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Role of Thymic Stromal Lymphopoietin (TSLP) in Palifermin-Mediated Immune Modulation and Protection from Acute Murine Graft-Versus-Host Disease

Cynthia A. Ellison,

Department of Pathology, Faculty of Medicine, University of Manitoba, 401 Brodie Center, 727 McDermot Avenue, Winnipeg, MB, Canada R3E 3P5

Yuri V. Lissitsyn,

Department of Pathology, Faculty of Medicine, University of Manitoba, 401 Brodie Center, 727 McDermot Avenue, Winnipeg, MB, Canada R3E 3P5

Juliet A. Packiasamy,

Department of Pathology, Faculty of Medicine, University of Manitoba, 401 Brodie Center, 727 McDermot Avenue, Winnipeg, MB, Canada R3E 3P5

Warren J. Leonard, and

Laboratory of Molecular Immunology, National Heart, Lung and Blood Institute, National Institute of Health, 10 Center Dr, Building 10, 7B05, MSC-1674, Bethesda, MD 20892, USA

John G. Gartner

Department of Pathology, Faculty of Medicine, University of Manitoba, 401 Brodie Center, 727 McDermot Avenue, Winnipeg, MB, Canada R3E 3P5

Cynthia A. Ellison: ellisonc@ms.umanitoba.ca

Abstract

Using the C57BL/6→(C57BL/6 × DBA/2)F₁-hybrid model of acute graft-versus-host disease (GVHD), we previously showed that treating the donor mice with palifermin provides protection from morbidity and a shift from Th1 to Th2 cytokine production. To determine whether thymic stromal lymphopoietin (TSLP) is involved in palifermin-mediated immune modulation, we used donors from the following groups: (1) untreated wild-type donors, (2) palifermin-treated wild-type donors, (3) untreated TSLPR^{-/-} donors, and (4) palifermin-treated TSLPR^{-/-} donors. Survival in the recipients was 0%, 100%, 31%, and 0%, for groups 1–4, respectively, indicating that TSLP responsiveness is required for palifermin-mediated protection from GVHD. We also found that the increases in Th2 cytokine levels that are induced by palifermin treatment are obviated in TSLPR^{-/-} donors, and that protection from GVHD (group 2) is associated with a higher percentage of CD4⁺CD25⁺Foxp3⁺ cells in the graft. Collectively, our findings show that when palifermin and TSLP act in concert, the predominant effect is protection in this model.

Keywords

Graft-versus-host disease; keratinocyte growth factor; palifermin; thymic stromal lymphopoietin

Introduction

Graft-versus-host disease (GVHD) is a complication of clinical allogeneic hematopoietic stem cell transplantation (HSCT). It develops when T cells from the donor recognize alloantigens expressed in the recipient. In its acute form, GVHD is characterized by tissue injury occurring primarily in the skin, liver, thymus, and lung, as well as the gastrointestinal tract [1]. Epithelial cells, the principal target of injury, undergo apoptosis. In the intestine, epithelial cell injury permits the entry of endotoxin into the host. Mice with acute GVHD succumb to very low levels of endotoxin, a phenomenon attributed to the secretion of cytokines such as IFN- γ , which prime macrophages to release large amounts of TNF α and NO [2]. Both of these factors are thought to mediate intestinal epithelial cell injury associated with acute GVHD, creating a vicious cycle of epithelial injury and endotoxin influx within the host [3,4].

Keratinocyte growth factor (KGF) is an epithelial cell growth factor that is produced by both mesenchymal cells and intraepithelial $\gamma\delta$ T cells [5–7]. It is also known as fibroblast growth factor 7. Human KGF is active on cells from several other species including mice, monkeys, and pigs [6,8,9]. Its receptor (KGFR/FGF7R) [10] is an alternatively spliced form of FGFR2/*bek* found on epithelial cells in the intestine [11], mammary glands [12], ovaries [13], and urinary tract [8], and on hepatocytes [11], keratinocytes [14], and alveolar type II cells [15]. Recombinant human KGF, otherwise known as palifermin, has been shown to protect against injury induced in the lung [16], bladder [17], and intestine [18] by chemicals or irradiation. This has been attributed to the ability of KGF to reduce oxidative damage and enhance DNA repair [19,20]. Palifermin can also prevent irradiation-induced hyposalivation in mice through a mechanism that involves the expansion of a pool of stem/progenitor cells [21]. A number of studies have shown that palifermin protects mice from acute, lethal GVHD. Some have employed models in which the recipients were conditioned prior to transplantation using irradiation either alone or in combination with cyclophosphamide [22,23]. Our own study, and one other, showed that palifermin protects against the development of acute GVHD in models that do not require a conditioning regimen [24,25]. This suggested that palifermin mediates protection from acute GVHD itself, and that the protective effect is not simply a result of its ability to mitigate mucosal injury induced by conditioning agents. Administering palifermin before allogeneic HSCT has also been shown to have important effects on the thymus such as preserving thymopoiesis and regenerating peripheral T cells in the host following allogeneic HSCT [26–28]. In normal mice, exogenously administered KGF enhances thymopoiesis and reverses age-related thymic involution [29].

