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Post-Transplantation Cyclophosphamide and Ixazomib Combination Rescues Mice Subjected to Experimental Graft-versus-Host Disease and Is Superior to Either Agent Alone

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A B S T R A C T

Lapses in the prevention of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (HSCT) warrant novel approaches. Such approaches include, among others, the use of post-transplantation cyclophosphamide (PTC) and proteasome inhibitors. Although PTC alone consistently produces low rates of chronic GVHD, the incidence of acute GVHD remains significant. Inversely, prolonged post-transplantation administration of proteasome inhibitors carries a risk of paradoxical aggravation of GVHD. We examined whether the combination of cyclophosphamide and ixazomib addresses the limitations of each of these agents when used alone to prevent GVHD in mice subjected to allogeneic HSCT across MHC barriers. We chose ixazomib, an orally bioavailable proteasome inhibitor, because of its favorable physiochemical characteristics. The combination of cyclophosphamide and ixazomib improved overall survival of mice in comparison to an untreated control group and to groups receiving either cyclophosphamide alone or ixazomib alone. Furthermore, cyclophosphamide prevented the surge of IL-1β, GVHD aggravation, and sudden death associated with prolonged administration of ixazomib after HSCT. Finally, we demonstrated that although ixazomib was administered before cyclophosphamide, it did not impair the preferential depletion of proliferating as opposed to resting donor T cells. Our data suggest that the combination of cyclophosphamide and ixazomib for the prevention of GVHD after allogeneic HSCT is promising and merits further investigation in clinical trials.

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INTRODUCTION

Graft-versus-host disease (GVHD) and disease relapse account for the majority of treatment failures after allogeneic hematopoietic stem cell transplantation (HSCT) [1-3]. Current GVHD prevention strategies that exclusively and broadly suppress or deplete T cells are only partially effective and abrogate the graft-versus-tumor effect [2,3]. Emerging alternative pharmacologic strategies to prevent GVHD employ post-transplantation cyclophosphamide (PTC) [4,5], costimulation blocking agents [6], chemo-cytokine antagonists [7], proteasome inhibitors (recently reviewed in [8]), and

epigenetic modulators [9,10]. Some of these approaches have been claimed to maintain the graft-versus-tumor effect or incorporate cytotoxic activity to prevent disease relapse.

PTC selectively depletes rapidly proliferating alloreactive T cells and fosters regulatory T cell (T_{reg}) expansion [11,12]. First introduced in the setting of haploidentical HSCT to omit the need for ex vivo T cell depletion, PTC has now been extensively studied in matched related and unrelated donor HSCT. These investigations have consistently yielded noticeably low rates of chronic GVHD [13-15]. However, the incidence of acute GVHD remains significant, particularly after reduced-intensity conditioning and peripheral blood transplantation [16].

Proteasome inhibitors possess immune-modulatory effects that span a wide variety of cellular processes of dendritic cells (DC) that are crucial for the development of GVHD [8]. In animal models and early clinical studies, proteasome inhibitors ameliorate GVHD when administered early in the post-transplantation period [8]. Additionally, proteasome inhibitors have cytotoxic effects and are active against a variety of

hematological malignancies. The latter effects, however, cannot be exploited to prevent disease relapse because of a phenomenon of paradoxical GVHD acceleration that is encountered after protracted post-transplantation administration of proteasome inhibitors [17,18]. The mechanisms of this phenomenon remain to be elucidated. Enhanced release of IL-1β by prestimulated DCs represents a plausible explanation [17,18]. Additionally, we have demonstrated that this phenomenon is associated with an accumulation of donor T cells [19].

We hypothesized that combining cyclophosphamide and a proteasome inhibitor might address the limitations of either drug administered alone by targeting GVHD at different stages of development (ie, DC activation and T cell activation and proliferation) and preventing GVHD acceleration induced by the prolonged administration of proteasome inhibitors via concomitant depletion of alloreactive donor T cells. We specifically chose ixazomib as a proteasome inhibitor because of its oral availability and unique physiochemical characteristics [20].

Our findings suggest that the combination of cyclophosphamide and ixazomib is superior to either agent alone in ameliorating experimental GVHD in mice and that cyclophosphamide prevents the phenomenon of GVHD acceleration associated with prolonged post-transplantation administration of ixazomib.

