THE ROLE OF BILIARY EPITHELIAL CELLS IN THE IMMUNOPATHOGENESIS OF NON-SUPPURATIVE DESTRUCTIVE CHOLANGITIS IN MURINE HEPATIC GRAFT-VERSUS-HOST DISEASE

JOHN M. VIERLING, MD, FACP, and (*by invitation*) GABRIELLA HREHA, PhD, HAIMEI WANG, DDS, and MARIUS BRAUN, MD

HOUSTON, TEXAS

ABSTRACT

Non-suppurative destructive cholangitis (NSDC) is characterized by T-cell infiltration of the biliary epithelia of small-to medium-caliber bile ducts, causing apoptosis of biliary epithelial cells (BEC) and, ultimately, ductopenia. NSDC is the primary histopathologic process in the autoimmune disease known as primary biliary cirrhosis (PBC) and in alloimmune graft-versus-host disease (GVHD) and hepatic allograft rejection. The onset of NSDC in the B10.D2→BALB/c murine model of hepatic GVHD is preceded by hepatic production of pro-inflammatory cytokines, accumulation of lipopolysaccharide (LPS), and expression of chemokine genes. To explain the curious restriction of NSDC to small- and medium-caliber intrahepatic bile ducts, we hypothesized that BEC lining these bile ducts secrete chemokines and cytokines that chemoattract, activate, and polarize the effector T cells mediating NSDC. To test this hypothesis we stimulated BALB/c immortalized BEC (IBEC) *in vitro* with pro-inflammatory mouse recombinant cytokines with and without LPS and determined the expression of chemokines and cytokines by IBEC using a polymerase chain reaction (PCR), quantitative protein enzyme-linked immunosorbent assays (ELISAs), and microarrays. The capacity of stimulated IBEC to chemoattract activated T cells was assessed in the presence and absence of inhibitors of specific chemokine receptors. We found that pro-inflammatory cytokines, especially the combination of IFNγ and TNFα, induced IBEC gene expression and the secretion of chemokine ligands for the chemokine receptors CCR1, CCR3, CCR5, and CXCR3. Chemokines secreted by IBEC stimulated with IFNγ plus TNFα chemoattracted activated T cells. Inhibition of CCR1, CCR3, CCR5, or CXCR3 significantly reduced the chemoattraction of activated T cells. We conclude that BEC probably play an active role in the immunopathogenesis of NSDC by mediating the chemoattraction and terminal activation of effector T cells

Correspondence and reprint requests: Dr. John M. Vierling, 1709 Dryden, Suite 1500, Houston, TX 77030, Tel: 713-798-8355, Fax: 713-798-8319, E-mail: vierling@bcm.tmc.edu

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responsible for apoptosis of BECs and ductopenia. Selective chemokine expression by BEC lining small- and medium-caliber bile ducts could explain the restriction of NSDC to ducts of this caliber. Inhibition of CCR1, CCR3, CCR5, and CXCR3 to block the chemoattraction and terminal activation of alloreactive T cells represents a potential therapeutic strategy for preventing NSDC after hematopoietic stem-cell transplantation or orthotopic liver transplantation.

Non-suppurative destructive cholangitis (NSDC) is a histopathologic process characterized by: 1) T-cell infiltration of the biliary epithelia restricted to small- and medium-caliber intra-hepatic bile ducts; 2) variation in the extent of intra-epithelial T-cell infiltration along the course of individual bile ducts; 3) patchy apoptosis of biliary epithellia cells (BEC, also known as cholangiocytes); and 4) progressive destruction of bile ducts, resulting in ductopenia. First described in primary biliary cirrhosis (PBC), NSDC (Figure 1) was later identified as the primary histopathologic process in human and experimental hepatic graft-versus-host disease (GVHD) and hepatic allograft rejection (HAR) (1–3). Thus, understanding the immunopathogenesis of NSDC could provide important insights for therapeutic advances in each of these diseases.

