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Etanercept blocks inflammatory responses orchestrated by TNF- α to promote transplanted cell engraftment and proliferation in rat liver

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Abstract

Engraftment of transplanted cells is critical for liver-directed cell therapy but most transplanted cells are rapidly cleared from liver sinusoids by proinflammatory cytokines/chemokines/receptors after activation of neutrophils or Kupffer cells. To define whether TNF- α served roles in cell-transplantation-induced hepatic inflammation, we used TNF- α antagonist, etanercept, for studies in syngeneic rat hepatocyte transplantation systems. After cell transplantation, multiple cytokines/chemokines/receptors were overexpressed, whereas etanercept prior to cell transplantation essentially normalized these responses. Moreover, ETN downregulated cell transplantation-induced intrahepatic release of secretory cytokines, such as high mobility group box 1. These effects of etanercept decreased cell transplantation-induced activation of neutrophils but not of Kupffer cells. Transplanted cell engraftment improved by several-fold in etanercept-treated animals. These gains in cell engraftment were repeatedly realized after pretreatment of animals with etanercept before multiple cell transplantation sessions. Transplanted cell numbers did not change over time indicating absence of cell proliferation after etanercept alone. By contrast, in animals preconditioned with retrorsine and partial hepatectomy, cell transplantation after etanercept pretreatment significantly accelerated liver repopulation compared with control rats. We concluded that TNF- α played a major role in orchestrating cell transplantation-induced inflammation through regulation of multiple cytokines/chemokines/receptor expression. As TNF- α antagonism by etanercept decreased transplanted cell clearance, improved cell engraftment and

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accelerated liver repopulation, this pharmacological approach to control hepatic inflammation will help optimize clinical strategies for liver cell therapy.

Keywords

Cell transplantation; Chemokine; Cytokine; Tumor necrosis factor; Liver repopulation

Introduction

Considerable efforts have been devoted to understanding mechanisms by which liver may be repopulated after cell transplantation. Such liver-directed cell therapy is of major significance for multiple enzymatic or protein deficiency states and other liver conditions (1,2). However, creating an appropriate mass of transplanted cells in the liver remains a hurdle for effective cell therapy, but remains critical for cell therapy outcomes in people (3,4). This accomplishment requires more insights into engraftment and proliferation of transplanted cells in the liver. Many critical steps have been elucidated in the process by which transplanted cells engraft in liver, including necessity for depositing cells in liver sinusoids and integration of transplanted cells in parenchyma before liver repopulation may proceed through survival or proliferation disadvantages to native cells versus transplanted cells (5–9). Nonetheless, the majority (70–80%) of transplanted cells is rapidly lost due to deleterious events in hepatic sinusoids including vasoconstriction with endothelin-1 or other regulators (8,9), and inflammatory chemokines, cytokines or receptors (10,11). The former process, i.e., hepatic ischemia-reperfusion (IR), could assist cell engraftment, e.g., by disrupting liver sinusoidal endothelial cells (LSEC) (12), inhibiting macrophage activation (13), or activating hepatic stellate cells (HSC) (11,14), which promotes cell survival and entry of transplanted cells into liver parenchyma, whereas the latter process, i.e., activation of polymorphonuclear leukocytes (PMN) or Kupffer cells (KC) may expose transplanted cells to inflammatory chemokines/cytokines/receptors, including those capable of recruiting cell types involved in innate immune responses (10). Cell transplantation-induced tissue injury may involve cyclooxygenase pathways and thromboembolic processes related to instant blood-mediated reaction (IBMR) (11,15), thereby offering opportunities for other interventions to improve cell engraftment. Whereas depletion of PMN and KC improved cell engraftment, loss of these important cell types is unsuitable for clinical applications, which is better advanced by discrete drug targets. However, as individual cytokine and chemokine receptors may engage single or multiple ligands, the underlying nature of inflammatory responses in various conditions is generally complex. Nonetheless, harnessing the potential of protective paracrine signaling, e.g., antagonism of cell transplantation-induced cyclooxygenase pathways by naproxen or celecoxib produced release of hepatoprotective paracrine signals from HSC, and improved cell engraftment (11). Therefore, cytokine-specific interventions seemed particularly significant in controlling cell transplantation-induced inflammation for clinical applications.

Here, we focused on tumor necrosis factor (TNF)- α , which serves major roles in inflammation, and is neutralized by well-characterized drugs, e.g., etanercept (ETN) (16), which is a dimeric soluble form of TNF- α receptor, type 2, and interferes with binding of

both TNF- α and - β to cell surface receptors. We considered that if TNF- α drove cell transplantation-induced inflammation, prophylactic ETN should have improved cell engraftment and proliferation. Our studies were facilitated by availability of dipeptidyl peptidase IV-deficient (DPPIV-) rats for assays of transplanted cell engraftment, as well as liver repopulation, e.g., by hepatic preconditioning with the pyrrolizidine alkaloid, retrorsine, plus two-thirds partial hepatectomy (PH) (5–14). Recent delineation of cell types contributing in cell transplantation-induced inflammation, such as LSEC, KC, PMN or HSC (10–14), allowed development of mechanisms underlying ETN-mediated TNF- α antagonism.

