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Host-derived CD70 suppresses murine GVHD by limiting donor T cell expansion and effector function[1]

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Abstract

Allogeneic hematopoietic cell transplantation (allo-HCT) is a potentially curative treatment for hematologic and immunologic diseases. However, graft-versus-host disease (GVHD) may develop when donor-derived T cells recognize and damage genetically distinct normal host tissues. In addition to T cell receptor signaling, co-stimulatory pathways are involved in T cell activation. CD27 is a TNF receptor family member expressed on T cells and its ligand, CD70, is expressed on APCs. The CD27/CD70 co-stimulatory pathway was shown to be critical for T cell function and survival in viral infection models. However, the role of this pathway in allo-HCT is previously unknown. In this study, we have examined its contribution in GVHD pathogenesis. Surprisingly, antibody blockade of CD70 following allo-HCT significantly increases GVHD. Interestingly, while donor T cell- or BM-derived CD70 plays no role in GVHD, host-derived CD70 inhibits GVHD as $CD70^{-/-}$ hosts show significantly increased GVHD. This is evidenced by reduced survival, more severe weight loss, and increased histopathologic damage compared to WT hosts. In addition, $CD70^{-/-}$ hosts have higher levels of proinflammatory cytokines TNF-α, IFN-γ, IL-2, and IL-17. Moreover, accumulation of donor $CD4^{+}$ and $CD8^{+}$ effector T cells is increased in $CD70^{-/-}$ versus WT hosts. Mechanistic analyses suggest that CD70 expressed by host hematopoietic cells is involved in the control of alloreactive T cell apoptosis and expansion. Together, our findings demonstrate that host CD70 serves as a unique negative regulator of allogeneic T cell response by contributing to donor T cell apoptosis and inhibiting expansion of donor effector T cells.

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Introduction

Graft-versus-host disease (GVHD) remains a major obstacle to successful allogeneic hematopoietic cell transplantation (allo-HCT). It has been recognized that alloreactive T cells are the culprits behind this adverse side effect (1). T cells are also beneficial following allo-HCT working to facilitate engraftment (2), provide graft-versus-leukemia effect (3), and ward off infectious diseases (4, 5). Therefore, ideal treatments to reduce GVHD do not completely eliminate T cell function. This idea has led to the study of T cell co-stimulation in GVHD. T cell co-stimulation is an essential component to T cell activation and constitutes a multitude of receptor/ligand interactions that play unique roles in activation. This provides a target by which T cell responses can be tuned down, instead of turned off.

CD27/CD70 is a co-stimulatory receptor ligand pair in the TNF receptor family that is important for $CD4^+$ and $CD8^+$ T cell function (6-13). CD27 is present on naïve T cells and transiently up-regulated after activation (7). CD70 expression is more tightly regulated and is expressed by mature antigen presenting cells (APCs) (14), intestinal non-hematopoietic APCs (15), thymic medulla (11) and activated T cells (14). For $CD8^+$ T cells, CD27 signaling provides a signal that enhances survival (16) and proliferation (17). CD27 is also important for $CD4^+$ T cells, providing survival signals for regulatory T cells (T_{regs}) in the thymus (11) and periphery (13), increasing Th1 development (18), and decreasing Th17 differentiation (10).

The CD27/CD70 co-stimulatory pathway has been studied in allo- and autoimmune responses. Antibody blockade of CD70 improved cardiac allograft survival compared to isotype controls (19). In autoimmunity, blockade and/or genetic deletion of CD27 has shown to be capable of decreasing symptoms of inflammatory bowel disease (20) and rheumatoid arthritis (21). These studies emphasize the important role of CD27/CD70 co-stimulation in T cell mediated diseases. In addition, CD70 mediated co-stimulation has also been implicated in immune regulation. In this regard, CD27 signaling can induce Fas-mediated activation induced cell death (AICD) in T cells encountering high antigen loads (22). Fas/FasL interactions are essential in controlling T cell AICD and subsequent expansion following allo-HCT (23). Furthermore, $CD27^{-/-}$ mice control solid tumor growth better than their WT controls (13). This study highlighted an important role for CD27 signaling in T_{reg} survival (13) and it is well-established the T_{regs} play a prominent role in the control of GVHD (24, 25). Together, these results suggest that CD27/CD70 can function to promote as well as regulate T cell responses.

T cell co-stimulation has been intensely studied in GVHD (26, 27). Previous work has employed blocking antibodies to receptor or ligand (28, 29), knockout donor T cells (30), or hosts which are deficient for co-stimulatory ligands (31, 32). While CD27/CD70 is known to be important for both $CD4^+$ and $CD8^+$ T cell responses in other models, the role for this co-stimulatory interaction has yet to be evaluated in GVHD. CD70 expression is primarily restricted to hematopoietic cells (14), with the exception of a non-hematopoietic APC population in the intestine (15) and thymic epithelial cells (11). Our work focuses on genetic deletion of recipient CD70, providing an environment in which host hematopoietic and non-hematopoietic APCs, which are paramount for initiation of GVHD (33-35), would lack this

co-stimulatory molecule. In this study, we define a unique suppressive role for host-expressed CD70 following allo-HCT. The presence of host-derived CD70 inhibits expansion of donor effector T cells, leading to concordant decreases in GVHD.

Materials and Methods

Mice

Male and female 8-16 week old C57BL/6 and BALB/c mice were purchased from the National Cancer Institute and Charles River–Frederick. C57BL/6 CD70$^{-/-}$ mice were kindly provided by Jonathan Ashwell (National Cancer Institute) (36). CD70$^{-/-}$ mice were bred in house and maintained in specific pathogen-free conditions. All experiments were conducted in accordance with protocols approved by the animal studies committee at Roswell Park Cancer Institute.

Allogeneic hematopoietic cell transplantation

On day -1 WT or CD70$^{-/-}$ C57BL/6 hosts received 965 cGy from a Cesium-137 source (Mark I, J.L. Shepherd and Associates) at a rate of 114cGy/min. C57BL/6 hosts were transplanted day 0 with BALB/c inoculum as indicated. Mice were weighed twice weekly and considered moribund when body weight reached below 80% of initial weight. In the C57BL/6→BALB/c model, host mice received 792 cGy irradiation on day -1 and were transplanted day 0 with indicated doses of BM + splenocytes or BM + CD25- PanT. T cell depletion was performed using CD90.2 microbeads and LS columns (Miltenyi), resulting in <5% of original T cell composition of BM. PanT and CD25- PanT cells were isolated using Mouse PanT isolation kit II and LS columns (Miltenyi). PanT antibody cocktail was supplemented with biotinylated anti-CD25 for depletion of CD25^{+} cells in CD25- PanT sorts. T cell sorts resulted in >97% purity of desired cell types.

Flow cytometry

Cells were stained with antibodies to H-2K^{d} (SF1-1.1), H-2K^{b} (AF6-88.5.5.3), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD25 (PC61), Foxp3 (FJK-16s), IFN-γ (XMG1.2) and fixable Live/Dead Aqua (Invitrogen) or Zombie UV™ (Biolegend). For intracellular staining, cells were fixed using the Foxp3 / Transcription Factor Staining buffer set (eBioscience). All samples were run on either a LSRFortessa (BD Biosciences) or a LSRII (BD Biosciences). All data were analyzed with FlowJo (Tree Star). For IFN-γ staining, mice were injected i.p. with 250μg BFA (Sigma) diluted in PBS 6 hours prior to harvest as previously described (37, 38). All steps prior to fixation were performed in BFA containing PBS. Active caspase-8 staining was performed using CaspGLOW™ Fluorescein Active Caspase-8 staining kit (eBioscience).

CFSE dilution

Single cell suspensions of sorted PanT cells were resuspended in 5mL of 37°C PBS. Equal volume of 2μM CFSE in 37°C PBS was added to PanT suspension and incubated for 10 min at 37°C. After 10 min, 5mL of 10% FBS containing RPMI was added and cells were washed. Cells were then washed twice in PBS before injection.

Histopathology scoring

Mice were sacrificed day 65 post allo-HCT and liver, large and small intestines were removed, formalin-fixed, sectioned, and stained with H&E. Intestine tissues were examined using a previously established semi-quantitative scoring system (39, 40). Blinded assessments were made for the presence of crypt epithelial cell apoptosis, crypt loss, surface colonocyte vacuolization, surface colonocyte attenuation, lamina propria inflammatory cell infiltrate, mucosal ulceration, and luminal sloughing of cellular debris, leukocyte infiltration of the lamina propria, and villous blunting. Liver samples were evaluated using the clinical GVHD score system (41). Assessment for the percentage of pathologic small bile ducts designated 0 as normal, 1 as less than 25%, 2 as 25~49%, 3 as 50~75%, and 4 as above 75%. Representative pictures were captured at 100×. Combined score is the sum of the scores from the liver, small intestine and large intestine from each individual mouse.

