Lymphotoxin-β Receptor Signaling Regulates Hepatic Stellate Cell Function and Wound Healing in a Murine Model of Chronic Liver Injury

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Lymphotoxin-beta (LTβ) is a proinflammatory cytokine and a member of the tumor necrosis factor (TNF) superfamily known for its role in mediating lymph node development and homeostasis. Our recent studies suggest a role for LTβ in mediating the pathogenesis of human chronic liver disease. We hypothesize that LTβ co-ordinates the wound healing response in liver injury via direct effects on hepatic stellate cells. This study used the choline-deficient, ethionine-supplemented (CDE) dietary model of chronic liver injury, which induces inflammation, liver progenitor cell proliferation, and portal fibrosis, to assess (1) the cellular expression of LTβ, and (2) the role of LTβ receptor (LTβR) in mediating wound healing, in LTβR−/− versus wild-type mice. In addition, primary isolates of hepatic stellate cells were treated with LTβR-ligands LTβ and LTβ-related inducible ligand competing for glycoprotein D binding to herpesvirus entry mediator on T cells (LIGHT), and mediators of hepatic stellate cell function and fibrogenesis were assessed. LTβ was localized to progenitor cells immediately adjacent to activated hepatic stellate cells in the periportal region of the liver in wild-type mice fed the CDE diet. LTβR−/− mice fed the CDE diet showed significantly reduced fibrosis and a dysregulated immune response. LTβR was demonstrated on isolated hepatic stellate cells, which when stimulated by LTβ and LIGHT, activated the nuclear factor kappa B (NF-κB) signaling pathway. Neither LTβ nor LIGHT had any effect on alpha-smooth muscle actin, tissue inhibitor of metalloproteinase 1, transforming growth factor beta, or procollagen α1(I) expression; however, leukocyte recruitment-associated factors intercellular adhesion molecule 1 and regulated upon activation T cells expressed and secreted (RANTES) were markedly up-regulated. RANTES caused the chemotaxis of a liver progenitor cell line expressing CCR5. Conclusion: This study suggests that LTβR on hepatic stellate cells may be involved in paracrine signaling with nearby LTβ-expressing liver progenitor cells mediating recruitment of progenitor cells, hepatic stellate cells, and leukocytes required for wound healing and regeneration during chronic liver injury. (HEPATOLOGY 2009;49:227-239.)

Abbreviations: αSMA, alpha smooth muscle actin; BMOL, bipotent murine oval liver; CDE, choline deficient, ethionine supplemented; HCV, hepatitis C virus; HSC, hepatic stellate cell; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon gamma; IkBα, inhibitor of kappa B alpha; LIGHT, lymphotoxin-related inducible ligand competing for glycoprotein D binding to herpesvirus entry mediator on T cells; LPC, liver progenitor cell; LT, lymphotoxin; NF-κB, nuclear factor kappaB; PIL, p53 immortalized liver; RANTES, regulated upon activation T cells expressed and secreted; TGF-β, transforming growth factor beta; TIMP-1, tissue inhibitor of metalloproteinase 1; TNF, tumor necrosis factor.

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Despite being a quiescent organ under resting conditions, the liver retains a remarkable capacity for regeneration in response to injury. Repair of liver tissue can be accomplished by the repeated division of mature adult hepatocytes. Alternately, under injury conditions in which the replicative capacity of hepatocytes is compromised, the liver regenerates via a coordinated response of committed adult liver progenitor cells (LPCs). After injury, dormant LPCs become activated and subsequently undertake a program of proliferation, migration, and differentiation to replace lost parenchymal tissue. This response is typically associated with chronic or carcinogenic injury, such as hepatitis B virus or hepatitis C virus (HCV) infection, hereditary hemochromatosis, alcoholic liver disease, or nonalcoholic fatty liver disease. These diseases, although diverse in origin, show a similar progression of pathological conditions, characterized by three major cellular changes: inflammation, expansion of the LPC compartment, and fibrogenesis, involving activation of hepatic stellate cells (HSCs).1,2 Each of these cellular processes has been extensively studied in isolation, using cell culture systems or animal models designed to selectively invoke the pathological condition of interest. However, few studies have investigated how these cellular responses are cross-regulated and how interaction between cell types may contribute to the regenerative and fibrogenic response of the liver to chronic injury. Clouston et al.3 have proposed that in chronic liver disease associated with HCV or nonalcoholic fatty liver disease, hepatic fibrosis is driven by expanding and migrating LPCs, suggesting potential cross-talk between HSCs and LPCs. Work from our group using the choline-deficient, ethionine-supplemented (CDE) dietary model of chronic liver injury supports this concept, showing a close association between LPC proliferation and the activation of HSCs, which drive the portal fibrosis associated with this model.5–7 Although these studies suggest the potential for communication between LPCs and HSCs, the precise mechanisms responsible are unknown.