Using the C57BL/6 $\rightarrow$ (C57BL/6 $\times$ DBA/2)F₁-hybrid model of acute GVHD, we previously showed that treating the recipient mice with palifermin in the peri-transplantation period prevented the development of intestinal GVHD despite elevated levels of TNF α and NO. It also prevented endotoxemia and significantly improved survival. This protective effect was associated with both a cytoprotective effect on epithelial cells and redirection of the immune response from Th1 cytokine production, to a mixed profile of both Th1 and Th2 cytokines, in which Th2 cytokines predominated [24]. To further explore this immunomodulatory effect, we performed experiments in which we treated the donors rather than the recipients with palifermin. Recipients of grafts from palifermin-treated donors showed a significant improvement in survival and the cytokine profile was dominated by Th2 cytokines. The donor mice also had elevated levels of Th2 cytokines in the spleen [30]. These findings demonstrate that in mice with acute GVHD, palifermin mediates potent immunoregulatory effects that are independent of the cytoprotective effects that are seen when it is given to the recipient mice.

Erickson and colleagues showed that the expression of thymic stromal lymphopoietin (TSLP) increases in thymic tissue of fetal mice that have been depleted of thymocytes and exposed to palifermin [31]. TSLP is expressed primarily in the lung, skin, and gastrointestinal tract where its major source is epithelial cells [32]. The TSLP receptor (TSLPR), a member of the hematopoietic cytokine receptor family, binds to TSLP with high affinity. It consists of the IL-7R α chain and a unique TSLPR chain, which alone binds to TSLP with low affinity [32–34]. Murine TSLP was originally identified in supernatant from a murine thymic epithelial cell line and was found to support the growth of early T- and B-cell progenitors [35]. Several studies have also demonstrated the ability of TSLP to direct immune responses towards a Th2 pathway [36–41]. We therefore hypothesized that TSLP might be involved in the mechanism through which palifermin redirects immune responses from Th1 to Th2 in mice with acute GVHD. We showed previously that TSLP mRNA expression in the thymus increases in donor mice that have received palifermin treatment [30]. We wished to determine whether TSLP is indeed involved in the mechanism through which palifermin modulates immune responses and protects mice from developing acute GVHD. Here we report results from experiments in which we studied the effects of palifermin treatment in acute murine GVHD that was induced using TSLP-deficient (TSLPR^{-/-}) donor mice.

Materials and Methods

Mice

Female C57BL/6J (H-2^b), donors, and (C57BL/6J $\times$ DBA/2J)F₁-hybrid recipients (H-2^{b/d}, hereafter referred to as B6D2F₁) were obtained from The Jackson Laboratory and used at 13–16 weeks of age. The TSLPR^{-/-} mice used in these experiments are deficient in the TSLP receptor and have a C57BL/6 genetic background. They were provided by Dr. W. Leonard, bred in the Genetic Models Center at the University of Manitoba, and used at 13–16 weeks of age. All of the experiments were performed in accordance with the standards of the Canadian Council on Animal Care.

Induction and Monitoring of GVH Reactions

Methods used to harvest and prepare the grafts have been described in detail elsewhere [42]. Briefly, a suspension of pooled lymph node and spleen cells was adjusted to a final concentration of 2×10^8 cells/ml in HBSS. Recipients were injected via the tail vein with 60×10^6 donor cells suspended in 300 μ l of HBSS. Recipients were weighed and monitored twice between days 1 and 10 and two to three times per day on days 11–35. They were euthanized when the clinical signs of GVHD such as wasting, ruffled fur, hunched posture, and lethargy became apparent and signs of morbidity had started to develop. Control mice consisted of B6D2F₁-hybrid mice that did not receive a graft.

Palifermin Treatment

To study the effect of donor palifermin treatment on the evolution of acute GVHD, donor mice were injected s.c. with 5 mg/kg of palifermin for seven consecutive days, beginning on day-8 pre-transplantation. Amgen, Inc. (Thousand Oaks, CA, USA) kindly provided the palifermin, which was produced in *Escherichia coli*, refolded, purified to homogeneity by conventional chromatography, tested to be endotoxin free, and assayed using the BALB/MK keratinocyte line [18].