We and others have studied the immunopathogenesis of NSDC in the B10.D2 (H-2^d, $Mls^{1b,2b}$)→BALB/c (H-2^d, $Mls^{1b,2a}$) murine model of GVHD, in which undefined minor differences in histocompatibility induce GVHD result in reproducible NSDC and progressive ductopenia (4, 5). To conduct *in vitro* studies of the BALB/c BEC targets of NSDC

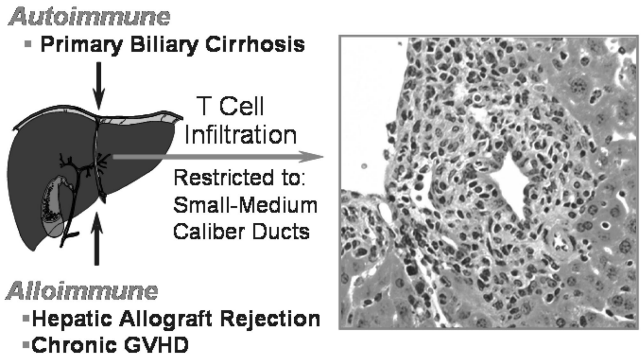

FIG. 1. Non-suppurative destructive cholangitis (NSDC) as the primary histopathologic process in the autoimmune disease primary biliary cirrhosis and in the alloimmune diseases chronic graft-versus-host disease (GVHD) and hepatic allograft rejection. Photomicrograph shows NSDC lesion in a BALB/c mouse recipient of a B10.D2 graft on day 7. Note infiltration of the biliary epithelium by lymphocytes.

at the cellular level, we developed SV40-transformed immortalized BEC (IBEC) from the BALB/c target strain, which exhibit characteristics of native BEC lining small- and medium-caliber bile ducts (6).

A curious feature of NSDC in PBC and of the alloimmune processes of GVHD and HAR is the restriction of the T-cell-mediated injury to BEC lining the small- and medium-caliber intrahepatic bile ducts, with sparing of the BEC lining larger-caliber intrahepatic and extrahepatic bile ducts as well as hepatocytes, liver sinusoidal endothelial cells, and tight-junction endothelial cells, despite their expression of identical allogeneic major histocompatibility complex (MHC) molecules (1).

Because bile ducts, hepatic arteries, and branches of the portal vein lie within the connective-tissue sleeve known as the portal tract, the selective inflammation of small- to medium-caliber bile ducts requires the trans-endothelial migration of alloactivated T cells from the portal veins or arterial capillaries into the portal tracts, and their subsequent trafficking to the biliary epithelia (7). Both transendothelial T-cell migration and tissue trafficking of the T cells to target tissues involve the recognition of chemoattractant cytokines, named chemokines, by chemokine receptors expressed on the activated T cells. Chemokines not only chemoattract activated T cells but also stimulate the terminal activation of effector-cell functions. Although chemokines are produced actively by mononuclear inflammatory cells in sites of inflammation, evidence indicates that BEC may also secrete chemokines and express chemokine receptors (8–10).

HYPOTHESIS

To explain the restriction of T-cell-mediated injury in NSDC, we hypothesized that pro-inflammatory cytokines and/or lipopolysaccharide (LPS) present in portal venous blood induce differential gene expression by BEC lining the small- and medium-caliber ducts within the liver, causing them to secrete chemokines that chemoattract and terminally activate the effector T cells that mediate NSDC.

EXPRESSION OF CHEMOKINE GENES BY BILIARY EPITHELIAL CELLS

To study our hypothesis, we used the B10.D2 (H-2^{d}, $Mls^{1b,2b}$)→BALB/c (H-2^{d}, $Mls^{1b,2a}$) murine model of GVHD (4). In this model, grafts containing spleen cells and bone-marrow cells from mice of the B10.D2 strain are injected intravenously into BALB/c mice that have been sublethally irradiated (600 rad, using ^{67}Cs as the radiation source) to prevent destruction of the B10.D2 graft by the BALB/c host-mouse strain. Controls

consisted of sublethally irradiated syngeneic BALB/c host mice injected with identical grafts from BALB/c mice. This controls for the effects of handling, irradiation, and graft injection. This model is characterized by reproducible kinetics of NSDC on days 7 and 14, preceded on day 4 by portal inflammation and production of interferon-gamma (IFNγ), tumor necrosis factor-alpha (TNFα), interleukin-1beta (IL-1β), and interleukin-6 (IL-6), and by accumulation of LPS within the liver. To study the BALB/c BEC targets of NSDC *in vitro,* we used previously described SV40-transformed IBEC (8).

Using a reverse transcriptase-polymerase chain reaction (RT-PCR) and RNase protection assays, we first demonstrated expression of mRNA for the chemokine ligands of the chemokine receptors CCR1, CCR3, CCR5, and CXCR3 in inflamed portal connective-tissue trees containing only small- or medium-caliber bile ducts obtained from mice with hepatic GVHD on days 7 and 14 (Figures 2A and 2B). Expression of mRNA for these chemokine ligands was absent in portal connective tissue trees obtained from syngeneic control mice (Figure 2A and 2B).