MATERIALS AND METHODS
Mice

Female BALB/c (H-2Kd), C57BL/6 (H-2Kb), and B6; FVB-Ptprc^a Tg(CAG-Luc,-GFP)L2G85Chco Thy1^a/J were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free environment at West Michigan Regional Laboratory (Grand Rapids, MI). The institutional animal care and use committee at West Michigan Regional Laboratory approved all animal protocols. All mice were 10 weeks old when the experiments were performed.

Mice Transplantation and GVHD Induction

Briefly, bone marrow (BM) cells were gently released from the femurs and tibias of donor C57BL/6 mice and suspended in phosphate buffer saline (PBS) (Fisher Scientific, Waltham, MA). Cell suspensions were then filtered through a 70-μm mesh filter and washed with PBS to obtain particulate-free, single-cell suspensions. GVHD inocula were obtained by gently crushing the spleens of either C57BL/6 or B6;FVB-Ptprca Tg(CAG-Luc,-GFP)L2G85Chco Thy1a/J mice. Splenocytes were then filtered using a 70-μm mesh filter, washed with PBS, and, in 1 experiment, labeled with carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies, Carlsbad, CA). Cell counts were performed on hemocytometers.

Recipient BALB/c mice were subjected to total body irradiation on day –1. Mice received 10 Grays (2 fractions, 3 hours apart) via Xstrahl SAARP irradiator (Camberley, United Kingdom). Irradiated mice received donor BM (5×10^6 cells) with splenocytes (5×10^6) by retro-orbital injections on day 0. Ixazomib (provided by Takeda, Cambridge, MA) was dissolved in 5% 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO), protected from light, and kept at room temperature for up to 14 days before use. Mice were either untreated or received cyclophosphamide (1 mg, intraperitoneally) alone, ixazomib (30 μg, subcutaneously) alone, or both at the indicated time points. Mice were monitored for weight and scored for GVHD 3 times weekly. GVHD scoring was based on weight loss, posture, activity, fur texture, skin integrity, and diarrhea and gut injury (severity score 0 to 2 for each variable, maximum index 12). Animals were euthanized if they lost >35% of their initial weight or reached a score ≥7. The experiments were terminated on day +60.

Cytokine Measurement, Cell Counts, and Gut Histology

Recipient animals alive on day +6 were sacrificed and exsanguinated. Serum cytokines were measured using EMD-Millipore (Billerica, MA) cytokine arrays according to the manufacturer's protocol. Splenocytes were separated from the spleens using dissociation medium (STEMCELL Technologies). Cells were then filtered using a 70-μm mesh filter and washed with PBS. Total splenocyte counts were performed on hemocytometers. Splenic donor CD4$^+$ cells were counted after staining with antibodies to H-2Kb and CD4 (BD Biosciences, St. Jose, CA) and analyzed on a FACSCalibur (BD Biosciences). CFSE-high and CFSE-low splenic donor CD4$^+$ cells were similarly

counted. Staining with 7-aminoactinomycin D (7-ADD, BD Biosciences) was used to limit analysis to nonapoptotic (7-ADD-negative) cells. Small intestines were harvested in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. They were examined and scored for GVHD on a standard Stanford scale from 0 to 4 by an independent pathologist.

Bioluminescence Imaging

B6;FVB-Ptprc^a Tg(CAG-Luc,-GFP)L2G85Chco Thy1^a/J splenocytes (5×10^6), which are ubiquitously transgenic for firefly luciferase, were used as graft-versus-host inocula. Mice were left untreated or received cyclophosphamide alone, ixazomib alone, or both as described above. Mice were injected with d-luciferin (PerkinElmer, Waltham, MA) 150 mg/kg intraperitoneally and imaged starting 20 minutes later using a Spectrum In Vivo Imaging System (PerkinElmer).

Statistical Analysis

Animal survival was analyzed using the Kaplan-Meier method via MedCalc software. Statistical significance was determined by a log-rank test. Cytokine measurement, cell counts, and gut histology experiments were repeated, more frequently for the untreated group and the group receiving ixazomib only, to achieve significant numbers of subjects because of the death of some of the mice before being euthanized. The results of all experiments were pooled together and analyzed by t-test, analysis of variance, or Kruskal-Wallis with Turkey-Kramer post hoc tests. Data was normalized using log transformation when necessary. A P value < .05 was considered significant.