Experimental Design

Our previous experiments showed that treating wild-type donor mice with palifermin in the pre-transplantation period protected the recipient mice from developing acute GVHD [30].

To determine whether TSLP responsiveness is an important feature in the mechanism of palifermin-mediated protection, we established the following four experimental groups: (1) recipients of grafts from untreated wild-type donors, (2) recipients of grafts from palifermin-treated wild-type donors, (3) recipients of grafts from untreated TSLPR^{-/-} donors, and (4) recipients of grafts palifermin-treated TSLPR^{-/-} donors. Recipients were monitored for weight loss and signs of clinical GVHD until they became moribund or the experiment was ended.

Our previous work also showed that treating the donor mice with palifermin induced the production of Th2 cytokines. To determine whether TSLP responsiveness might be involved in this phenomenon, we measured Th1 and Th2 cytokines in splenocyte cultures from untreated wild-type donors, untreated TSLPR^{-/-} donors, palifermin-treated wild-type donors, and palifermin-treated TSLPR^{-/-} donors. To further explore the immunoregulatory effects of palifermin in the donor mice, we determined the percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in grafts prepared from these four groups of donors. We also measured IFN- γ levels in the recipients on day 8 since this is the time at which a burst of IFN- γ is typically seen in this model. This response is typically associated with the development of morbidity associated with acute GVHD.

Cytokine Levels in Splenocyte Culture Supernatants

We used ELISA to quantify IL-4, IL-5, IL-13, and IFN- γ in spleen cell culture supernatants from untreated wild-type donors, untreated TSLPR^{-/-} donors, palifermin-treated wild-type donors, and palifermin-treated TSLPR^{-/-} donors. We also measured IFN- γ concentrations in splenocyte cultures prepared from the recipient mice from these four groups, 8 days after the induction of GVHD. Splenocyte suspensions were prepared as previously described [43]. Spleens were harvested aseptically in HBSS. A sterile suspension containing 2 ml of splenocytes at a concentration of 7.5×10^6 cells/ml was placed in a 24-well flat-bottom culture plate and incubated at 37°C in 5% CO₂. Samples of supernatants were drawn after 48 h for IFN- γ and IL-4 and after 72 h for IL-5 and IL-13, and stored at -70°C. IL-4 was captured with 11B11 mAb and detected with BVD6-24G2 mAb (BD Pharmingen, San Diego, CA, USA). IL-5 was captured with TRFK5 mAb and detected with TRFK4 mAb (BD Pharmingen). IL-13 was measured using the Mouse IL-13 Ready-SET-Go! kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. IFN- γ was captured with XMGI.2 mAb and detected with R4-6A2 mAb (American Type Culture Collection, Rockville, MD, USA).

Enumeration of Regulatory T Cells in the Graft

We used flow cytometry to determine whether palifermin treatment affects the percentages of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in grafts from untreated wild-type donors, untreated TSLPR^{-/-} donors, palifermin-treated wild-type donors, and palifermin-treated TSLPR^{-/-} donors. Spleens and lymph nodes were harvested from individual mice in each group of donors, and a single cell suspension was made by pressing the organs through a stainless steel mesh as previously described [43]. Cells from each mouse were resuspended in 5 ml of RBC Lysis Buffer (Stem Cell Technologies Inc., Vancouver, Canada) and incubated at room temperature for 5 min, with periodic re-suspension. Ten milliliters of PBS was added to stop the reaction, after which the cells were centrifuged and counted. Regulatory T cells were stained using the Mouse Regulatory T cell Staining kit #3 (eBioscience). Aliquots containing 10^6 cells were placed in 100 μ l of staining buffer and incubated with FITC-conjugated anti-mouse CD4 mAb (RM4-5) at a concentration of 1.25 μ g/ml and/or in PE-conjugated anti-mouse CD25 (PC61.5) at a concentration of 0.6 μ g/ml for 30 min at 4°C. FITC-conjugated rat IgG2a (eBR2a) and PE-conjugated rat IgG1 (eBRG1) were used as isotype controls. Cells were then incubated in fixation/

permeabilization buffer for 1 h, followed by FcR Block (affinity purified anti-mouse CD16/32; 2.4G2 from eBioscience) at a concentration of 5 µg/ml for 15 min at 4°C. This was followed by incubation in PE-Cy5-conjugated anti-mouse/rat Foxp3 mAb (FJK-16s) at a concentration of 5 µg/ml for 1 h at 4°C. PE-Cy5-conjugated rat IgG2a (eBR2a) was used as an isotype control. Cells were then washed twice in permeabilization buffer and resuspended in 0.5 ml of staining buffer. For flow cytometry analysis, forward versus side light scatter histograms were set up to define bit map gates for single intact lymphocytes, with acquisition based on 100,000 gated events. Analyses were performed using EXPO 32 Multi COMP software, version 1.2B, which was provided with the EPICS Ultra cell sorter (Beckman Coulter, Hialeah, FL, USA) instrument used for these experiments. Data was analyzed using WinList™ software.