Materials and Methods

Drugs and chemicals

D-galactosamine (GalN), nicotine, retrorsine and all reagents were from Sigma Chemical Co. (St. Louis, MO). ETN was from Amgen Inc. (Thousand Oaks, CA). Drugs were dissolved in normal saline for intravenous (iv) injection via tail vein (ETN) or intraperitoneal (ip) injection (GalN, nicotine, retrorsine).

Animals

Protocols were approved by Animal Care and Use Committee at Albert Einstein College of Medicine in compliance with NIH regulations. Donor F344 rats were from National Cancer Institute (Bethesda, MD). DPPIV- F344 rats of 8 to 10 weeks age and 120–200 g weight were from Special Animal Core of Marion Bessin Liver Research Center. Animals were kept with unrestricted access to pelleted food and water in Institute for Animal Studies at Einstein.

Hepatocyte isolation and transplantation

Hepatocytes were isolated by collagenase perfusion of liver (5–14). Cells with viability >80% by trypan blue dye exclusion were transplanted within 2 h from isolation. For cell engraftment studies, 1×10^7 cells suspended in 0.2 milliliters of Roswell Park Memorial Institute 1640 culture medium (RPMI) were injected into spleen over 9 to 12 s. Some rats received cells thrice at 14 d intervals. Cells were transplanted 2 h after nicotine or 16–20 h after ETN. For identifying effective dose of ETN for cell transplantation, we used 1–9 mg/kg ETN with extent of cell engraftment after 4 d as readout of efficacy (n=3 each). For liver repopulation, rats were preconditioned with 30 mg/kg retrorsine at 6 and 8 weeks of age followed 4 weeks later by PH and after another week 5×10^6 cells were transplanted in RPMI intrasplenically. For Kupffer cell (KC) activity, animals were given ^{99m}Tc -sulfur colloid followed by gamma imaging and quantitation of liver activity, or 1 h pulse of 0.1 mL carbon particles intrasplenically, as previously described (13). In some studies, rats were given 70 mg/kg GalN, as described previously (17). Major findings were reproduced by repeat studies.

Cell culture assays

For cytotoxicity assays, 1×10^5 hepatocytes were cultured per cm^2 plastic dishes in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and

antibiotics. Cells were pretreated for 2 h with 200 ng/mL actinomycin D before including 10 ng/mL TNF- α (Serologicals Corp., Atlanta, GA) for 16–18 h and cell viability was analyzed by thiazolyl blue dye (MTT) (8,9). For some studies, cells were incubated in serum-free DMEM or DMEM plus 10 μ g/ml ETN for 1 h on ice, followed by cell culture or transplantation assays. All conditions were in triplicate.

Gene expression analysis

Total RNAs were isolated from 3 replicate liver samples per condition by TRIzol Reagent (Life Sciences, Carlsbad, CA). After DNase treatment, 1 μ g RNAs were converted to cDNA by RT² PCR Array First Strand Kit (SABiosciences, Frederick, MD). Reverse-transcription polymerase chain reactions (RT-PCR) used TNF- α primers (Refseq number, NM_012675; PPR06411F; SABiosciences) or rat cytokine/chemokine/receptor array (PARN 011C; SABiosciences), according to the manufacturer and as previously described (8–11). The array probed for 84 chemokine, cytokine and receptor genes, 5 housekeeping genes, 1 genomic DNA contamination control, 3 RT controls for cDNA conversion, and 3 other positive controls. Array components are at: http://www.sabiosciences.com/rt_pcr_product/HTML/PARN-011A.html. PCR used Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Fold gene expression changes were determined by 2^{-Ct} method to compare experimental and control samples after gene expression was normalized with invariantly expressed housekeeping genes. Gene expression difference 2-fold along with $p < 0.05$ was considered significant.

Tissue staining

Samples from multiple liver lobes per animal were frozen in cold methylbutane at -80°C . Later, 5 μ m cryostat sections were prepared. For KC, sections were fixed in ice-cold ethanol and stained with hematoxylin (13). For neutrophils (PMN), ethanol-fixed sections were stained for myeloperoxidase (MPO) activity, as previously described (8,9). For DPPIV+ transplanted cells, sections were fixed in chloroform-acetone and stained histochemically with toluidine blue counterstain as described previously (5–14).

Morphometry included grading of carbon-containing KC in multiple areas centered on portal vein radicles under $\times 200$ magnification ($n=3$ per condition): grade 1, minimal carbon; grade 2, intermediate carbon; and grade 3, maximal carbon incorporation, as previously described (13). The numbers of MPO+ PMN and transplanted cells were counted in 100 consecutive areas centered on portal radicles per animal under $\times 100$ magnification.

Western blotting for high mobility group box 1 (HMGB1) protein

Serum samples were passed through Centricon100 device (EMD Millipore, Billerica, MA) under $3300\times g$ at 4°C and proteins were resolved by 10% SDS-PAGE followed by transfer to nitrocellulose membranes. The blots were incubated for 3 h with rabbit anti-HMGB1 (1:250, R204, gift of K. Tracey, Feinstein Institute, North Shore-LIJ Health System, NY) in PBS with 0.2% Tween20 and 5% nonfat milk at room temperature. After incubation with peroxidase-conjugated anti-rabbit donkey IgG (NA9340), blots were developed by chemiluminescence (ECL Plus, Amersham Biosciences, GE Healthcare, Port Washington, NY).

Statistical Analysis

Data are shown as means $\pm$ SEM. Significances were analyzed by t-test, Chi-square test or ANOVA with Tukey's test for pairwise comparisons of mean responses. P values <0.05 were considered significant.