RESULTS
Cyclophosphamide in Combination with Ixazomib Yields Favorable Outcomes and Abolishes GVHD Acceleration associated with the Prolonged Administration of Ixazomib

First, we sought to compare the effects of cyclophosphamide and ixazomib administered alone or in combination on the weight, GVHD score, and survival of mice subjected to lethal GVHD across MHC barriers. All treatment groups had improved outcomes when compared with the untreated group in terms of weight and GVHD scores (Figure 1A,B). Additionally, cyclophosphamide alone and ixazomib alone were superior to the control group in terms of survival ($P < .0001$ and $P = .012$, respectively) and the combination was superior to either drug alone ($P = .04$ for cyclophosphamide alone and $P = .004$ for ixazomib alone) (Figure 1C). Remarkably, the addition of cyclophosphamide completely abolished the phenomenon of sudden death after ixazomib administration on day +5.

The Addition of PTC to Ixazomib Suppresses the Production of Proinflammatory Cytokines in Vivo

Surges in IL-1β and secondarily TNF-α and IL-6 have been implicated in the phenomenon of GVHD acceleration associated with prolonged administration of proteasome inhibitors after allogeneic HSCT. Therefore, we measured serum cytokine levels in the surviving mice on day +6. IL-1β was higher in mice receiving ixazomib alone in comparison to the untreated group ($P < .05$) (Figure 2A). This effect was prevented by the addition of cyclophosphamide. The same was observed with IL-6 (Figure 2B). For TNF-α, serum levels were reduced in mice receiving cyclophosphamide alone in comparison to the control group ($P < .05$). This reduction was more significant in the group receiving the combination of the 2 drugs relative to the group receiving cyclophosphamide alone ($P < .05$) (Figure 2C).

Ixazomib Does Not Impair the Selective Depletion of Proliferating Donor T Cells Induced by Cyclophosphamide

Maximal T cell proliferation requires DC activation. Because ixazomib impairs DC maturation and function, we sought to
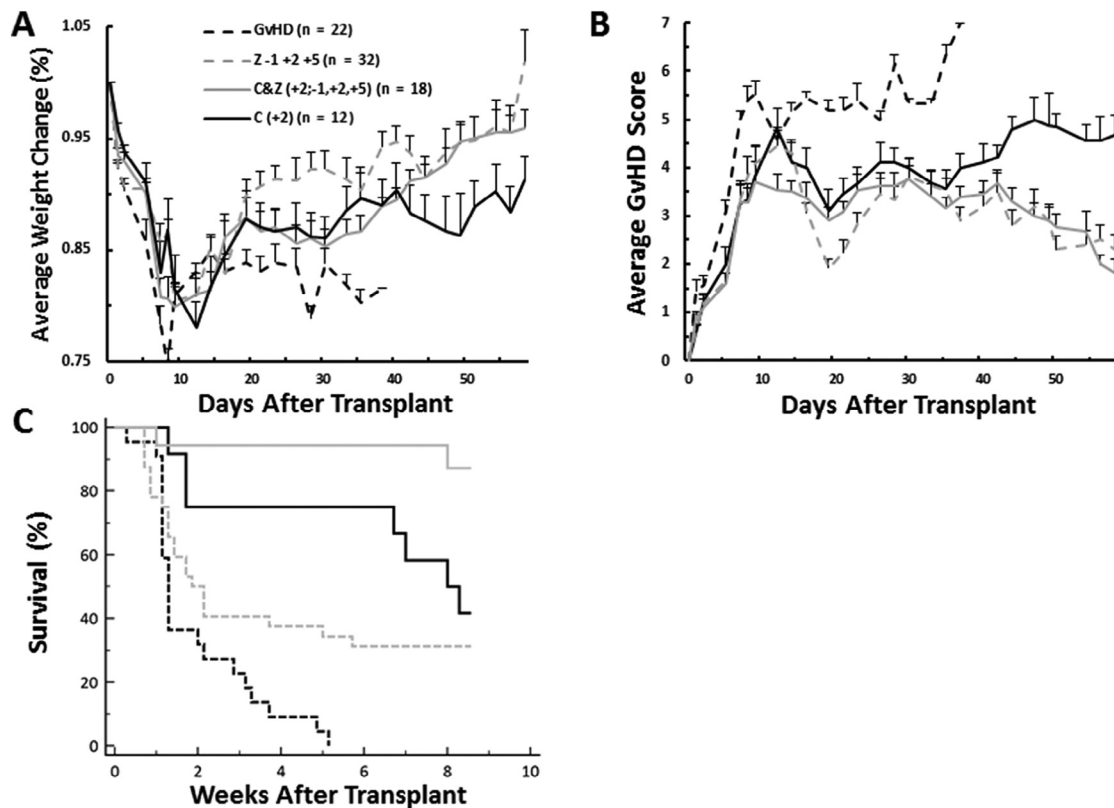
Figure 1. Effects of cyclophosphamide and ixazomib on GVHD in a murine model. Lethally irradiated 10-week old BALB/c mice received 5×10^6 BM cells from C57BL/6 mice. GVHD was induced by the administration of splenocytes (5×10^6). Results from independent sets of experiments with mice receiving ixazomib (30 μg, subcutaneously) on days –1, +2, and +5 (2 experiments with 8 and 24 mice), cyclophosphamide (1 mg, intraperitoneally) on day +2 (2 experiments, 6 mice each), or cyclophosphamide and ixazomib (3 experiments, 6 mice each) according to the same schedules were pooled together to generate weight (A), GVHD score (B), and Kaplan-Meier survival (C) curves. All experiments included a control group (2 to 8 mice per experiment). Survival P values: C to GVHD $< .0001$. Z to GVHD $=.012$. C and Z to C $=.04$. C and Z to Z $=.004$). C: cyclophosphamide. Z: ixazomib. Caps: SEM.