SV40-transformed IBEC were next used to study cytokine and LPS induction of chemokine expression *in vitro.* IBEC from the BALB/c host (target) strain of mice in the GVHD model were co-cultured with LPS or the mouse recombinant (r) cytokines, rIFNγ, rTNFα, rIL-1β, and rIL-6, levels of which are known to be elevated in the liver before the onset of NSDC *in vivo.* LPS induced the expression of mRNA for the chemokine MCP-1, and smaller levels of mRNA for the chemokine CCL5 (regulated on activation, normal T-cell expressed and secreted [RANTES]). An identical pattern of mRNA induction was observed for IL-1β. As expected, neither LPS nor IL-1β induced the expression of mRNA for interferon-inducible chemokine ligands of CXCR3 receptors. In contrast, mouse rIFNγ did induce expression of mRNA for the IFN-inducible chemokines CCL9 (MIG), CCL10 (IP-10), and CCL11 (I-TAC), which bind to CXCR3. In addition, rIFNγ induced small amounts of mRNA for CCL5 (RANTES) and for MCP-1. The combination of rIFNγ and rTNFα induced the expression of similar amounts of mRNA for the CXCR3 chemokine ligands, but resulted in a substantial increase in mRNA expression for CCL5 (RANTES).

Microarrays (Affymetrix mouse U74Av2 chip; assays Affy Metrix, Inc. Santa Clara, CA) were performed with RNA isolated from IBEC cultured with a combination of rIFNγ and rTNFα, to assess the kinetics and extent of mRNA induction. In accord with our hypothesis, we found that genes for the chemokine ligands of the chemokine receptors CCR2, CCR1, CCR3, CCR5, and CXCR3 (Table 1) were among the

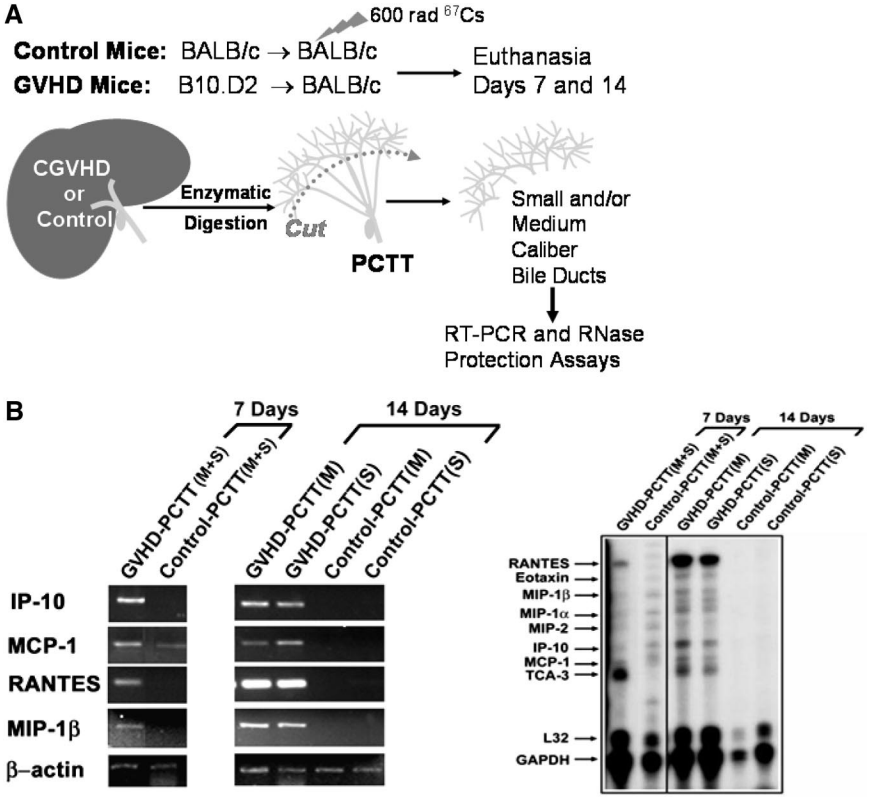

FIG. 2. Immunoisolation of portal connective-tissue trees (PCTTs) during hepatic GVHD and assessment of chemokine RNA expression in PCTTs containing small- and medium-caliber bile ducts. *Panel A:* Collagenase perfusion was used to remove parenchymal cells from the intrahepatic portal PCTTs of BALB/c mice with hepatic GVHD or BALB/c control mice. This permitted harvesting of proximal PCTT containing small- to medium-caliber intrahepatic bile ducts, which are the targets of NSDC. *Panel B:* Results of RT-PCR and RNase protection assays, demonstrating expression of chemokine mRNA in PCTT containing small-caliber (S) and/or medium-caliber (M) bile ducts in BALB/c mice with hepatic GVHD on days 7 and 14. Chemokine mRNA expression was absent in BALB/c mice.

earliest IBEC genes strongly induced by the combination of rIFNγ and rTNFα.