Results

Survival

Figure 1 shows that all of mice that received grafts from untreated wild-type donors (group 1) developed GVHD-associated morbidity. Recipients of grafts from palifermin-treated wild-type donors (group 2) were completely protected from morbidity associated with the development of acute GVHD, which is consistent with our previously published findings [30]. Interestingly, 69% of the mice that received grafts from untreated TSLPR^{-/-} donors (group 3) developed signs of morbidity, whereas the remaining 31% survived until the end of the experiment. No protection was seen recipients of grafts from palifermin-treated TSLPR^{-/-} donors (group 4), and they all developed GVHD-associated morbidity on or before day 14 post-induction. The difference in survival in groups 2 and 3 was significant ($p < 0.0001$, log rank test).

Cytokine Levels in Donor Splenocyte Cultures

Figure 2 shows that there were no significant differences in the levels of IFN-γ, IL-4, IL-5, or IL-13 in untreated wild-type donors when compared to untreated TSLPR^{-/-} donors. In contrast, some significant differences were seen following palifermin treatment. The mean concentration of IL-4 was >20-fold higher in palifermin-treated wild-type donors (244 pg/ml) when compared to palifermin-treated TSLPR^{-/-} donors (10 pg/ml; $p < 0.01$). The mean concentration of IL-5 in palifermin-treated wild-type donors (1,500 pg/ml) was nearly 3-fold higher than that seen in palifermin-treated TSLPR^{-/-} donors (500 pg/ml; $p < 0.05$). Similarly, the mean level of IL-13 was nearly 3-fold higher in palifermin-treated wild-type donors (8,500 pg/ml) when compared to palifermin-treated TSLPR^{-/-} donors (3,100 pg/ml; $p < 0.05$).

Percentages of CD4⁺CD25⁺Foxp3⁺ Regulatory T Cells in the Graft

Bruinsma and colleagues showed that keratinocyte growth factor induces the expansion of murine peripheral CD4⁺Foxp3⁺ cells in the blood [44]. Clinical studies have suggested that these regulatory T cells protect against the development of GVHD [45,46]. Findings from other animal models showed that adoptively transferring CD4⁺CD25⁺Foxp3⁺ cells at the time of transplantation reduces GVHD-associated mortality [27,47–50]. This is further supported by experiments showing that depleting CD25⁺ cells from donor T-cell infusions increases the lethality of GVHD [47,48]. We performed flow cytometry analyses to determine whether the absence of TSLP responsiveness in the donor mice might influence the percentages of CD4⁺CD25⁺Foxp3⁺ cells in the graft. Figure 3 shows that the mean percentage of these cells in palifermin-treated wild-type donors (2.75%) was significantly higher than the mean percentages seen any of the other donor groups. Significant differences were present amongst the percentages in the remaining three recipient groups, but these differences did not correlate with the different levels of survival observed in these groups.

IFN- γ Levels in Spleens from Recipient Mice

Our previous work showed that a burst of IFN- γ develops on day 8 in this model of acute GVHD, and that this is typically associated with the development of GVHD-associated morbidity [24,51] (Fig. 4). In our current study, we found that the highest levels of IFN- γ were seen in recipients of grafts from wild-type donors (group 1; 87 U/ml) and recipients of grafts from palifermin-treated TSLPR^{-/-} donors (group 4; 36 U/ml). Although the IFN- γ concentration in group 4 was less than half of that seen in group 1 ($p < 0.001$), all of the recipients in these two groups succumbed to acute GVHD, suggesting that the concentration of IFN- γ in these two groups was sufficient to induce morbidity. The lowest concentrations of IFN- γ were seen in recipients of grafts from palifermin-treated wild-type donors (group 2; 18 U/ml), in which all of the recipients survived, and in recipients of grafts from untreated TSLPR^{-/-} donors (group 3; 9 U/ml), in which 31% of the recipients survived. Our observation that the IFN- γ level was lower in group 3 compared to group 2 was unexpected, since the survival levels in these two groups were 100% and 31%, respectively ($p < 0.05$). The reason for this is not known.