determine whether the administration of ixazomib before cyclophosphamide would prevent T cell cycling and proliferation, thus impairing the ability of cyclophosphamide to deplete donor T cells. We enumerated donor CD4+ cells in the spleens of surviving mice on day +6. We also quantified resting (CFSE-high) versus proliferating (CFSE-low) donor CD4+ cells. As expected, cyclophosphamide alone induced profound depletion of donor CD4+ cells in comparison to the untreated group and to the group receiving ixazomib alone ($P < .05$). Importantly the addition of ixazomib did not impair this effect (Figure 3A). Furthermore, the selectivity of the effect of cyclophosphamide on proliferating, as opposed to quiescent, donor CD4+ T cells was preserved (Figure 3B).

The Addition of PTC to Ixazomib Prevents Small Intestine GVHD Aggravation

The gastrointestinal tract represents the primary target of GVHD acceleration after prolonged administration of proteasome inhibitors [18]. Therefore, we compared the effects of the different treatments on the histology of the small intestine in the surviving mice on day +6. The small intestines' GVHD scores were increased in the group receiving ixazomib only in comparison to the untreated group. When cyclophosphamide was added, this increase was abolished (Figure 3C). Notably, the group receiving cyclophosphamide only also exhibited worse histologic changes in comparison to the control group.

Cyclophosphamide and Ixazomib Combination Reduces Donor T Cell Expansion

To confirm our results, we tested the effects of each drug administered alone or in combination using GVHD inocula that are ubiquitously transgenic for firefly luciferase to quantify donor T cells by bioluminescence signal intensity. In this model that is more aggressive than the C57BL/6→BALB/c model, mice receiving the combination of the 2 drugs exhibited improved survival in comparison to the untreated control ($P = .0001$), cyclophosphamide alone ($P = .004$), and ixazomib alone ($P < .0001$) groups (Figure 4A). All mice receiving ixazomib alone died abruptly after ixazomib administration on day +5. The addition of cyclophosphamide completely prevented this phenomenon. Furthermore, bioluminescence signal intensity increased sharply after day +5 in the control and ixazomib alone groups. Although the increase in bioluminescence signal intensity was delayed in the group receiving cyclophosphamide alone, the increase was stabilized after 4 weeks in the group receiving the combination (Figure 4B,C).