Results

Dose-ranging studies with 1, 3, 6, or 9 mg/kg i.v. ETN (n=3 ea) showed transplanted cell engraftment was most improved after 9 mg/kg ETN. The ETN doses were well tolerated with no overt morbidity or mortality. This was in agreement with ETN toxicology documented by Foods and Drugs Administration (Nonclinical Pharmacology and Toxicology Review of BLA 98-0286. <http://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/ucm088697.pdf>). The half-life of ETN in mice, rats and monkeys was 46h to 77h after 1–15 mg/kg dose. No adverse effects were observed in monkeys despite 15 mg/kg ETN twice weekly for 28d. Therefore, we chose 9 mg/kg ETN for subsequent studies.

Analysis of cell engraftment change and other parameters in animals 6 h, 1 d, 2 d, 4 d or 7 d after cell transplantation (n=3–6 each), plus untreated control rats (n=3), was informative (Fig. 1A). Hepatic expression of TNF- α increased after cell transplantation, as RT-PCR showed greater mRNA levels within 6 h, which was 5–20-fold higher than untreated control rats over 48 h (Fig. 1B). Greater TNF- α expression was significant because administration of ETN before cell transplantation improved cell engraftment. In ETN-treated rats, we observed more transplanted cells in portal vein radicles and also liver parenchyma at early as well as later times (Fig. 1C). Morphometry indicated transplanted cell numbers per 100 liver lobules after 1, 2, 4 and 7 days in control rats of 130 ± 9 , 135 ± 13 , 112 ± 8 , and 122 ± 23 . In ETN-treated rats, transplanted cell numbers were greater at all times. Taken together, transplanted cell numbers increased 3.2 ± 0.2 -fold (range 2.7 to 3.6-fold), $p<0.05$, ANOVA (Fig. 1D). This increase in transplanted cell numbers was unchanged over 3 mo without spontaneous proliferation of transplanted cells in these healthy DPPIV- rats (not shown), which was as expected.

To define effects of TNF- α on cell transplantation-induced cytokines/chemokines/receptors, we examined gene expression profiles. In control rats, cell transplantation-induced differences were greatest after 6 h and 25 of 84 (29.8%) genes were 2-fold upregulated, whereas in ETN-treated rats at that time, only 3 of 84 (3.6%) genes were upregulated, $p<0.05$, Chi-square (Fig. 2A). This indicated ETN affected expression of multiple cytokines/chemokines/receptors. Gene expression increased after cell transplantation by median of 6-fold (range, 2–123) in control rats versus median of 225-fold (range, 74–518) in ETN-treated rats. Upregulated genes after cell transplantation belonged mostly in categories of chemokines (9 of 27 array probes) or cytokines (5 of 25 array probes) (Fig. 2B). Remarkably, in ETN-treated rats, expression of upregulated genes became normal, except for IL1r2, TNF- α , and Tnfrsf1a (TNF- α receptor-1), which were expressed at higher levels than after cell transplantation without ETN (Fig. 2C). Therefore, during initial transplanted

cell clearance, expression of multiple cytokines/chemokines/receptors was orchestrated by TNF- α .

As activated PMN and KC express or otherwise regulate many chemokines and cytokines (10), we examined whether ETN altered activation of these cell types. Cell transplantation resulted after 6 h in appearance of 20 ± 1 MPO+ PMN per periportal area versus 2.0 ± 0.2 MPO+ PMN in untreated controls, a 10-fold increase, $p < 0.05$ (Fig. 3A). By contrast, cell transplantation in ETN-treated rats produced 16 ± 2 MPO+ PMN per periportal area, which was less than after cell transplantation alone, 8-fold versus 10-fold $p < 0.05$, and indicated ETN had some inhibitory effect on activation of PMN. The number of KC with carbon uptake increased from 54 ± 8 per periportal area in normal controls to 125 ± 17 per periportal area after cell transplantation, 2.3-fold increase, $p < 0.05$. Moreover, phagocytotic activity was of higher grade (Fig. 3B). However, in ETN-treated rats, cell transplantation-induced KC activation was unchanged, since 110 ± 25 activated KC per periportal area were observed, 2-fold increase versus normal controls, $p < 0.05$. Quantitative analysis of ^{99m}Tc -sulfur colloid uptake by gamma imaging confirmed ETN did not alter cell transplantation-induced KC activity (not shown). Therefore, ETN likely exerted its effects largely by TNF- α -mediated expression of cytokines/chemokines/receptors within liver, as indicated by mRNA expression analysis, possibly by recruiting circulating cells from blood, e.g., PMN, and likely by regulating secreted signals from cell types.

To examine contribution of mediators released after cell transplantation by activated PMN or KC, we focused on secreted HMGB1, which may be released by inflammatory cells as well as damaged or necrotic hepatocytes, and potentiates TNF- α -related liver injury (18). Interestingly, HMGB1 responsiveness was found to be controlled by parasympathetic nervous system via nicotinic cholinergic receptors regulated by vagal nerve (19). We found by western blots that HMGB1 blood levels increased shortly after cell transplantation (Fig. 3C), although HMGB1 blood levels increased more in animals with GalN-induced liver necrosis, which was of course much greater than liver injury with cell transplantation (not shown). Remarkably, HMGB1 was not detected in blood when cells were transplanted after ETN, indicating TNF- α regulated release of this cytokine from cells. To determine whether HMGB1 release was controlled locally, i.e., by intrahepatic TNF- α , or systemically at the level of parasympathetic nervous system, we treated animals with nicotine before cell transplantation. In case of local control, cell engraftment should not have changed after nicotine, whereas in case of systemic control, cell engraftment should have improved after nicotine. In nicotine-pretreated animals, cell engraftment neither improved nor worsened (Fig. 3D), indicating ETN improved cell engraftment through effects on intrahepatic TNF- α and not secondarily via cytokines regulated by additional controls, such as HMGB1.