Luminex assay

Serum was collected by retro-orbital eye bleed on the indicated days following allo-HCT. Blood was immediately placed on ice until all samples were collected. Once the final sample was collected, all samples were incubated at room temperature for 20 min to allow for clotting. After incubation, vials were centrifuged at 4°C for 10 min at 2000g. Serum was removed and vials were then frozen at -80°C. Luminex: Mouse/Cytokine/Chemokine 11-plex was performed by the Flow and Image Cytometry, Luminex Divison at Roswell Park Cancer Institute as per manufacturer's instructions.

BM chimera

Chimeras were generated between C57BL/6 WT or $CD70^{-/-}$ hosts with intravenous injection of 5×10^6 BM plus 5×10^6 splenocytes one day after the hosts received irradiation with 965 cGy. After 3 months, the chimeric hosts were irradiated with 800 cGy and transplanted with 4×10^6 BM alone or combined with 3×10^6 PanT cells isolated from BALB/c donors. Hosts are then monitored for GVHD and survival as previously described (42, 43).

BM derived dendritic cells (BMDCs) and mixed lymphocyte reaction (MLR)

C57BL/6 WT and $CD70^{-/-}$ BMDCs were cultured in RPMI medium containing 5% GM-CSF for 7 days. LPS (100ng/ml) was added to culture on day 6 to fully mature the BMDCs, which were then used as stimulators for MLR. PanT cells as responders were isolated from splenocytes of BALB/c mice. 0.25×10^6 responders and 0.05×10^6 stimulators were then co-cultured in 96-well plate for 4 to 5 days.

Results

Antibody blockade of CD70 increases GVHD

Working under the hypothesis that co-stimulation is required for full effector function of T cells, we reasoned that either blocking or eliminating CD27/CD70 co-stimulation would decrease GVHD. To determine if the CD27/CD70 co-stimulatory interaction played a role in GVHD we first utilized antibody blockade of CD70. Antibody blockade of co-stimulation has been used previously in murine GVHD models (29, 44) and CD70 blockade has been

performed in models of auto- and allo-immunity (19, 21, 45). Figure 1A shows the survival of BALB/c (allogeneic) and C57BL/6 (syngeneic) host mice after transplant of 2×10^6 bone marrow (BM) ± 3×10^6 total splenocytes of C57BL/6 origin. Post-HCT administration of a CD70 blocking antibody (FR70) in doses ranged 25μg - 250μg in separate experiments surprisingly yet consistently increased GVHD in allogeneic recipients as evidenced by significantly decreased survival versus IgG treated controls (Fig. 1A and supplemental Fig. 1). In contrast, no lethal or otherwise severe GVHD was observed in syngeneic recipients (C57BL/6→C57BL/6) of equivalent transplants treated with control IgG or anti-CD70 antibody.

CD70 deficiency in donor T cells and BM does not impact GVHD

Since our antibody blockade could be affecting multiple cell types, we first sought to determine if CD70 derived from the donor or host was important for suppressing GVHD. To this end, we transplanted mice with WT BM + WT splenocytes or $CD70^{-/-}$ BM + $CD70^{-/-}$ splenocytes. In this setting, CD70 is absent from both the donor BM and splenocytes. We found that $CD70^{-/-}$ BM + $CD70^{-/-}$ splenocytes mediated identical GVHD compared to WT controls (Fig. 1B-C). Due to the difference in T_{reg} numbers in the splenic T cell compartment of WT and $CD70^{-/-}$ mice (11), we examined if the function of conventional T cells in the absence of T_{reg} were affected by CD70. We observed no difference in GVHD between recipients of WT or $CD70^{-/-}$ T_{reg}-depleted splenic T cells (CD25- PanT) (Fig. 1D-E). These data indicate that donor-derived CD70 is not contributing to GVHD.

$CD70^{-/-}$ hosts have increased GVHD following allo-HCT

We next hypothesized that, due to the importance of host APCs in GVHD (33), host-derived CD70 may be responsible for suppressing GVHD. We employed an MHC-disparate HCT system using WT and $CD70^{-/-}$ C57BL/6 (H-$2K^b$) host mice transplanted with BALB/c (H-$2K^d$) bone marrow (BM) with or without sorted splenic T cells (PanT). Based on our antibody blockade data, we hypothesized that GVHD would be increased in $CD70^{-/-}$ hosts compared to WT controls. Indeed, we found that $CD70^{-/-}$ mice had increased GVHD as evidenced by greater weight loss (Fig. 2A) and increased lethality (Fig. 2B) compared to WT controls. This difference in GVHD was more evident at higher numbers of PanT cells (Fig. 2C-D).

Interactions between CD27 and CD70 have been implicated in T_{reg} survival (13) and it is well-established the donor T_{regs} play a prominent role in the control of GVHD (24, 25). Therefore, we hypothesized that host-derived CD70 could be providing a survival signal to donor-derived T_{regs}, allowing for better control of GVHD by donor T_{regs} in WT versus $CD70^{-/-}$ recipients. To test this hypothesis, we purified CD25- PanT cells and transplanted these in combination with T cell depleted BM (TCD-BM) into WT and $CD70^{-/-}$ recipients. We found that increased GVHD in $CD70^{-/-}$ recipients was not dependent on donor T_{regs}, as transplant with T_{reg}-depleted PanT cells still resulted in increased GVHD in $CD70^{-/-}$ recipients (Fig. 2E-F). Together, these data suggest that host-derived CD70 suppresses GVHD mediated by conventional donor T cells.

Pathologic GVHD is significantly increased in CD70$^{-/-}$ hosts

To confirm that the observed weight loss and survival differences between WT and CD70$^{-/-}$ hosts were due to GVHD, we sacrificed mice following allo-HCT and assessed the large intestine, small intestine and liver. As expected, mice that had more weight loss and death also had increased levels of pathologic GVHD (Fig. 3A). While some difference in pathology was observed in the large, but not small intestine (Fig. 3B-C), we found a significant increase in damage in the livers of CD70$^{-/-}$ versus WT hosts (Fig. 3D-E). These data confirmed that GVHD was responsible for decreased survival of CD70$^{-/-}$ hosts following allo-HCT.

Pro-inflammatory cytokines are increased in CD70$^{-/-}$ hosts following allo-HCT

Following allo-HCT pro-inflammatory cytokines play an essential role in mediating tissue damage and providing signals to enable allogeneic T cells to mediate GVHD (46). Due to the differences in GVHD between WT and CD70$^{-/-}$ hosts, we speculated that pro-inflammatory cytokines would be increased in CD70$^{-/-}$ hosts compared to WT controls. We evaluated levels of TNF-α, IL-2, IFN-γ and IL-17 five days following allo-HCT. In hosts that received BM alone, cytokine levels were low and no difference was observed between WT and CD70$^{-/-}$ hosts (Fig. 4A-D). In contrast, CD70$^{-/-}$ mice that received allogeneic donor PanT cells had significantly higher levels of TNF-α, IL-2, IFN-γ and higher levels of IL-17 compared to WT controls.

Donor T cell expansion is increased in CD70$^{-/-}$ hosts following allo-HCT

Since donor T cells are mediators of GVHD, our data suggest that host CD70$^{-/-}$ may be increasing donor T cell expansion, function, or both following allo-HCT. As the spleen is a primary site of T cell activation following allo-HCT (47, 48) we evaluated the spleen of WT and CD70$^{-/-}$ hosts following allo-HCT. In host mice receiving BM only, we observed very few donor-derived T cells and no significant differences in total cell number or the number of donor CD4^{+} and CD8^{+} T cells between WT and CD70$^{-/-}$ hosts (data not shown). In contrast, in hosts that received PanT cells, we found that the total number of splenocytes, as well as the percentage and number of donor CD4^{+} and CD8^{+} T cells were significantly increased in CD70$^{-/-}$ hosts compared to WT hosts from day 5 after allo-HCT (Fig. 5A-B), a time when substantial lethality occurred to CD70$^{-/-}$ hosts. In fact, absolute numbers of donor CD4^{+} and CD8^{+} were 26-fold and 20-fold higher, respectively, in CD70$^{-/-}$ hosts at day 5 after allo-HCT. These data indicate that the absence of CD70 provides an environment that is conducive to marked donor T cell expansion after allo-HCT.

CD70 does not dominate host residual T cell number or NK-mediated anti-donor function

While the hosts received lethal irradiation conditioning, we consistently observed residual host T cells present after allo-HCT. To test whether CD70 may affect the residual host T cell pool, we first analyzed the number and accumulation of host T cells in WT and CD70$^{-/-}$ mice. Compared to T cell numbers pre-HCT, both CD4^{+} and CD8^{+} host T cells were reduced to lower than 1% between days 3 and 7 post-HCT, yet there was no significant difference between WT and CD70$^{-/-}$ hosts either pre- or post-HCT (Fig. 6A). Furthermore, since host NK cells have been shown to mediate anti-donor function and inhibit GVHD (49),

we examined whether CD70 affects host NK cell number and function. While WT and CD70$^{-/-}$ mice have equivalent NK cell numbers (Fig. 6B), we further performed NK depletion to determine the role of NK cells in this CD70-dependent phenotype. We injected NK1.1 antibody to deplete host NK cells prior to irradiation and allo-HCT. Depletion of host NK cells significantly expedited GVHD for both WT and CD70$^{-/-}$ hosts, which presented similar death curves as a result of acute GVHD (Fig. 6C). These data show that the anti-donor response mediated by residual host NK cells plays an important role in slowing down GVHD progression. However, this response does not appear to be the dominant mechanism by which host-derived CD70 suppresses GVHD because NK cell depletion in CD70$^{-/-}$ hosts also significantly expedited GVHD.