Lymphotoxin beta (LTβ) is a proinflammatory cytokine of the tumor necrosis factor (TNF) family. It has a well-characterized role in mediating inflammatory cell responses, especially those involving B-lymphocytes. It signals as a cell surface–anchored heterotrimer with lymphotoxin alpha (LTα) and thus is involved in autocrine and paracrine signaling to adjacent cells. The LTα1:β2 heterotrimer binds specifically to the LTβ receptor (LTβR), which also can be activated by another TNF family member, LTβ-related inducible ligand competing for glycoprotein D binding to herpes virus entry mediator on T cells (LIGHT). Once activated, LTβR initiates a signaling cascade resulting in activation of nuclear factor kappaB (NF-κB). We have observed up-regulated LTβ expression after chronic liver injury induced by bile duct ligation9 and the CDE diet10,11 in rodents, as well as chronic HCV infection in humans.12 In HCV-induced liver disease, a significant positive correlation was observed between the level of hepatic LTβ transcript present and the extent of fibrosis.12 LTβ expression was detected on inflammatory cells, LPCs, and occasional small hepatocytes adjacent to fibrous septa. The localization of LTβ-expressing cells to the perportal regions in which fibrosis occurs supports a possible role for LTβR-initiated signaling in controlling the function of HSCs. In accordance, LTβ has been reported to modulate the function of several fibrogenic cell types, including fibroblast-like synoviocytes,13 lymph node fibroblastic reticular cells,14 and fibrosarcoma cells.15 It also has been shown to activate intracellular signaling in primary human fibroblasts.16

In 2005, Akhurst et al.11 documented a reduced LPC response to chronic liver injury induced by the CDE diet in mice lacking the LTβR. Although not examined, the authors postulate that this is mediated by direct proliferative effects of LTβ. Anders et al.17 also reported a role for LTβ in mediating the liver’s regenerative response in acute liver injury.17 We hypothesize that LTβR signaling may represent a coordinating pathway, controlling the wound healing and regenerative responses of inflammatory, progenitor, and fibrogenic cell types. Moreover, we hypothesize that signaling initiated by LTβR may facilitate cross-talk between these cell types. To test these hypotheses, we investigated the role of LTβR-initiated signaling in mediating the function of HSCs and its potential role in the proinflammatory events associated with chronic liver injury and wound healing. The CDE model of chronic liver injury was used in this study as an ideal means of investigating potential cross-talk attributable to the extensive LPC expansion, HSC activation, and portal fibrosis that accompanies this model.18

Materials and Methods

Animals. C57Bl/6 (wild-type) mice were obtained from the Animal Resources Centre of Western Australia. LTβR−/− mice, created on the 129 background and back-crossed onto C57Bl/6, were from Prof. Klaus Pfeffer (The Technical University of Munich, Germany). Male mice, 4 to 6 weeks of age, were fed the CDE diet for 1 to 3 weeks exactly as described previously.11 All animal experiments were performed in strict accordance with the guidelines set by the National Health and Medical Re-
search Council of Australia and the University of Western Australia Animal Ethics Committee.

