

Correlation of Immune Markers With Outcomes in Biliary Atresia Following Intravenous Immunoglobulin Therapy

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Biliary atresia is a progressive fibroinflammatory cholangiopathy of infancy that is associated with activation of innate and adaptive immune responses targeting bile ducts. A recently completed multicenter phase I/IIA trial of intravenous immunoglobulin in biliary atresia did not improve serum total bilirubin levels at 90 days after hepatoportoenterostomy or survival with the native liver at 1 year. A mechanistic aim of this trial was to determine if the peripheral blood immunophenotype was associated with clinical outcomes. Flow cytometry of peripheral blood cell markers (natural killer [NK], macrophage subsets, T- and B-cell subsets, regulatory T cells), neutrophils, and activation markers (clusters of differentiation [CD]38, CD69, CD86, human leukocyte antigen-DR isotype [HLA-DR]) was performed on 29 patients with biliary atresia at baseline and at 60, 90, 180, and 360 days after hepatoportoenterostomy. Plasma cytokines and neutrophil products were also measured. Spearman correlations of change of an immune marker from baseline to day 90 with change in serum bilirubin revealed that an increase in total bilirubin correlated with 1) increased percentage of HLA-DR⁺CD38⁺ NK cells and expression of NK cell activation markers CD69 and HLA-DR, 2) decreased percentage of regulatory T cells, and 3) increased interleukin (IL)-8 and associated neutrophil products (elastase and neutrophil extracellular traps). Cox modeling revealed that the change from baseline to day 60 of the percentage of HLA-DR⁺CD38⁺ NK cells and plasma IL-8 levels was associated with an increased risk of transplant or death by day 360. *Conclusion:* Poor outcomes in biliary atresia correlated with higher peripheral blood NK cells and IL-8 and lower regulatory T cells. Future studies should include immunotherapies targeting these pathways in order to protect the biliary tree from ongoing damage. (*Hepatology Communications* 2019;3:685-696).

Biliary atresia (BA) is a progressive fibroinflammatory cholangiopathy of infancy that results in obstruction of the biliary tree within 3-4 months of age. If no therapy is implemented, portal hypertension and end-stage liver disease ensue, leaving liver transplantation as the only therapeutic option for

long-term survival. Hepatoportoenterostomy (HPE) is the operative procedure used currently to improve bile drainage in infants with BA.⁽¹⁾ Although prompt diagnosis and surgical intervention may restore bile flow, progression to end-stage liver disease occurs in almost 80% of patients by age 20 years, with over

Abbreviations: BA, biliary atresia; CD, clusters of differentiation; CI, confidence interval; CS&T, cytometer setup and tracking; FACS, fluorescent activated cell sorted; FBS, fetal bovine serum; Foxp3, forkhead box P3; HLA-DR, human leukocyte antigen-DR isotype; HPE, hepatoportoenterostomy; HR, hazard ratio; IFN, interferon; IL, interleukin; IQR, interquartile range; IVIg, intravenous immunoglobulin; MFI, mean fluorescent intensity; MPO, myeloperoxidase; NET, neutrophil extracellular trap; NK, natural killer cells; O.D., optical density; PBMC, peripheral blood mononuclear cell; PRIME, Safety Study of Intravenous Immunoglobulin Post-Portoenterostomy in Infants With Biliary Atresia; Th, T helper; TNF, tumor necrosis factor; Treg, regulatory T cell.

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50% of the patients requiring liver transplantation by 2 years of age.⁽²⁾

The biological basis for the progression of liver disease after HPE is not fully understood, but the presence of inflammation and proinflammatory cytokines in the liver and bile ducts at the time of diagnosis suggests that the host immune response, at least in part, mediates the progressive injury. Both innate and adaptive immune responses have been implicated in the pathogenesis of bile duct injury in BA.⁽³⁻²²⁾ Given these observations, the immunosuppressant intravenous immunoglobulin (IVIg) was recently tested in a phase I/IIa trial following HPE in infants with BA (Safety Study of Intravenous Immunoglobulin Post-Portoenterostomy in Infants With Biliary Atresia [PRIME] study).⁽²³⁾ IVIg has been used in a number of immune-mediated and autoimmune diseases to attenuate the inflammatory response and reduce disease severity,⁽²⁴⁻²⁹⁾ including in the mouse model of BA.⁽³⁰⁾ IVIg has a multitude of effects on the immune system, including inhibition of T-cell activation, antibody and cytokine production, dendritic cell maturation, natural killer (NK) cell trafficking, and neutrophil function.^(28,29) Furthermore, IVIg is associated with expansion and activation of anti-inflammatory regulatory T cells (Tregs).⁽²⁹⁾

In the PRIME study, IVIg was administered to 29 infants with BA at 3-5, 30, and 60 days post-HPE.