DISCUSSION

We demonstrated that the combination of cyclophosphamide and ixazomib was superior to either drug administered alone in rescuing mice subjected to a lethal GVHD and that the combination overcomes some of the limitations of administering either agent alone.
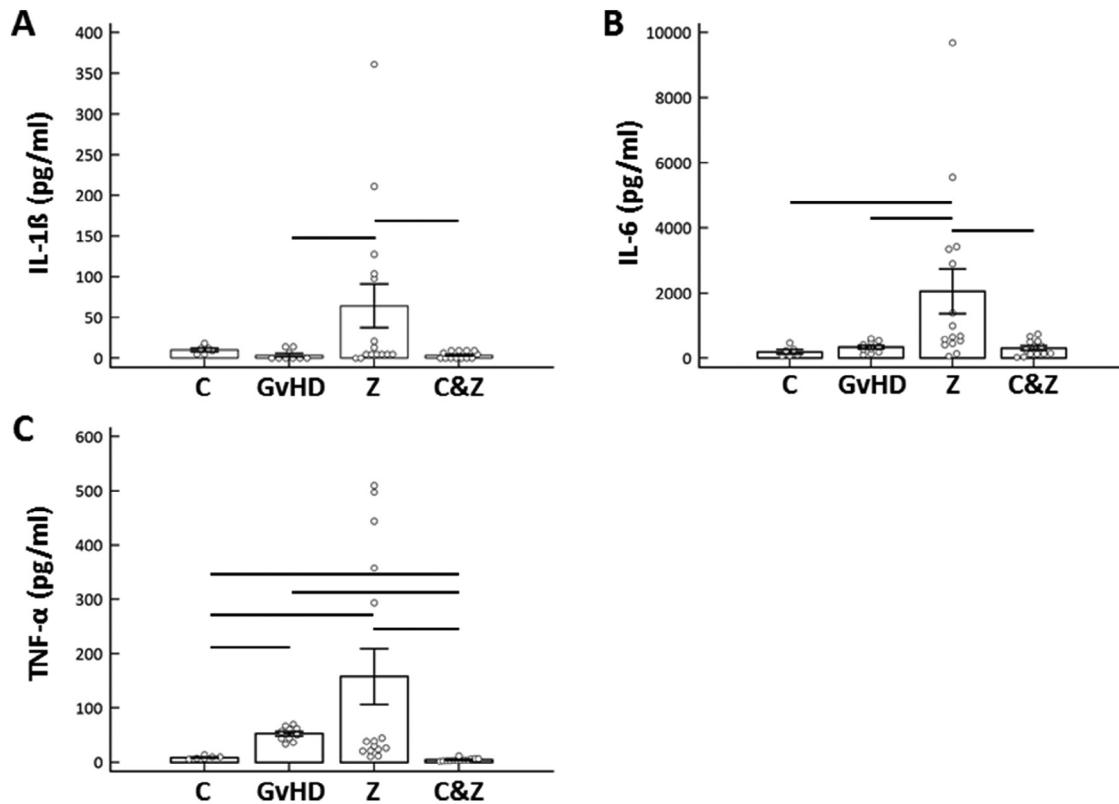
Figure 2. Effects of PTC and ixazomib on serum cytokine levels in vivo. Lethally irradiated 10-week old BALB/c mice received BM cells (5×10^6) from C57BL/6 mice. GVHD was induced by the administration of splenocytes (5×10^6). Mice were left untreated (GVHD) or received cyclophosphamide (1 mg intraperitoneally) on day +2, ixazomib (30 µg subcutaneously) on days −1, +2, and +5, or both according to the same schedules. Surviving mice were sacrificed on day +6 and exsanguinated. Serum cytokines were measured. (A) IL-1β, (B) IL-6, and (C) TNF-α. These data were combined from 3 separate experiments. Each circle represents a subject. C: cyclophosphamide. Z: ixazomib. Caps: SEM. Bars: *P* < .05.

PTC has been recently established as a clinical platform for the prevention of GVHD (reviewed in [15]) including in HLA-matched related and unrelated HSCT. However, unfavorable rates of acute GVHD have been reported when PTC is used alone, in particular in the setting of reduced-intensity conditioning and peripheral blood grafts [16]. This has prompted several investigators to examine the role of PTC in combination with cyclosporine [21], sirolimus [22,23], or bortezomib [24].

We believe that the addition of a proteasome inhibitor to PTC is attractive for several reasons. Proteasome inhibitors suppress DC maturation and function (reviewed in [8]). Consequently, as opposed to the conventional calcineurin inhibitor-based prophylactic regimens that affect T cells exclusively, the combination of PTC and a proteasome inhibitor targets GVHD development at 2 key stages: DC activation and T cell activation and proliferation. Furthermore, omitting the need for calcineurin and mTOR inhibitors has practical implications, given the need for prolonged administration and drug level monitoring of these agents, their unfavorable toxicity profile, and the need for strict patient compliance.