Next, to determine whether ETN could improve cell engraftment on repeated occasions, we studied animals with cells transplanted thrice, each time with or without ETN (Fig. 4A). Significantly more cells engrafted after three versus one session of cell transplantation (Fig. 4B). In control animals, where ETN was not given, we observed 140 ± 30 transplanted cells per 100 liver lobules after one cell transplantation session and 510 ± 48 cells per 100 liver lobules after three cell transplantation sessions, which was 3.6 ± 0.3 -fold increase, $p < 0.05$, *t*-test. In ETN-treated rats, after three cell transplantation sessions, we observed 1538 ± 96

transplanted cells per 100 liver lobules, which was 11 ± 0.7 - or 3 ± 0.2 -fold greater than control rats receiving cells either once or thrice, respectively, $p<0.05$, ANOVA (Fig. 4C). When only one dose of ETN was given followed by transplantation of cells thrice, cell engraftment improved much less (not shown). Therefore, cell engraftment improved successively when ETN was given before each cell transplantation session.

To demonstrate whether ETN would also increase liver repopulation kinetics, we used retrorsine-PH-preconditioned rats (Fig. 5A). Significant liver repopulation was seen in control rats and this further increased in ETN-treated rats (Fig. 5B). Morphometry showed 1 mo and 3 mo after cell transplantation $15\pm5\%$ and $35\pm15\%$ of liver was replaced in control rats and $45\pm5\%$ and $90\pm6\%$ of liver was replaced in ETN-treated rats, respectively, $p<0.05$, ANOVA (Fig. 5C).

Finally, we determined whether cells modified by incubation with ETN in vitro will be protected from cytotoxicity. Primary hepatocytes were susceptible to TNF- α cytotoxicity in culture conditions with viability declining to $70\pm2\%$ of untreated controls (Fig. 6A). Incubation of cells for 1 h with as little as $0.1\text{ }\mu\text{g/ml}$ ETN abolished this TNF- α cytotoxicity (Fig. 6B). However, transplantation of hepatocytes incubated with $10\text{ }\mu\text{g/ml}$ ETN did not improve cell engraftment (Fig. 6C). We found 90 ± 10 and 98 ± 20 transplanted cells in recipients of control cells and ETN-treated cells, respectively, $p>0.05$ (Fig. 6D). Therefore, we did not pursue liver repopulation studies with ETN-incubated cells.

Discussion

This work established that TNF- α orchestrated cell transplantation-induced expression of cytokines/chemokines/receptors since these events were inhibited by pretreatment with ETN, that cytokines/chemokines/receptors were responsible for clearing transplanted cells since cell engraftment improved >3-fold after ETN, and that liver repopulation was accelerated after inhibition by ETN of cytokines/chemokines/receptor expression. Similarly, cell transplantation in other vascular beds, e.g., coronary vessels, included cytokine/chemokine/receptor responses (20), indicating our results are of broader relevance. Whether TNF- α will have central roles in clearance of transplanted stem/progenitor cells or other cell types of interest will require further studies.

Transplantation of cells in liver sinusoids reproduces some events of IR injury, since temporary occlusion of sinusoidal blood flow by transplanted cells produces hepatic ischemia, while signaling between transplanted and native cells produces different types of events. These changes resulting from cell transplantation differed from those produced by biologically inert particles, e.g., macroaggregated albumin (MAA) of sizes similar to cells (21). For instance, despite sharing IR events to some extent, cytokine/chemokine/receptor responses differed after injection of MAA or cell transplantation in liver sinusoids (10,21). It was understandable that cell transplantation-induced hepatic inflammation was specifically related to mediators emanating from cell types involved in innate immunity, i.e., PMN and KC or monocytes, but not lymphocytes, NK cells or dendritic cells in the setting of syngeneic cell transplantation (10). Of course, allogeneic or xenogeneic cell transplants activate adaptive immune responses (22). After cell transplantation, injury to hepatocytes,

LSEC and HSC cause additional changes, e.g., induction of cyclooxygenase pathways, which are deleterious and interfere with cell engraftment, or expression of substances, e.g., VEGF or HGF, which are cytoprotective and assist in cell engraftment (11). These inflammatory mechanisms yielded pharmacological approaches, i.e., naproxen and celecoxib, which improved cell engraftment through cytoprotective signaling from HSC and surprisingly not by dampening cell transplantation-induced inflammation (11,23). Therefore, efficacy of TNF- α antagonism by ETN, which could synergize with other interventions, should be of great interest for cell therapy. Moreover, this mechanism of TNF- α antagonism in the cell transplantation setting will be applicable to additional drug development. We obtained similar gains in engraftment of transplanted rat hepatocytes in DPPIV- rats that had been pretreated with Infliximab, which is a chimeric monoclonal antibody against TNF- α , and is widely used in clinical medicine (not shown). These studies used a rat version of Infliximab kindly provided by Centocor Biotech. Inc., which eventually merged with other commercial entities.