Host CD70 contributes to activation-induced cell death of donor T cells

Since previous work indicated that CD27 signaling can promote T cell proliferation and survival (8, 17), we sought to determine why donor T cell expansion was increased in CD70$^{-/-}$ hosts after allo-HCT. We first evaluated the proliferation of donor T cells. Using CFSE-stained donor T cells, we identified day 3 after allo-HCT as a time point when we could observe robust, but not complete, dilution of CFSE (Fig. 7A). At this time point, we found identical CFSE dilution in both CD4^{+} and CD8^{+} donor T cells in WT and CD70$^{-/-}$ hosts (Fig. 7B), ruling out an increase in T cell proliferation accounting for higher expansion in CD70$^{-/-}$ hosts.

CD27/CD70 co-stimulation can promote survival (8) but can also drive T cell AICD in the presence of abundant antigen (22, 50). We hypothesized that host-derived CD70 caused an increase in AICD, thus accounting for decreased T cell expansion in WT hosts. Therefore, we monitored T cell apoptosis 4 days post allo-HCT. Since CD27/CD70 co-stimulation has been shown to up-regulate Fas mediated AICD (22), we measured active levels of an apoptotic effector downstream of Fas signaling termed caspase-8 (51, 52). We found that 4 days post allo-HCT donor CD4^{+} and CD8^{+} T cells in WT hosts had more active caspase-8 than donor T cells in CD70$^{-/-}$ hosts (Fig. 7C). When adding together all donor T cells with activated caspase-8 (active caspase-8^{+}dead^{-} + active caspase-8^{+} dead^{+}), we found that donor T cells within WT hosts had significantly more caspase-8 dependent AICD than donor T cells in CD70$^{-/-}$ hosts (Fig. 7D). Although only a moderate difference (5-10%) is consistently observed, it may be due to the fast turnover of dead cells. These data suggest that host CD70 increases caspase-8 dependent AICD in donor T cells in WT hosts. Therefore, donor T cells in CD70$^{-/-}$ hosts gain a survival advantage that leads to marked accumulation (Fig. 5).

CD70 expressed by host hematopoietic APCs contributes to AICD of alloreactive T cells

Because CD70 is expressed by hematopoietic cells including mature APCs, B cells and T cells (14) as well as a population of non-hematopoietic intestinal APCs (15), we generated BM chimeras to determine whether hematopoietic CD70 or non-hematopoietic CD70 dominantly contributes to the suppression of GVHD. We first performed syngeneic transplants generating BM chimeras that have normal CD70 in the hematopoietic compartment but are deficient for CD70 in non-hematopoietic cells (WT → CD70$^{-/-}$) and chimeras in which CD70 deficiency is confined to the hematopoietic compartment (CD70$^{-/-}$

→ WT). After allo-HCT to these chimeras, we observed significantly increased lethal GVHD in hosts that lack CD70 in hematopoietic compartment ($CD70^{-/-}$ → WT) compared to hosts that lack CD70 in non-hematopoietic compartment (WT→ $CD70^{-/-}$) (Fig. 8A). This result suggests that it is hematopoietic CD70 that makes the dominant contribution to the suppression of GVHD. To further define whether hematopoietic APCs are responsible for this CD70-dependent mechanism, we first compared dendritic cell number and subset composition in WT versus $CD70^{-/-}$ mice. We found that CD70 deficiency does not affect these parameters (Supplemental Fig. 2). Next we generated BM derived dendritic cells (BMDCs) from C57BL/6 WT and $CD70^{-/-}$ mice, and used them as stimulators in a mixed lymphocyte reaction (MLR) to mimic the alloreactive T cell response. Indeed, more than 40% of mature BMDCs expressed substantial levels of CD70 (Fig. 8B). When BALB/c T cells were used as responders in the MLR, WT BMDCs induced significantly higher levels of caspase-8 activation than $CD70^{-/-}$ BMDCs (Fig. 8C). These data indicate that CD70 expressed by host hematopoietic APCs stimulates caspase-8 dependent AICD in alloreactive donor T cells.

Host CD70 inhibits accumulation of donor effector T cells

Our data indicate that T cell expansion is increased in $CD70^{-/-}$ hosts following allo-HCT (Fig. 5). We wondered whether the decrease in AICD resulted in an increase in effector T cells. To evaluate effector T cells following allo-HCT, we utilized an *in vivo* assay to determine the amount of IFN-γ producing T cells (37). The percentage of donor $CD4^{+}$IFN-γ^{+} and $CD8^{+}$IFN-γ^{+} T cells was significantly increased in $CD70^{-/-}$ versus WT hosts (Fig. 9A-B). When accounting for the increased T cell numbers in $CD70^{-/-}$ recipients, the absolute number of IFN-γ-producing $CD4^{+}$ and $CD8^{+}$ effector T cells were increased 83-fold and 34-fold, respectively, in $CD70^{-/-}$ versus WT hosts (Fig. 9B). Together these results suggest that host-derived CD70 limits the number of donor effector T cells following allo-HCT.

Discussion

Our study provides evidence showing that in the absence of host-derived hematopoietic CD70, expansion of donor effector T cells is increased leading to concordant increases in GVHD. This increase in GVHD was also observed in a second system in which we used a CD70 blocking antibody. Our results are in contrast to the studies that have shown decreased GVHD when hosts are deficient for ligands to other TNF family receptors such as OX40L (44), 4-1BBL (53), and CD30L (54). Therefore, this work suggests that host-derived CD70 plays a unique role by suppressing GVHD.

Similar to the other TNF family receptor interactions, early studies evaluating the role of CD27/CD70 co-stimulation showed this pairing to be essential for optimal T cell responses (6, 8). However, after further dissecting this pair in multiple disease models it became evident that it could also serve as a negative regulator of T cell responses. Perhaps most similar to our study is the fact that $CD70^{-/-}$ mice have increased experimental autoimmune encephalomyelitis (EAE) compared to WT controls (10). EAE is highly dependent on Th17 responses and CD27 signaling works to dampen Th17 differentiation (10). In line with this data, we found that IL-17 is increased in $CD70^{-/-}$ hosts (Fig. 4D), which show increased

GVHD (Fig. 2). Therefore, the ability of CD27/CD70 co-stimulation to decrease GVHD may be in part due to the reduction in Th17 response since Th17 cells have been shown to contribute to GVHD severity (55-57).

Also consistent with the literature is the ability of CD27/CD70 co-stimulation to drive AICD in T cells (22). AICD is of marked importance in GVHD, as it provides a regulatory checkpoint to prevent T cell expansion (23). Our data suggest that CD27/CD70 co-stimulation is essential for governing T cell expansion following allo-HCT. Work from others has shown that CD27/CD70 co-stimulation increases AICD in the presence of abundant antigen (22). It appears that the abundant antigens present in our GVHD model highlight the role of CD27/CD70 co-stimulation in AICD. Similar to our study, GITR (another member of the TNF receptor family) stimulation increases AICD of donor $CD4^+$ T cells, which results in a decrease in GVHD (58). Both CD27 and GITR have been shown to increase Fas mediated AICD (22, 58), but have also been shown to interact with the pro-apoptotic protein Siva-1 (59, 60). Interestingly, both Fas and Siva-1 can increase levels of active caspase-8 (51, 52, 60). Though OX40, CD40, and 4-1BB have similar cytoplasmic tails to CD27 and GITR (59), it appears that GITR and CD27 may be fundamentally different from other members of the TNF receptor family because of this association with Siva-1. This may help explain the seemingly paradoxical roles of GITR/GITRL and CD27/CD70 co-stimulation in GVHD.

Previous work from others has shown that CD27/CD70 co-stimulation promotes IFN-γ production (12, 18, 61). In contrast, we find a decrease in IFN-γ production in the presence of CD27/CD70 co-stimulation. We believe that the ability of host-derived CD70 to drive AICD may help to explain the increase in IFN-γ producing T cells in our $CD70^{-/-}$ hosts. WT APCs may be better at activating T cells than $CD70^{-/-}$ APCs due to the fact that they can provide CD27/CD70 co-stimulation. However, in the context of allo-HCT with abundant allogeneic antigens, T cell activation may become overabundant, meaning that the full repertoire of co-stimulatory molecules may drive T cells past the point of adequate activation to over-activation (i.e. AICD). Similar to viral models when CD27/CD70 co-stimulation works to eliminate dominant T cell clones (50), CD27/CD70 co-stimulation may be eliminating highly alloreactive T cells. These may be the same effector T cells that are expressing IFN-γ and other inflammatory cytokines, resulting in the selective loss of IFN-γ^+ T cells in the presence of CD27/CD70 co-stimulation. This mechanism therefore spares alloreactive IFN-γ producing T cells in $CD70^{-/-}$ hosts, resulting in increased donor T cells and IFN-γ production.

