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Immune Features of Disparate Liver Transplant Outcomes in Female Hispanics With Nonalcoholic Steatohepatitis

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Background. Nonalcoholic steatohepatitis (NASH) is a severe immune-mediated stage of nonalcoholic fatty liver disease that is rapidly becoming the most common etiology requiring liver transplantation (LT), with Hispanics bearing a disproportionate burden. This study aimed to uncover the underlying immune mechanisms of the disparities experienced by Hispanic patients undergoing LT for NASH. **Methods.** We enrolled 164 LT recipients in our institutional review board-approved study, 33 of whom presented with NASH as the primary etiology of LT (20%), with 16 self-reported as Hispanic (48%). We investigated the histopathology of prereperfusion and postreperfusion biopsies, clinical liver function tests, longitudinal soluble cytokines via 38-plex Luminex, and immune cell phenotypes generated by prereperfusion and postreperfusion blood using 14-color flow cytometry and enzyme-linked immunosorbent assay. **Results.** Hispanic LT recipients transplanted for NASH were disproportionately female (81%) and disproportionately suffered poor outcomes in the first year posttransplant, including rejection (26%) and death (38%). Clinically, we observed increased pro-inflammatory and apoptotic histopathological features in biopsies, increased AST/international normalized ratio early posttransplantation, and a higher incidence of presensitization to mismatched HLA antigens expressed by the donor allograft. Experimental investigations revealed that blood from female Hispanic NASH patients showed significantly increased levels of leukocyte-attracting chemokines, innate-to-adaptive switching cytokines and growth factors, HMGB1 release, and TLR4/TLR8/TLR9/NOD1 activation, and produced a pro-inflammatory, pro-apoptotic macrophage phenotype with reduced CD14/CD68/CD66a/TIM-3 and increased CD16/CD11b/HLA-DR/CD80. **Conclusions.** A personalized approach to reducing immunological risk factors is urgently needed for this endotype in Hispanics with NASH requiring LT, particularly in females.

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Obesity and metabolic syndrome constitute a worldwide epidemic with Hispanics bearing a disproportionate burden.[1–4] Nonalcoholic fatty liver disease (NAFLD) is the most common liver pathology associated with obesity, diabetes, and metabolic syndrome. NAFLD represents a large, growing global public health concern, and policies to mitigate the disease burden are urgently needed.[5] NAFLD includes the entire spectrum of fatty liver disease, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which can progress to liver cirrhosis and end-stage liver disease, requiring orthotopic liver transplantation (LT).

NASH is associated with a 50% higher death rate than NAFLD, and it is poorly understood why only a portion of patients progress,[6] making it a significant healthcare concern. In recent years, the proportion of adults on the waiting list for LT with a diagnosis of HCV has declined, whereas the proportion of adults with NASH has continually increased.[7–9] The European Liver Transplant Registry reported that NAFLD/NASH is likely underrecognized as an indication for LT, with many etiologies classified simply as cirrhosis or hepatocellular carcinoma (HCC).[10]

LT success is hampered by the immune-mediated cellular damage elicited by ischemia-reperfusion injury (IRI), which lowers both short- and long-term allograft survival. There is growing experimental and clinical evidence that recipient steatosis,

such as that seen in NASH, exacerbates injury-inducing mechanisms active during IRI and thus may be a major contributor to racial and ethnic disparities related to LT outcomes.[11]

Therefore, we investigated IRI, recipient steatosis, evolving immune profiles, and outcomes in a cohort of Hispanic NASH LT recipients, to better define the targetable immune mechanisms of this endotype.

MATERIALS AND METHODS

Study Design, Approval, and Sample Collection

One hundred sixty-four adult primary orthotopic LT recipients were recruited between May 10, 2013, and September 17, 2020 (Table 1). All studies were reviewed and approved by the University of California, Los Angeles institutional review board (UCLA IRB) (#13-000143). All patients provided informed consent before participation in the study. Routine standard-of-care triple noninduction regimen immunosuppression was administered according to the UCLA LT protocol. Study data were collected and managed using REDCap electronic data capture tools hosted at UCLA.[12] AST, ALT, bilirubin, and international normalized ratio (INR) levels were measured as part of the standard of care. Donor organs were procured from donations after brain death or circulatory death using standardized techniques. Organs were perfused and stored in cold University of Wisconsin solution (ViaSpan; Bristol-Meyers Squibb Pharma, Garden City, NY). Cold ischemia time was defined as the time from the perfusion of the donor with the preservation solution to the removal of the liver from cold storage. Tru-cut needle biopsies were obtained from donor allografts approximately 2 h before transplantation (PRE biopsies). Recipient venous blood was collected with an acid-citrate-dextrose anticoagulant during 2 main phases relative to the transplant: preoperative (PO), and postoperative at 1 d (D1), 1 w (W1), and 1 m (M1). Portal blood was collected from the recipient portal vein (PV) before reperfusion and after being flushed through the vena cava of the donor's liver during reperfusion (LF). Protocol Tru-Cut needle biopsies were obtained from the left lobe after complete revascularization of the allograft (2 h postreperfusion; POST biopsies) before surgical closure of the abdomen and graded for IRI and related histopathological features as previously described.[13] Whole glass hematoxylin and eosin-stained slides were digitally scanned at a continuum of 0.4–40× magnification using Aperio ScanScope AT, and images were acquired using Aperio ImageScope software version 12.3.3.5048.

HLA Typing and Evaluation of HLA Sensitization

HLA typing of recipients and donors was performed using molecular methods for HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQA1, -DQB1, -DPA1, and -DPB1 loci by LABType SSO at intermediate resolution, according to the manufacturer's specifications, and the results were analyzed using HLA Visual software (One Lambda, Canoga Park, CA). Pretransplant sera were tested for antibodies directed against HLA class I (A, B, C) and class II (DRB, DQA1, DQB1, DPA1, DPB1) antigens using a combination of Luminex PRA antibody identification (Immucor, Norcross, GA) and single-antigen bead (SAB) antibody identification assays (One Lambda). For SAB, a normalized cutoff value >1000 median fluorescence intensity (MFI) was used to assign positive specificity for HLA-A, -B, -DRB, and -DQ antigens. A normalized value of >2000 MFI was used to assign HLA antibodies to HLA-C and -DP antigens. These thresholds were based on our previous experience.[14]

Cytokine/Chemokine Luminex Assay

Plasma samples were prepared by centrifugation at 500*g* for 12 min and stored at −80°C. Human 38-plex magnetic cytokine/chemokine kits (EMD Millipore, HCYTMAG-60K-PX38) were used according to the manufacturer's instructions and as previously described.[13] Fluorescence was quantified using a Luminex 200 instrument. Cytokine/chemokine concentrations were calculated using Milliplex Analyst software version 4.2 (EMD Millipore). Luminex assays and analyses were performed using the UCLA Immune Assessment Core.

HMGB1 Detection in Patient Plasma Samples

HMGB1 enzyme-linked immunosorbent assay (IBL International) was performed according to the manufacturer's instructions.

Human Primary Monocyte Isolation and Immunophenotyping

Monocytes were enriched from healthy third-party donor blood samples using negative selection with RosetteSep technology (StemCell Tech) at a final purity of >92.6%. For monocyte stimulation, 30,000 cells were exposed to either RPMI-1640 medium with 10% FBS alone or 10 ng/mL M-CSF (R&D Systems), 50 ng/mL lipopolysaccharide (InvivoGen), 200 ng/mL all-thiol HMGB1 (HMGBiotech), 200 ng/mL disulfide-HMGB1 (HMGBiotech), or 10% patient LF samples and incubated at 37°C for 3 d. Monocyte phenotypes were assessed using flow cytometry as previously described.[15]

Pattern Recognition Receptor-activation Assay

Human TLR-2/-3/-4/-5/-7/-8/-9/Null, NOD1/-2, and Dectin1b-specific HEK-Blue reporter cells (InvivoGen, San Diego, CA) were grown, maintained, and used as previously described.[16]

Hierarchical Clustering Analysis

Cytokine abundance, irrespective of condition, was normalized using robust Z-scaling with the median and median absolute deviation. The medians of the scaled values for each group were color-coded and plotted on heatmaps. Unsupervised hierarchical clustering was performed on rows and columns using Euclidean distance as a similarity measure with Ward's linkage.

Statistics

For correlations between demographic data and IRI, Student's *t*-test was used for continuous variables, and Fisher's exact test was used for categorical variables. Two-way analysis of variance with Sidak's multiple comparisons test was used to determine differences among categories of time and/or NASH status unless otherwise indicated. All *P*-values were 2-sided and a value <0.05 was considered significant.