Evidences in support of cell transplantation-induced PMN and KC activation included expression of chemokines, e.g., CCL3 (ligand for CCR1 and CCR5), CCL4 (ligand for CCR5), CXCL1 and CXCL2 (ligands for CXCR1/2) (24,25). Among these, CXCL1 and CXCL2 attract PMN, CCL3, monocytes, and, CXCL4, both PMN and monocytes, to sites of inflammation (26–29). Other chemokines, e.g., CCL17, CCL22, CXCL9 or CXCL10, which may be released by macrophages or endothelial cells also amplify inflammation (30,31). Morphological evidences of MPO+ PMN and carbon-containing KC further verified these cell types regulated cell transplantation-induced hepatic inflammation. Similarly, involvement of PMN and KC in IR injury through TNF- α or IL-6 is well accepted (32). After cell transplantation, receptors, such as Tnfrsf1b, Ltb and ABCF1, are associated with TNF- α -induced inflammation (33,34). Previously, this TNF- α group of genes was normalized when cells were transplanted after depletion of PMN by a polyclonal antibody but not after depletion of KC by gadolinium chloride (10). Our results in ETN-treated rats showing less PMN activation versus unchanged KC activation reinforced the possibility that PMN served early roles in cell transplantation-induced inflammation. This was in agreement with induction by PMN of TNF- α or IL6 release from macrophages (35). As cell transplantation-induced cytokine/chemokine/receptor expression was normalized in ETN-treated rats, this confirmed role of TNF- α in regulating gene expression, and was in agreement with previous studies showing that TNF- α may induce chemokine expression in parenchymal cells either by itself, e.g., regulation of CCL3, CCL4 or CCR1/2 (36), or in concert with other cytokines, such as IL4 or interferon, e.g., regulation of CCL9, CCL10, CCL17 or CCL22 (30,31).

The paradigm of TNF- α and HMGB1 regulation re-illustrated the importance of TNF- α in cell transplantation-induced inflammation. Recently, considerable information has accumulated about the role of HMGB1 in IR and other liver injuries (18). Rapid rise in serum HMGB1 after cell transplantation was in agreement with liver injury, perhaps destruction of less viable transplanted cells, and TNF- α -HMGB1 interaction. Here, normalization of serum HMGB1 levels in ETN-treated rats reiterated central role of TNF- α . This TNF- α effect was at intrahepatic level and not at systemic or central neural level

involving cholinergic stimulation of TNF- α release from macrophages, as during sepsis in animals and humans (19).

The plethora of cytokine/chemokine/receptor expression in inflammatory states often poses difficulties in dissecting governing roles of individual molecules. Because drug targeting of cytokines/chemokines/receptors was of therapeutic value for several conditions, including tissue or organ transplantation (37), such insights are relevant for clinical medicine. Under allograft setting, acquired immune mechanisms involving lymphocytes and related cells serve additional roles but innate immune responses related to other cell types, including neutrophils and macrophages, remain important in transplanted cell clearances. Therefore, role of TNF- α in orchestrating cell transplantation-induced chemokine/cytokine/receptor responses should be exciting. As ETN acts by neutralizing soluble form of p75 Tnfrsf1b (or TNF- α receptor type 2) but not p55 Tnfrsf1a (or TNF- α receptor type 1), this likely explained greater upregulation of TNF- α and Tnfrsf1a after cell transplantation in ETN-treated rats. Previously, in ETN-responsive arthritis or hepatic acute phase response, expression of multiple chemokines was also normalized (38,39). Moreover, role of PMN in contributing to benefits of ETN was recognized, which will be in agreement with our results showing some decreases in MPO+ PMN in ETN-treated rats. As ETN may modulate leukocyte migration, e.g., by downregulating cell adhesion molecules, such as integrins, selectins, etc., as well as other cytokines, e.g., IL-6, this likely helped in cell engraftment (40).

Another aspect of TNF- α antagonism by ETN was improvement in cell engraftment on repeated occasions. Previously, repeated cell transplantation provided greater liver repopulation, e.g., after hepatocyte transplantation in large numbers, nearly 10% of the liver was repopulated (7). Moreover, ETN did not interfere with liver repopulation, whereas drugs, e.g., rapamycin, the mTOR activator, or bosentan, blocker of endothelin-1 receptors, type A and B, increased cell engraftment but inhibited cell proliferation and liver repopulation (8,22). This should help further investigation of the role of TNF- α in liver regeneration or cytoprotection since animal studies incriminated TNF- α in hepatocyte proliferation and hepatic preconditioning (41). On the other hand, blockade of endothelin receptors by bosentan before transplantation provided cytoprotection and improved cell engraftment as well as proliferation (8). We considered ETN could similarly be helpful, including because TNF- α may be released from cells under settings of cytotoxicity (42), but incubating cells with ETN before cell transplantation was ineffective. Nevertheless, TNF- α antagonism by ETN offers important cell therapy strategies, either by itself or in conjunction with additional drugs to block deleterious processes interfering with cell engraftment and/or proliferation. As cell therapy will require single or only few doses of ETN depending on cell transplantation sessions required, this should be accomplished relatively simply and safely. Parallel advances in methods to accomplish liver repopulation with cells and to track transplanted cells in people will be helpful.