**Cytokine Expression Analysis.** LTβ, LTα, TNF, and interferon gamma (IFN-γ) messenger RNA (mRNA) levels in whole liver from CDE-fed animals were determined using the RiboQuant ribonuclease protection assay system (BD Pharmingen) with the Mck-3b probe set, exactly as the manufacturer recommended, and additionally, as described previously.21,22 Cells were cultured at 37°C, 95% humidity in William’s E medium supplemented as previously described.20

**LPC Lines.** Three LPC lines were used in these studies: p53 immortalized liver 2 (PIL-2), PIL-4, and bipotent murine oval liver (BMOL). These have been previously described.19,20 Cells were cultured at 37°C, 95% humidity in William’s E medium supplemented as previously described.20

**Hepatic Stellate Cell Isolation.** Rat HSCs were isolated from the livers of normal male Sprague-Dawley rats (600 g ± 50 g) by sequential perfusion with pronase and collagenase as previously described.21,22 HSCs were seeded onto either plastic wells (activated HSCs) or Teflon-coated membranes (quiescent HSCs) (Millipore) in Medium 199 (M199; Invitrogen) supplemented with 10% fetal bovine serum, 10% horse serum, 50 mM ascorbic acid, and penicillin/streptomycin (100 U and 100 µg/mL, respectively). Cells were maintained at 37°C in a humidified atmosphere of 5% CO2. For cytokine stimulation experiments, cells were transferred to serum-free M199 overnight before addition of recombinant murine LTα1:β2 (herewith: rmLT) or recombinant human LIGHT (rhLIGHT). Medium was changed every 48 hours during the experimental period. Except where specified, all HSC experiments were performed on day 5 cultures that had undergone auto-activation as demonstrated previously21 and confirmed in the current manuscript.

**Western Blotting.** Cells were lysed in 1× sodium dodecyl sulfate sample buffer [62.5 mM Tris HCl pH 6.8, 2% wt/vol sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, 0.01% wt/vol bromophenol blue]. Equal volumes of whole cell protein were separated by 9% sodium dodecyl sulfate polyacrylamide gel electrophoresis at 100 V for 1.5 hours before transfer onto nitrocellulose (GE). After blockade of nonspecific protein binding, blots were incubated for 1 hour with primary antibodies [diluted in phosphate-buffered saline (PBS)] containing 3% to 5% skim milk powder or bovine serum albumin. Blots were then washed four times with phosphate-buffered saline/Tween after incubation for 1 hour with secondary antibodies conjugated to horseradish peroxidase. After extensive washing in phosphate-buffered saline/Tween 20 (0.05%), blots were processed to distilled water for detection of antigen using the enhanced chemiluminescence system (GE). Digital images of resulting chemiluminescent signals were captured using the Fujifilm LAS3000 system and analyzed using Fujifilm Multi Gauge V3.0 software.

**RNA Isolation and Real Time Reverse Transcription Polymerase Chain Reaction.** RNA was isolated from cultured HSCs or whole liver tissue using the RNeasy mini kit (Qiagen) according to the manufacturer’s instruction. Total RNA was DNase treated (DNA Free, Ambion) and quantified, and 1 to 3 µg total RNA reverse transcribed using the Thermoscript or Superscript III systems (Invitrogen). Negative control samples were generated by omission of the reverse transcriptase enzyme from reverse transcription (RT) reactions.

Real-time RT polymerase chain reaction (PCR) analysis was performed in accordance with a standard protocol incorporating SYBR Green1 (Molecular Probes) and using Platinum Taq DNA Polymerase (Invitrogen) and primers specific for the target of interest (Table 1). Fluorescence detection and PCR cycling was performed using a RotorGene RG-3000 thermal cycler (Corbett Research, Australia). Fluorescence measurements of unknowns were back-referenced to a standard curve to relate fluorescence to concentration in arbitrary units. These results were then normalized to the concentration of a house-
keeping gene: either basic transcription factor 3 or β-actin in the same sample, to give final values as normalized expression of the gene of interest.

**Cell-Proliferation-Assay.** HSC proliferation was determined in the presence or absence of rmLTβ for up to 5 days. After treatments, relative cell number was determined using the CellTitre 96 AQueous One Solution Cell Proliferation Assay (Promega), exactly as the manufacturer recommended.

**Immunostaining of Mouse Liver.** Immunostaining was performed on formalin-fixed, paraffin-embedded, or cryosectioned tissue. Staining was performed according to a standard two-step indirect immunofluorescent or immunoperoxidase method, with or without pretreatment for antigen recovery. Primary antibodies used were mouse anti-human alpha smooth muscle actin (α-SMA; Sigma), rabbit anti-mouse LTβ (Santa Cruz), rat anti-mouse A6 (gift from Dr. Valentina Factor), anti-mouse CD45 (BD Pharmingen), anti-mouse regulated upon activation T cells expressed and secreted (RANTES; Serotec). Cell counts were performed at 40× magnification on adjacent, nonoverlapping fields of view. Ten fields of view were counted per tissue sample and averages pooled for each group to give final values as mean ± standard error for the treatment group.