RESULTS

LT Recipient, Donor, and Transplant Characteristics

We analyzed prereperfusion and postreperfusion biopsies and plasma samples from 164 LT recipients. Of these, 33 (20%) patients required LT for NASH (Table 1). NASH patients requiring LT were significantly older at the time of transplantation (NASH+, 60 ± 9 y) than those with other etiologies (NASH−, 55 ± 12 y; $P = 0.02$). This was largely driven by

TABLE 1.

Recipient and donor clinical and demographic data

Clinical and demographic data	All	NASH–					NASH+					
		All	Non-Hispanic (NH)		Hispanic (H)		All	Non-Hispanic (NH)		Hispanic (H)		
			Male (M)	Female (F)	Male (M)	Female (F)		Male (M)	Female (F)	Male (M)	Female (F)	
	n = 164	n = 131	n = 42	n = 28	n = 39	n = 22	n = 33	n = 10	n = 7	n = 3	n = 13	
Characteristic	**Value**	**Value**	**Value**	**Value**	**Value**	**Value**	**Value**	**Value**	**Value**	**Value**	**Value**	***P*[a]**
Recipient												
Age (y), mean ± SD	56 ± 11	55 ± 12	57 ± 12	56 ± 9	53 ± 13	53 ± 11	60 ± 9	57 ± 8	58 ± 12	64 ± 7	63 ± 8	0.02*
Race, n (%)												0.32
Asian	14 (9%)	10 (8%)	7 (17%)	2 (7%)	0 (0%)	1 (5%)	4 (12%)	2 (2%)	2 (29%)	0 (0%)	0 (0%)	
Black/African American	10 (6%)	10 (8%)	5 (12%)	5 (18%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
White/Caucasian	95 (58%)	74 (56%)	29 (69%)	21 (75%)	14 (36%)	10 (45%)	21 (63%)	7 (70%)	5 (71%)	2 (67%)	7 (54%)	
Other/unknown	45 (27%)	37 (28%)	1 (2%)	0 (0%)	25 (64%)	11 (50%)	8 (24%)	1 (10%)	0 (0%)	1 (33%)	6 (46%)	
Liver disease etiology, n (%)												
Alcoholic	50 (30%)	49 (37%)	13 (31%)	5 (18%)	25 (64%)	6 (27%)	1 (3%)	0 (0%)	0 (0%)	1 (33%)	0 (0%)	<0.0001****
HBV	8 (5%)	7 (5%)	6 (14%)	0 (0%)	1 (3%)	0 (0%)	1 (3%)	1 (10%)	0 (0%)	0 (0%)	0 (0%)	>0.99
HCV	48 (29%)	44 (34%)	16 (38%)	8 (29%)	11 (28%)	9 (41%)	4 (12%)	2 (2%)	1 (14%)	1 (33%)	0 (0%)	0.02*
AIH	6 (4%)	6 (5%)	1 (2%)	2 (7%)	2 (5%)	1 (5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.60
PBC	4 (2%)	4 (3%)	0 (0%)	1 (4%)	0 (0%)	3 (14%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.58
PSC	7 (4%)	7 (5%)	2 (5%)	3 (11%)	1 (3%)	1 (5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.35
ALF	7 (4%)	7 (5%)	2 (5%)	4 (14%)	0 (0%)	1 (5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.35
Other	12 (7%)	11 (8%)	5 (12%)	4 (14%)	2 (5%)	0 (0%)	1 (3%)	0 (0%)	0 (0%)	0 (0%)	1 (8%)	0.46
w/HCC	52 (32%)	47 (36%)	14 (33%)	11 (39%)	13 (33%)	9 (41%)	5 (15%)	2 (2%)	2 (29%)	0 (0%)	1 (8%)	0.02*
MELD, at list, mean ± SD	25 ± 12	24 ± 12	25 ± 12	24 ± 13	27 ± 12	23 ± 11	27 ± 11	23 ± 12	30 ± 11	28 ± 10	29 ± 10	0.12
MELD, at transplant, mean ± SD	34 ± 8	34 ± 8	33 ± 9	34 ± 9	35 ± 6	36 ± 7	36 ± 6	33 ± 5	36 ± 4	37 ± 4	36 ± 8	0.22
Transplant(s), n (%)												0.08
Isolated liver	143 (87%)	111 (85%)	34 (81%)	25 (89%)	32 (82%)	20 (91%)	32 (24%)	9 (90%)	7 (100%)	3 (100%)	13 (100%)	
Liver-kidney or liver–heart	21 (13%)	20 (15%)	8 (19%)	3 (11%)	7 (18%)	2 (9%)	1 (1%)	1 (10%)	0 (0%)	0 (0%)	0 (0%)	
Donor												
Age (y), mean ± SD	39 ± 17	39 ± 17	41 ± 17	36 ± 15	39 ± 15	38 ± 20	40 ± 19	35 ± 18	51 ± 16	35 ± 19	40 ± 20	0.63
Gender, n (%)												0.85
Male	90 (55%)	71 (54%)	27 (64%)	13 (46%)	23 (59%)	8 (36%)	19 (58%)	9 (90%)	3 (43%)	2 (67%)	5 (38%)	
Female	74 (45%)	60 (46%)	15 (36%)	15 (54%)	16 (41%)	14 (64%)	14 (42%)	1 (10%)	4 (57%)	1 (33%)	8 (62%)	
Race, n (%)												>0.99
Asian	11 (7%)	8 (6%)	2 (5%)	2 (7%)	3 (8%)	1 (5%)	3 (9%)	1 (10%)	1 (14%)	0 (0%)	1 (8%)	
Black/African American	12 (7%)	10 (8%)	3 (7%)	2 (7%)	4 (10%)	1 (5%)	2 (6%)	0 (0%)	2 (29%)	0 (0%)	0 (0%)	
White/Caucasian	84 (51%)	67 (51%)	24 (57%)	17 (61%)	16 (41%)	10 (45%)	17 (52%)	6 (60%)	2 (29%)	2 (67%)	7 (54%)	
Other/undisclosed	57 (35%)	46 (35%)	13 (59%)	7 (25%)	16 (41%)	10 (45%)	11 (33%)	3 (30%)	2 (29%)	1 (33%)	5 (38%)	
Ethnicity, n (%)												0.65
Hispanic/Latino	89 (54%)	43 (33%)	12 (29%)	7 (25%	15 (38%)	9 (41%)	11 (33%)	3 (30%)	2 (29%)	1 (33%)	5 (38%)	
Non-Hispanic/Latino	38 (23%)	87 (66%)	30 (71%)	21 (75%)	23 (59%)	13 (59%)	22 (64%)	7 (70%)	5 (71%)	2 (67%)	8 (62%)	
Unknown/undisclosed	37 (23%)	1 (1%)	0 (0%)	0 (0%)	1 (3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Status, n (%)												0.35
DBD	158 (96%)	126 (96%)	39 (93%)	28 (100%)	37 (95%)	22 (100%)	31 (94%)	9 (90%)	7 (100%)	3 (100%)	12 (92%)	
DCD	6 (4%)	5 (4%)	3 (7%)	0 (0%)	2 (5%)	0 (0%)	2 (6%)	1 (10%)	0 (0%)	0 (0%)	1 (8%)	
Warm ischemia, min, mean ± SD	52 ± 14	51 ± 15	49 ± 12	51 ± 20	53 ± 15	53 ± 10	54 ± 10	57 ± 11	55 ± 10	57 ± 7	50 ± 9	0.42
Cold ischemia (h), mean ± SD	8 ± 3	8 ± 2	7 ± 2	7 ± 2	8 ± 5	7 ± 2	8 ± 3	8 ± 2	8 ± 1	8 ± 1	9 ± 3	0.24
Recipient + donor												
ABO, n (%)												0.74
Identical	149 (91%)	117 (89%)	36 (86%)	21 (75%)	39 (100%)	21 (95%)	31 (94%)	9 (90%)	6 (86%)	3 (100%)	13 (100%)	
Compatible	15 (9%)	14 (11%)	6 (14%)	7 (25%)	0 (0%)	1 (5%)	2 (6%)	1 (10%)	1 (14%)	0 (0%)	0 (0%)	
Donor Risk Index, mean ± SD	1.52 ± 0.39	1.51 ± 0.36	1.59 ± 0.33	1.40 ± 0.29	1.51 ± 0.33	1.53 ± 0.37	1.56 ± 0.50	1.29 ± 0.36	1.76 ± 0.55	1.46 ± 0.31	1.68 ± 0.54	0.10

t-test for continuous variables; Fisher's exact test for categorical variables.

* *P*-value < 0.05.