In summary, this study shows a suppressive role for CD27/CD70 co-stimulation in GVHD, suggesting that targeting this interaction may be beneficial for ameliorating GVHD in the clinic. This work also highlights the importance of studying CD27/CD70 co-stimulation in different contexts, as this co-stimulatory pairing has been found to have both stimulatory and inhibitory potential. In addition, this study suggests that antibody blockade of T cell co-stimulation does not always reduce T cell responses and can have unexpected deleterious effects. In fact, strategies eliminating host-derived CD70 may lead to selection for alloreactive T cells that are pathogenic. Thus, preclinical work must ensure that co-

stimulatory blockade strategies, in particular combined blockade strategies, are truly decreasing T cell responses and are not selecting for highly alloreactive T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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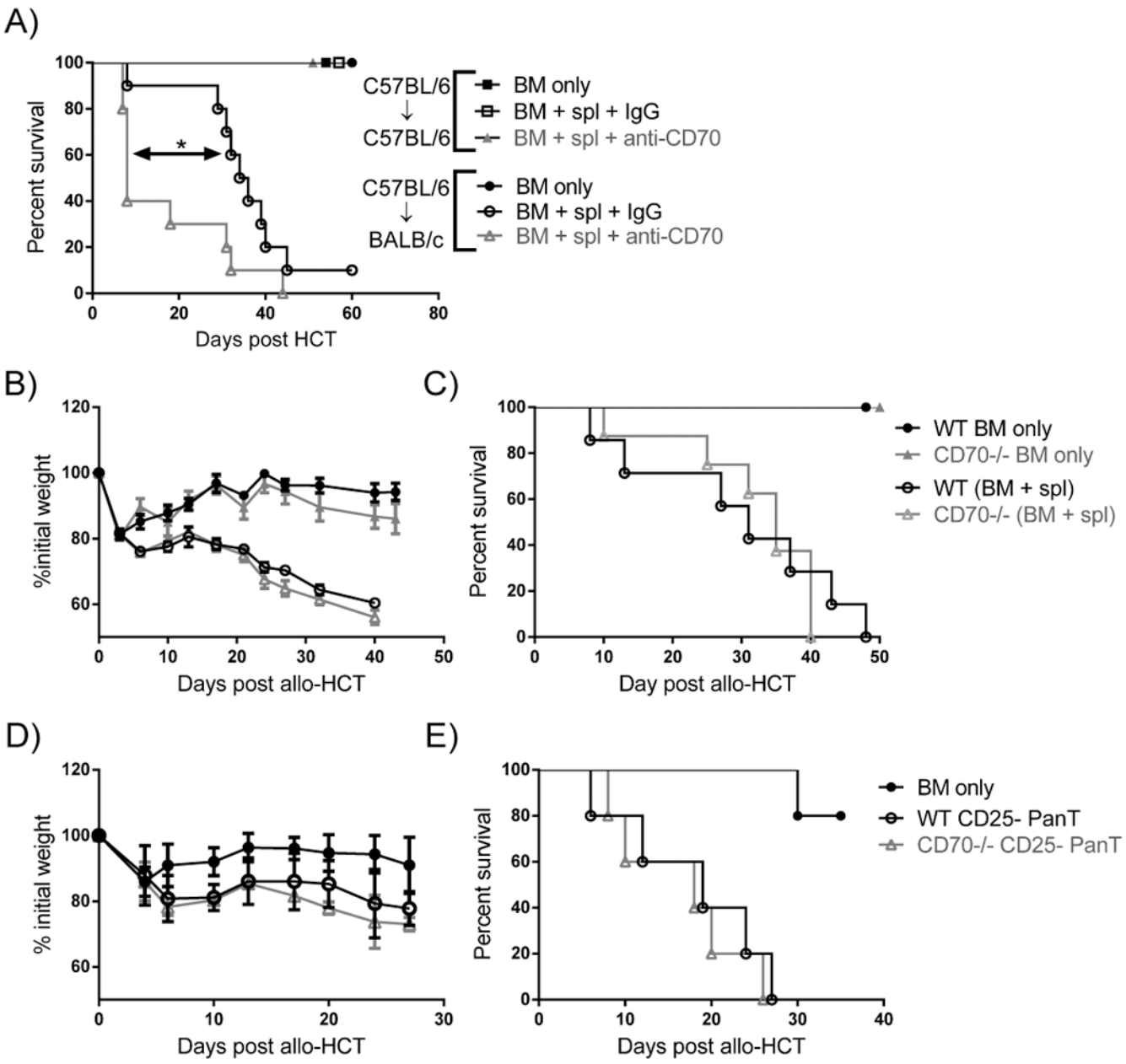

Figure 1. Blockade of CD70 exacerbates GVHD

(A) BALB/c and C57BL/6 mice were given 792 and 965 cGy irradiation, respectively, on day -1 and transplanted day 0 with 2×10^6 WT C57BL/6 BM only (n=2-4) ± 3×10^6 C57BL/6 splenocytes (spl) (n=4-10). Recipient mice were either treated with 25μg control IgG or 25μg anti-CD70 blocking antibody 4 and 6 days post-transplant and then monitored for survival. Results are pooled from two individual experiments. (B-C) BALB/c mice were given 792 cGy irradiation on day -1 and transplanted day 0 with 2×10^6 WT BM (n=4) ± 3.5×10^6 WT splenocytes (n=7) or 2×10^6 $CD70^{-/-}$ BM (n=4) ± 3.5×10^6 $CD70^{-/-}$ splenocytes (n=8). Mice were then monitored for weight loss (B) and survival (C). (D-E) BALB/c mice were given 792 cGy irradiation on day -1 and transplanted with 2×10^6 WT

BM (n=5) ± 0.4×10^6 WT or $CD70^{-/-}$ CD25- PanT (n=5). Mice were then monitored for weight loss (D) and survival (E). In (A, C, E) data are presented as percent survival. In (B, D) data are presented as mean ± SEM. Statistical significance evaluated by log rank (Mantel-Cox) test. *$P<0.05$.

