to mitochondrial stress and cytokine stimulation with IL-1β and TNF identified as key inducers.
- HSC and HPC require autophagy to overcome stressors in the GVHD setting in order to successfully engraft and reconstitute the bone marrow.

Autophagy is an intracellular survival process that has established roles in the long-term survival and function of hematopoietic stem cells (HSC). We investigated the contribution of autophagy to HSC fitness during allogeneic transplantation and graft-versus-host disease (GVHD). We demonstrate in vitro that both tumor necrosis factor and IL-1β, major components of GVHD cytokine storm, synergistically promote autophagy in both HSC and their more mature hematopoietic progenitor cells (HPC). In vivo we demonstrate that autophagy is increased in donor HSC and HPC during GVHD. Competitive transplant experiments demonstrated that autophagy-deficient cells display reduced capacity to reconstitute the hematopoietic system compared to wild-type counterparts. In a major histocompatibility complex-mismatched model of GVHD and associated cytokine dysregulation, we demonstrate that autophagy-deficient HSC and progenitors fail to establish durable hematopoiesis, leading to primary graft failure and universal transplant related mortality. Using several different models, we confirm that autophagy activity is increased in early progenitor and HSC populations in the presence of T-cell–derived inflammatory cytokines and that these HSC populations require autophagy to survive. Thus, autophagy serves as a key survival mechanism in HSC and progenitor populations after allogeneic stem cell transplant and may represent a therapeutic target to prevent graft failure during GVHD.

Introduction

Allogeneic hematopoietic stem cell transplantation (SCT) is a well-established treatment for a number of hematopoietic malignancies that cannot be cured by conventional chemotherapy. Transplantation success relies on the proliferative potential of donor hematopoietic stem cells (HSC) and their unique ability to reconstitute the recipient's entire hematopoietic system and restore immunity. The curative property of SCT relies on naïve donor T cells within the graft being able to recognize recipient antigens as foreign and target residual malignant cells for destruction. This is referred to as the

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All materials, data sets, and protocols are available on request from the corresponding authors, Kelli P. A. MacDonald (Kelli.MacDonald@qimrberghofer.edu.au) and Katie E. Lineburg (Katie.Lineburg@qimrberghofer.edu.au).

The full-text version of this article contains a data supplement.

graft-versus-leukemia (GVL) effect. For this reason, donor T cells are an important component within the graft, but these same T cells are also the primary mediators of an early cytokine storm that drives the establishment of a highly inflammatory milieu resulting in graft-versus-host disease (GVHD), a life-threatening complication of SCT.[2] Establishing transplant protocols that maximize the efficiency of HSC engraftment and GVL, whereas minimizing GVHD has been a long-term goal in the field.

The stem cell graft contains a variety of pluripotent cell types, including HSC in addition to more differentiated hematopoietic progenitor cells (HPC).[3-5] HPC and HSC (collectively called HSPC) are responsible for providing almost immediate, short-term hematopoiesis to protect the host after myeloablation and stem cell transplantation.[6] Engraftment after SCT is defined by the reconstitution of the host hematopoeitic compartment and is measured by an absolute neutrophil count (ANC) greater than 0.5×10^9 cells per liter, lasting 3 consecutive days.[7] Primary graft failure is characterized by an absence (<95%) of donor cell engraftment 28 days after allo-SCT. In contrast, secondary graft failure is characterized by loss of hematopoiesis after successful primary engraftment and recurrent ANC ($<0.5 \times 10^9$/L).[8-10] Studies have reported that after acute lethal irradiation, primary bone marrow (BM) reconstitution is primarily mediated by committed myeloid progenitors.[11]

Allogeneic SCT represents perhaps the most stressful therapeutic procedure undertaken in regard to BM stem cells and the BM microenvironment. Indeed GVHD itself causes T-cell–mediated destruction of hematopoietic niches resulting in impaired BM haematopoiesis.[12] Studies determined that distinct biological regulation is required for stress hematopoiesis, which includes that after SCT, and have identified that the HSPC that maintain hematopoiesis after a SCT may indeed differ from those required to sustain naïve steady-state hematopoiesis.[13,14]

Macroautophagy (herein referred to as autophagy) is a highly conserved and tightly regulated catabolic process that involves the sequestration and lysosomal degradation of cellular components including mitochondria, endoplasmic reticulum, peroxisomes, misfolded proteins, and damaged organelles.[15-18] More than 30 autophagy related genes (Atg) are known to contribute to this process.[19] Studies to date have demonstrated that autophagy is required for the survival and function of HSC in the steady state.[20] Conditional ablation of *Atg7* in all hematopoietic cells, using the VAV^{cre} system, led to an accumulation of mitochondria and reactive oxygen species in HSC. $VAV^{cre}Atg7^{fl/fl}$ mice displayed a significant reduction in HSC number within the $Lin^{neg}c\text{-}Kit^{+}Sca\text{-}1^{+}$ (LKS^{+}) compartment. Furthermore, production of both lymphoid and myeloid progenitors was impaired.[21,22]

To date, little has been described with regard to HSPC in the setting of allogeneic SCT and a possible role of autophagy in stem cell survival and differentiation during the early posttransplant period when the cytokine storm is induced and HSC are critically required for primary engraftment.

Materials and methods

Mice

Female mice aged 8 to 14 weeks were used for all transplants. Strains used are listed in supplemental Table 1. Mice were housed in sterilized microisolator cages and received acidified autoclaved water (pH, 2.5) after transplantation. Animal studies were performed in accordance with QIMR Berghofer Ethics Protocols.

BM staining

BM cells were stained using antibodies purchased from BioLegend; lineage negative cells were identified by staining with a lineage cocktail comprised of biotinylated antibodies against B220 (RA3-6B2), CD3ε (145-2C11), CD5 (53-7.3), Gr-1 (GB6-8C5), Mac-1 (M1/70), and Ter-119 (TER-119) and tertiary staining with streptavidin (SA) PE/Cy7. Staining with anti-c-Kit and anti-Sca-1 allowed gating the HSC containing $Lineage^{neg}Kit^{+}Sca\text{-}1^{+}$ ($LKS^{+)}$ population and HPC containing LSK^{-} population. As Sca-1 can be upregulated broadly on leukocytes during inflammation, for post-transplant samples endothelial cell-selective adhesion molecule (ESAM) was substituted for Sca-1 staining to identify HSC as previously described.[23] Staining with CD48 and CD150 antibodies identified long-term HSC (LT-HSC) within LKS^{+}. Addition of anti-CD34 and anti-CD16/32 mAb permitted HPC to be divided into progenitor subpopulations: granulocyte macrophage progenitors (GMP) defined as FcγR $CD16/CD32^{high}$ $CD34^{+}$; common myeloid progenitors (CMP) defined as $CD16/CD32^{mid}$ $CD34^{+}$; and megakaryocyte erythroid progenitors (MEP) defined as CD16/CD32 negative $CD34^{-}$.[24] Antibody incubations were performed for 20 to 30 minutes at 4°C before flow cytometric analysis.

Flow cytometry analysis

Flow cytometric analysis was performed using a LSR Fortessa cytometer (BD Biosciences) using BD FACSDiva (v8.0) and analyzed using FlowJo software (v10).

Imaging flow cytometry

Imaging flow cytometry was used to identify and simultaneously image single cells in real time. BM from LC3-GFP mice was stained and LKS^{+} and LKS^{neg} populations gated. Imagestream^{X} (Amnis) imaging software was used to measure the accumulation of LC3 II protein on the autophagosome membrane (as a readout of autophagy activity). Images (×60) and statistics were processed using the IDEAS software (version 6.0, Amnis).