In comparison to a historical cohort, IVIg therapy did not improve outcome in BA based on the outcome measures of total serum bilirubin levels of <1.5 mg/dL at 90 days post-HPE and transplant-free survival at 360 days post-HPE.⁽²³⁾ A component of the PRIME study included immunophenotyping patients with BA over time in the setting of IVIg therapy. The aim of this study was to characterize the peripheral blood immunophenotype of patients with BA at diagnosis (baseline) and at 60, 90, 180, and 360 days post-HPE in order to determine potential changes over time in response to IVIg and correlations of specific immune markers with outcomes (serum bilirubin, transplant-free survival).

Participants and Methods

PRIME STUDY

PRIME⁽²³⁾ was a multicenter, single-arm, open-label phase I/IIa trial of IVIg therapy following HPE in infants with BA. It was conducted at eight clinical sites in the Childhood Liver Disease Research Network (ChiLDRen) funded by the National Institute of Diabetes and Digestive and Kidney Diseases (clinicaltrials.gov NCT01854827). Ethical approval was obtained at each site and at the Data

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Coordinating Center; parents or legal guardians of the infants provided written informed consent. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the appropriate institutional review committees. Enrollment began in October 2013 and ended in July 2015, with the 360-day follow-up completed in July 2016. We refer the reader to Mack et al.⁽²³⁾ for complete information on the PRIME study. Inclusion criteria included age ≤ 120 days, enrollment within 3 days of diagnosis of BA and HPE, postconception age ≥ 36 weeks, and weight $\geq 2,000$ g. Exclusion criteria included BA with extrahepatic congenital manifestations (i.e., BA splenic malformation syndrome) or another concurrent chronic condition prohibiting the use of IVIg. Participants received three infusions of 1 g/kg body weight of IVIg (Gamunex-C; Grifols, Los Angeles, CA) at days 3-5, 30, and 60 days after HPE. Routine clinical care guidelines for postoperative care included oral ursodeoxycholic acid for 360 days after HPE, trimethoprim-sulfamethoxazole for 180 days after HPE, and vitamin supplementation. Corticosteroid treatment was prohibited.

PERIPHERAL BLOOD IMMUNOPHENOTYPING

Whole blood and plasma were obtained at the time of diagnosis (baseline; prior to IVIg infusion #1), 60 days post-HPE (prior to IVIg infusion #3), and 90, 180, and 360 days post-HPE. Blood or plasma was not collected for immunophenotyping after a patient received a liver transplant. Whole blood was shipped overnight express at room temperature to the University of Colorado-Anschutz Medical Campus for Ficoll-gradient purification of peripheral blood mononuclear cells (PBMCs) and dextran-gradient purification of neutrophils according to standard protocols (BD Biosciences, San Jose, CA). PBMCs and neutrophils were frozen in 90% fetal bovine serum (FBS)-10% dimethyl sulfoxide in liquid nitrogen until the time of analysis. Plasma was aliquoted and frozen at -20°C at the local site and then shipped in batches to the Children's Hospital Colorado Clinical Translational Research Center (CTRC) Core Laboratory for analysis. The specific peripheral blood immune cells and plasma cytokines analyzed were chosen based on those immune markers described in the literature to be associated with BA or known to be altered by IVIg.

Fluorescence-Activated Cell Sorting Analysis

Fluorescence-activated cell sorting (FACS) analysis was performed by the ClinImmune Laboratories Flow Cytometry Core, University of Colorado-Anschutz Medical Campus, according to standard protocol. All samples were processed over a 2-week time period using identical conditions for staining and FACS analysis. Cryopreserved PBMCs were thawed in complete media (1% phosphosilicate glass, 10% FBS, 89% Roswell Park Memorial Institute media), washed with staining buffer (phosphate-buffered saline containing 2% FBS), and $>1.0 \times 10^6$ cells were surfaced stained with PBMC markers, including NK cell subsets (clusters of differentiation [CD]3, CD56), macrophage subsets (CD14, CD16, CD11b), T-cell subsets (CD3, CD4, CD8), B cells (CD19), activation markers on macrophages, B cells and T cells (CD38, CD69, CD86, CD32, interferon [IFN]- γ R1 [CD119], human leukocyte antigen-DR isotype [HLA-DR]); Tregs (CD3, CD4, CD25, CD127, and forkhead box P3 [Foxp3]); and neutrophil markers (CD66b, CD15, CD14; activation markers CD62L, chemokine (C-X-C motif) receptor 4 [CXCR4], CD54). Cells were fixed with 1% paraformaldehyde and analyzed using a Canto II cytometer (BD Immunocytometry Systems). Similarly, for the Treg panel, cells were washed, fixed, surface stained, permeabilized (eBioscience), and then intracellularly stained with Foxp3, washed and resuspended in staining buffer, and analyzed using an LSR II cytometer (BD Immunocytometry Systems). Fluorescence minus one (FMO) or isotype controls were used in all experiments.