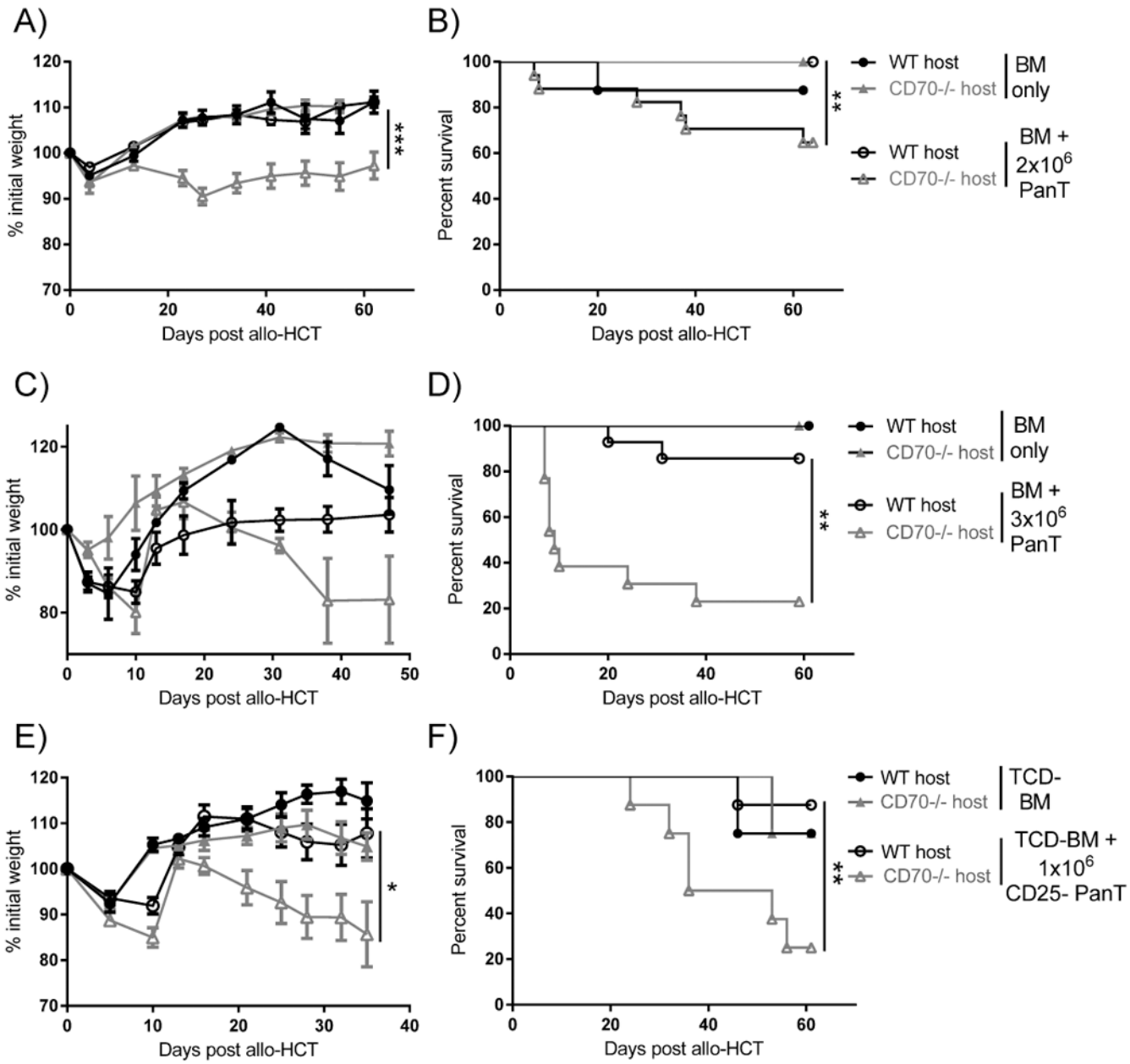

Figure 2. Absence of host CD70 significantly increases GVHD

(A-D) WT or CD70$^{-/-}$ C57BL/6 mice received 965 cGy irradiation on day -1 and were subsequently transplanted day 0 with (A-B) 4×10^6 BALB/c BM (n=5-8) ± 2×10^6 PanT (n=17-20), or (C-D) 4×10^6 BALB/c BM (n=5-8) ± 3×10^6 PanT (n=13-14). Data are pooled from 2 individual experiments. Weight loss (A, C) and survival (B, D) are shown. (E-F) WT or CD70$^{-/-}$ C57BL/6 mice received 965 cGy irradiation on day -1 and were subsequently transplanted day 0 with 4×10^6 BALB/c TCD-BM (n=4) ± 1×10^6 CD25- PanT (n=8). Mice were then monitored for weight loss (E) and survival (F). In (A, C, E) data are presented as mean ± SEM. Statistical significance determined by ANOVA in (A, C, E) and log-rank (Mantel Cox) in (B, D, F); *P<.05; **P<.01;***P<.001.

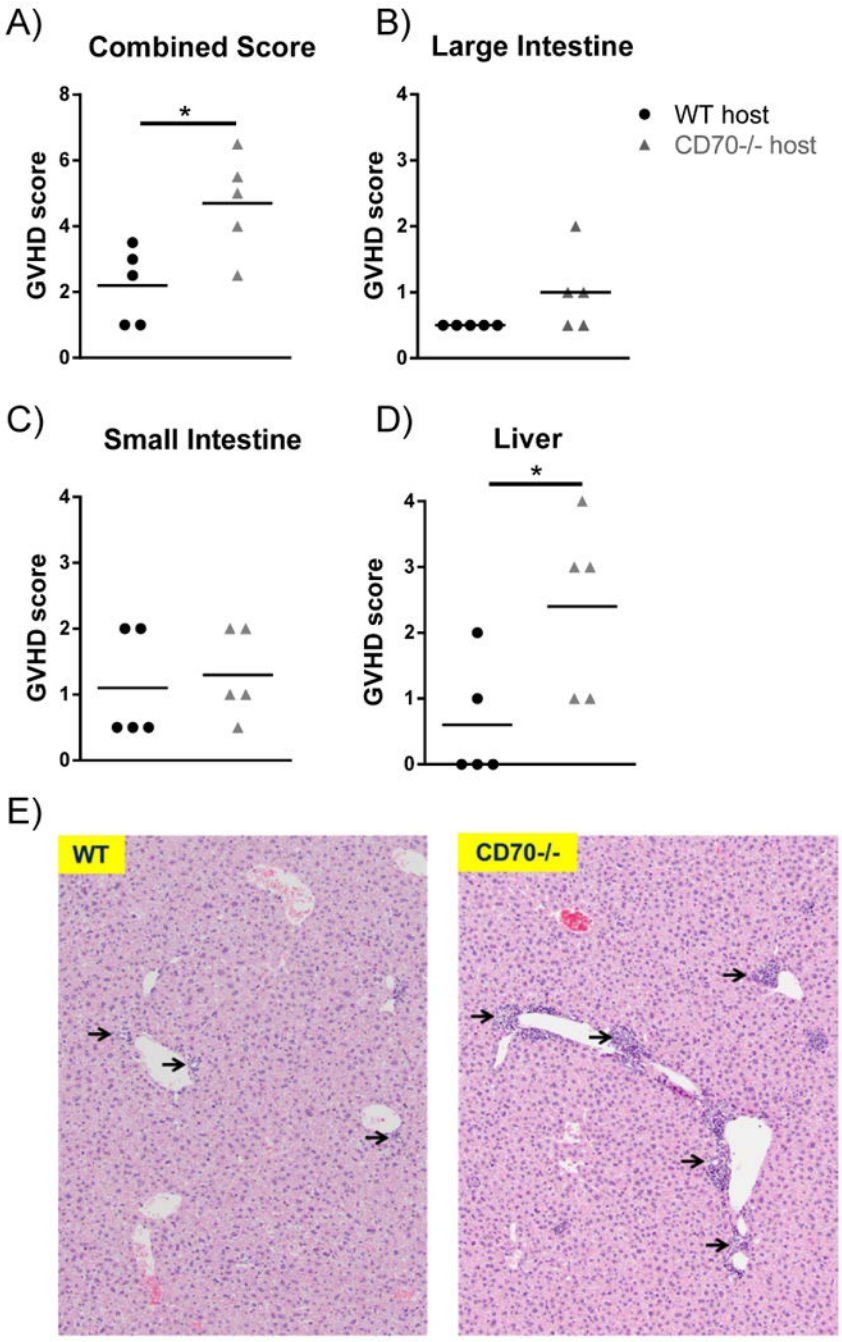

Figure 3. Absence of host CD70 results in significantly increased pathologic GVHD

WT or CD70$^{-/-}$ C57BL/6 mice received 965 cGy irradiation on day -1 and were subsequently transplanted day 0 with 4×10^6 BALB/c BM + 2×10^6 PanT. 65 days following allo-HCT mice were sacrificed and small, large intestines, and liver formalin fixed, embedded in paraffin, and stained with hematoxylin and eosin. Samples were scored for GVHD on a scale of 0-4 by a blinded pathologist using an established scoring system (39-41), with mice chosen from one of two individual experiments described in Fig. 2A-B. (A) Scores from all three organs were added to create a combined score. Scores of the (B)

large intestine, (C) small intestine, and (D) liver are shown. Each point represents an individual sample and data were analyzed by student's t test. *P<.05. (E) Representative areas of liver tissue (Scale bar 100μm) from WT (left) and CD70$^{-/-}$ (right) hosts. Arrows indicate damaged bile ducts.