**Indirect Immunofluorescence and Confocal Microscopy of Activated Rat HSCs.** Rat HSCs were culture-activated on plastic tissue culture wells in M199 media for 5 days. Indirect immunofluorescence analysis and confocal microscopy was performed as described, with minor modifications (see Supporting Methods, online). Goat anti-mouse LTβR antibody (30 μg/mL; R&D Systems) was incubated with α-SMA antibody (1:400; Sigma), as a marker of activated HSCs. The intracellular localization of LTβ-R in HSCs was assessed using indirect immunofluorescence and confocal microscopy (see Supporting Methods, online).

**Chemotaxis Assay.** Chemotaxis of PIL-4 or BMOL LPC lines in the presence of 10 or 100 ng/mL recombinant murine RANTES (R&D Systems) was assayed using the Chemicon Cell Migration Assay system (Chemicon), per the manufacturer’s instructions. Results are expressed as relative fluorescence units. Resultant values were confirmed to be within the linear range of the assay. Fetal bovine serum was used as a positive control.

**Statistical Analysis.** Data are presented as mean values ± standard error of the mean (SEM). Groups were compared using one-way analysis of variance (ANOVA) with Dunnett’s multiple comparison post hoc test or the Student t test (where applicable) using Prism 4.0 (GraphPad Software, San Diego, CA). A P value of less than 0.05 was considered statistically significant.

**Results**

**LTβ Is Expressed by LPCs Adjacent to HSCs/Myofibroblasts in Murine CDE Liver Injury Model.** Immunofluorescent staining for LTβ in CDE-fed mouse livers demonstrated a pattern of expression similar to that reported in chronic hepatitis C. LTβ was expressed primarily in periportal liver regions (Fig. 1A, B). Most cells positive for LTβ were LPCs, as demonstrated by their co-expression of the marker A6 (Fig. 1C, E), although
some signal was also seen in what appeared to be small hepatocytes (Fig. 1E). Additionally, a subset of CD45+ lymphocytes were positive for LTβ in CDE liver (Fig. 1G), but this was not seen in normal liver (Supporting Fig. 1B, D, F). A6-expressing mature bile ducts were also positive for LTβ in normal liver; these accounted for most of the cells stained positively with the anti-LTβ antibody (Supporting Fig. 1).

Double staining for LTβ (Fig. 1B) and αSMA (Fig. 1D), a marker of activated HSCs and myofibroblasts, showed an intimate spatial relationship between LTβ and αSMA-positive cells (Fig. 1F). No individual cells co-stained for the two proteins; however, cells positive for LTβ were routinely present adjacent to cells positive for αSMA (Fig. 1F).

Regression analysis was used to compare whole liver mRNA levels of LTβ with the number of αSMA-positive cells in mice fed the CDE diet for 7, 14, or 21 days. The results show a significant positive correlation between the two parameters (Fig. 1H: \( r^2 = 0.7; P = 0.0057 \)).

**Signaling via LTβR Is Required for Fibrogenesis in Murine CDE Liver Injury Model.** LTβR-/- mice fed the CDE diet for 14 or 21 days showed reduced collagen \( \alpha_1 \) mRNA levels compared with wild-type controls (Fig. 2A). Sirius red (Fig. 2B, C, D) and αSMA staining (Fig. 2E, F) confirmed a reduction in the extent of fibrosis and the number of activated HSCs/myofibroblasts in LTβR-/- (Fig. 2B, D, F) animals, compared with wild-type (Fig. 2B, C, E).

**LTβR-/- Disrupts the Inflammatory Response to CDE-Induced Chronic Liver Injury.** Levels of key proinflammatory cytokines, LTα, LTβ, TNF, and interferon gamma (IFNγ), were examined in whole liver from 2-week CDE or control diet-fed wild-type and LTβR-/- mice (Fig. 3A-D). LTα expression was elevated in LTβR-/- livers (Fig. 3A). In wild-type mice, levels of TNF, LTβ, and IFN-γ were induced by feeding mice a CDE diet for 2 weeks (Fig. 3B, C). In LTβR-/- mice, the CDE diet produced a less substantive increase in hepatic growth factor mRNA, on average only approximately 50% of that seen in wild-type (Fig. 3B-D).