Cell counts

Blood counts were determined with the Hemavet 950FS (Drew Scientific).

Atg5$^{-/-}$ fetal liver (FL) chimeras

To generate FL chimeras, mice heterozygous for the Atg5 deficiency were time-mated and at E17.5 pregnant mice were culled, pups removed and the FL dissociated into a single cell suspension. Female $Atg5^{-/-}$, $Atg5^{+/-}$, or wild-type (WT) pups were identified by DNA genotyping and 2×10^6 to 3×10^6 FL cells from each were transferred into lethally irradiated (1000 cGy) B6.SJL-*Ptprc*^{a} recipient mice. Chimeric mice were allowed 3 months to reconstitute the immune system. Donor engraftment was confirmed to be >98%.

Stem cell and BM transplantation

On day 1, recipient mice received 1000 cGy (B6) or 1100 cGy (B6D2F1) total body irradiation (TBI, 137Cs source at ~85 cGy per minute), split into 2 doses. In FL transplant experiments, B6D2F1 recipients underwent transplantation on d0 with 3×10^6 FL cells

with or without 1 × 10^6 purified T cells from CD45.1$^+$ B6.SJL-*Ptprc*^{a} donors. Non-GVHD control groups were injected with FL-only grafts. For bone marrow transplant (BMT) experiments, B6D2F1 recipients underwent transplantation on d + 0 with 5 × 10^6 BM from either *Atg7*$^{-/-}$*Lyz2*^{cre}, *Atg7*$^{-/-}$*VAV*^{cre}, or appropriate WT littermate controls ± 1 × 10^6 purified T-cells from CD45.1$^+$ B6.SJL-*Ptprc*^{a} donors.

Cyclosporine administration

Transplant recipients were treated with cyclosporine (CsA) or saline based on animal weight (25 μg/gram body weight; Novartis Pharma, Basel, Switzerland)[25] daily from day 0 to day 14 through intraperitoneal injection.

Assessment of GVHD

Systemic GVHD was assessed using a cumulative scoring system measuring changes in 5 clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity (maximum index 10). Transplanted mice were monitored daily, and those with GVHD clinical scores of ≥6[26,27] were culled (date of death registered as the next day).

In vitro cytokine analysis

BM was isolated from B6.LC3-GFP mice and 5 × 10^6 cells cultured for 4 hours at 37°C in media supplemented with 20 ng/mL recombinant murine cytokine (IL-1β, tumor necrosis factor [TNF], IFNγ, granulocyte colony stimulating factor (G-CSF), CCL2, IL-6, or IL-1β+TNF) or nonsupplemented media. After 4 hours of incubation, cells were washed and surface-stained.

Serum cytokine analysis

Serum cytokine concentrations were determined using the BD cytometric bead array system (BD Biosciences Pharmingen) according to the manufacturer's protocol.

LC3 Western blot analysis

CD34$^+$ cells were isolated from umbilical cord blood by density gradient centrifugation, followed by magnetic bead selection (Miltenyi Biotech). Purified CD34$^+$ cells were cryogenically stored until use. The use of human cord blood for research purposes was approved by the QIMR Berghofer Medical Research Institute Human Research Ethics Committee (P1580) and the Mater Misericordiae Ltd Human Research Ethics Committee (26472) and was performed according to the principles of the Declaration of Helsinki. Thawed human CD34$^+$ cells were stimulated for 6 hours at 37°C in the presence or absence of cytokines TNF+IL-1β (100 ng/mL each) and +/− chloroquine (80 uM). Samples were snap frozen in radioimmunoprecipitation assay buffer. Protein separation and staining is described in supplemental Methods.

Mitochondrial analysis

Posttransplant BM cells or human CD34$^+$ stimulated in vitro +/− TNF + IL1β, were stained with a murine HSC mAb panel or human CD34 respectively, washed and resuspended in Hanks Balanced Salt Solution with 20 nM TMRM (Thermofisher, cat#T668) and 25 nM MitoTracker Green (Thermofisher, cat# M7514) and incubated at 37°C for 45 minutes before flow-cytometric analysis.

Statistical analysis

Survival curves were plotted using Kaplan-Meier estimates and compared by log-rank analysis using PRISM 6 (GraphPad software). *P* values < .05 were considered statistically significant. A 2-tailed Mann-Whitney *U* test or an unpaired *t* test was used to evaluate significant differences between groups, and all data are represented as mean ± standard error of the mean.

Results

Autophagy is active in hematopoietic and progenitor cells at steady state

To determine whether autophagy is an active process in HSPC, we used flow cytometry and multispectral imaging flow cytometry to compare autophagosome formation in HSC and HPC. Using BM from LC3-GFP mice, which express GFP driven off the LC3 promoter and permit the identification of LC3-GFP autophagosomes,[28] we were able to quantify the number of autophagosomes as a measure of autophagic activity in subsets of cells. Gating on lineage negative BM cells, these were divided into LKS$^+$ and LKS^{neg} populations (Figure 1A). Steady-state expression of LC3-GFP was greater in LKS$^+$ (90.6%), including both long-term (LT)-HSC (99.1%) and short-term (ST)-HSC (79.2%), than LSK^{neg} HPC (41.7%) cells (Figure 1B). Furthermore, imaging flow cytometry analysis demonstrated the intracellular localization of LC3 and its accumulation on the autophagosome membrane, thus confirming the constitutive activity of this pathway in both HSC and HPC (Figure 1C). Quantification of LC3-GFP punctae revealed a significantly higher number of autophagosomes in HSC compared to HPC at steady state (Figure 1D). Thus, at steady state, HSC demonstrate a significantly greater level of autophagy than their myeloid progenitor counterparts.

Autophagy is dispensable for HSC and HSPC development

Hematopoietic ablation of *Atg7*, under the *VAV*^{cre} promoter, was previously shown to be essential for HSC maintenance.[21] To examine the impact of ablation of *Atg5*, another core autophagy protein, on HSC and progenitor cells, we utilized mice harboring a global deficiency in *Atg5*.[29] Because germinal deletion of *Atg5* results in neonatal lethality,[30] we examined the impact of *Atg5* deficiency on the HSC compartment in FL harvested from pups at embryonic day 18 (E17.5). Although, the absolute cell numbers in *Atg5*$^{-/-}$ FL were significantly reduced compared to WT littermates (Figure 2A), the absolute number of LKS$^+$ did not differ between WT and *Atg5*$^{-/-}$ FLs (Figure 2B). Similarly, LKS^{neg} HPC numbers and the GMP, CMP, and MEP subsets within this population did not differ between WT and *Atg5*$^{-/-}$ mice (Figure 2C). Thus, we confirmed that autophagy is not required for the development of HSC or HPC in FL.