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References

1. Viswanathan P, Gupta S. New directions for cell-based therapies in acute liver failure. *J Hepatol.* 2012; 57:913–915. [PubMed: 22709618]
2. Vogel KR, Kennedy AA, Whitehouse LA, Gibson KM. Therapeutic hepatocyte transplant for inherited metabolic disorders: functional considerations, recent outcomes and future prospects. *J Inherit Metab Dis.* 2014; 37:165–176. [PubMed: 24085555]
3. Huebert RC, Rakela J. Cellular therapy for liver disease. *Mayo Clin Proc.* 2014; 89:414–424. [PubMed: 24582199]
4. Filippi C, Dhawan A. Current status of human hepatocyte transplantation and its potential for Wilson's disease. *Ann N Y Acad Sci.* 2014 Mar 7. [Epub ahead of print].
5. Rajvanshi P, Kerr A, Bhargava KK, Burk RD, Gupta S. Efficacy and safety of repeated hepatocyte transplantation for significant liver repopulation in rodents. *Gastroenterology.* 1996; 111:1092–1102. [PubMed: 8831605]
6. Gupta S, Rajvanshi P, Sokhi RP, Slehra S, Yam A, Kerr A, Novikoff PM. Entry and integration of transplanted hepatocytes in liver plates occur by disruption of hepatic sinusoidal endothelium. *Hepatology.* 1999; 29:509–519. [PubMed: 9918929]
7. Wu YM, Joseph B, Berishvili E, Kumaran V, Gupta S. Hepatocyte transplantation and drug-induced perturbations in liver cell compartments. *Hepatology.* 2008; 47:279–287. [PubMed: 17935178]
8. Bahde R, Kapoor S, Bandi S, Bhargava K, Palestro C, Gupta S. Directly acting drugs prostacyclin or nitroglycerine and endothelin receptor blocker bosentan improve cell engraftment in rat liver. *Hepatology.* 2013; 57:320–330. [PubMed: 22899584]
9. Bahde R, Kapoor S, Viswanathan P, Spiegel HU, Gupta S. Endothelin-1 receptor A blocker darusentan decreases cell transplantation-induced hepatic perturbations and improves liver repopulation. *Hepatology.* 2013 Oct 1. [Epub ahead of print].
10. Krohn N, Kapoor S, Enami Y, Follenzi A, Bandi S, Joseph B, Gupta S. Hepatocyte transplantation-induced liver inflammation is driven by cytokines-chemokines associated with neutrophils and Kupffer cells. *Gastroenterology.* 2009; 136:1806–1817. [PubMed: 19422086]
11. Enami Y, Bandi S, Kapoor S, Krohn N, Joseph B, Gupta S. Hepatic stellate cells promote hepatocyte engraftment in rat liver after prostaglandin-endoperoxide synthase inhibition. *Gastroenterology.* 2009; 136:2356–2364. [PubMed: 19303017]
12. Joseph B, Kumaran V, Berishvili E, Bhargava KK, Palestro CJ, Gupta S. Monocrotaline promotes transplanted cell engraftment and advances liver repopulation in rats via liver conditioning. *Hepatology.* 2006; 44:1411–1420. [PubMed: 17133480]
13. Joseph B, Malhi H, Bhargava KK, Palestro CJ, McCuskey RS, Gupta S. Kupffer cells participate in early clearance of syngeneic hepatocytes transplanted in the rat liver. *Gastroenterology.* 2002; 123:1677–1685. [PubMed: 12404242]
14. Benten D, Kumaran V, Joseph B, Gupta S. Hepatocyte transplantation activates hepatic stellate cells with beneficial modulation of engraftment. *Hepatology.* 2005; 42:1072–1081. [PubMed: 16250034]
15. Lee JH, Cho JH, Yeo J, Lee SH, Yang SH, Sung YC, Kang JH, Park CS. The pharmacology study of a new recombinant TNF receptor-hyFc fusion protein. *Biologicals.* 2013; 41:77–83. [PubMed: 23190454]
16. Gustafson EK, Elgue G, Hughes RD, Mitry RR, Sanchez J, Haglund U, Meurling S, Dhawan A, Korsgren O, Nilsson B. The instant blood-mediated inflammatory reaction characterized in hepatocyte transplantation. *Transplantation.* 2011; 91:632–638. [PubMed: 21289595]
17. Gupta S, Rajvanshi P, Irani AN, Palestro CJ, Bhargava KK. Integration and proliferation of transplanted cells in hepatic parenchyma following D-galactosamine-induced acute injury in F344 rats. *J Pathol.* 2000; 190:203–210. [PubMed: 10657020]
18. Chen R, Hou W, Zhang Q, Kang R, Fan XG, Tang D. The emerging role of HMGB1 in liver diseases. *Mol Med.* 2013; 19:357–366. [PubMed: 24306421]
19. Wang H, Liao H, Ochani M, Justiniani M, Lin X, Yang L, Al-Abed Y, Wang H, Metz C, Miller EJ, Tracey KJ, Ulloa L. Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. *Nat Med.* 2004; 10:1216–1221. [PubMed: 15502843]