**** *P*-value < 0.0001.

ABO, blood group; AIH, autoimmune hepatiti; ALF, acute liver failure; DBD, donation after brain death; DCD, donation after brain death; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MELD, model for end-stage liver disease; NASH, nonalcoholic steatohepatitis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

the NASH+ Hispanic population, which was the oldest (H = 64 ± 12). Despite the overall cohort being predominantly male (94/164 = 57%), NASH+ recipients were mostly female (20/33 = 61%), and most female NASH+ recipients were Hispanic. Although HCC is a common covariate for patients with end-stage liver disease due to NASH progression, our population of NASH+ patients requiring LT had significantly less HCC than NASH recipients (15% NASH+ with HCC versus 36% NASH– with HCC). A similar pattern was observed for HCV (12% NASH+ with HCV versus 34% NASH– with HCV). Donor Risk Index (DRI) was highest in NASH+ females regardless of ethnicity. Multivariate analysis did not reveal any correlation between NASH etiology and any other recipient or donor demographic or clinical parameters.

Although NASH+ patients overall did not have the highest incidence of worsened outcomes within the first-year posttransplant, Hispanics with NASH (H) had a significantly higher incidence of for-cause biopsies, chronic inflammation, AMR, and death during this period (Table 2) compared with non-Hispanics with NASH (NH). Upon further investigation, female Hispanics with NASH (F) suffered disproportionately, with the poorest outcomes within the first-year posttransplant, compared with their male (M) counterparts (Table 3). The cause of death for female Hispanics with NASH was categorized predominantly due to infection (n = 4/6; 67%), similar to Hispanics without NASH. NASH– non-Hispanics cause of death were predominantly cardiovascular (n = 3; 75%).

Hispanic Females Requiring LT for NASH Disproportionately Suffer From IRI

To determine the incidence of IRI in this cohort, intraoperative biopsies of the liver allograft were obtained 2 h postreperfusion and assessed according to our standard practice at UCLA.[13] IRI scoring is evaluated by the amount of neutrophilic infiltration and hepatocellular necrosis occurring at this early timepoint. Four additional histopathological features are also scored, as they are indicative of an overall poorer quality organ, including ballooning degeneration of hepatocytes, biliary cholestasis, microsteatosis, and sinusoidal congestion. We found it is common for patients with significant necrosis/inflammation (IRI+) to also have 1–3 of these additional histopathological features, particularly those recipients who experienced rejection episodes within the first-year posttransplant.[13] Of the 146 enrolled LT recipients scored for IRI severity by histopathology, 78 had biopsy-proven IRI (IRI+, 53%), and 68 did not (IRI–, 47%). There was no difference in IRI scores among the LT recipients based on NASH status (Figure 1A, left panel). However, NASH+ recipients classified as Hispanic in the UNOS disproportionately suffered from IRI, with 67% (H = 10/15) identified as IRI+ and 33% (NH = 5/15) identified as IRI– of those scored (Figure 1A, middle panel). Notably, this pattern was conserved in female Hispanics, with 67% of female Hispanics with NASH being IRI+ (F = 8/12) compared with only 33% of male Hispanics with NASH being IRI+ (M = 1/3) (Figure 1A, right panel). Taken together, these results indicate that female Hispanics with NASH have a higher incidence of IRI than other NASH+ LT recipients.

NASH+ recipients had increased severity of 2 histopathological features posttransplant compared with NASH recipients (Figure 1B), including macrovesicular steatosis and hepatocellular ballooning. Among NASH+ recipients, Hispanics (H) had an increased incidence of cholestasis and congestion posttransplantation. Female NASH+ Hispanics (F) had the highest incidence of all 4 detrimental histopathological features (Figure 1B and C), which were previously associated with poorer outcomes in LT-IRI+ recipients.[13] Importantly, female NASH+ Hispanics were the only demographic group

TABLE 2.

Female Hispanics with NASH have poor outcomes in the first year posttransplant

Recipients, n = 164	EAD (n = 34)	Chronic inflammation (n = 11)	ACR (n = 22)	AMR (n = 5)	Death (n = 15)
NASH – non-Hispanic male (n = 42)	7 (17%) [21%]	1 (2%) [9%]	6 (14%) [27%]	1 (2%) [20%]	3 (7%) [20%]
NASH – non-Hispanic female (n = 28)	7 (25%) [21%]	0 (0%) [0%]	8 (29%) [36%]	2 (7%) [40%]	1 (4%) [7%]
NASH – Hispanic male (n = 39)	11 (28%) [32%]	1 (3%) [9%]	2 (5%) [9%]	0 (0%) [0%]	1 (3%) [7%]
NASH – Hispanic female (n = 22)	5 (23%) [15%]	0 (0%) [0%]	2 (9%) [9%]	0 (0%) [0%]	2 (9%) [13%]
NASH + non-Hispanic male (n = 10)	1 (10%) [3%]	3 (30%) [27%]	1 (10%) [5%]	0 (0%) [0%]	2 (20%) [13%]
NASH + non-Hispanic female (n = 7)	0 (0%) [0%]	1 (14%) [9%]	1 (14%) [5%]	0 (0%) [0%]	0 (0%) [0%]
NASH + Hispanic male (n = 39)	0 (0%) [0%]	0 (0%) [0%]	0 (0%) [0%]	0 (0%) [0%]	0 (0%) [0%]
NASH + Hispanic female (n = 16)	3 (19%) [9%]	5 (31%) [45%]	2 (13%) [9%]	2 (13%) [40%]	6 (38%) [40%]

ACR, acute cellular rejection; AMR, antibody-mediated rejection; EAD, early allograft dysfunction; NASH, nonalcoholic steatohepatitis.

TABLE 3.

Cause of death for liver transplant recipients

Recipients, n = 15	Cardiovascular (n = 4)	Hepatic (n = 2)	Infection (n = 9)
NASH – non-Hispanic male (n = 3)	2 (67%) [50%]	0 (0%) [0%]	1 (33%) [11%]
NASH – non-Hispanic female (n = 1)	1 (100%) [25%]	0 (0%) [0%]	0 (0%) [0%]
NASH – Hispanic male (n = 1)	0 (0%) [0%]	0 (0%) [0%]	1 (100%) [11%]
NASH – Hispanic female (n = 2)	0 (0%) [0%]	0 (0%) [0%]	2 (100%) [22%]
NASH + non-Hispanic male (n = 2)	0 (0%) [0%]	1 (50%) [50%]	1 (50%) [11%]
NASH + non-Hispanic female (n = 0)	0 (0%) [0%]	0 (0%) [0%]	0 (0%) [0%]
NASH + Hispanic male (n = 0)	0 (0%) [0%]	0 (0%) [0%]	0 (0%) [0%]
NASH + Hispanic female (n = 6)	1 (17%) [25%]	1 (17%) [50%]	4 (67%) [44%]

NASH, nonalcoholic steatohepatitis.