Autophagy-deficient cells display delayed engraftment and reduced competitive fitness

Having established that autophagy was not required for the generation of FL HSPC, we next examined the functional requirement for autophagy in these cells. HSC from conditional *Tie2*^{Cre}/*Fip200*$^{-/-}$ and *VAVc*^{re}/*Atg7*$^{-/-}$ adult mice are not capable of reconstituting lethally irradiated recipient mice.[20,21] To determine whether *Atg5*$^{-/-}$ FL were capable of functional engraftment, we transplanted B6.*Atg5*$^{-/-}$ or littermate B6.WT (CD45.2$^+$) FL cells into lethally irradiated congenic CD45.1$^+$ B6.SJL WT recipients and monitored engraftment in the BM, spleen, and peripheral blood at 6 weeks posttransplant (Figure 2D). These experiments showed that in a noncompetitive engraftment setting, *Atg5*$^{-/-}$ FL cells were

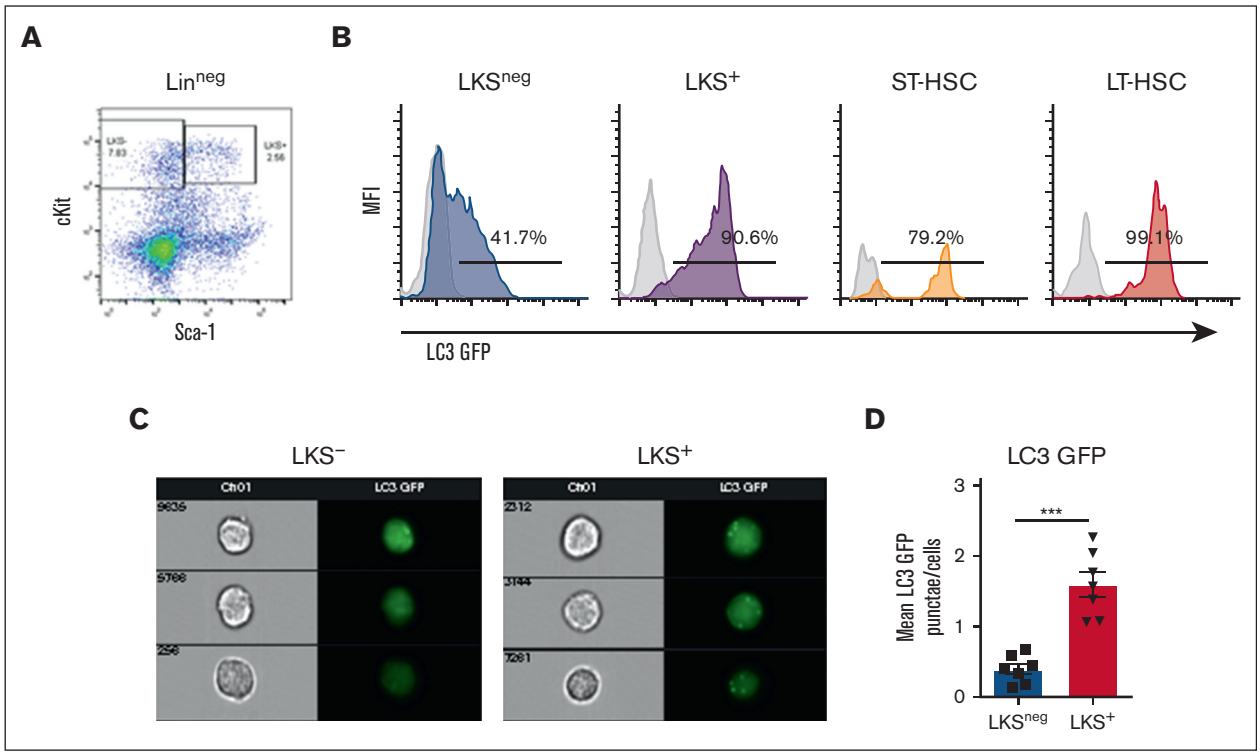

Figure 1. LC3-GFP in naïve BM stem-cell populations. (A-D) Analysis of freshly isolated BM cells from LC3-GFP mice. (A) Lineage negative (Lin^-) cells were divided into $cKit^+Sca\text{-}1^+$ (LKS^+) and $cKit^+Sca\text{-}1^-$ (LKS^{neg}) populations. (B) Representative histograms of flow cytometric analysis for LC3 expression. Frequency of $LC3^+$ cells in LKS^{neg}, LKS^+, short-term HSC (ST-HSC and LKS^+ $Flt3^-CD150^-CD48^-$), and long-term HSC (LT-HSC and LKS^+ $Flt3^-CD150^+CD48^-$) (2 independent experiments; n = 6). (C) Representative images from imaging flow cytometry analysis of LC3-GFP BM. (D) Quantification of LC3-GFP punctae in LKS^{neg} and LKS^+ BM populations (n = 7, from 2 independent experiments). Data are shown as mean ± SEM. Statistical significance was determined using an unpaired 2-tailed Mann-Whitney test *U* test (***$P = .006$). SEM, standard error of the mean.

capable of engrafting and reconstituting the recipient hematopoietic system, albeit with a significantly reduced engraftment compared with WT FL cells.

We next compared the competitive fitness of autophagy-deficient HSC with WT cells. Thus, lethally irradiated *Rag1*-deficient recipients, which lack mature B and T lymphocytes,[31] were transplanted with grafts containing equivalent numbers of 50:50 ratio of WT ($CD45.1^+$) and autophagy-deficient *Atg5*$^{-/-}$ or hemizygous *Atg5*$^{+/-}$ ($CD45.2^+$) FL (Figure 2E). Six weeks posttransplant, we used flow cytometry to distinguish $CD45.1^+$ WT cells from $CD45.2^+$ *Atg5*-deficient cells and observed significantly reduced reconstitution of both *Atg5*$^{-/-}$ and *Atg5*$^{+/-}$ compared with WT (Figure 2E). Because $CD45.1^+$ cells exhibit an engraftment disadvantage compared with $CD45.2^+$ cells,[32] these results support a role for autophagy in engraftment posttransplant. Furthermore, the ratio of donor *Atg5*$^{-/-}$ to WT cells was further reduced compared to that of Atg5$^{+/-}$ to WT cells, demonstrating that there is a dose response to the level of autophagy required for these cells to engraft early posttransplant. This superior engraftment by WT cells demonstrates that after congenic transplantation, autophagy is required for optimal engraftment.

Autophagy is upregulated after allogeneic transplant in the setting of GVHD

After allogeneic transplantation, the inflammatory milieu that ensues may elicit additional requirements for autophagy within HSC and HPC to facilitate engraftment. To determine the requirement for autophagy in this setting, we used the well-established C57BL/6 into B6D2F1 model of GVHD to examine autophagic activity in HSC and HPC at d7 posttransplant when acute GVHD has been initiated and GVHD clinical scores diverge significantly from non-GVHD control mice.[33] Here, we utilized the transgenic B6.LC3-GFP reporter mice and transplanted 5 × 10^6 LC3-GFP BM cells together with or without 2 × 10^6 LC3-GFP $CD3\varepsilon^+$ T cells to induce GVHD. At day 7 posttransplant, we analyzed the level of autophagy in HSC and HPC in the BM (Figure 3A). BM resident lineage negative cells were selected and HSC and HPC populations gated as in Figure 1. Using both conventional flow cytometry and imaging flow cytometry, we quantified the level of autophagy in these populations by measuring the intensity of LC3-GFP fluorescence and the number of LC3-GFP punctae in individual cells respectively. Conventional flow cytometry analysis confirmed that LC3-GFP mean fluorescent intensity was significantly higher in Lin^{neg} in the setting of GVHD compared to that of non-GVHD recipient BM (Figure 3B). Furthermore, imaging flow cytometry (Figure 3C) confirmed an increased number of autophagosomes in both $ESAM^+$ HSC and $ESAM^-$ HPC in the setting of GVHD. Additionally, autophagic activity was significantly increased in CMP and MEP subsets in the setting of GVHD however there was no difference observed in the GMP subset (Figure 3D). Thus, we demonstrate that autophagy is