20. Solheim S, Seljeflot I, Lunde K, Aukrust P, Yndestad A, Grøgaard HK, Aakhus S, Forfang K, Arnesen H. Inflammatory responses after intracoronary injection of autologous mononuclear bone marrow cells in patients with acute myocardial infarction. *Am Heart J*. 2008; 155:55.e1–55.e9. [PubMed: 18082489]
21. Gupta S, Rajvanshi P, Malhi H, Sokhi RP, Slehria S, Vasa SRG, Dabeva M, Shafritz DA, Kerr A. Cell transplantation causes loss of gap junctions and activates GGT expression permanently in host liver. *Am J Physiol Gastroint Liver Physiol*. 2000; 279:G815–G826.
22. Wu YM, Joseph B, Gupta S. Immunosuppression using the mTOR inhibition mechanism affects replacement of the rat liver with transplanted cells. *Hepatology*. 2006; 44:410–419. [PubMed: 16871590]
23. Bahde R, Kapoor S, Gupta S. Nonselective inhibition of prostaglandin-endoperoxide synthases by naproxen ameliorates acute or chronic liver injury in animals. *Exp Mol Pathol*. 2014; 96:27–35. [PubMed: 24220607]
24. Moreno C, Gustot T, Nicaise C, Quertinmont E, Nagy N, Parmentier M, Le Moine O, Devière J, Louis H. CCR5 deficiency exacerbates T-cell-mediated hepatitis in mice. *Hepatology*. 2005; 42:854–862. [PubMed: 16175603]
25. Li X, Klintman D, Liu Q, Sato T, Jeppsson B, Thorlacius H. Critical role of CXC chemokines in endotoxemic liver injury in mice. *J Leukoc Biol*. 2004; 75:443–452. [PubMed: 14673016]
26. Rittner HL, Mousa SA, Labuz D, Beschmann K, Schäfer M, Stein C, Brack A. Selective local PMN recruitment by CXCL1 or CXCL2/3 injection does not cause inflammatory pain. *J Leukoc Biol*. 2006; 79:1022–1032. [PubMed: 16522746]
27. Gaga M, Ong YE, Benyahia F, Aizen M, Barkans J, Kay AB. Skin reactivity and local cell recruitment in human atopic and nonatopic subjects by CCL2/MCP-1 and CCL3/MIP-1alpha. *Allergy*. 2008; 63:703–711. [PubMed: 18070228]
28. Xiao Z, Visentin GP, Dayananda KM, Neelamegham S. Immune complexes formed following the binding of anti-platelet factor 4 (CXCL4) antibodies to CXCL4 stimulate human neutrophil activation and cell adhesion. *Blood*. 2008; 112:1091–1100. [PubMed: 18539895]
29. Mueller A, Meiser A, McDonagh EM, Fox JM, Petit SJ, Xanthou G, Williams TJ, Pease JE. CXCL4-induced migration of activated T lymphocytes is mediated by the chemokine receptor CXCR3. *J Leukoc Biol*. 2008; 83:875–882. [PubMed: 18174362]
30. Liu LY, Bates ME, Jarjour NN, Busse WW, Bertics PJ, Kelly EA. Generation of Th1 and Th2 chemokines by human eosinophils: evidence for a critical role of TNF-alpha. *J Immunol*. 2007; 179:4840–4848. [PubMed: 17878383]
31. Lombardi A, Cantini G, Piscitelli E, Gelmini S, Francalanci M, Mello T, Ceni E, Varano G, Forti G, Rotondi M, Galli A, Serio M, Luconi M. A new mechanism involving ERK contributes to rosiglitazone inhibition of tumor necrosis factor-alpha and interferon-gamma inflammatory effects in human endothelial cells. *Arterioscler Thromb Vasc Biol*. 2008; 28:718–724. [PubMed: 18239149]
32. Kuboki S, Shin T, Huber N, Eismann T, Galloway E, Schuster R, Blanchard J, Edwards MJ, Lentsch AB. Hepatocyte signaling through CXC chemokine receptor-2 is detrimental to liver recovery after ischemia/reperfusion in mice. *Hepatology*. 2008; 48:1213–1223. [PubMed: 18688883]
33. Takasaki J, Kawauchi Y, Masuho Y. Synergistic effect of type II phospholipase A2 and platelet-activating factor on Mac-1 surface expression and exocytosis of gelatinase granules in human neutrophils: evidence for the 5-lipoxygenase-dependent mechanism. *J Immunol*. 1998; 160:5066–5072. [PubMed: 9590257]
34. Richard M, Drouin R, Beaulieu AD. ABC50, a novel human ATP-binding cassette protein found in tumor necrosis factor-alpha-stimulated synoviocytes. *Genomics*. 1998; 53:137–145. [PubMed: 9790762]
35. Wilsson A, Lind S, Ohman L, Nilsson-Augustinsson A, Lundqvist-Setterud H. Apoptotic neutrophils containing *Staphylococcus epidermidis* stimulate macrophages to release the proinflammatory cytokines tumor necrosis factor-alpha and interleukin-6. *FEMS Immunol Med Microbiol*. 2008; 53:126–135. [PubMed: 18400011]