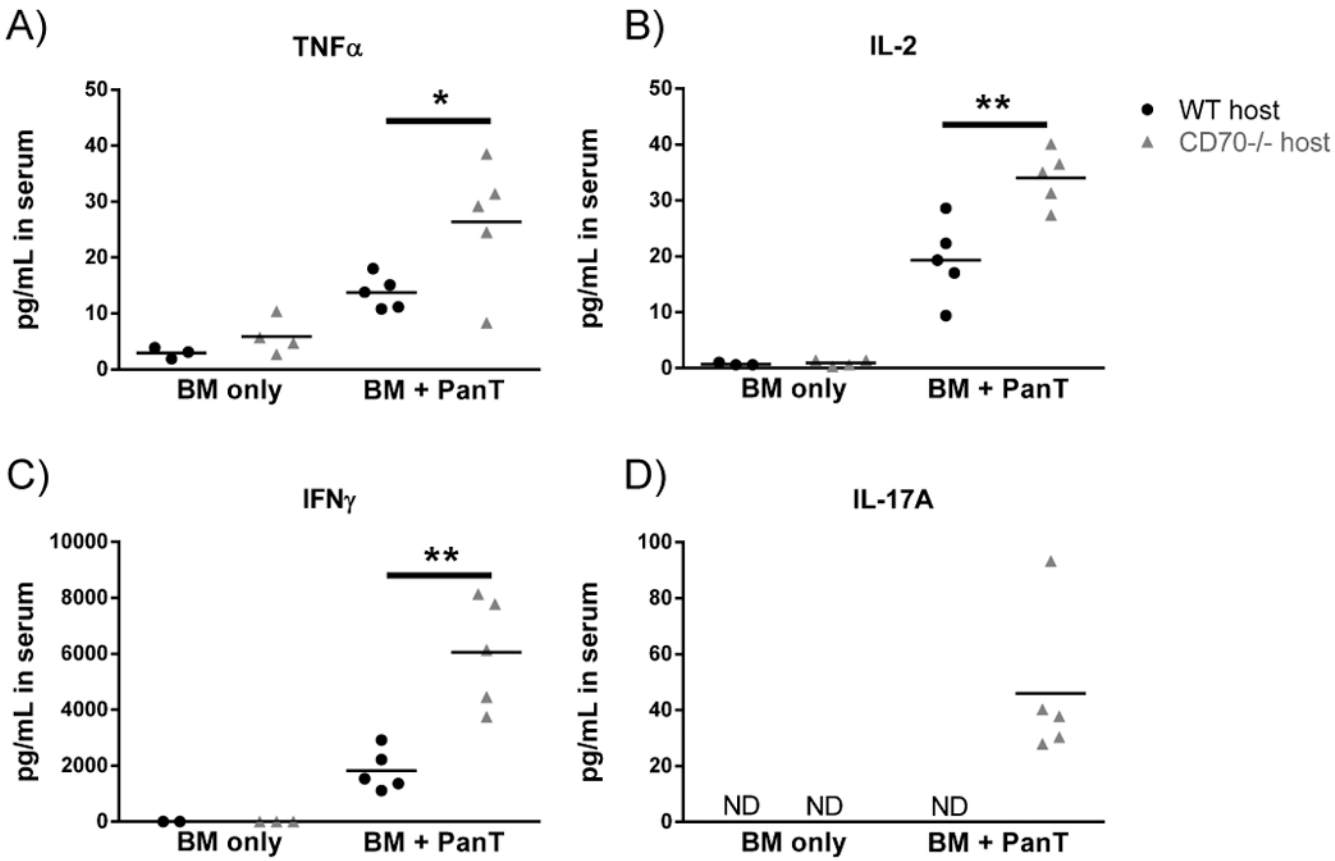

Figure 4. Serum levels of pro-inflammatory cytokines are significantly increased following allo-HCT in CD70$^{-/-}$ hosts

WT or CD70$^{-/-}$ C57BL/6 mice received 965 cGy irradiation on day -1 and were subsequently transplanted on day 0 with 4×10^6 BALB/c BM ± 5×10^6 PanT. 5 days post allo-HCT serum was collected via retro-orbital eye bleed and frozen immediately. Luminex was performed to evaluate (A) TNFα, (B) IL-2, (C) IFN-γ, and (D) IL-17A as per manufacturer's instructions. Shown are representative data from one of two individual experiments. Each point represents an individual sample and data were analyzed by student's t test. *P<.05; **P<.01.

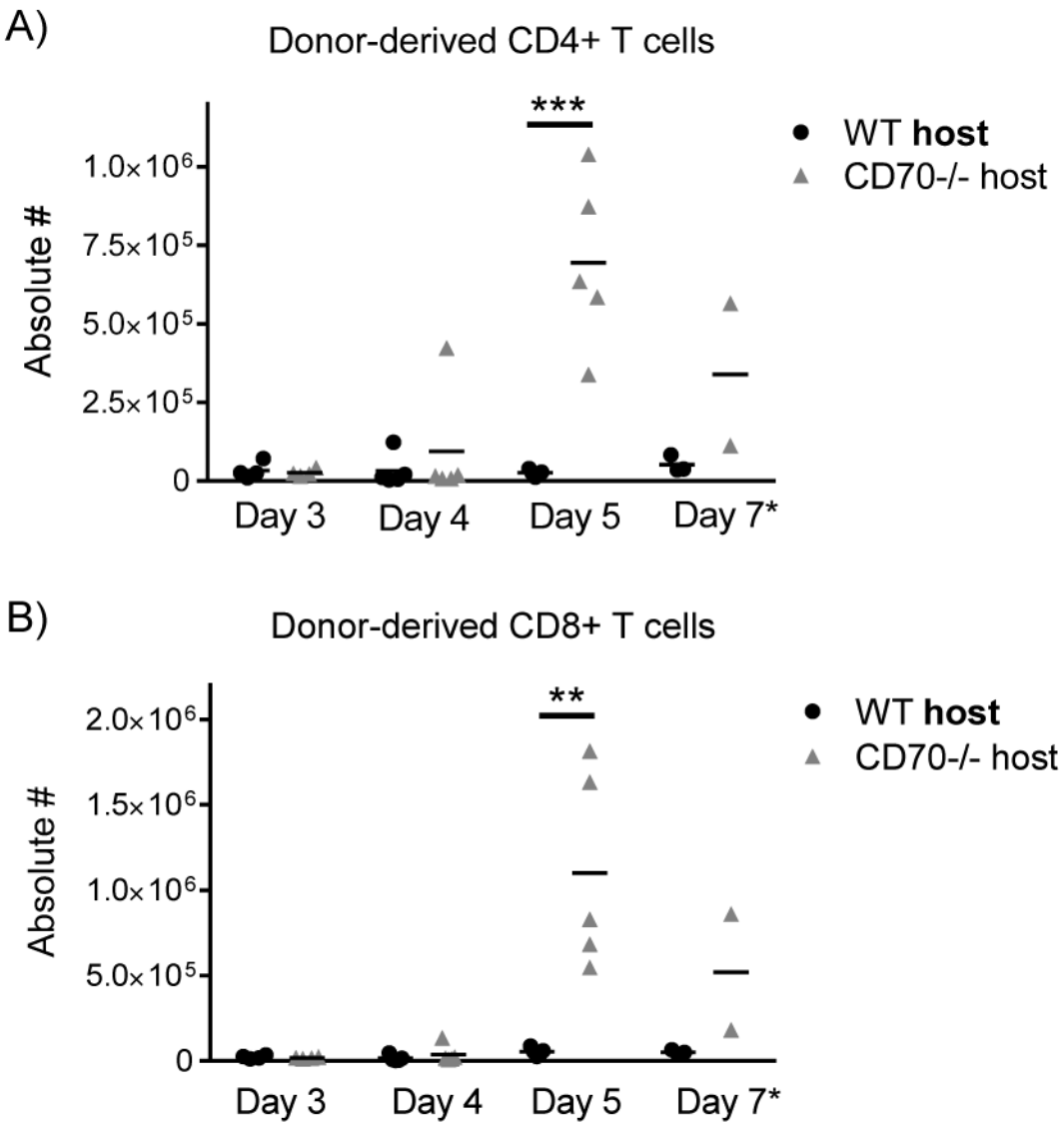

Figure 5. Donor T cell expansion is increased in CD70$^{-/-}$ hosts

WT or CD70$^{-/-}$ C57BL/6 mice received 965 cGy irradiation on day -1 and were subsequently transplanted day 0 with 4×10^6 BALB/c BM ± PanT. For early time points (days 3, 4, and 5), 5×10^6 PanT were injected in order to harvest sufficient T cells for reliable analysis. For the late time point (day 7), 2×10^6 PanT were injected to minimize untimely host lethality. At days 3, 4, 5, and 7 following allo-HCT, spleens were harvested, counted and stained for flow cytometry. Absolute numbers of donor-derived CD4$^+$ (A) and CD8$^+$ (B) T cells in WT and CD70$^{-/-}$ hosts receiving BM + PanT were acquired via the following equation: (total number of splenocytes) × (percentage of live H-2K^{d+}CD3$^+$CD4$^+$ or H-2K^{d+}CD3$^+$CD8$^+$ T cells). Summary data from three individual experiments are shown (n=2-5 for each time point). Statistical significance was analyzed by student's t test. ** P<.01; *** P<.001.