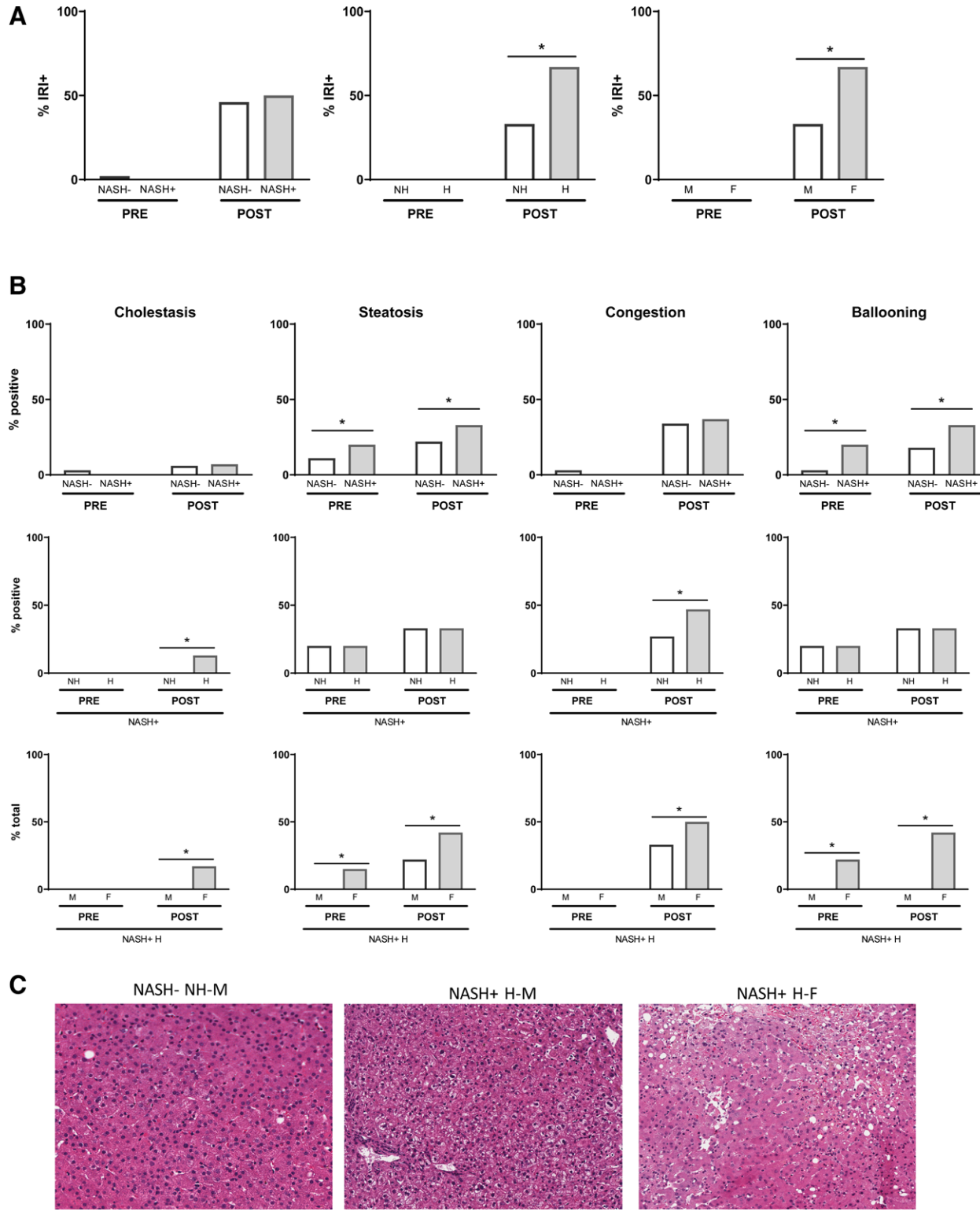

FIGURE 1. Histopathological features are prominent in female Hispanic NASH patients at 2 h postreperfusion. Donor allograft biopsies were obtained 2 h pre- (PRE) or post- (POST) reperfusion and evaluated for IRI (A) or related histopathological features (B). B, Percentage of total NASH– vs NASH+ patients (n = 146, 116 NASH– and 30 NASH+; left column), percentage of non-Hispanic (NH) vs Hispanic (H) NASH+ patients (n = 30, 15 NH and 15 H; middle column), or percentage of total male (M) vs female (F) Hispanic NASH patients (n = 15, 3 M and 12 F; right column) that scored positive for any of the 4 histopathological features of cholestasis, steatosis, congestion, and/or ballooning. Data are presented as bar graphs representing mean values for each group of patients as indicated. *$P < 0.05$. Statistical significance was determined using 2-way ANOVA with post-hoc Sidak's for multiple comparisons. C, Representative images of postreperfusion biopsies from a non-Hispanic NASH– male (NASH– NH-M), a Hispanic male with NASH (NASH+ H-M), and a female Hispanic with NASH (NASH+ H-F). Scale bar = 40 μm. ANOVA, analysis of variance; IRI, ischemia-reperfusion injury; NASH, nonalcoholic steatohepatitis.

that received donor organs with increased severity of histopathological features including both steatosis and ballooning, predisposing them to increased injury.

Clinical Liver Function Tests Are Elevated Early Posttransplant for Female Hispanic NASH Patients

Our previous data suggested that disturbances in bilirubin levels early in the first week posttransplant and AST levels later in the same week are mediated by IRI.[13] Transplant recipients were evaluated for liver function using standard-of-care tests, including serum AST, ALT, total bilirubin, and INR (Figure 2). We did not find any association between these clinical liver function tests and NASH etiology (Figure 2A). However, we found that Hispanics with NASH (H, Figure 2B) had significantly elevated ALT, AST, and INR on the first day posttransplant and increased ALT and INR on the second day posttransplant, predominantly driven by the female cohort (F, Figure 2C).

Hispanic Females With NASH Have Increased Presensitization to Mismatched Donor HLA Antigens

Highly sensitized patients face longer waiting times in most organ allocation programs, increased graft rejection, immunosuppressed side effects, and poorer outcomes. Nevertheless, clinical testing for HLA sensitization in LT candidates has unclear implications because of the intrinsically tolerogenic microenvironment of the organ. We found that the incidence of HLA presensitization in NASH+ LT recipients was significantly higher than that in non-NASH recipients (Figure 3A, left panel). This incidence was higher in Hispanics with NASH (H; Figure 3A, middle panel) and was the highest in females from this group (F; Figure 3A, right panel). Although most LT recipients were sensitized to class I, female Hispanics with NASH were more frequently sensitized to only class II or both class I/II HLA antigens than their male counterparts (F versus M; Figure 3A). Furthermore, presensitization to HLA donor-specific antigens (DSA) was also the highest in this

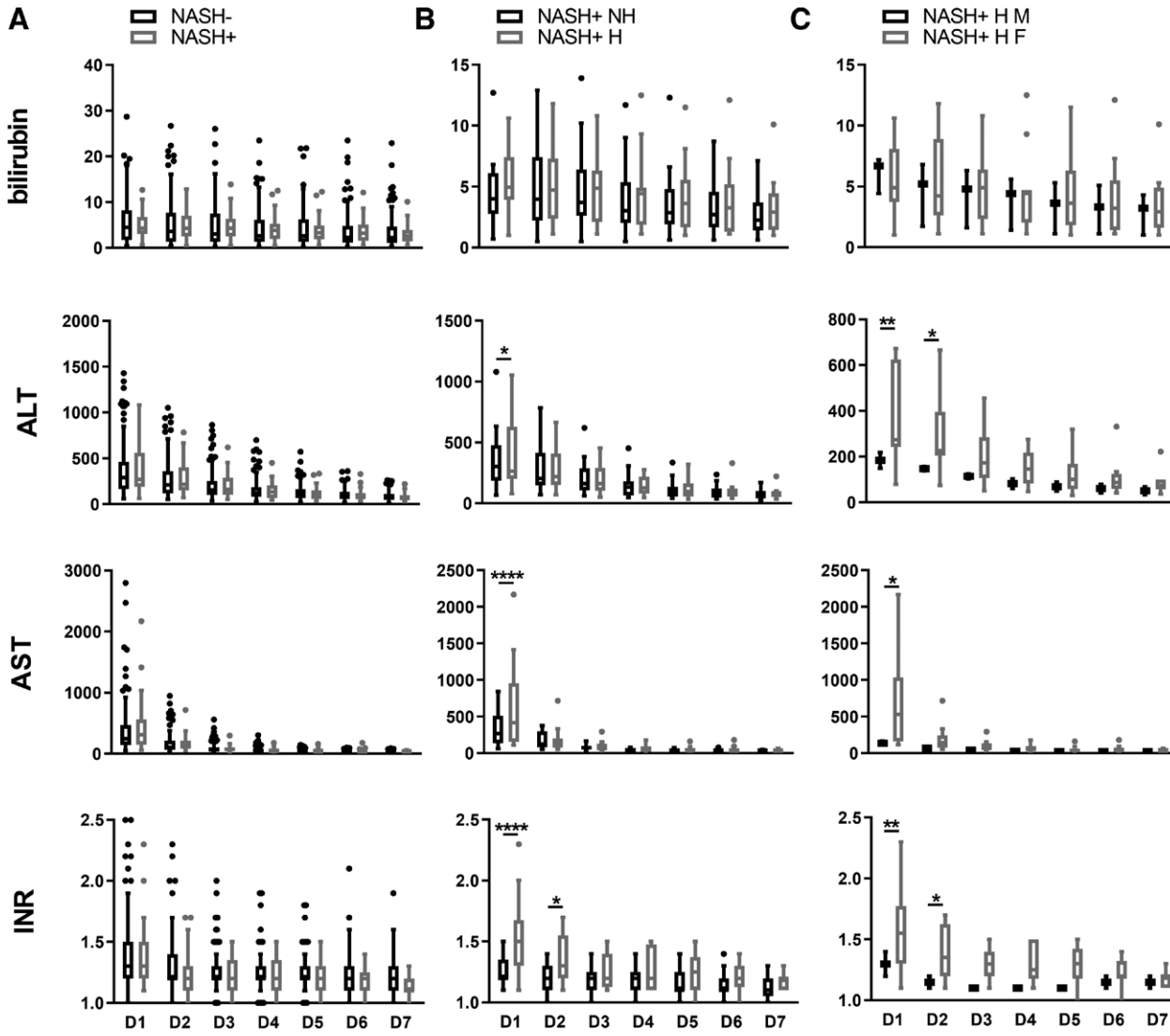

FIGURE 2. Liver function deteriorates in female Hispanic NASH patients in the first-week posttransplant. Four clinical liver function tests were performed on recipient circulating peripheral blood taken days 1–7 posttransplant (D1–D7) and evaluated for (A) NASH– vs NASH+ recipients (n = 146; 116 NASH–, 30 NASH+), (B) non-Hispanic vs Hispanic NASH+ recipients (n = 30; 15 NH, 15 H), or (C) male vs female Hispanic NASH+ recipients (n = 15; 3 M, 12 F). Data are presented as Tukey box and whisker plots with the box representing the interquartile range, whiskers are inner fences reaching 1.5 times the interquartile range, dots indicating outlying values, and line representing median values for each day: Bilirubin (mg/mL), ALT and AST (U/L), or INR. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. The Wilcoxon rank-sum test was used for comparison between patient groups. INR, international normalized ratio; NASH, nonalcoholic steatohepatitis.