In our study, we specifically chose ixazomib as the proteasome inhibitor to combine with cyclophosphamide. Ixazomib is a novel proteasome inhibitor that has been recently approved by the Food and Drug Administration for the treatment of multiple myeloma. Ixazomib is orally bioavailable and possesses several potential advantages over bortezomib that are relevant to the prevention of GVHD. Equally potent, ixazomib's proteasome dissociation half-life is 6 to 8 times

faster than that of bortezomib [20]. Therefore, cells treated with ixazomib are more capable of recovering their proteasome activity than cells treated with bortezomib [20]. We have demonstrated that ixazomib suppresses naïve human DC maturation and cytokine production with a limited impact on cell viability [19]. Several studies indicate that host DCs, necessary for the initiation of GVHD, wane off rapidly after transplantation [25-27]. On the other hand, donor DCs are inadequate for the initiation of alloreactivity and can inversely inhibit GVHD development and contribute to immune reconstitution [25-28]. Therefore, a transient proteasome inhibition that suppresses host DCs before their disappearance while allowing prompt recovery of the engrafting donor DCs after allogeneic HSCT may be preferred.

We addressed 2 limitations of the use of proteasome inhibitors for the prevention of GVHD. Although early administration of proteasome inhibitors after allogeneic HSCT ameliorates GVHD in an experimental murine model, prolonged administration has been associated with worsening gastrointestinal alloreactivity-dependent toxicity and abrupt death [17,18]. We have shown that ixazomib rescues a proportion of mice subjected to lethal experimental GVHD when administered on days +1 and +4 or on days −1, +2, and +5 [19]. However, GVHD aggravation and sudden death occurred in a proportion of mice receiving the prolonged schedule, albeit less frequently than what is reported with bortezomib [19]. In the current study, the addition of cyclophosphamide completely abrogated the small intestine's GVHD acceleration and sudden death, possibly because of the concomitant deple-
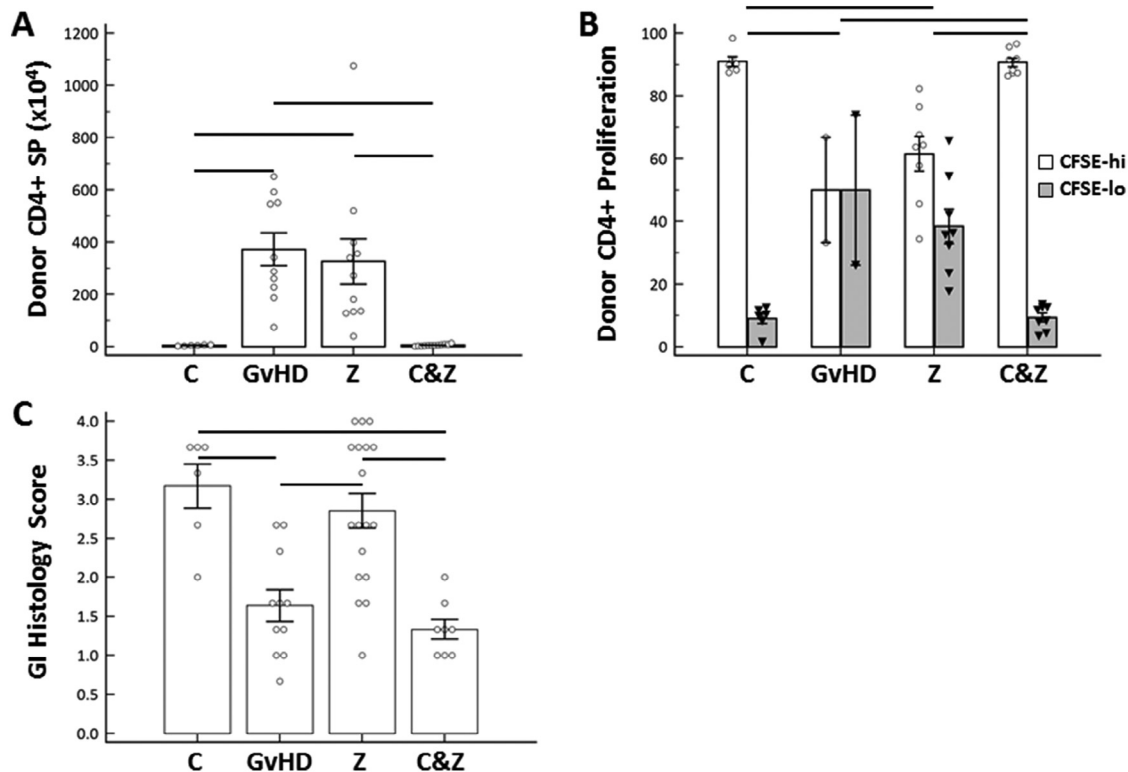
Figure 3. Effects of PTC and ixazomib on donor CD4+ cells and gut histology in vivo. Lethally irradiated 10-week old BALB/c mice received BM cells (5×10^6) from C57BL/6 mice. GVHD was induced by the administration of splenocytes (5×10^6). In 1 experiment, splenocytes were CFSE-labeled. Mice were left untreated (GVHD) or received PTC (1 mg intraperitoneally) on day +2, ixazomib (30 µg subcutaneously) on days −1, +2, and +5, or both according to the same schedules. Surviving mice were sacrificed on day +6. Splenic total and donor CD4+ T cells were stained and counted using FACS analysis. Analysis was restricted to nonapoptotic (7-AAD-negative) cells. Small intestines were harvested and GVHD scores were assigned after staining. (A) Donor CD4+ cell counts. (B) Percentage of CFSE-high and CFSE-low donor CD4+ cells. (C) GVHD histologic scores. These data were pooled from 3 independent experiments for donor CD4+ cells and small intestine histology and 1 experiment for CD4+ proliferation. Each circle or triangle represents a subject. C: cyclophosphamide. Z: ixazomib. Hi: high. Lo: low. Caps: SEM. Bars: $P < .05$.