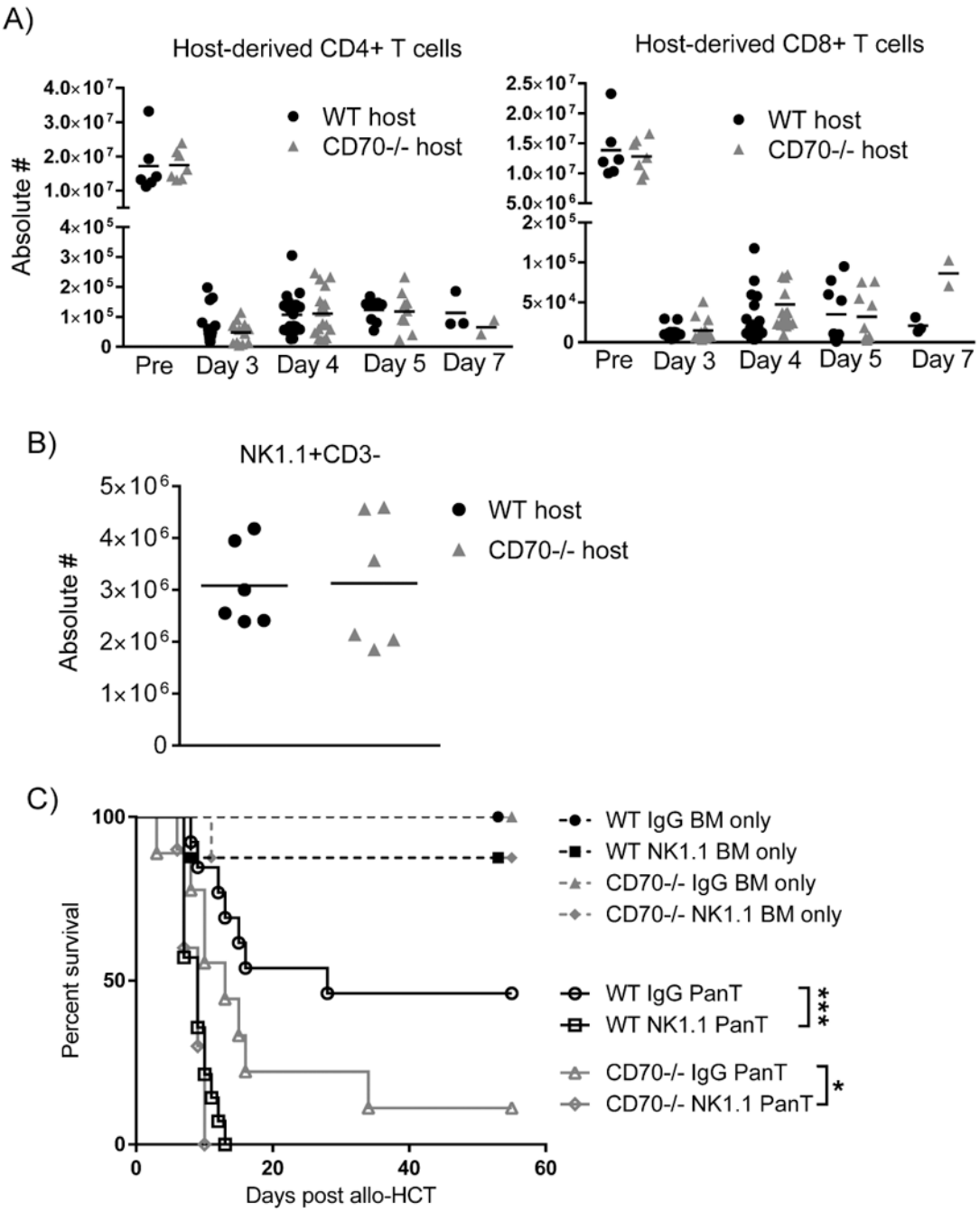

Figure 6. Residual host lymphocyte number and anti-donor function are not affected by CD70

(A) WT or CD70$^{-/-}$ C57BL/6 mice were transplanted and spleen cells were analyzed as described in Fig. 5 legend on days 3, 4, 5 and 7 after allo-HCT. Absolute numbers of host-derived CD4^{+} and CD8^{+} T cells in WT and CD70$^{-/-}$ hosts were acquired via the following equation: (total number of splenocytes) × (percentage of live H-2K^{b+}CD3^{+}CD4^{+} or H-2K^{b+}CD3^{+}CD8^{+} T cells). Summary data from three individual experiments are shown (n=2-12 for each time point). Host T cells (A) and NK cells (B) in non-irradiated WT or CD70$^{-/-}$ C57BL/6 mice were also provided. (C) One dose of 200μg NK1.1 antibody

(PK136) or IgG control was injected on day -2. The host mice then received 965 cGy irradiation on day -1 and were subsequently transplanted on day 0. Survival data of hosts receiving 4×10^6 BALB/c BM ± 3×10^6 PanT are pooled from two individual experiments (n=8-14 for each group). Statistical significance evaluated by log rank (Mantel-Cox) test. $*P<0.05$; $***P<.001$.

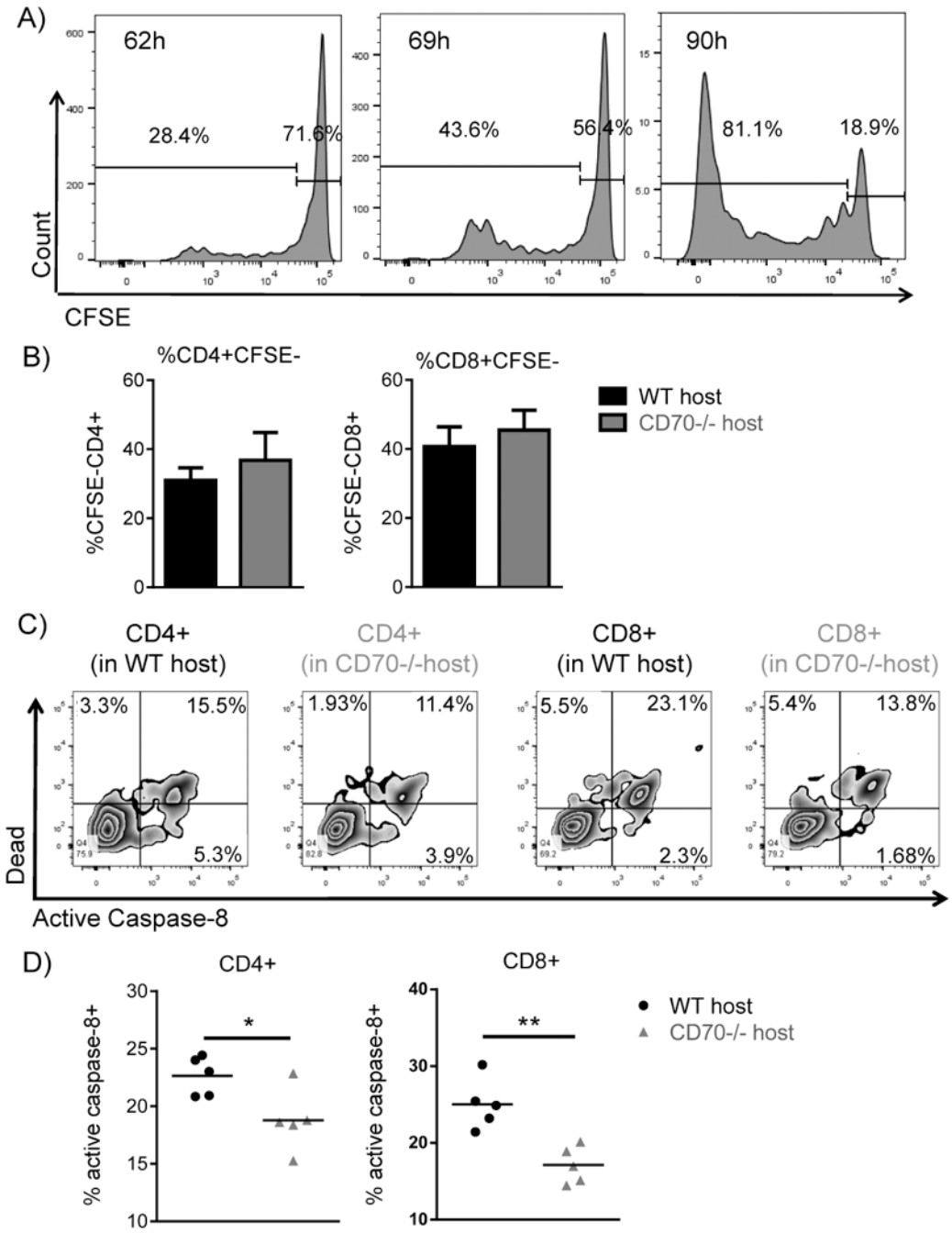

Figure 7. Donor T cells in CD70$^{-/-}$ hosts have similar proliferation, but significantly decreased caspase 8-dependent activation induced cell death

(A-B) WT or CD70$^{-/-}$ C57BL/6 mice received 965 cGy irradiation on day -1 and were subsequently transplanted on day 0 with 4×10^6 BALB/c BM + 5×10^6 CFSE labeled PanT. (A) Represented histograms of CFSE dilution in donor-derived live H-2K^{d+}CD8$^+$ T cells in WT hosts at the indicated hours after allo-HCT. (B) 69 hours after allo-HCT, CFSE dilution was assessed in donor-derived live H-2K^{d+}CD4$^+$ and H-2K^{d+}CD8$^+$ T cells. Summary data from 4 mice of each genotype. (C-D) WT or CD70$^{-/-}$ C57BL/6 mice received 965 cGy irradiation on day -1 and were subsequently transplanted day 0 with 4×10^6 BALB/c BM

+ 5×10^6 PanT. 4 days post allo-HCT spleens were harvested and active caspase-8 was evaluated in H-2K^{d+}H-2K^{b-}TCRβ$^+$CD4$^+$ and H-2K^{d+}H-2K^{b-}TCRβ$^+$CD8$^+$ T cells. (C) Flow diagrams depicting T cell expression of live/dead viability dye versus active caspase-8 in WT and CD70$^{-/-}$ hosts. (D) Summary data from 5 mice of each genotype. Representative data from one of three individual experiments is shown. Data were analyzed by student's t test. *P<0.05; **P<0.01.