CD45 staining was used to examine lymphocyte numbers in CDE livers. This confirmed previous reports that LTβR-/- mice have a pathological condition characterized by the presence of large clusters of inflammatory cells in periporal liver regions.24 This phenotype is
present in all LTBR−/− livers, regardless of treatment with CDE diet (Fig. 3E). Immunohistochemical staining shows that most of the cells in these clusters are CD45+ (Fig. 3F).

LTBR Is Expressed in Quiescent and Activated Primary Cultures of HSCs. LTBR expression was demonstrated in both quiescent and activated HSCs. Quantitative RT-PCR demonstrated expression of LTBR mRNA by quiescent HSCs with LTBR mRNA levels remaining unchanged over a 6-day time course as HSCs became activated (Fig. 4A). Increasing expression of αSMA (Fig. 4B), procollagen α1(I) (Fig. 4C), and tissue inhibitor of metalloproteinases 1 (TIMP-1) (Fig. 4D) are shown, demonstrating the progressive activation of HSCs with time in culture.

Confocal microscopy was used to demonstrate the presence of LTBR protein expression in primary cultures of αSMA-expressing activated HSCs (Fig. 4E-G). LTBR was localized to the cell surface and in endosomal vesicles (see Supporting Fig. 2).

LTBR Is Activated in HSCs by Exposure to rmLTβ. LTβ (rmLTβ) was tested at various concentrations for its ability to initiate intracellular signaling through NF-κB in HSCs. A time-dependent and dose-dependent increase in expression of IkBα mRNA, an NF-κB-responsive gene, was observed after rmLTβ stimulation (Fig. 5A, B). Based on this, an effective dose of approximately 25 ng/mL was determined. Protein levels of IkBα were also increased significantly with LTβ treatment (Fig. 5C). Phosphorylation of a regulator of both IkBα and NF-κB activation, was rapidly induced by rmLTβ stimulation, with similar or greater efficacy than TNF (Fig. 5D).

LTβ Does Not Augment HSC Activation. Expression of the HSC activation markers αSMA, procollagen α1(I), TIMP-1, and transforming growth factor beta1 (TGF-β1) have been shown to be inducible by cytokine treatment25 and are associated with HSC contractility and collagen synthesis. To determine whether LTβ directly enhances expression of these markers, HSCs were treated with rmLTβ (1-100 ng/mL) for up to 12 hours. Recombinant LTβ did not augment the expression of either αSMA, procollagen α1(I), TIMP-1, or TGF-β1 (Supporting Figs. 3A-H) at any dose examined, although there was a 30% decrease in αSMA mRNA expression at 12 hours.

To test for longer-term, possibly indirect effects of LTβ on the activated HSCs phenotype, HSCs were treated with 50 ng/mL rmLTβ for up to 5 days. LTβ treatment was associated with a modest reduction in the expression of both αSMA protein (Fig. 6A, B) and collagen α1(I) mRNA (Fig. 6C); however, these differences
did not reach statistical significance. Proliferation, as judged by proliferating cell nuclear antigen protein levels (Fig. 6A, D) or CellTiter-96-Aqueous-One-Solution-Cell-Proliferation-Assay assay (Fig. 6E), was also not significantly different between treatment groups.

**rmLTβ Induces Expression of Intercellular Adhesion Molecule 1 and RANTES in Activated HSCs.** Treatment of activated HSCs with rmLTβ induced expression of two chemotaxis-related molecules: intercellular adhesion molecule 1 (ICAM-1) (Fig. 7A, B) and RANTES (Fig. 7C, D). Both were up-regulated in a time-dependent (Fig. 7A, C) and dose-dependent fashion (Fig. 7B, D). Examination of ICAM-1 mRNA showed peak expression after 3 hours; however, western blot analysis showed continual increases in ICAM-1 protein for up to 48 hours (Fig. 7E).