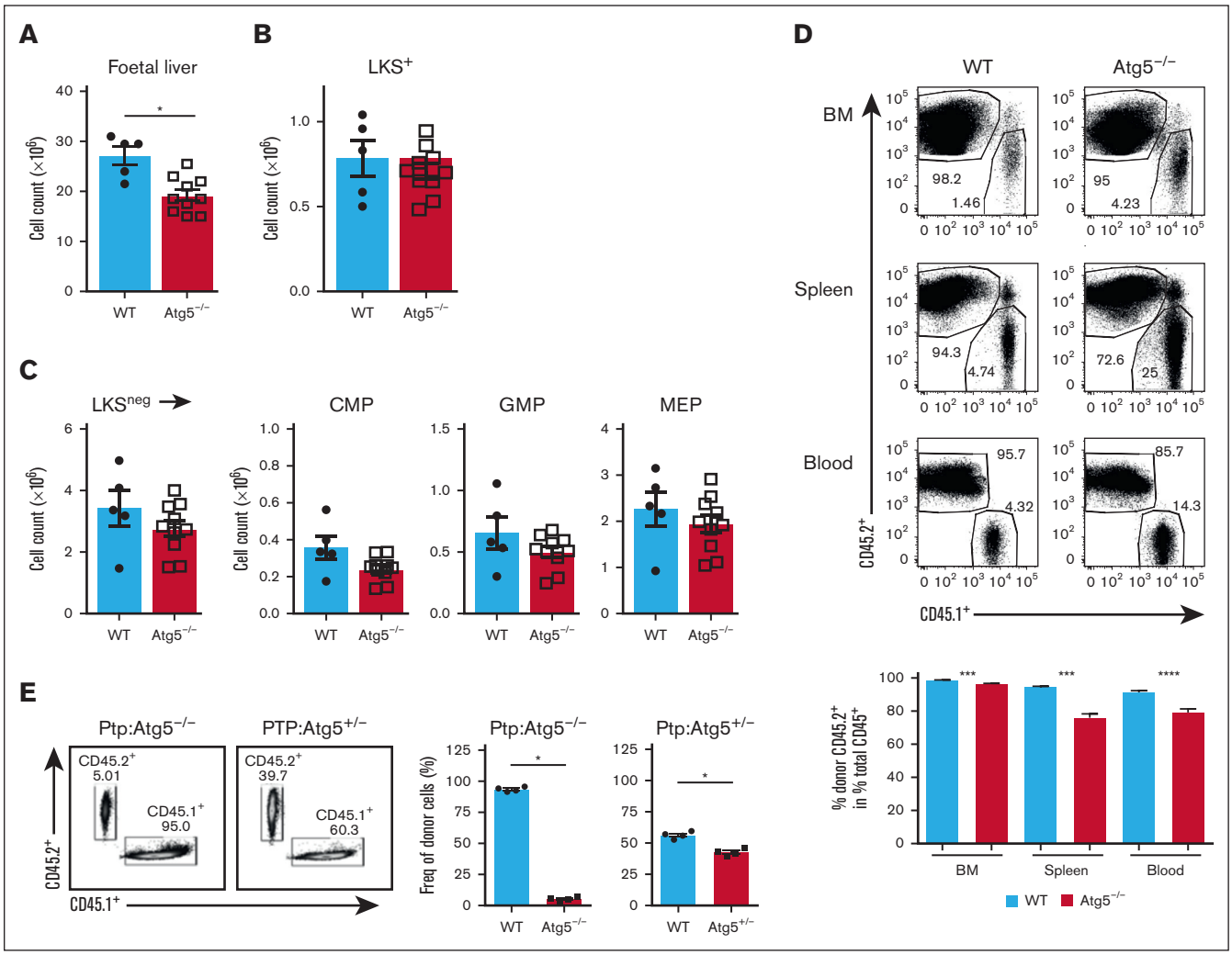

Figure 2. Autophagy-deficient cells demonstrate delayed engraftment. (A-C) Analysis of freshly isolated FL cells from embryonic day 17.5 (E17.5) (A) Absolute cell number in *Atg5*$^{-/-}$ FL compared with that in WT FL. (B) Comparison of absolute cell number in LSK^{+} cells between *Atg5*$^{-/-}$ and WT. (C) Absolute cell numbers of LKS^{neg} cells compared between *Atg5*$^{-/-}$ and WT followed by LKS^{neg} subsets: GMP, CMP, and MEP. (D-E) Analysis of engraftment in chimeric mice. Recipients were lethally irradiated and reconstituted with FL cells in a 1:1 ratio comprised of B6.SJL-*Ptprc*^{a} (CD45.1^{+}):*Atg5*$^{-/-}$ (CD45.2^{+}) cells. (D) Analysis of competitive engraftment in BM, spleen, and blood using flow cytometry to differentiate WT.B6.SJL-*Ptprc*^{a} (CD45.1^{+}) donor cells from *Atg5*$^{-/-}$ (CD45.2^{+}) donor cells with relative donor frequencies (%) of total CD45^{+} cells displayed below (n = 7 from 2 repeat experiments). (E) Representative plots of competitive engraftment between WT.B6.SJL-*Ptprc*^{a} (CD45.1^{+}) and either *Atg5*$^{-/-}$ (total KO) or *Atg5*$^{+/-}$ (heterozygous KO) both (CD45.2^{+}) followed by relative donor frequencies (n = 4 representative of results from 2 repeat experiments). Data are shown as mean ± SEM. Statistical significance was determined using an unpaired 2-tailed Mann-Whitney test *U* test (*$P = .0286$; ***$P = .0006$; ****$P < .0001$).

invoked in HSC and HPC populations early posttransplant in the setting of GVHD.

Autophagy is required for HSPC engraftment and survival during GVHD

Given the elevated autophagic activity observed in donor cells in the setting of GVHD, we next examined the functional impact of this process on stem cell fitness after allogeneic SCT. We first transplanted B6D2F1 recipients with 1.5 × 10^{6} to 3 × 10^{6} FL cells from either WT or *Atg5*$^{-/-}$ FL (CD45.2^{+}) together with 1×10^{6} B6.SJL-*Ptprc*^{a} (CD45.1^{+}) CD3ε^{+} T cells (FL+T). At d10 post-transplant, absolute numbers of platelets and neutrophils and hemoglobin concentration were dramatically reduced in the blood, as well as BM and spleen cellularity and myeloid cells in the recipients of *Atg5*$^{-/-}$ FL+ WT T grafts compared with recipients of WT FL+ WT T grafts (Figure 4A). To examine the impact of autophagy deficiency within the donor graft in the setting of GVHD, we compared the survival of irradiated recipients of either WT or *Atg5*$^{-/-}$ FL+ WT T-cell grafts (to induce GVHD) to recipients of *Atg5*$^{-/-}$ FL cells only (non GVHD). Recipients of FL only (non-GHVD) donor grafts survived long-term (day 60+) whereas more than 75% of recipients given WT FL + T grafts succumbed to GVHD by day 60 (Figure 4B). However, we observed significantly ($P < .0001$) accelerated mortality in the *Atg5*$^{-/-}$ FL+T recipient group with the majority succumbing by day 20 posttransplant. We next examined HSPC engraftment in the BM of recipients early (day 10) posttransplant. Gating on lineage negative cells we