tion of alloreactive donor T cells. Another potential culprit in the phenomenon of sudden death is a surge in IL-1β and, subsequently, IL-6 and TNF-α [17,18]. We demonstrated that the addition of cyclophosphamide prevented the rise in IL-1β and IL-6 and induced a more profound reduction in TNF-α than was observed with either drug alone.

Intriguingly, PTC alone was also associated with worsening histological findings of the small intestine in mice sacrificed on day +6. However, contrary to the effect of ixazomib, there was no increase in signal intensity on bioluminescent imaging. This argues against alloreactivity-dependent cyclophosphamide toxicity. Further studies are warranted to elucidate this observation.

Since rapid proliferation of alloreactive donor T cells is required for maximal efficacy of PTC [29], proteasome inhibitors may, in theory, interfere with PTC activity by suppressing DC maturation and function and consequently impair alloreactive donor T cell proliferation. In our experiment, the combination of cyclophosphamide and ixazomib still induced a profound depletion of donor CD4+ cells. Furthermore, the selectivity of this effect on rapidly proliferating, as opposed to resting cells, was preserved.

Our study did not examine the effects of PTC and ixazomib on T$_{regs}$. Preliminary data suggest that proteasome inhibitors promote the generation of suppressor T cells [30]. Because of the well-established role that T$_{regs}$ play in the prevention of GVHD [31], it would be of interest to examine the effect of the combination of PTC and ixazomib on T$_{regs}$ expansion and on the balance between effector T cells and T$_{regs}$.