**Quiescence Does Not Alter the Response of HSCs to LTβ.** To ascertain whether the observed effects of rmLTβ treatment were limited to activated HSCs, gene expression experiments were repeated using HSCs cultured on Teflon, which has previously been shown to inhibit the auto-activation of isolated HSCs and thereby retain them in a quiescent state.21 Quiescent HSCs remained responsive to simulation with rmLTβ, as evidenced by increased IκBα mRNA levels (Fig. 8A). Expression of αSMA and procollagen α1(I) remained at control levels after 25 ng/mL LTβ stimulation, and culture of cells on Teflon did not alter this result (Fig. 8B, C). ICAM-1 (Fig. 8D) and RANTES expression (Fig. 8E) was significantly up-regulated after LTβ stimulation in both activated and quiescent HSC cultures.

**rhLIGHT Mimics the Effects of rmLTβ on HSCs.** LIGHT is another member of the TNF family, also capable of initiating intracellular signaling via LTβR. Stimulation of HSCs by rhLIGHT induced both time-dependent (Fig. 9A, G) and dose-dependent (Fig. 9B, H) expression of IκBα (Fig. 9A, B) and RANTES (Fig. 9G, H). Recombinant human LIGHT induced a dose-dependent increase in ICAM-1 gene expression, which approached statistical significance (Fig. 9F). Treatment of HSCs with LIGHT had no effect on αSMA (Fig. 9C, D) or procollagen α1(I) mRNA levels (results not shown). However, the extent of up-regulation of all three NF-κB-responsive genes by LIGHT was consistently twofold to fivefold lower than that induced by rmLTβ, potentially because of the use of human LIGHT. Gene expression of the LIGHT receptor herpes entry viral...
Fig. 5. Treatment of day 5 HSC cultures with rmLTβ induced expression of the NF-κB-responsive gene IkBα in a time-dependent (ANOVA, $P = 0.0005$; A) and dose-dependent (ANOVA, $P < 0.0001$; B) fashion. Western blotting illustrated a steady increase in IkBα protein over a 2-day time course (C). A regulator of both IkBα and NF-κB activation, which regulates NF-κB activation-dependent signaling, showed rapid phosphorylation after stimulation with LTβ (D), further illustrating its ability to initiate intracellular signaling in isolated HSCs. Data represent mean ± SEM, $n = 3$. Time-response experiments were performed using 100 ng/mL LTβ; dose-response experiments were analyzed at 3 hours. Asterisks denote a significant difference of indicated mean values; *$P < 0.05$; **$P < 0.01$.

Fig. 6. Treatment of transforming HSCs for extended periods with 50 ng/mL LTβ did not modulate their auto-activation, as evidenced by levels of αSMA protein (A, B) and procollagen α(I) mRNA (C). A modest down-regulation in both markers was observed in LTβ-treated cultures; however, this did not reach statistical significance (t test, $P > 0.05$). Proliferating cell nuclear antigen protein levels were not affected by LTβ treatment, indicating no effect on proliferation (A, D). Additionally, LTβ had no significant effect on HSC numbers as judged by CellTiter-96-Aqueous-One-Solution-Cell-Proliferation-Assay (E). Data represent mean ± SEM, $n = 3$. 

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mediator was not detected in either quiescent or activated HSCs (results not shown).

**RANTES Receptor (CCR5) Is Expressed by LPCs and Facilitates Their Chemotaxis.** RANTES and CCR5 expression were examined in control and CDE-fed mouse liver as well as in isolated LPCs and immortalized LPC cell lines. RANTES expression was modestly up-regulated in CDE liver compared with control (Fig. 10A), whereas CCR5 was significantly up-regulated (Fig. 10B). Primary isolates of LPCs from CDE livers expressed both RANTES (Fig. 10A) and CCR5 (Fig. 10B). The BMOL LPC cell line was negative for both receptor and ligand. PIL-2 expressed RANTES (Fig. 10A) but not CCR5 (Fig. 10B), whereas the PIL-4 LPC cell line expressed CCR5 at levels comparable to primary LPCs (Fig. 10B).

Fluorescence immunostaining showed expression of RANTES in periportal liver regions. A portion of RANTES-positive cells were also αSMA positive (Fig. 10C); another population of RANTES-expressing cells were CD45+ (Fig. 10D). RANTES-expressing cells were demonstrated adjacent to A6-positive LPCs (Fig. 10E).