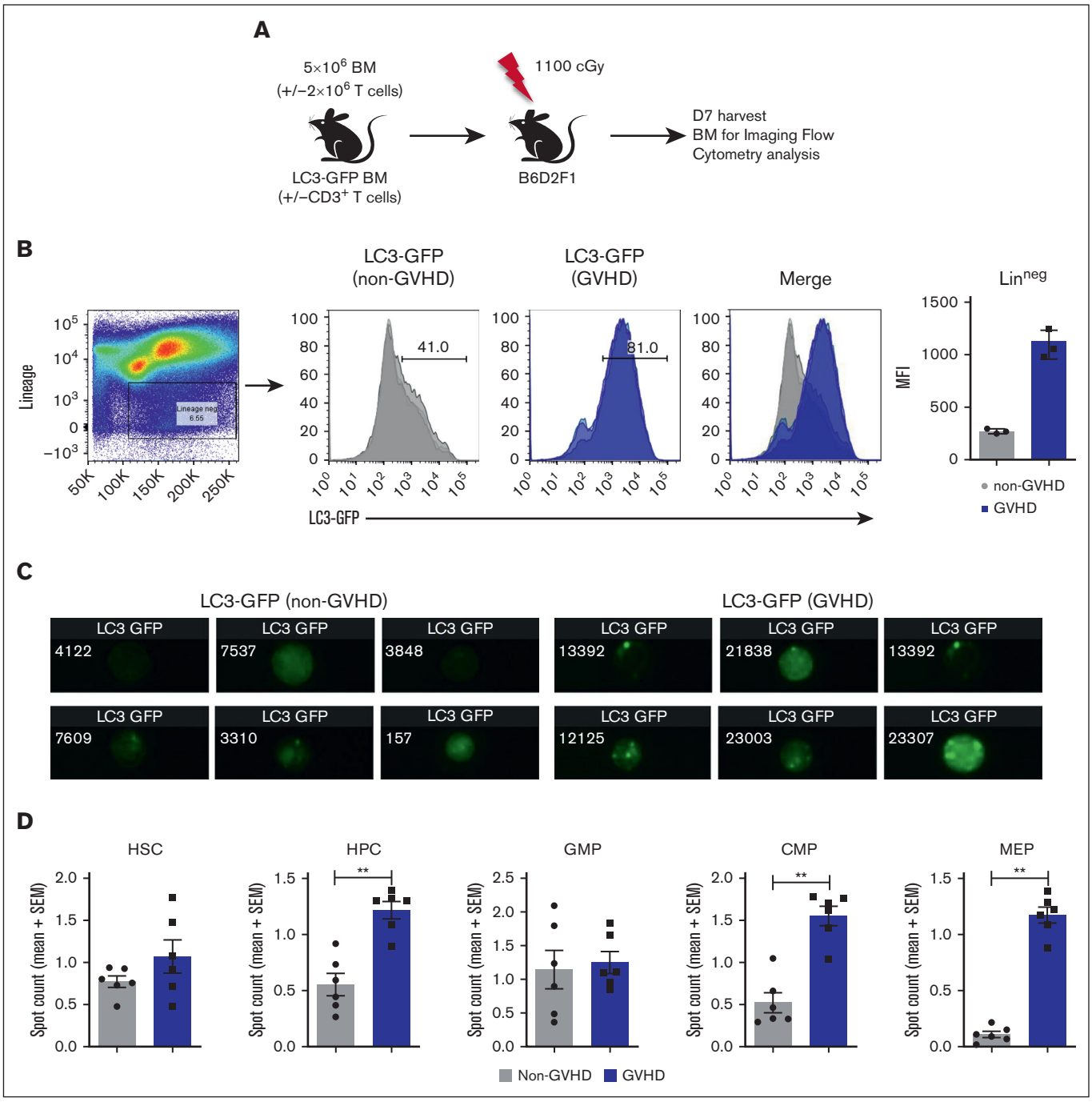

Figure 3. Autophagy is upregulated in the GVHD setting. (A) Lethally irradiated B6D2F1 mice were transplanted with 5 × 10^6 BM + 2 × 10^6 $CD3^+$ T cells (GVHD) or without T cells (non-GVHD) from LC3-GFP mice. BM was harvested at day 7 posttransplant. (B) Flow cytometry was used to identify lineage negative (Lin^-) BM cells and compare LC3-GFP expression between non-GVHD vs GVHD groups. Representative histograms comparing LC3-GFP MFI between non-GVHD and GVHD groups (n = 3; representative of 2 experiments). (C) Representative pictures of imaging flow cytometry (Amnis) analysis of LC3-GFP punctae in individual cells from the lineage negative BM population. (D) Mean spot count was quantified from imaging flow cytometric analysis. Posttransplant analysis uses the stem cell marker ESAM in place of Sca-1 to identify HSC and HPC subsets. Thus LKE^+ (previously LKS^+); LKE^{neg} (previously LKS^-); followed by progenitor subsets: GMP, CMP, and MEP (n = 6, representative of 2 repeat experiments). Data are shown as mean ± SEM. Statistical significance was determined using an unpaired 2-tailed Mann-Whitney test *U* test (**P = .0043; except MEP **P = .0022).

separated these into $Lin^-cKit^+ESAM^+$ (HSC) and $Lin^-cKit^+ESAM^-$ (HPC) populations (Figure 4C). Total lineage negative, HSC and HPC populations were all significantly reduced in recipients of *Atg5*$^{-/-}$ FL+T grafts. Further subsetting within the HPC compartment revealed significantly reduced frequency and absolute numbers in the GMP, CMP, and MEP populations in recipients of *Atg5*$^{-/-}$ FL+T grafts (Figure 4C). Notably, the transfer of *Atg5*$^{+/-}$ FL + T, in which autophagy is reduced but not ablated, also