In summary, our study suggests that the combination of PTC and ixazomib is effective in ameliorating experimental GVHD in mice and that the addition of PTC prevents the phenomenon of GVHD acceleration encountered with prolonged administration of proteasome inhibitors. This provides justification to study the combination in prospective clinical trials. In addition, our data may justify studying a possible strategy to prevent disease relapse after allogeneic HSCT by adding cyclophosphamide to a sustained course of ixazomib to alleviate the risk of GVHD aggravation.
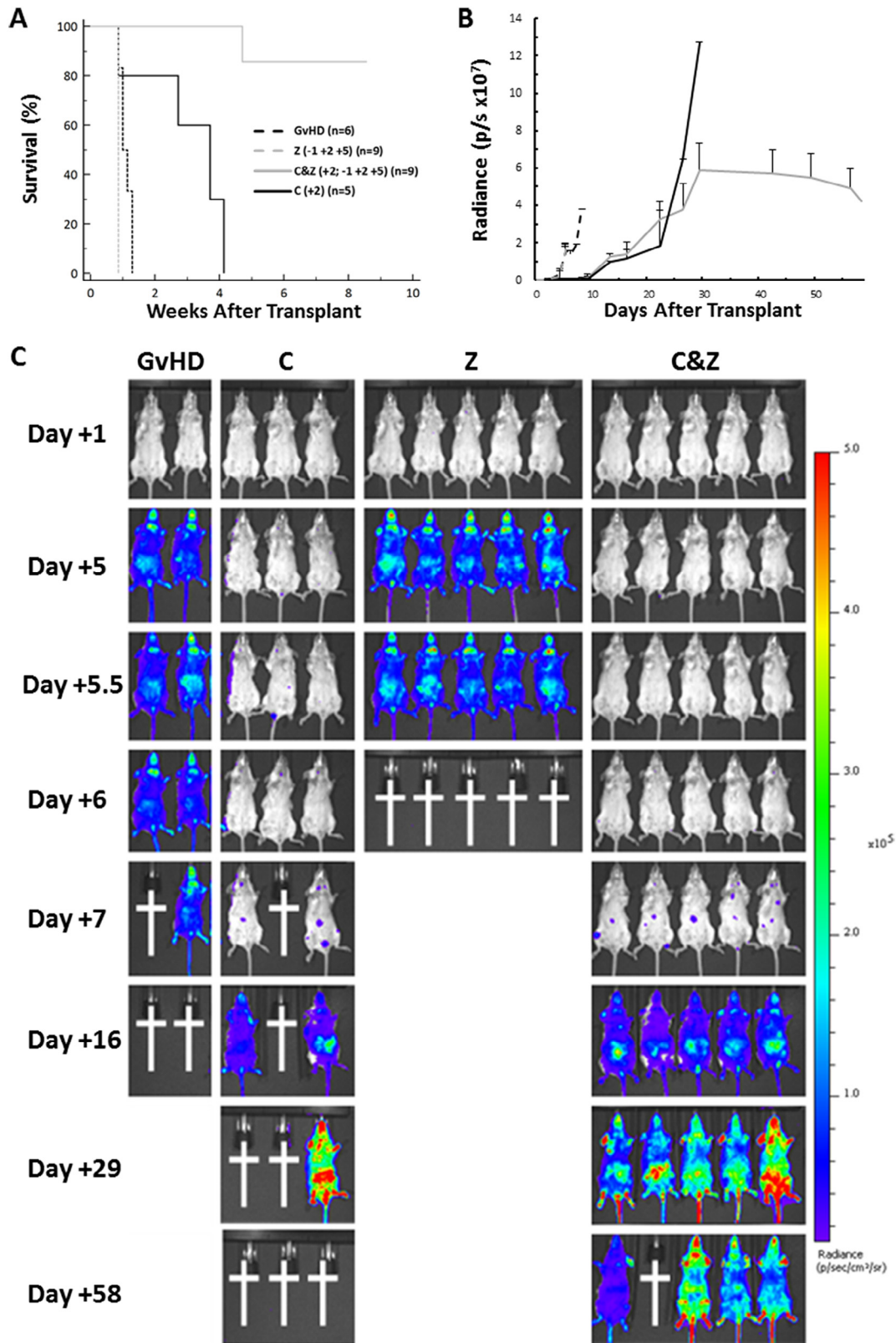
Figure 4. Effects of PTC and ixazomib on survival and donor T cell burden in vivo. Lethally irradiated 10-week old BALB/c mice received BM cells (5×10^6) from C57BL/6 mice. GVHD was induced by the administration of B6; FVB-Ptprca Tg(CAG-luc,-GFP)L2G85Chco Thy1a/J (ubiquitously transgenic for firefly luciferase) splenocytes (5×10^6). Mice were left untreated (GVHD) or received PTC (1 mg intraperitoneally) on day +2, ixazomib (30 μg subcutaneously) on days −1, +2, and +5, or both according to the same schedules. Mice were imaged 20 minutes after intraperitoneal injection with d-luciferin at 150 mg/kg. (A) Kaplan-Meier survival curves. *P* values C and Z to GVHD, C, and Z =.0001, =.004, and <.0001, respectively. (B) Kinetics of bioluminescence signal intensity (radiance). (C) Bioluminescence images at serial time points. Color bar represents signal intensity. All images are shown relative to each other on a logarithmic scale. (A) and (B) were pooled from 3 independent experiments. C represents a typical experiment. C: cyclophosphamide. Z: ixazomib. Caps: SEM. †: Subject died before imaging time point.

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