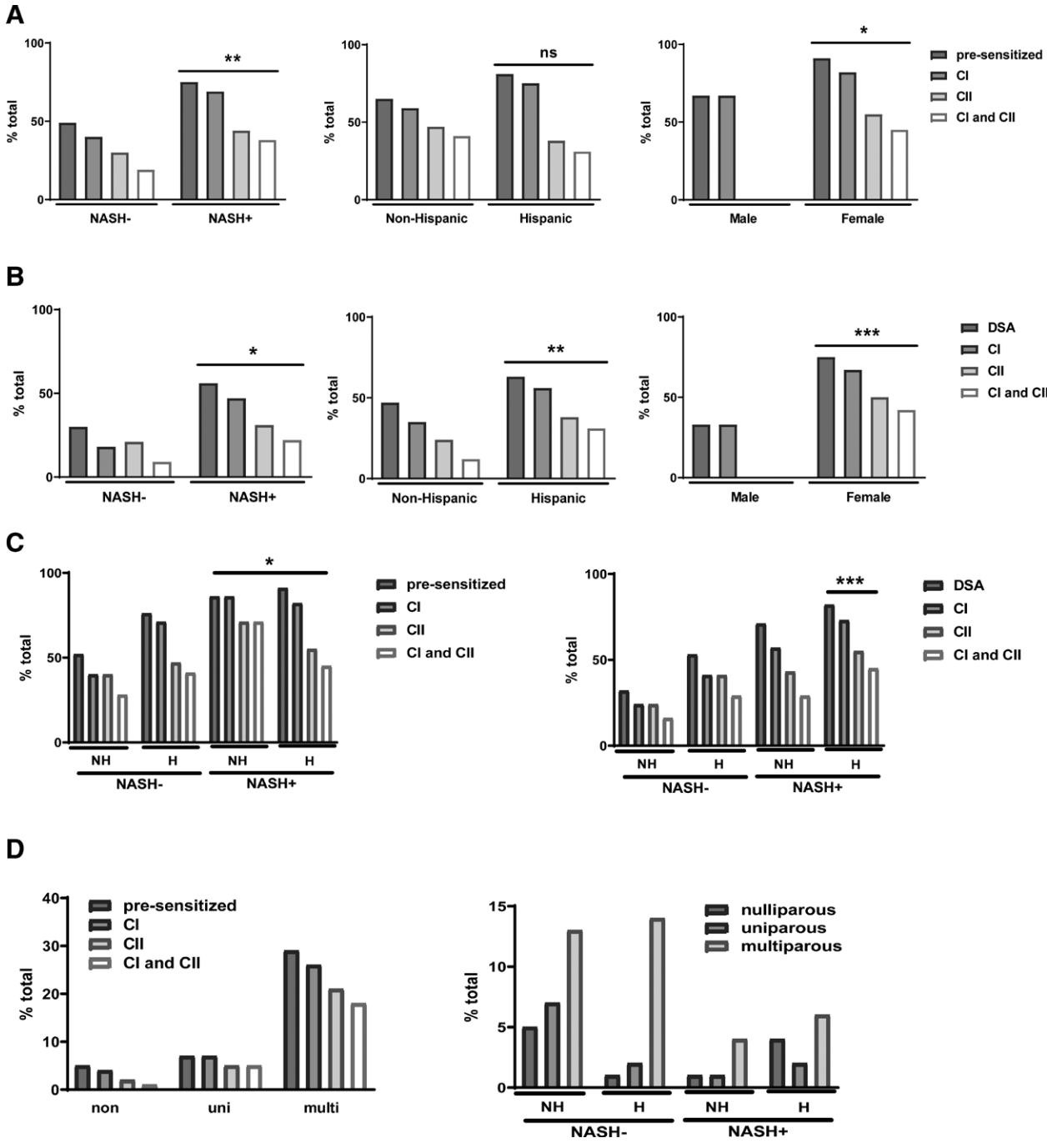

FIGURE 3. Female Hispanics with NASH are more frequently sensitized to donor-specific HLA antigens pretransplant. Recipient blood samples obtained pretransplant were tested by single-antigen bead (SAB) based assay for antibodies to either (A) third party or (B) donor-specific HLA antigens for NASH– vs NASH+ recipients (n = 146; 116 NASH–, 30 NASH+; left panels), non-Hispanic vs Hispanic NASH+ recipients (n = 30; 15 NH, 15 H; middle panels), or male vs female Hispanic NASH+ recipients (n = 15; 3 M, 12 F; right panels). C, SAB data for females that are presensitized to third-party HLA antigens (top row), or HLA DSA (bottom row) specific for NASH– non-Hispanics (NASH– NH, n = 25), NASH– Hispanics (NASH– H, n = 17), NASH+ non-Hispanics (NASH+ NH, n = 7) or NASH+ Hispanics (NASH+ H, n = 12). (D) parity data for females that are presensitized (left panel) and specific for NASH– non-Hispanics (NASH– NH, n = 25), NASH– Hispanics (NASH– H, n = 17), NASH+ non-Hispanics (NASH+ NH, n = 7) or NASH+ Hispanics (NASH+ H, n = 12) (right panel). Data are presented as percent of total patients with positive antibodies. Statistical significance was determined using 2-way ANOVA with post-hoc Sidak's for multiple comparisons. ANOVA, analysis of variance; DSA, donor-specific antigen; NASH, nonalcoholic steatohepatitis.

group (F versus M; Figure 3B). DSA evaluated for NASH+ female Hispanics was again specific to only class II or both class I/II HLA antigens compared with class I only in male Hispanics with NASH (F versus M; Figure 3B). Female transplant recipients are often presensitized by prior pregnancies, and we evaluated presensitization in all females from our cohort (Figure 3C) to determine whether female recipients, in general, were similarly presensitized. We found the highest incidence of female presensitization to HLA among NASH+ recipients regardless of ethnicity (NASH+ versus NASH–; Figure 3C, left panel), but the highest DSA-specific presensitization in female Hispanics with NASH (NASH+ H; Figure 3C, right panel). Most liver allograft recipients receive multiple blood products before transplantation; however,

female parity is well-known to increase sensitization, and that is true in our cohort as well (Figure 3D, left panel). Of the 61 females included in this study, there were 12 nulliparous, 12 uniparous, and 37 multiparous at the time of transplantation. Of the 12 nulliparous, 4 were Hispanic females with NASH (33%). Of the 12 uniparous, 2 were Hispanic females with NASH (17%), and of the 36 patients who were multiparous, only 6 were Hispanic females with NASH (16%). Therefore, the immune phenotype associated with Hispanic females with NASH is not likely to be due to increased parity alone (Figure 3D, right panel).

Hispanic Female LT Recipients With NASH Have a Unique Longitudinal Cytokine Profile Capable of Shaping an Adaptive Immune Response

We investigated the evolution of the immune response over time by evaluating 38 cytokines, chemokines, and growth factors in the blood samples obtained before, during, and up to 1 mo after transplantation in our cohort of patients undergoing LT (Figure 4). Unsupervised hierarchical clustering performed on systemic blood samples obtained from pretransplant and posttransplant revealed 6 analyte groups (Figure 4A). The first cluster identified CCL11 and sCD40L as the highest in NASH+ patients before transplantation (PO) and in NASH patients at 1-mo posttransplant (M1). The second cluster was specific to the M1 time point and showed an increase in GRO and decrease in Flt3L in NASH+ patients. The third cluster contained increased levels of cytokines, chemokines, and growth factors in NASH+ patients PO including IL-1b, IFNa2, TGFa, IL-6, IL-12p70, IL-17A, CCL22, IL-7, IL-12p40 and GM-CSF. The fourth cluster contained increased levels of analytes in NASH+ patients PO and 1-d posttransplant (D1), including G-CSF, CXCL10, CX3CL1, and IL-8. The fifth cluster identified CCL4, TNFa, and IL-3 as increased in NASH+ patients at all time points compared with NASH recipients. The final cluster showed CCL3, IL-15, IL-10, IL-1RA, and IL-2 as the most influential mediators on D1.