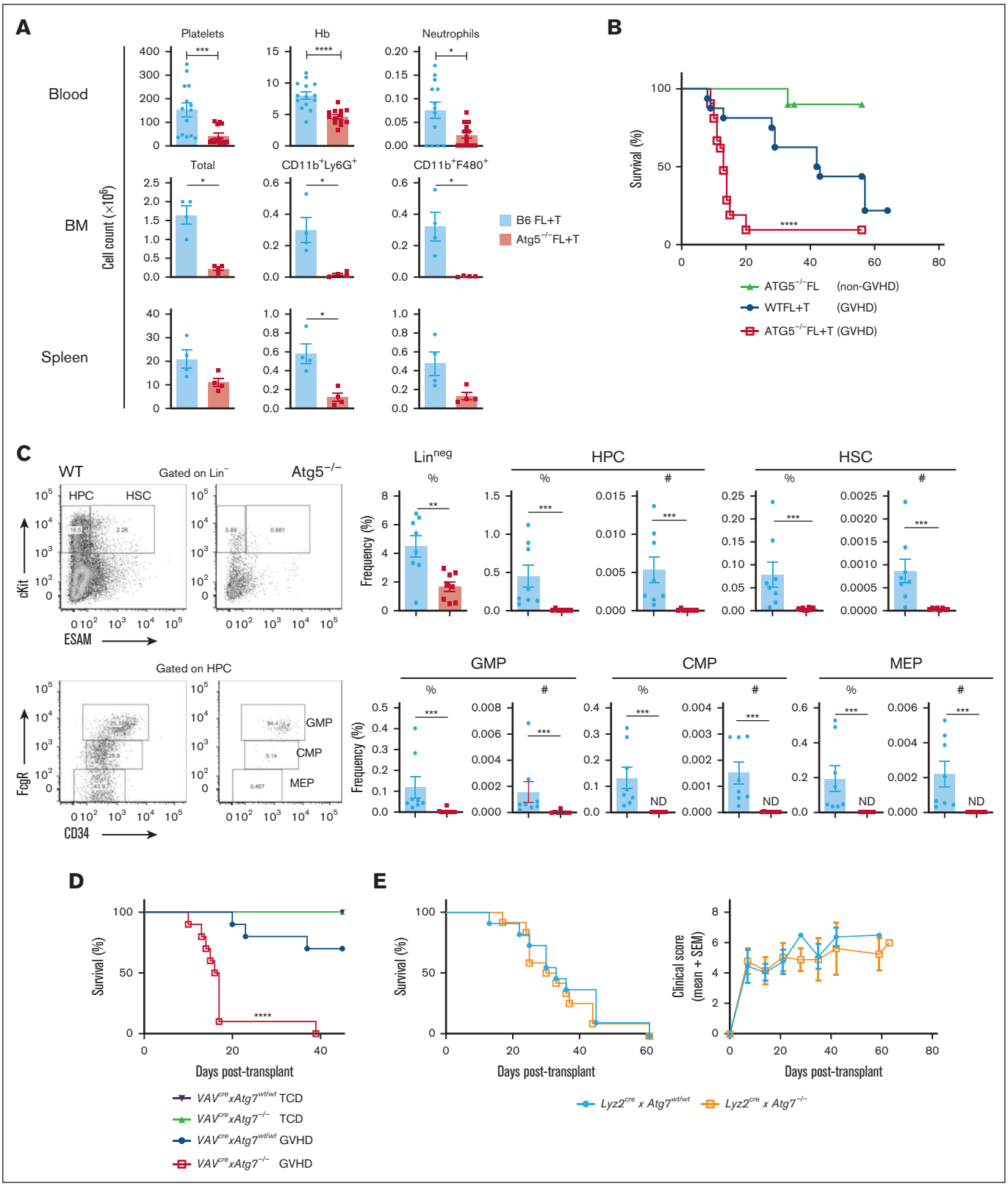

Figure 4. Autophagy is required during primary engraftment in the GVHD setting. (A-C) Lethally irradiated B6D2F1 mice were transplanted with WT or *Atg5*$^{-/-}$ FL cells together with CD3ε^{+} T cells (GVHD) or without (non-GVHD). (A) Engraftment, determined by total cell number of platelets, Hb (hemoglobin) and neutrophils in peripheral blood and total cell numbers together with CD11b^{+}Ly6G^{+} (granulocytes) and CD11b^{+}F480^{+} (macrophages) in BM and spleen (blood n = 14, representative of 2 repeat experiments; BM and spleen n = 4 taken from 1 representative experiment). Data are shown as mean ± SEM. Statistical significance was determined using an unpaired 2-tailed Mann-Whitney *U* test

impaired engraftment of HSC and HPC populations at d12 post-transplant (supplemental Figure 1). This result highlights the critical requirement for autophagy during HSC and HPC engraftment after allogeneic SCT. To confirm the role of autophagy, rather than off target effects of *Atg5* deficiency, we utilized a second model in which VAV^{cre} promoter expression drives the deletion of *Atg7* in all hematopoietic cells. Again, we observed significantly reduced survival in mice receiving *VAV*^{cre}*Atg7*$^{fl/fl}$ donor grafts (Figure 4D). To determine if this critical requirement for autophagy was restricted to HSC or additionally required by the more mature myeloid HPC, we examined the effect of ablating autophagy specifically in myeloid cells. Here, we crossed *Atg7*-floxed mice with Lyz2^{cre} to delete *Atg7* from the GMP stage of myeloid differentiation onward.[34] Lethally irradiated B6D2F1 recipients were administered a BM+T donor graft with either WT or *Lyz2*^{cre}*Atg7*$^{fl/fl}$ donor BM together with WT CD3$^+$ T-cell to induce GVHD. In this system, we observed no significant difference in clinical scores or survival between recipients of WT vs *Lyz2*^{cre}*Atg7*$^{fl/fl}$ donor grafts (Figure 4E), suggesting that although autophagy is critical in HSPC for successful primary engraftment posttransplant in the context of GVHD, it is dispensable in more mature myeloid cells.

Calcineurin inhibition suppresses inflammatory cytokine production and reduces the HSPC survival requirements for autophagy after allogeneic BMT

Having observed a critical requirement for autophagy in HSPC specifically in the setting of GVHD, we next examined whether inhibition of T-cell function could impact HSPC autophagy and the need for autophagy to engraft in the context of GVHD. To test this, we administered the calcineurin inhibitor cyclosporin (CsA) early after BMT to prevent the acquisition of donor T-cell effector function. Here, BM+T grafts from LC3-GFP donors were transferred into lethally irradiated B6D2F1 recipients, after which either saline or CsA were administered daily (Figure 5A). As expected, levels of inflammatory cytokines including IFNγ, TNF, and CCL2 (BM+T saline vs CsA, trend only; $P = .093$) in recipient BM+T mice treated with saline were elevated compared to T-cell depleted controls and the BM+T recipients treated with CsA (Figure 5B). Thus, CsA effectively reduced proinflammatory cytokine production in vivo. Next, we analyzed autophagy by LC3-GFP expression in lineage negative (HSC/HPC) cells at d7 posttransplant. BM+T recipients receiving CsA over the early posttransplant period displayed significantly reduced LC3-GFP signal compared to saline treated BM+T recipients (Figure 5C). Strikingly, CsA administration reduced LC3-GFP signals to levels equivalent to non-GVHD controls suggesting that activated alloreactive T cells drive autophagy in HSPC. We next examined the contribution of elevated proinflammatory cytokines to graft failure that occurs in the recipients of autophagy-deficient donor grafts. Cohorts of mice received WT or *Atg5*$^{-/-}$ FL ± WT T grafts, and either saline or CsA was administered daily posttransplant. D7 serum cytokine analysis confirmed elevated cytokine levels (IFNγ, TNF, and also CCL2) in recipients of both WT or *Atg5*$^{-/-}$ grafts, and also that levels were significantly reduced in mice administered CsA (Figure 5D). To determine whether the alloreactive T-cell–mediated cytokine storm could directly contribute to HSPC autophagy, freshly isolated LC3-GFP BM cells were cultured for 4 hours with or without recombinant TNF, IL-1β, G-CSF, IFNγ, CCL2, or IL-6 (each 10 ng/mL). Flow cytometric analysis demonstrated that TNF, IL-1β, and to a lesser extent G-CSF elicited significantly increased LC3-GFP fluorescence in LT-HSC (LKS$^+$Flt3$^-$CD48$^-$CD150$^+$), ST-HSC (LKS$^+$Flt3$^-$CD48$^-$CD150$^-$), LKS$^-$ HPC, GMP, and CMP populations (Figure 5E). IFNγ treatment also increased LC3-GFP in LKS$^+$ and GMP, CMP, and MEP subsets. Notably, simultaneous treatment with IL-1β+TNF resulted in the greatest increase in LC3-GFP fluorescence in LKS$^+$, ST-HSC, LT-HSC, GMP, and CMP populations (but not in MEP) demonstrating the cumulative impact of cytokine exposure on autophagy activity in HSC and HPC populations. Furthermore, using in vitro culture in the presence or absence of chloroquine, we demonstrate constitutive basal autophagy activity in human CD34$^+$ stem cells and confirm that stimulation with IL-1β+TNF upregulates autophagy activity (Figure 5F), which is in line with our observations in murine HSC and HPC cells. Finally, we found CsA administration for the first 14 days posttransplant significantly improved survival in recipients of *Atg5*$^{-/-}$ BM + WT T grafts (Figure 5G) demonstrating that inhibition of alloreactive T-cell activation not only reduces the cytokine storm but relieves the need for autophagy in HSPC after allogeneic transplant.