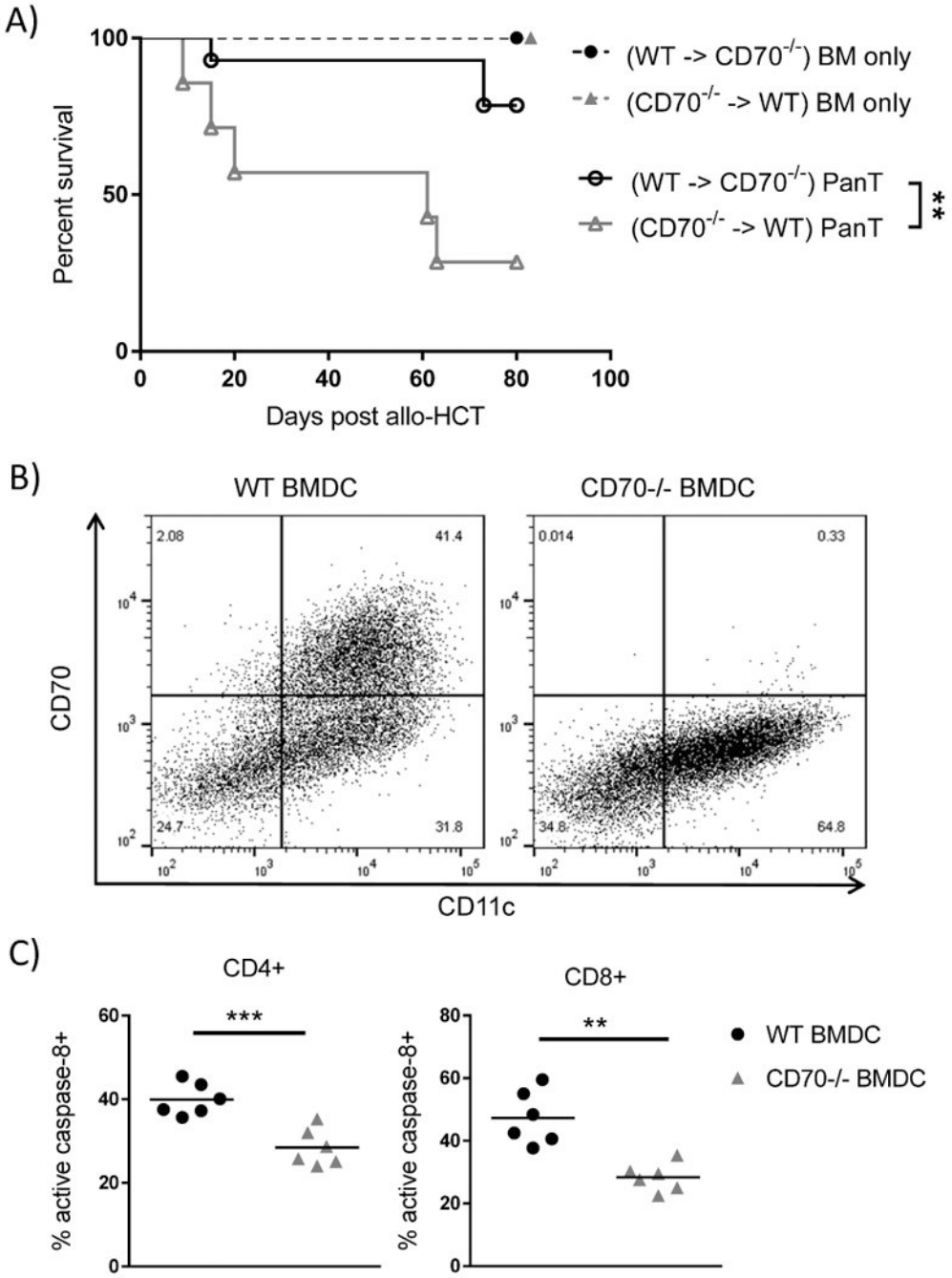

Figure 8. CD70 expressed by host hematopoietic APCs suppresses GVHD by inducing AICD in alloreactive donor T cells

(A) Chimeras were generated between C57BL/6 WT and CD70$^{-/-}$ mice through syngeneic transplants with 5×10^6 BM plus 5×10^6 splenocytes. After 3 months, the chimeric hosts were lethally irradiated with 800 cGy for allo-HCT. Survival data of chimeras receiving 4×10^6 BALB/c BM ± 3×10^6 PanT are pooled from two individual experiments (n=7-14 for each group). Statistical significance evaluated by log rank (Mantel-Cox) test. * P<0.05. (B) WT and CD70$^{-/-}$ BMDCs were cultured in RPMI medium containing GM-CSF for 6 days. LPS was added to culture on day 6 to fully mature the BMDC culture. On day 7 CD70 expression

on $CD11b^{+}CD11c^{+}$ BMDCs was assessed by flow cytometry. (C) Active caspase-8 was analyzed on day 5 after culture of WT or $CD70^{-/-}$ BMDCs mixed at a 1:5 ratio with BALB/c PanT cells. Shown are representative data from three experiments. Statistical significance for caspase-8 activation was analyzed by student's t test. $^{**}P<0.01$; $^{***}P<0.001$.

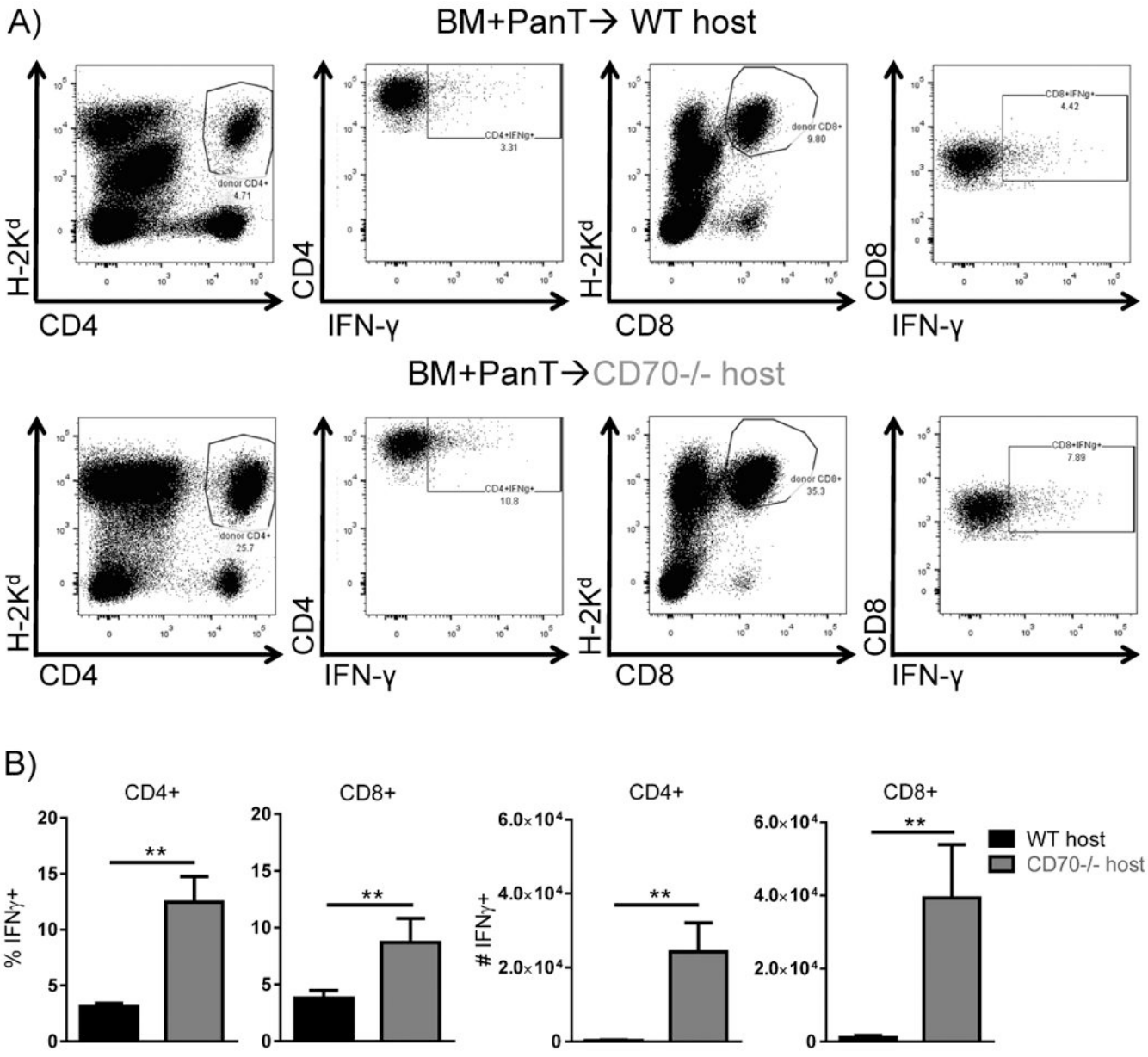

Figure 9. Donor effector T cells are significantly increased in CD70$^{-/-}$ hosts

WT or CD70$^{-/-}$ C57BL/6 mice received 965 cGy irradiation on day -1 and were subsequently transplanted day 0 with 4×10^6 BALB/c BM + 5×10^6 PanT. On day 5 post allo-HCT mice were injected with 250μg of BFA. 6 hrs post-injection mice were sacrificed, spleens were harvested and assessed for IFN-γ production by flow cytometry. (A) Depicted is IFN-γ expression within live H-2K^{d+}CD4$^+$ and live H-2K^{d+}CD8$^+$ T cells. (B) The percentage and absolute numbers of donor CD4$^+$ and CD8$^+$ producing IFN-γ. Representative data from one of two individual experiments is shown. Data were analyzed by student's t test. $^{**}P<0.01$.