Unsupervised hierarchical clustering of the portal blood samples produced 3 main clusters (Figure 4B). The first cluster identified strong correlations between several analytes in the PV sample obtained just before reperfusion and NASH etiology, including IL-6, IL-8, IL-12p40, CCL2, IL-9, IL-12p70, IFNa2, sCD40L, GM-CSF, IL-17A, CCL3, IL-5, VEGF, TNFb, IFNg, CCL7, IL-1a, IL-4, TGFa, TNFa, CCL11, CCL4, CXCL10, and CCL22. The second cluster was specific to NASH+ patients, with G-CSF, IL-7, IL-2, IL-3, and IL-1b all increasing in these patients both prereperfusion and postreperfusion. The third cluster correlated with cytokines present in the LF sample obtained immediately following reperfusion, with GRO, CX3CR1, FLT3L, and IL-1Ra increased in non-NASH patients, and FGF2 and IL-15 increased in NASH+ patients.

Further investigation into cytokine profiles of Hispanic NASH patients showed that 10 mediators associated with NASH in systemic blood samples were highest in female Hispanics (F versus M; Figure 4C) at several time points including 2 chemokines: CX3CL1, CXCL10; 4 cytokines, IL-1b, IL-7, IL-17A, and TNFa; and 3 growth factors: FGF2, G-CSF, GM-CSF, and GRO. Portal blood samples from female Hispanics with NASH had increased levels of 10 slightly different mediators associated with NASH etiology (F versus M; Figure 4D) in PV, LF, or both, including 5 chemokines: CCL2, CCL4, CCL11, CCL22, and CXCL10; 4 cytokines: IL-12p40, sCD40L, IL-4, and IL-15; and 1 growth factor: FGF2.

Hispanic Females With NASH Have a Unique Pattern Recognition Receptor Activation Profile Produced by Their Intraoperative Blood Samples

Pattern recognition receptor (PRR) recognition by damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) is a critical component of the immune system's ability to recognize and respond to cellular stress/damage or pathogens, including switching from an innate immune response to a prolonged adaptive immune response. We profiled the intratransplant blood of LT recipients (Figure 5) for their ability to activate a panel of PRRs using HEK-Blue cells transfected with a specific PRR.[16] We found that NASH patients had a slightly increased ability to activate TLR4 and a decreased ability to activate TLR5/9 prereperfusion compared with patients with other etiologies (NASH+ versus NASH–; Figure 5A). Postreperfusion samples showed an increased ability to activate TLR2/4/5/8/9 and Dectin-1b. Among NASH patients, Hispanics had much lower pretransplant activation levels of TLR2/4/5/NOD2, reminiscent of the phenotype we previously found to be associated with an increased incidence of IRI.[16] Hispanic and non-Hispanic patients were similarly capable of activating TLR4/9 with their LF samples (Hispanic versus non-Hispanic; Figure 5B), recapitulating the IRI PRR phenotype, but had a decreased ability to activate TLR5/7/NOD2 and Dectin-1b, which we previously showed to be associated with IRI– patient samples. Female Hispanics with NASH had a much higher incidence of TLR3/4/7/8/9/NOD1 and Dectin-1b activation by PV samples (female versus male; Figure 5C). Following reperfusion, female Hispanics with NASH had an increased ability to activate TLR4, 8, and NOD1, and a decreased ability to activate TLR3 and Dectin-1b.

Hispanic NASH Patients Have More DAMP Release With the Potential for Monocyte Activation During LT

PRR activation is driven by the release of DAMPs following cellular injury such as that incurred during allograft IRI. We previously showed that the DAMP HMGB1 is increased in the LF of IRI+ LT recipients.[15] As NASH patients, particularly female Hispanic NASH patients, induced high TLR4/9 activation, we investigated the release of HMGB1 in these patients (Figure 6A). We found no differences in the levels of HMGB1 between NASH patients and other LT-requiring etiologies, but Hispanic NASH patients, especially females, had a significantly higher increase in HMGB1 in their LF than in their baseline PV samples.

PRR-expressing innate immune cells can be activated in patients with a higher DAMP release and increased PRR activation potential. Therefore, we investigated the potential of blood samples from LT recipients to alter the immune phenotype of third-party monocytes obtained from healthy donors (Figure 6B). Although we did not find a monocyte phenotype associated with NASH in our cohort, we found that Hispanic NASH patients consistently and significantly increased CD16, CD11b, CD80, and HLA-DR levels and decreased the expression of CD14, CD68, CD66a, GAL-9, and TIM-3, indicating a pro-inflammatory rather than a regulatory program had been initiated. Owing to sample limitations, we were unable to evaluate sex as a variable in this assay; however, it should

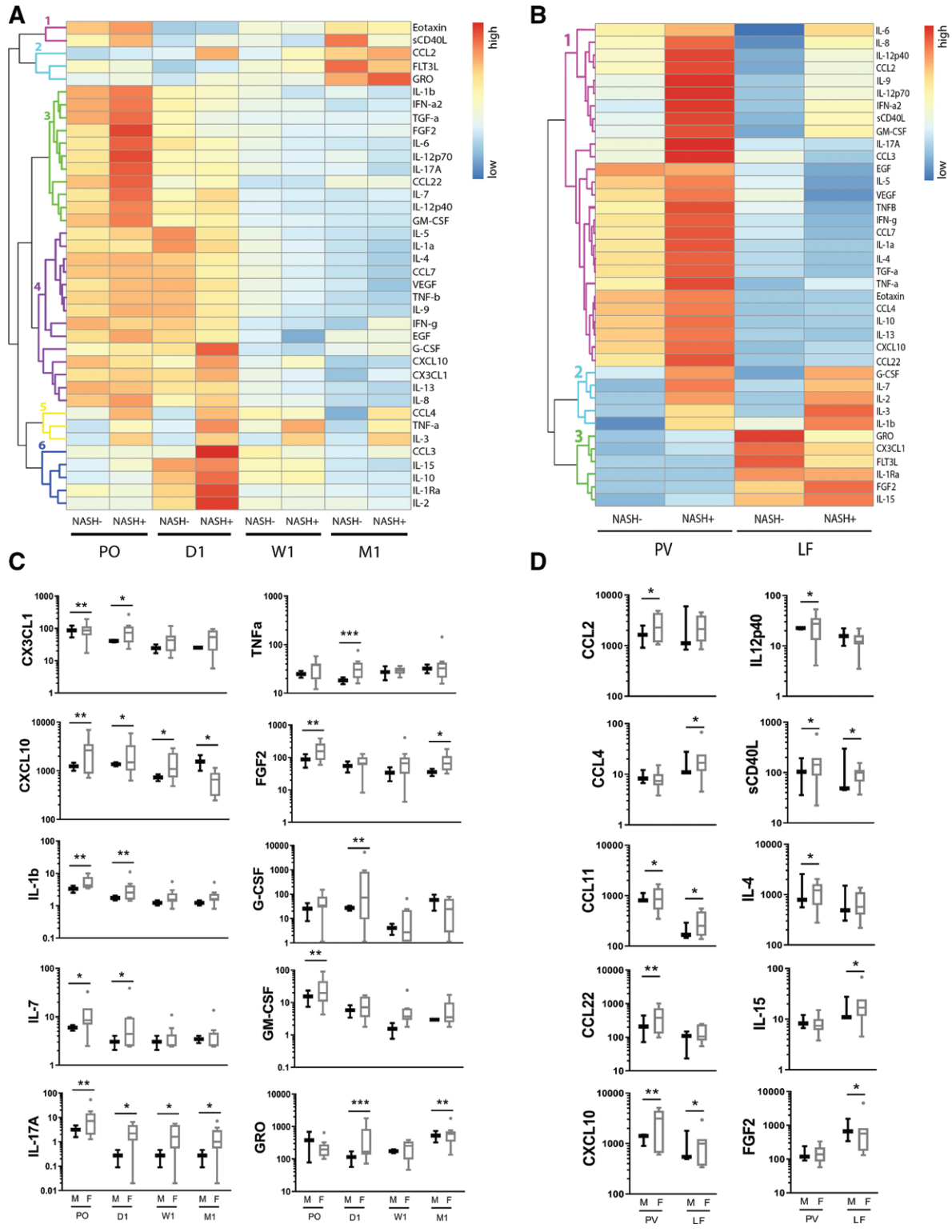

FIGURE 4. Longitudinal NASH-associated cytokine profiles are highest in Hispanic females. Recipient cytokines, chemokines, and growth factors were evaluated for (A and C) recipient circulating blood samples at preoperative (PO), or postoperative (day 1 [D1], week 1 [W1], or month 1 [M1]) time points, or (B and D) portal blood samples obtained prereperfusion (PV), or postreperfusion (LF). A and B, Shown are heatmaps in which the rows represent cytokines, the columns represent patient NASH status at the time of transplant, and the colors represent normalized median cytokine concentration values (white-low, red = high). The rows and columns are ordered based on the results of unsupervised hierarchical clustering, with dendrograms for the cytokines and patient groups shown on the vertical and horizontal axes, respectively. C and D, Of 38 cytokines, chemokines, and growth factors tested by Luminex Multiplex assay, 13 were significantly higher in female (F) vs male (M) NASH+ patients either (C) preoperatively (PO) or at postoperative day 1 (D1), week 1 (W1), or month 1 (M1) time points, or (D) at PV or LF time points. Data are presented as Tukey box-and-whisker plots: whiskers are inner fences reaching 1.5 times the interquartile range, and boxes represent the interquartile ranges, dots indicate outlying values, and lines represent median values for each time point. (A,B) NASH–/+ (n = 114, NASH– = 91, NASH+ = 24), and (c and d) NASH+ Hispanic males vs females (M = 3, F = 10) *$P < 0.05$. The Wilcoxon rank-sum test was used for comparison between patient groups. NASH, nonalcoholic steatohepatitis.