36. Glabinski AR, Bielecki B, Kolodziejewski P, Han Y, Selmaj K, Ransohoff RM. TNF- α microinjection upregulates chemokines and chemokine receptors in the central nervous system without inducing leukocyte infiltration. *J Interferon Cytokine Res.* 2003; 23:457–466. [PubMed: 13678434]
37. Schnickel GT, Bastani S, Hsieh GR, Shefizadeh A, Bhatia R, Fishbein MC, Belperio J, Ardehali A. Combined CXCR3/CCR5 blockade attenuates acute and chronic rejection. *J Immunol.* 2008; 180:4714–4721. [PubMed: 18354195]
38. Campbell SJ, Jiang Y, Davis AE, Farrands R, Holbrook J, Leppert D, Anthony DC. Immunomodulatory effects of etanercept in a model of brain injury act through attenuation of the acute-phase response. *J Neurochem.* 2007; 103:2245–2255. [PubMed: 17883399]
39. Schmidt EM, Davies M, Mistry P, Green P, Giddins G, Feldmann M, Stoop AA, Brennan FM. Selective blockade of tumor necrosis factor receptor I inhibits proinflammatory cytokine and chemokine production in human rheumatoid arthritis synovial membrane cell cultures. *Arthritis Rheum.* 2013; 65:2262–2273. [PubMed: 23784528]
40. Chandrasekharan UM, Siemionow M, Unsal M, Yang L, Poptic E, Bohn J, Ozer K, Zhou Z, Howe PH, Penn M, DiCorleto PE. Tumor necrosis factor alpha (TNF- α) receptor-II is required for TNF- α -induced leukocyte-endothelial interaction in vivo. *Blood.* 2007; 109:1938–1944. [PubMed: 17068152]
41. Teoh N, Leclercq I, Pena AD, Farrell G. Low-dose TNF- α protects against hepatic ischemia-reperfusion injury in mice: implications for preconditioning. *Hepatology.* 2003; 37:118–128. [PubMed: 12500196]
42. Biton S, Ashkenazi A. NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF- α feed forward signaling. *Cell.* 2011; 145:92–103. [PubMed: 21458669]

Figure 1. Effect of ETN on transplanted cell engraftment

(A) Protocol for analyzing gene expression, cell engraftment and tissue changes in rats after cell transplantation alone or cell transplantation after treatment of rats with ETN or vehicle. Studies used 3–6 rats per time-point. (B) RT-PCR showing cell transplantation rapidly and persistently induced hepatic expression of TNF. Lane 1, untreated control liver sample and lanes 2–4, liver samples 6, 24 and 48 h after cell transplantation. (C) Representative liver sections showing DPPIV+ transplanted hepatocytes (red color) 1 d or 7 d after cell transplantation in controls and ETN-treated rats. Orig. mag., $\times 100$. (D) Morphometric data showed cell engraftment was superior in ETN-treated rats at all times.

Figure 2. Differences in hepatic expression of chemokines/cytokines/receptors after cell transplantation

(A) Graphic depiction indicating 25 of 84 genes were upregulated after cell transplantation versus 3 of 84 genes in animals after ETN and cell transplantation. (B) Distribution of upregulated genes in ontology groups. (C) Differences in normalized expression against housekeeping genes of cytokines/chemokines/receptors after cell transplantation with or without prior ETN. Genes indicated in bold letters were upregulated only in ETN-treated rats after cell transplantation.

Figure 3. Cell transplantation-induced activation of inflammatory cells and soluble cytokine expression

(A) Cell transplantation increased number of MPO+ PMN compared with untreated control rats but this increase in MPO+ PMN was less in ETN-treated rats. (B) KC showed greater carbon uptake after cell transplantation. This was unchanged in ETN-treated rats. (C) Western blot for serum HMGB1 showed increased expression after GalN and also cell transplantation. In ETN-treated rats, serum HMGB1 was not detected. Lane 1, HMGB1 protein; lanes 2–4, animals 5 d after GalN as positive controls; lanes 5–10, replicate animals 6, 24 and 48 h after cell transplantation; lanes 11–12, replicate ETN-treated animals 24 h after cell transplantation with no HMGB1. (D) Morphometric data showing cell engraftment did not increase in nicotine-treated animals (n=3 ea).

Figure 4. ETN and repeated cell transplantation

(A) Protocol for administering ETN each time before 3 cell transplantation thrice at intervals followed by cell engraftment analysis in 3–6 rats after 38 d. (B) Representative tissue examples showing cell engraftment after 3 sessions of cell transplantation in control rat and ETN-treated rat with the latter showing more DPPIV+ transplanted cells (red color). (C) Morphometric analysis showing significant differences in number of transplanted cells in animals after only one session of cell transplantation versus after 3 sessions of cell transplantation with or without prior ETN treatments.

Figure 5. Effect of ETN on kinetics of liver repopulation

(A) Protocol indicating use of retrorsine and PH preconditioning for liver repopulation analysis. Animals were given cells followed by analysis of liver repopulation after 1 mo and 3 mo. (B) Tissue staining for DPPIV showing extent of liver repopulation in control rat and ETN-treated rat after 3 mo. (C) Morphometric data indicating liver repopulation was significantly greater after 1 mo as well as 3 mo in ETN-treated rats.

Figure 6. Antagonism of TNF in cultured primary rat hepatocytes

(A) Shows protocols for studies of cells including cytotoxicity assays in the short-term in vitro as well as transplantation of ETN-treated cells for engraftment analysis after 1 week. (B) Chart indicates abrogation of TNF cytotoxicity when cells were cultured after incubation for 1 h with ETN. (C) Engraftment of control DPPIV+ transplanted cells and those cells treated with ETN in vitro. (D) Morphometric analysis of cell engraftment indicating no benefit of ETN treatment of cells in vitro.