To examine whether RANTES is capable of inducing LPC chemotaxis, BMOL and PIL-4 cells were cultured in the presence of 10 or 50 ng/mL RANTES and cell migration assayed using a commercially available system. PIL-4 cells migrated toward medium containing recombinant murine RANTES, in a dose-dependent fashion (Fig. 10F). In contrast, no migration of BMOL cells was observed at either concentration of RANTES (data not shown).

**Discussion**

LTβ is a TNF family member originally identified for its role in immune signaling in B lymphocytes. The finding that LTβ is expressed in chronically injured liver, and moreover by LPCs, suggests a role for LTβ-initiated signaling in mediating pathogenesis in diseases such as chronic hepatitis C. Recent evidence suggests there may be cross-talk between the liver’s fibrogenic HSC and the progenitor cell compartment; however, the mechanism by which this interaction is mediated and the resultant biological implications have not previously been investigated.

In the current study we identify LTβ as one potential mediator of the interaction between HSCs and LPCs. Hepatic LTβ mRNA levels correlated with numbers of...
activated HSCs/myofibroblasts in mice fed a CDE diet to induce chronic liver injury. LTβ expression in CDE liver was predominantly localized to A6-positive LPCs residing immediately adjacent to activated HSCs positive for αSMA. LTβ protein was also detected in a proportion of CD45-expressing inflammatory cells and in occasional small hepatocytes. In normal liver, almost all LTβ-positive cells were A6-positive bile duct cells. Because LTα1:β2 signals exclusively as a cell-surface–anchored heterotrimer, its mechanism of action must be via paracrine signaling only to adjacent cells. Previous reports have suggested that LTβ may be an autocrine growth factor within the LPC population; however, LTβ did not promote proliferation of LPC cell lines at any concentration tested (30-300 ng/mL) (results not shown).

Mice lacking LTβR were found to have a significantly attenuated wound healing response to the CDE diet. This is characterized by reduced LPC numbers as previously reported, abrogation of fibrosis development, and a dysregulated inflammatory response. Thus, we propose that LTβ coordinates the wound healing response to chronic injury by facilitating cell–cell communication and mediating cross-talk between different regenerative cell types.

LTβ exists as two alternative heterotrimers: LT-α1:β2 and α2:β1. This study focuses on LTα1:β2, because this heterotrimer signals through LTβR. In this study, we demonstrate that both quiescent and activated HSCs express the LTβR. A second ligand was also examined, LIGHT, which is capable of signaling either through LTβR or its own receptor, herpes entry viral mediator. However, the herpes entry viral mediator was not detected in HSCs, suggesting that the effects of LIGHT on HSCs were mediated via LTβR. Confocal microscopy illustrated that LTβR localized to the cell surface of HSCs as well as around the perinuclear region, where receptor recycling typically occurs. Stimulation of isolated HSCs with recombinant LTα1:β2 (herewith LTβR) or LIGHT invoked downstream signaling consistent with that expected after activation of LTβR, including induction of a regulator of both IkBα and NF-κB activation phosphorylation and IkBα expression, molecular markers of NF-κB activation.

Interestingly, treatment of HSCs with LTβ did not affect proliferation or the expression of activation markers αSMA, procollagen α1(I), TIMP-1, and TGF-β1. Culture of HSCs on Teflon membranes to inhibit their auto-activation did not affect these results. These findings are of considerable interest, because the results obtained in LTβR−/− mice illustrated a reduction in the number of αSMA-positive cells as well as in collagen mRNA and

Fig. 8. Day 5 HSCs cultured on plastic were compared with cells maintained on Teflon (to retain their quiescence) to determine whether the activation state of the cells affected their response to LTβ. ICAM-1 (A), ICAM-1 (D), and RANTES (E) mRNA levels were up-regulated in response to 25 ng/mL LTβ in both activated (plastic) and quiescent (Teflon) HSCs. Procollagen α1(I) (B) and αSMA (C) expression remained unchanged after LTβ stimulation, and culture of HSCs on Teflon did not alter this finding. Data represent mean ± SEM, n = 3. Asterisk denotes a significant difference by Student’s t test of the indicated mean values; *P < 0.05.
protein. Thus, LTβ plays a role in mediating hepatic fibrogenesis, however, not as a direct mediator of collagen production by HSCs. Rather, we hypothesize that LTβ may function to recruit inflammatory cells, LPCs, and HSCs to the site of injury and thereby promote wound healing and regeneration by facilitating communication between these cell types.