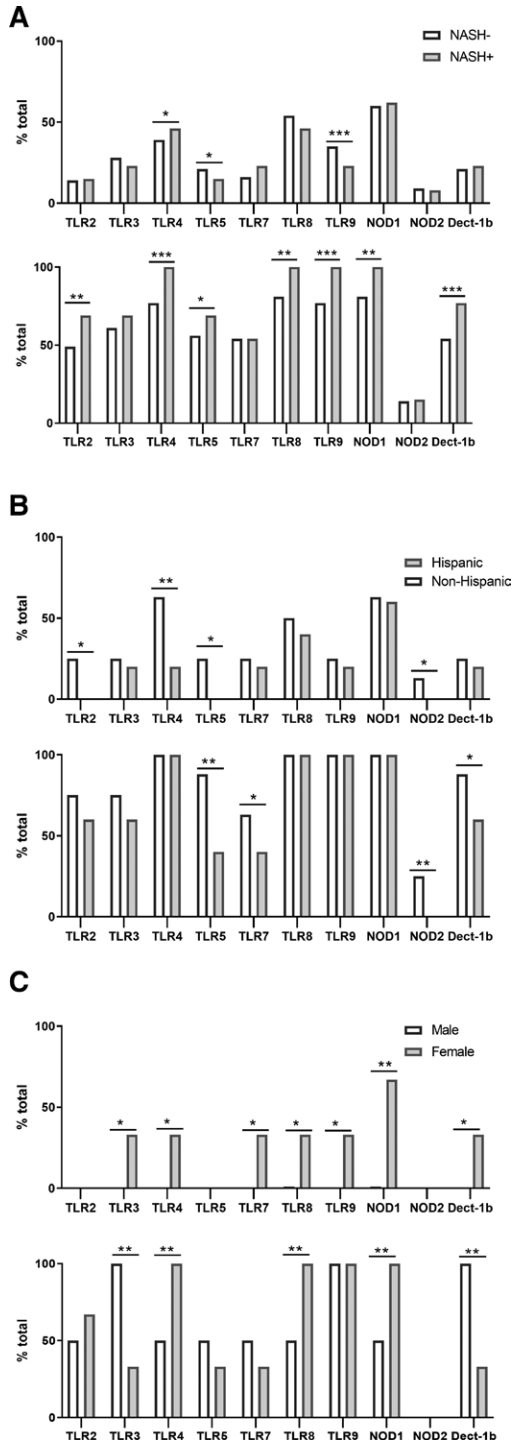

FIGURE 5. Pattern recognition receptor reactivity screening of NASH patients reveals increased activation of pattern recognition receptors (PRRs). Patient plasma samples obtained from the portal vein prereperfusion (portal vein [PV]) or just after being flushed through the liver during reperfusion (liver flush [LF]) were tested for reactivity to PRRs using a panel of 10 human HEK-Blue transfected cell lines (hTLR2, hTLR3, hTLR4, hTLR5, hTLR7, hTLR8, hTLR9, hNOD1, hNOD2, and hDectin1b). Shown are the percent total of (A) NASH– vs NASH+ recipients (n = 146; 116 NASH–, 30 NASH+), (B) non-Hispanic vs Hispanic NASH+ recipients (n = 30; 15 NH, 15 H), or (C) male vs female Hispanic NASH+ recipients (n = 15; 3 M, 12 F) with activation of indicated PRRs above the EC_{max} of their positive control ligand. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ by 2-way ANOVA with Tukey's multiple comparisons test. ANOVA, analysis of variance; NASH, nonalcoholic steatohepatitis.

be noted that our cohort of Hispanic NASH patients requiring LT was predominantly female.

DISCUSSION

Our results provide the first detailed longitudinal pretransplant, intratransplant, and posttransplant immune profiles of female Hispanic patients with NASH undergoing LT. It is widely appreciated that NASH is becoming the leading cause of LT.[17] Similarly, the disadvantages that females and Hispanics experience in LT are well known.[18] Our cohort of NASH patients requiring LT contained a disparate number of female Hispanics, who experienced disproportionate rates of poor outcomes, including ACR, AMR, and death, within the first year posttransplant. Here, we describe the immunological endotype specific to NASH+ LT recipients who are both female and Hispanic.

Analysis of clinical testing revealed that female Hispanics with NASH had significantly higher liver function tests during the first 2 d posttransplant. Female Hispanic NASH patients in our cohort also had increased cardinal histopathological features that are detrimental to both allograft and patient survival including increased macrovesicular steatosis, hepatocellular ballooning, biliary cholestasis, and congestion, in addition to an increased incidence of IRI at the postreperfusion timepoint. Female Hispanic NASH patients were also the only demographic group that received donor organs with significant steatosis and ballooning features. Although they did not reach significance, DRI scores for female Hispanics had the highest DRI, indicating a donor quality issue independent of recipient phenotype or other perioperative factors. Female patients may wait longer because of their smaller body habitus, thus needing smaller livers from less-abundant smaller donors, resulting in the acceptance of more marginal donors for these patients to facilitate transplantation in a timely manner. This has recently been addressed in the new MELD 3.0 scoring system, now incorporating recipient sex, which was determined to be closely linked to their size.[19] SAB-based antibody testing of pretransplant sera from LT patients showed that these patients were frequently presensitized to mismatched, donor-specific HLA antigens, which was not simply due to increased multiparity. Although both liver biopsy and SAB data are commonly reviewed by relevant clinical teams posttransplant, these data support the utility of pretransplant testing, especially in selecting high-risk LT candidates such as female Hispanics. Longitudinal monitoring of HLA antibody trends over time in these patients is urgently required to better understand their role in the worsened outcomes.

The experimental investigation confirmed a heightened pretransplant immune status with the potential to prime the immune system of NASH+ patients toward the pro-inflammatory and pro-apoptotic responses seen in their histopathology. Again, we found the strongest association among the female Hispanics. We found several key NASH-related cytokines that were upregulated pretransplant, including the hallmark innate-to-adaptive switching cytokines: IL-1b, IL-7, and IL-17A. Prereperfusion portal blood from female Hispanic NASH LT recipients contained chemokines responsible for immune cell infiltration including CCL2, CCL11, CCL22, and CXCL10, as well as additional innate-to-adaptive switching cytokines IL-12p40, sCD40L, and IL-4.