Although autophagy is known to be important in steady state stem cell maintenance, our data demonstrate an additional protective role for autophagy in HSC and HPC in response to the inflammatory stress and altered BM microenvironment encountered during GVHD. Functionally, autophagy allows cells to survive harsh environments through the removal of damaged organelles and misfolded or aggregated proteins. Mitochondria have key functions in HSC homeostasis and mitophagy (the specific removal of damaged or dysfunctional mitochondria through autophagy) represents a key mechanism by which healthy mitochondrial activity is maintained.[35] By examining mitochondrial functionality posttransplant, we observed modest changes in the mitochondrial profile of donor HSC in the BM of GVHD mice compared to non-GVHD controls. During GVHD, BM HSC exhibited a subtle reduction in mitochondrial density and membrane potential (supplemental Figure 2A) indicative of mitochondrial stress. Moreover, this shift in mitochondrial profile was recapitulated in human

Figure 4 (continued) (*$P = .0286$; **$P = .0437$; ***$P = .0003$; and ****$P < .0001$). (B) Survival analysis of transplant recipients given *Atg5*$^{-/-}$ or WT FL cells together with WT T cells (GVHD) or without (non-GVHD) (*Atg5*$^{-/-}$ GVHD n = 21; WT GVHD n = 16; *Atg5*$^{-/-}$ non-GVHD n = 10). (C) Flow cytometry analysis of HSC (LKE$^+$: Lin^{neg}cKit$^+$ESAM$^+$) and myeloid HPC (LKE^{neg}: Lin^{neg}cKit$^+$ESAM^{neg}) populations: CMP, GMP, and MEP from recipients of WT of *Atg5*$^{-/-}$ donor (GVHD) grafts at day 10 posttransplant (n = 8 representative of 2 repeat experiments). (D) In a second model: lethally irradiated B6D2F1 mice were transplanted with WT or *VAV*^{cre}*Atg7*$^{fl/fl}$ BM together with WT T cells (GVHD) or without them (non-GVHD). Survival analysis of transplant recipients given WT or *VAV*^{cre}*Atg7*$^{fl/fl}$ donor grafts with WT T cells or without (GVHD n = 10; non-GVHD n = 6 representative of 2 repeat experiments). (E) In a third model: lethally irradiated B6D2F1 mice were transplanted with WT or *Lyz2*^{cre}*Atg7*$^{fl/fl}$ BM together with WT T cells (GVHD) or without (non-GVHD). Survival and clinical scores comparing WT GVHD and *Lyz2*^{cre}*Atg7*$^{fl/fl}$ GVHD groups (n = 11 and 12, respectively; representative of 2 repeat experiments). Transplant data are shown as mean ± SEM. Statistical significance was determined using an unpaired 2-tailed Mann-Whitney test *U* test (**$P = .0070$; ***$P = .0003$). Survival curves were analyzed using a log-rank (Mantel-Cox) test (****$P < .0001$).

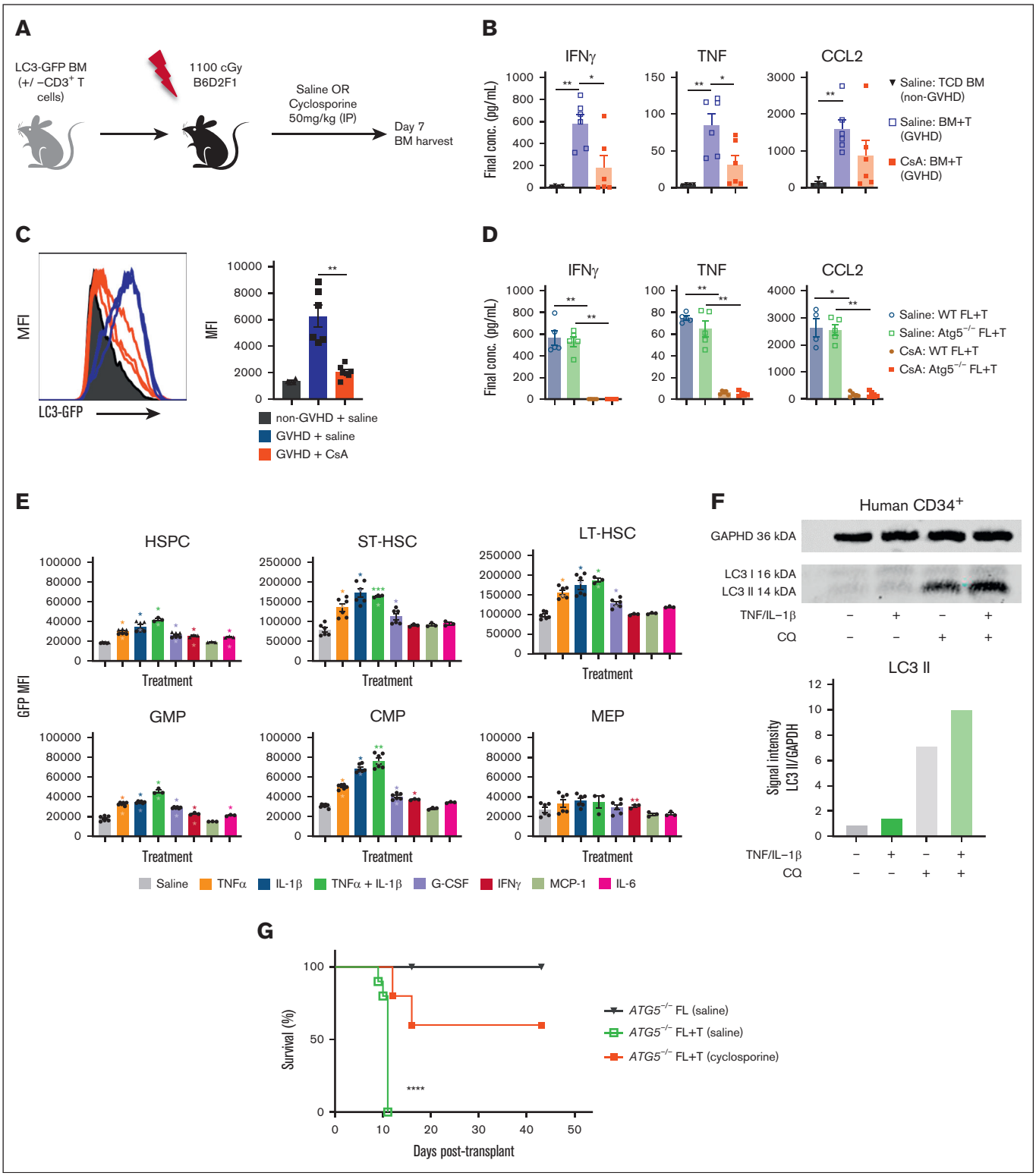

Figure 5. Autohagy is upregulated in HSC and HPC in response to proinflammatory cytokines. (A) Lethally irradiated B6D2F1 mice were transplanted with 5 × 10^6 BM + T cells (GVHD) or without T cells (non-GVHD) from LC3-GFP mice. Recipients were treated with either saline or cyclosporine (CsA) from day 0 to 6 posttransplant and BM was harvested at day 7 (D7) posttransplant. (B) Serum cytokine analysis at day 7 demonstrated that administration of CsA reduced serum concentrations of the cytokines IFNγ, TNF, and CCL2 to levels comparable to non-GVHD controls (n = 4-6, representative of 2 repeat experiments). (C) Mean fluorescent intensity of LC3-GFP expression in HSPC. (D) Lethally irradiated B6D2F1 mice were transplanted with 5 × 10^6 *Atg5*$^{-/-}$ or WT FL cells + WT T cells (GVHD) or without T cells (non-GVHD). Day 7 serum cytokine analysis of GVHD groups administered with either saline or CsA from day 0 to 6 posttransplant and non-GVHD controls administered saline (n = 5 per group). (E) Analysis of LC3-GFP

cord-blood–derived CD34$^+$ cells in response to in vitro stimulation with IL-1β+TNF (supplemental Figure 2B). Together, these data implicate mitophagy as a contributing mechanism by which autophagy supports HSC/HPC engraftment after allogeneic SCT. Altogether, these results suggest that the proinflammatory cytokines released during GVHD contribute to the increased requirement for autophagy in HSC and HPC during the period of engraftment and hematopoietic reconstitution. Thus, effective immune suppression in the early posttransplant period enhances survival of autophagy-deficient HSPC and improves recipient engraftment.