Supporting this, we demonstrated that inflammatory cytokine expression was significantly altered in LTβR−/− livers. In control-diet fed mice, cytokine levels were routinely twofold to threefold higher than for wild-type controls. However, unlike their wild-type counterpart, LTβR−/− mice failed to induce expression of LTβ, TNF, and IFN-γ in response to the CDE diet, suggesting that the inflammatory response to CDE-induced liver injury/regeneration is lacking in these animals. We observed extramedullary lymphopoiesis in our LTβR−/− colony, as has previously been described. Interestingly, levels of LTα correlated with numbers of inflammatory cells present in the livers of wild-type and LTβR−/− mice. However, the other proinflammatory cytokines examined showed no such relationship. This suggests that leukocyte numbers do not reflect the inflammatory status of the liver in this model.

Both LTβ and LIGHT have been shown to invoke chemokine expression in various cell types. In lymphocytes, LTβR-mediated expression of adhesion factors is believed to control lymph node organization; loss of LTβR-initiated signaling impairs this process. Our data clearly demonstrate a strong, sustained up-regulation of the chemokine RANTES and significant expression of the adhesion factor ICAM-1 by HSCs after LTβR stimulation. Both ICAM-1 and RANTES are known to facilitate leukocyte recruitment to remote sites, including the liver. ICAM-1 has been extensively studied in the context of chronic liver injury, and its expression has also been reported to increase after CDE-induced injury. In contrast, the role of RANTES in chronic liver disease has been significantly less well-characterized.

Gene expression analysis of RANTES, and its receptor CCR5, showed a significant increase in CCR5 but not RANTES mRNA levels in CDE-treated liver compared with control, suggesting regulation of this pathway by receptor expression in chronic injury. RANTES immunostaining illustrated its expression by CD45+ inflammatory cells, as well as αSMA-positive cells located immediately adjacent to LPCs. Additionally, primary isolates of LPCs were found to express CCR5, indicating their potential responsiveness to stimulation with RANTES. In accordance, PIL-4 cells (a CCR5+ LPC line) were found to migrate toward medium containing recombinant murine RANTES in a dose-dependent manner, whereas the BMOL cell line (CCR5-) did not. RANTES was not pro-proliferative to PIL-4 cells at any concentration tested (data not shown). HSCs are also known to express CCR5; however, RANTES, as well as inducing a migratory effect on HSCs, is also mitogenic. This suggests that chemokine expression by HSCs may facilitate recruitment of LPCs as well as inflammatory cells and mediate their adhesion to HSCs at the site of injury. It also highlights RANTES and ICAM-1 as potential mediators of the close spatial relationship observed between these cell populations in chronically injured liver.
The response of LPCs after chronic injury is characterized by a coordinated program of cellular activity to ultimately mediate regeneration of lost tissue. The migration of LPCs is an often overlooked component of this process; however, it is essential because these cells arise in periporal regions of the liver but are often required for regeneration in central regions of the liver acinus where damage is most severe. Very little is known about how this process is mediated; however, it follows that LPCs would require matrix degradation (and reconstruction) to facilitate their migration. Thus, we propose that the interaction between HSCs and progenitor cells may mediate this process. Finally, our data indicate that LPCs are involved in the process of early fibrogenesis in chronic injury, supporting the conclusions of Clouston et al. and Richardson et al. as well as our own previous findings.

In conclusion, we have identified LTβ/H9252 as an important signaling molecule mediating the liver’s wound healing response to chronic injury. Paracrine signaling between LPCs and HSCs exists, as evidenced by a clear spatial association between LTβ-expressing cells and HSCs, and the responsiveness of HSCs to stimulation with LTβ. We propose that chemokine production by HSCs in response to LTβR stimulation may facilitate recruitment of leukocytes, LPCs, and HSCs, and thereby promote wound healing and regeneration. In accordance, LTβR deletion caused disruption to the fibrogenic and inflammatory responses to chronic injury, as well as inhibiting the regenerative response of LPCs. Taken together, this suggests that the role of LTβ is not restricted to one cellular compartment; rather, LTβ appears to mediate cross-talk between different cell types involved in repair of the liver after severe or recurrent hepatic injury.

References


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