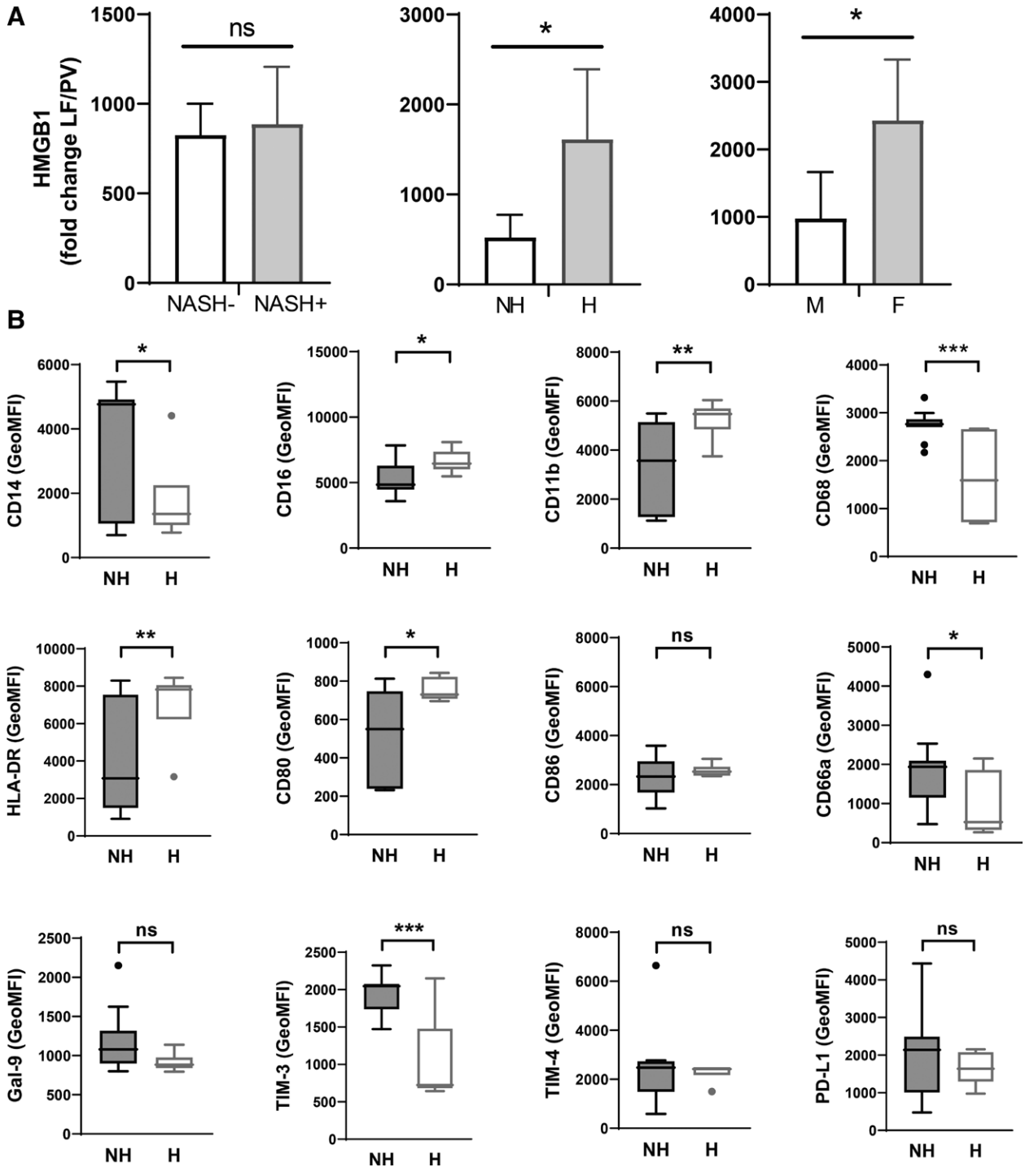

FIGURE 6. Female Hispanics with NASH have increased DAMP release and Hispanics have pro-inflammatory monocyte activation (A) paired analysis of fold change of HMGB1 levels in LF over those in PV for each patient by NASH+/–, NASH+ non-Hispanic vs Hispanic (NH vs H), or NASH+ Hispanic male vs female (M vs F) status. Data are presented as Tukey box-and-whisker plots: Whiskers are inner fences reaching 1.5 times the interquartile range, and boxes represent the interquartile ranges; dots indicate outlying values, and lines represent median values. *P < 0.05 by 2-way ANOVA with Sidak's multiple comparisons test was used to determine differences among time and/or patient status. B, Stimulated monocytes were multiplex stained with a panel of directly labeled antibodies and run on a flow cytometer to determine surface expression levels of CD14, CD16, CD11b, CD68, HLA-DR, CD80, CD86, CD66a, Gal-9, TIM-3, TIM-4, and PD-L1. GeoMFI for the respective surface marker on cells stimulated by specific patient groups are shown: NASH– vs NASH+ recipients (n = 146; 116 NASH–, 30 NASH+), non-Hispanic vs Hispanic NASH+ recipients (n = 30; 15 NH, 15 H), or (C) male vs female Hispanic NASH+ recipients (n = 15; 3 M, 12 F). Data are presented as Tukey box-and-whisker plots: Whiskers are inner fences reaching 1.5 times the interquartile range, and boxes represent the interquartile ranges; dots indicate outlying values, and lines represent median values. *P < 0.05; **P < 0.01; ***P < 0.001 by Student t-test. ANOVA, analysis of variance; DAMP, damage-associated molecular pattern; LF, liver flush; NASH, nonalcoholic steatohepatitis.

The innate immune response to damaged hepatocytes is a major component of NASH pathogenesis, given that damaged hepatocytes recruit inflammatory cells that promote remodeling and fibrosis. The key cells involved in this immune response include tissue-resident macrophages (Kupffer cells) and infiltrating myeloid cells that respond to cellular damage. ROS production by these cells leads to the synthesis of lipid peroxidation products, which can cause a self-perpetuating and chronic immune response.[20] Recruited CD11b+ macrophages promote lipogenesis and insulin resistance, connecting metabolic responses with inflammatory recruitment in NASH.[21] Several cytokines, chemokines, and growth factors that are increased in NASH+ female Hispanics are important in myeloid cell infiltration and activation of a pro-inflammatory phenotype, including CX3CL1,[22,23] CCL2,[24–27] GM-CSF,[28] and CXCL10,[29,30] confirming the role of inflammatory myeloid cells in these individuals. Postreperfusion LF blood from Hispanics with NASH increased the pro-inflammatory phenotype of third-party monocytes, and samples from female Hispanics with NASH were significantly more capable of activating TLR4, a PRR implicated in NASH progression[31,32] as well as TLR8, TLR9, and NOD1, PRRs with relatively few studies regarding their role in transplantation. PRR activation increases the pro-inflammatory and profibrotic cytokine IL-1b, which leads to increased DAMP release and further allograft inflammation. The TLR4/9-specific DAMP HMGB1 was significantly increased in female Hispanics requiring LT for NASH, supporting the role of this pathway in unfavorable outcomes. Unfortunately, we were unable to specifically test the effect of blood samples from female Hispanics with NASH on macrophage activation and polarization because of the limited numbers; therefore, mechanistic studies to polarize and skew macrophages are imperative when large numbers of patient samples are available for testing.

Hepatic IL-12 is linked to the development of steatosis and NAFLD through NKT cell depletion and promotion of Th1-associated cytokine production.[33] We found the highest levels of IL-12p40 in the portal blood of female Hispanics with NASH, who also showed signs of steatosis in their postreperfusion allografts. Paired with reduced IL-12p40 in patient LF samples immediately following reperfusion, it is tempting to speculate that this cytokine remained in these allografts to promote posttransplant NAFLD/NASH recurrence.

Several factors orchestrate the switch from the innate immune response to an adaptive program. The liver is an important site for primary T-cell activation, which normally provides an environment that is biased toward tolerance. IRI may represent a situation in which tolerance is escaped and immunological activation becomes pathogenic. IL-15 increases the frequency of $CXCR6^{+}PD1^{high}$ $CD8^{+}$ T cells and is associated with increased liver damage.[34,35] However, the major contributor to long-term chronic allograft damage is $CD4^{+}$ effector T cells, and many of the soluble mediators we found to be increased in female Hispanics with NASH can profoundly impact the function and survival of these cells, such as IL-7, IL-17A, and sCD40L.[36–43] Taken together, female Hispanics with NASH have a heightened innate immune response that quickly switches to an adaptive immune response upon transplantation, with the capacity to drive chronic inflammation-induced damage, leading to poorer outcomes.

Studies in animal models may provide insight into the enhanced immunological risk observed in female Hispanics with NASH requiring LT for survival. Female mice develop exacerbated NAFLD when housed in thermoneutral conditions owing to increased signaling via the IL-17 and TLR4 pathways,[44,45] 2 main pathways implicated in worsened outcomes in our female Hispanic patients with NASH. Immune cells also express multiple sex hormone receptors, which modulate hepatic immune responses in a sex-specific manner.[46] For example, Kupffer cells from female mice express higher levels of MyD88 and greater p38 mitogen-activated protein kinase phosphorylation and, therefore, show greater activation following lipopolysaccharide challenge than macrophages from male mice.[47]

Given the heterogeneity of NAFLD/NASH, evidence-based tailored clinical care is crucial to reduce the burden of the NASH epidemic. Additional studies are needed to understand which elements, recipient endotype and/or donor quality, are most influential on transplant outcome. Proper consideration of sex, age, size, hormonal status, and sociocultural gender differences will allow for better appreciation of differences in therapeutic targets and treatment responses and will aid in achieving precision medicine for this high-risk population. We found that reduced quality organs coupled with heightened recipient immune status combined in female Hispanics with NASH produced worsen outcomes compared with other demographic groups undergoing LT at our center. Therefore, the reduction of immunological risk factors, such as specific pro-inflammatory cytokines or cellular subsets, should be carefully considered to achieve personalized medicine for this immunological endotype of female Hispanics with NASH requiring LT.

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