SECRETION OF CHEMOKINES BY BILIARY EPITHELIAL CELLS

To assess whether the induced mRNA resulted in the actual secretion of ligands for CCR2, CCR1, CCR3, CCR5, and CXCR3, we collected the supernatants of LPS-and/or cytokine-stimulated IBECs and performed

TABLE 1

Kinetics of mRNA expression by immortalized biliary epithelial cells (IBECs) stimulated with a combination of mouse recombinant interferon-γ and tumor necrosis factor-α. Note the fold increases of the chemokine ligands between 2 and 24 h after cytokine stimulation.

Gene	0.25 Hours	.5 Hours	2 Hours	6 Hours	24 Hours	Chemokine	Receptors
CCL2	1.0	1.0	3.1	3.8	3.2	MCP1	CCR2
CCL5	1.0	1.0	1.0	4.2	42.8	RANTES	CCR1, CCR3, CCR5
CXCL9	1.0	1.0	28.3	91.5	111.2	MIG	CXCR3
CXCL10	1.0	2.0	25.3	17.4	14.1	IP-10	CXCR3

All values 2-24 hours $P < 0.05$ as compared with baseline value.

ELISA on them to quantify the amounts of chemokine ligands secreted by IBEC (Table 2). In accord with the results of the gene-expression studies, LPS- and rIL-1β-induced the IBEC secretion of MCP-1 and of small amounts of CCL5 (RANTES), while rIFNγ or rTNFα induced the IBEC secretion of MCP-1 and of larger amounts of CCL5 (RANTES). The combination of rIFNγ and rTNFα stimulated a substantial increase in the secretion of the CXCR3 ligands CXCL9 (MIG) and CXCL10 (IP-10).

CHEMOATTRACTION OF ACTIVATED T CELLS BY CHEMOKINES SECRETED BY BILIARY EPITHELIAL CELLS

The key component of our hypothesis is that effector T cells are chemoattracted and terminally activated by chemokines secreted by BEC that line small- to medium-caliber bile ducts. To study the ability of IBEC to chemoattract activated T cells, we immersed transwells (Colc-Parmer, Inc., Vernon Hills, IL) containing concanavalin-A (Con-A)-stimulated spleen cells into culture wells containing confluent cultures of IBEC (Figure 3). The transwells containing Con-A-activated T cells were separated from the wells containing confluent cultures of

TABLE 2

Secretion of chemokines by immortalized biliary epithelial cells (IBECs) stimulated with LPS or mouse recombinant cytokines. Mean values, with all SEM ≤4%.

	MCP-1 pg/mL	CCL5 (RANTES) pg/mL	CXCL9 (MIG) pg/mL	CXCL10 (IP-10) pg/mL
Medium alone	0	0	0	0
LPS	1,465	138	5.6	3.8
IL-1β	1,314	263	4.1	2.5
IFNγ	1,045	651	2210	790
TNFα	1,334	1,476	1.1	1.3
IFNγ + TNFα	1,377	3,473	2,290	991

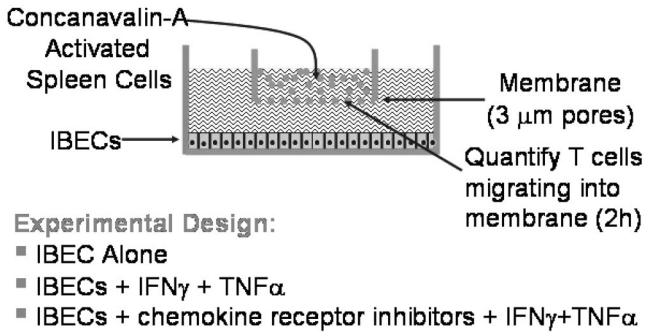

FIG. 3. Transwell method for assessing chemoattraction of activated T cells by immortalized biliary epithelial cells (IBEC) cultured in the presence or absence of mouse recombinant interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα)

IBEC by a permeable membrane with 3-μm-diameter pores. To stimulate chemokine secretion, IBEC were cultured in either the presence or absence of IFNγ plus TNFα. To study the impact of chemokines specific for CCR as opposed to CXCR3 receptors, we added specific chemokine-receptor inhibitors to the cultures. To inhibit the ligands of CCR1, CCR3, and CCR5, we used inhibitory CCL5 (RANTES) ligands (kind gifts of Oliver Hartley, PhD, of the University of Geneva, Switzerland). These included the methionyl variant (Met-RANTES) and the aminooxypentane variant of RANTES (AOP-RANTES) (11, 12). To inhibit the effect of CXCR3 ligands, we used a specific non-cytotoxic monoclonal antibody directed against the CXCR3 receptor (13) (a kind gift of Robert Strieter, M.D.). Table 3 shows that inhibitors of the chemokine receptors CCR1, CCR3, CCR5, and CXCR3 significantly reduced the chemoattraction of activated T cells caused by chemokines secreted by IBEC stimulated with a combination of IFNγ and TNFα.