Discussion

In this study, we confirmed our previous finding that GVHD-associated morbidity is obviated in the recipients when grafts from palifermin-treated wild-type donors are used (group 2). We further showed that there were no survivors when grafts from palifermin-treated TSLPR^{-/-} donors were used to induce GVHD (group 4), thereby demonstrating that TSLP responsiveness plays an integral role in the protective, immunomodulatory effects of palifermin treatment in this model. One unexpected finding was the observation that 31% of the recipients survived when untreated TSLPR^{-/-} donors were used (group 3). This suggests that some of the effects of TSLP alone can promote the development of GVHD. Because none of the recipients of grafts from palifermin-treated TSLPR^{-/-} donors survived (group 4), whereas 31% of the recipients of grafts from untreated TSLPR^{-/-} donors did survive (group 3), it is likely that, when the donors are unresponsive to TSLP, some of the effects of the palifermin treatment promote the development of acute GVHD. Collectively, these results show that when palifermin and TSLP act in concert, they deliver a potent immunomodulatory effect that protects the recipients from developing GVHD-associated morbidity.

We found that the ability of palifermin to promote the production of Th2 cytokines in the donor mice is mitigated in the absence of TSLP responsiveness. This further underscores a role for TSLP in palifermin-mediated immune modulation in this model. We further observed that grafts from palifermin-treated wild-type donors (group 2) contained the highest percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells among the four recipient groups. This finding is important because recipients in group 2 were completely protected against the development of GVHD-associated morbidity, suggesting that these cells might play a role in the immunological mechanism responsible for the protective effects of palifermin in this model.

To summarize, our findings support the hypothesis that, in this model of acute GVHD, palifermin can modulate donor immune responses and deliver a protective effect to the recipients through a mechanism that involves TSLP. It is important to emphasize that the purpose of these experiments was not to test the efficacy of treating donors with palifermin as a means of preventing clinical GVHD, but rather to use this mouse model to gain a better understanding of the possible mechanisms through which palifermin can mediate protective, immunomodulatory effects that are independent of its well-established cytoprotective effects in acute GVHD.

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Fig. 1.

Survival curves are shown for recipients of grafts from (1) untreated wild-type donors ($n=12$, squares), (2) palifermin-treated wild-type donors ($n=14$, circles), (3) recipients of grafts from untreated TSLPR^{-/-} donors ($n=16$, triangles), and (4) recipients of grafts from palifermin-treated TSLPR^{-/-} donors ($n=13$, diamonds). A log rank test was used to compare survival in the two groups of recipients that still had survivors at the end of the experiment (groups 2 and 3)

Fig. 2. Mean concentrations of IFN- γ , IL-4, IL-5, and IL-13 in splenocyte culture supernatants from wild-type donors (*black bars*) and TSLPR^{-/-} donors (*gray bars*) that either had or had not received palifermin treatment are shown. *Error bars* represent the SEM for the concentrations observed in three individual mice. The mean concentrations of each cytokine observed in the four groups of donors were compared using an ANOVA followed by Tukey's multiple comparison test

Fig. 3.

Mean percentages of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in grafts consisting of pooled spleen and lymph node cells from the donor mice. Grafts from wild-type (*black bars*) and TSLPR^{-/-} donors (*gray bars*) that either had or had not received palifermin treatment were enumerated. A representative example of data from a graft harvested from palifermin-treated wild-type donors is shown in (a), (b), and (c). The gate in (a) (R1) delineates the population that was analyzed. The gate in (b) (R10) delineates the population co-expressing CD4 and CD25. The gate on the histogram in (c) (R9) delineates the CD4⁺CD25⁺ cells that also express Foxp3. The *bar graph* (d) shows the percentages of CD4⁺CD25⁺Foxp3 cells in grafts from each group of donors. *Error bars* represent the SEM for the percentages observed in three individual mice. The mean percentages were compared using an ANOVA followed by Tukey's multiple comparison test.

Fig. 4.

Mean concentrations of IFN- γ in splenocyte culture supernatants from recipients of grafts from wild-type donors (*black bars*) and recipients of grafts from TSLPR^{-/-} donors (*gray bars*) that either had or had not received palifermin treatment are shown. Spleens were harvested on day 8 post-induction. *Error bars* represent the SEM for the concentrations observed in three individual mice. The mean concentrations IFN- γ observed in the four groups of donors were compared using an ANOVA followed by Tukey's multiple comparison test