Discussion

Efficient engraftment posttransplant is critical to protect the recipient from infection and permit the effective T-cell–mediated clearance of tumor burden via GVL. Studies to date have identified a key role for T-cell–derived proinflammatory cytokines including TNF[36] and IL-1[37] as negative regulators of hematopoiesis, which induce the production of reactive oxygen species in HSPC and have been linked to BM failure syndromes.[38-40] In the setting of aplastic anemia (AA), excessive production of TNF by BM lymphocytes in tandem with higher expression of tumor necrosis factor receptors on HSC populations in AA patients, resulted in BM failure.[38] This demonstrates that HSC are highly responsive to proinflammatory cytokine stimulation and susceptible to BM failure in response. Furthermore, a recent study identified that median plasma levels of proinflammatory cytokines, including IL-1β and IL-6, were significantly elevated in pediatric patients with engraftment complications, supporting a role for proinflammatory cytokines as stressors in the hematopoietic compartment during the early posttransplant period.[41]

It is well established that myeloablative preconditioning and the presence of alloreactive T cells in the setting of GVHD elicits a cytokine storm resulting in systemic increase in proinflammatory cytokines including TNF, IL-1β, and IFNγ.[42,43] Our findings demonstrate that several proinflammatory cytokines, but most significantly TNF and IL-1β, induce autophagy in vitro in HSC and HPC. Furthermore, we demonstrate in vivo that allogeneic T cells induce an increase in autophagic activity in murine HSPC by d7 posttransplant.

When autophagy is ablated in the donor stem cell graft, donor HSPCs fail to support hematopoietic recovery resulting in significantly reduced reconstitution of all 3 lineages of the hematopoietic compartment reflected by neutropenia, thrombocytopenia, and anemia; and recipient mice succumb to engraftment failure by d10 posttransplant. Furthermore, our experiments using *Lyz2*^{cre}*Atg7*$^{fl/fl}$ stem cell grafts, which restrict autophagy deficiency to more terminally differentiated myeloid progenitor cells, failed to replicate the significantly reduced survival observed when autophagy was ablated in all hematopoietic cells (using *VAV*^{cre}*Atg7*$^{fl/fl}$ and *Atg5*$^{-/-}$ mice). This finding supports a critical role for autophagy in HSC and early myeloid progenitor populations (CMP and MEP) during primary engraftment in the setting of GVHD.

We confirmed that development of the HSC pool in autophagy (*Atg5*)-deficient mice is comparable to that of WT mice. After transplantation, in the absence of a proinflammatory milieu, the autophagy-deficient graft is indeed capable of functional engraftment and long-term hematopoietic recovery with however a competitive disadvantage compared to WT HSC in a competitive transplantation setting. However, during GVHD and the associated inflammatory cytokine storm, we demonstrated a critical requirement for autophagy in the donor HSC graft for primary engraftment and hematopoietic recovery. Furthermore, treatment with cyclosporine (CsA) during day 0 to day 14 posttransplant reduced systemic levels of donor T-cell–derived cytokines including IL-1β and TNF and rescued autophagy-deficient donor HSC from primary graft failure.

These findings support a role for autophagy in HSC and myeloid progenitor survival in the setting of alloreactive GVHD. We demonstrate that IL-1β and TNF are potent inducers of autophagy in HSC and HPC in vitro and that without autophagy activity these populations fail to engraft. A direct role for IL-1 in accelerating HSC cell division and myeloid differentiation has been described indicating that chronic exposure to IL-1 restricts HSC proliferation and self-renewal causing IL-1-exposed HSC to fail large-scale replicative demands such as transplantation.[44] Similarly, neutralizing donor T-cell–derived TNF in vivo is demonstrated to increase short-term stem and progenitor cell engraftment and to accelerate hematopoietic recovery.[45] In this study, we demonstrate that in response to these cytokine stressors, HSC and progenitor cells critically require autophagy to survive and engraft and that in the absence of autophagy, recipients succumb to primary graft failure. These findings identify IL-1β and TNF as key proinflammatory mediators of HSPC stress during the early posttransplant period and highlight the potential benefits of promoting autophagy activity within the donor graft or attenuating cytokine signaling to promote primary engraftment after SCT. Rapamycin is a commonly used immune suppressant after BMT and its ability to induce autophagy may have advantageous effects in relation to HSC fitness and survival during the treatment of GVHD.

Two biopharmaceutical agents currently used in the clinic for the suppression of proinflammatory cytokines include: (1) anakinra, which is a recombinant and slightly modified version of the human interleukin 1 receptor antagonist protein (IL1RN),[46] and (2) enbrel/etanercept, which is a TNF-receptor fusion protein.[47] Anakinra is an effective agent for the inhibition of both IL-1α and IL-1β dependent signaling and is currently used in the clinic for the treatment of chronic inflammatory diseases such as rheumatoid arthritis and IL1RN deficiencies and has an excellent safety profile.[48,49]

Figure 5 (continued) expression in specified BM HSPC subsets isolated from LC3-GFP mice and stimulated in vitro with cytokines (TNF, IL-1β, TNF+IL-1β, G-CSF, IFNγ, CCL2, and IL-6) or saline (n = 3-6, representative of 2 repeat experiments). Data are shown as mean ± SEM. Statistical significance was determined using an unpaired 2-tailed Mann-Whitney test *U* test (*P = .0152 and **P = .0022) for treatment conditions: TNF, IL-1β, and G-CSF (n = 6). A paired *t* test (*P = .0188; **P = .0027; ***P = .0001; and ****P < .0001) was used for treatment conditions: TNF + IL-1β, IFNγ, CCL2 and IL-6. Survival curves were analyzed using a log-rank (Mantel-Cox) test (****P < .0001). (F) Western blot analysis of LC3II expression in purified cord blood CD34$^+$ stem cells stimulated in vitro with or without TNF and IL-1β and chloroquine. Total cell lysates were immunoblotted with anti-GAPDH and anti-LC3B antibodies and LC3II band intensity expressed relative to GAPDH. (G) Survival analysis of recipients of *Atg5*$^{-/-}$ FL+ WT T grafts treated with either saline or CsA from day 0 to 14 compared to saline treated non-GVHD controls (n = 10 per group, representative of 2 repeat experiments).

Importantly for SCT, IL1RN is dispensable for normal HSC function, even under conditions of hematopoietic stress suggesting that treatment with anakinra after SCT could indeed improve engraftment outcome by reducing IL-1β signals to the donor HSC graft.[44]

In summary, we have identified candidate cytokine stressors that trigger the requirement for autophagy in the donor HSC graft. Further investigation into the impact of both clinically available cytokine inhibitors and pretransplant manipulation techniques (presented above) may provide improved strategies for the preparation of the HSC graft and posttransplant regimes to maximize donor engraftment.

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Authorship

Contribution: K.P.A.M. conceptualized the project; K.E.L. designed and performed experiments, analyzed data, and prepared figures; L.L.-E.M., C.R.H., L.L.T., C.M., J.-P.L., and B.T. performed experiments and analyzed data; B.R.B., S.W.L., G.R.H., and J.-P.L. provided intellectual input and interpreted data; and K.E.L. and K.P.A.M. wrote the manuscript with input from all authors.

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