CONCLUSIONS

To explain the restriction of NSDC to BEC lining only small- to medium-caliber intrahepatic bile ducts in the alloimmune diseases

TABLE 3

Chemotaxis of activated T cells induced by stimulation of immortalized biliary epithelial cells (IBEC) with a combination of mouse recombinant interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) is inhibited by blocking chemokine receptors CCR1, CCR3, CCR5 and CXCR3. Data are expressed as the percent of maximal chemotaxis.

	No Inhibitors	MET-RANTES	AOP-RANTES	Anit-CXCR3
IBEC alone	No chemotaxis	No chemotaxis	No chemotaxis	No chemotaxis
IBEC+IFNγ+TNFα	100%	42%	22%	22%

CVGHD and HAR, we hypothesized that BEC were not inherently targeted for destruction by their expression of allo-MHC molecules or tissue-specific antigens recognized by effector CD4+ or CD8+ T cells, but that these BEC instead contribute to their inflammatory destruction by T cells. The results of *in vivo* studies of portal connective-tissue trees from mice undergoing hepatic GVHD, conducted with the B10.D2 ($H\text{-}2^d$, $Ms^{1b,2b}$)→BALB/c ($H\text{-}2^d$, $Mls^{1b,2a}$) model of hepatic GVHD, supported this hypothesis. Similarly, *in vitro* studies of BALB/c IBEC confirmed that pro-inflammatory cytokines or LPS, known to be present before the onset of NSDC, can induce the expression of chemokine genes and secretion of biologically active chemokine ligands for the T cell chemokine receptors CCR1, CCR3, CCR5, and CXCR3. Our results indicate that BEC play an active role in the immunopathogenesis of NSDC by mediating the chemoattraction and terminal activation of effector T cells responsible for the apoptosis of BEC, resulting in ductopenia. Selective chemokine expression by BEC lining small- and medium-caliber bile ducts may explain the restriction of NSDC to ducts of this caliber; however, studies of BEC lining larger intrahepatic and extrahepatic bile ducts will be required to assess this fully. Inhibition of the chemokine receptors CCR1, CCR3, CCR5, and CXCR3 to block the chemoattraction and terminal activation of allo-reactive T cells represents a potential therapeutic strategy for preventing, NSDC after hematopoietic stem-cell transplantation or orthotopic liver transplantation.

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DISCUSSION

Criley, Torrance: Is this seduction or victimization?

Vierling, Houston: I think that if you're the biliary epithelial cell, you would feel victimized. Biologically, our results tell us that inflammation in the human body involves a strong component of target-cell participation that deserves increasing attention to understand how target cells seduce their own injury by effector cells.

Sudthanthiran, New York: John, that was a beautiful set of studies, and it's so striking how much your expression profiles are similar to what I would find in my transplant patients who undergo rejection in terms of RANTES, IP-10, and other CXCR3 ligands. I have three inter-related questions. First, did you see a pattern of TH17 expression, because this is now becoming prominent in the context of epithelial cell-related injury? Second, did you also see any evidence for granzyme B or perforin cytotoxic attack molecules being expressed? My third and related question is whether the pro-inflammatory process is counter-regulated by FOXP3 regulatory T-cell expression in this model.

Vierling, Houston: Those are all excellent questions. With respect to your third question, the T regulatory-cell participation in this model of GVHD has yet to be investigated. We do know that part of the activation contributing to the cytokine milieu stimulating the cholangiocytes comes from NK and NKT cells. Whether or not there is a TH17 component during the evolution of NSDC to ductopenia has not been studied, but one would expect it to occur later than 7 to 14 days. Because of the costs of these experiments, we have not extended the period of observation required to study TH17 cells, but that deserves to be looked at because it could augment inflammation in an

antigen-, as well as a cytokine-independent mechanism. T cells mediate cytotoxicity against biliary epithelial cells primarily through a Fas-Fas ligand mechanism, rather than through a granzyme-perforin mechanism.