Between 0.25 million and 0.5 million events were collected. Electronic compensation was performed with antibody capture beads (BD Biosciences) stained separately with individual monoclonal antibodies used in the test samples. To ensure the accuracy and precision of the measurements taken from day to day, quality control was performed daily on the LSR-II using the cytometer setup and tracking (CS&T) feature within BD FACSDiva software. The program uses standardized CS&T beads (BD Biosciences) to determine voltage, laser delays, and area scaling and to track these settings over time. A manual quality control using rainbow beads was also performed daily to verify the laser delay and area scaling determined by the CS&T.

The data files were analyzed using Diva software (BD Biosciences) and FlowJo Software (Treestar Inc.). Acquired data were analyzed using FlowJo software using appropriate templates created for each of the panels. Gates were adjusted on a patient-specific basis using the FMO or isotype controls. The percentage of each cell type was determined and reported as a percentage of the parent cell type. For studies pertaining to activation markers, the mean fluorescent intensity (MFI) of the activation marker on the particular cell type was recorded. The frequencies and MFI for each gated population was exported into an excel spreadsheet for additional statistical analysis. The gating strategy for FACS analysis is provided in Supporting Fig. S1.

Plasma Protein Analysis

Quantification of cytokines was performed by the Children's Hospital Colorado CTSC Core Laboratory using the Luminex T helper (Th)1/Th2 multiplex assay (interleukin [IL]-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor, IFN- γ , tumor necrosis factor [TNF]- α) and the IL-17 enzyme-linked immunosorbent assay (Thermo Fisher Scientific, Waltham, MA) according to standard protocols. Quantification of neutrophil function was performed with assays available from Cayman Chemical (Ann Arbor, MI) for plasma elastase, myeloperoxidase (MPO), and neutrophil extracellular traps (NETs) according to protocols. Both positive and negative controls were performed with each assay, and individual samples were analyzed in duplicate.

CLINICAL OUTCOMES

In this study, measures of interest included the PBMC and neutrophil subsets, cell activation markers, plasma cytokines and neutrophil functional assays (detailed above), and clinical outcomes as defined by 1) changes in total bilirubin at 90 days and 180 days and 2) liver transplant or death. Considering these two endpoints together, a composite clinical outcome was defined as follows: If a patient had no liver transplant/death and serum bilirubin was <1.5 mg/dL at 360 days post-HPE, then the patient was grouped into the Good Outcome group; otherwise, if a patient had liver transplant/

death or serum bilirubin persisting above 1.5 mg/dL up to 360 days post-HPE, then the patient was grouped into the Poor Outcome group. Total bilirubin was determined directly using standard laboratory methods or calculated by the addition of direct plus indirect bilirubin.⁽³¹⁾ Total bilirubin levels after transplant were not used for the analysis.

STATISTICAL ANALYSIS

The analysis population is the modified intention-to-treat (mITT) population; all PRIME participants who maintained eligibility and received at least 80% of one dose of IVIg were considered. Participants with missing immune markers were excluded from the given analyses. If total bilirubin levels were missing at 360 days, good bile drainage was imputed if the total bilirubin value was <1.5 mg/dL at 270 days, the visit immediately prior to the 360-day time point.

Changes in immune biomarkers and total bilirubin at 90 days and 180 days were calculated relative to baseline. Spearman's correlation coefficients and accompanying *P* values are presented to describe the relationship between a change in biomarker and a change in total bilirubin at 90 and 180 days. To assess the impact of changes in markers to 60 days in relation to transplant/patient death, Cox proportional hazards models were fitted among the patients who survived up to 60 days (hence, completed all three doses of IVIg). Logistic regression was used to assess the dichotomous Poor Outcome at 360 days. Two models were assessed for each biomarker, one modeling Poor Outcome associated with a change from baseline to 60 days post-HPE and the second modeling Poor Outcome associated with a change from baseline to 90 days post-HPE. All analyses were performed using SAS version 9.3 (SAS Institute Inc.).

Results

PATIENT COHORT

There were 29 participants in the PRIME study; 25 of these received all three IVIg infusions and 4 received only two infusions. The mITT population had a mean \pm SD age at HPE of 60 \pm 19 days.

There were more female (62%) than male participants, and the population was predominantly white and non-Hispanic (Table 1). Serum total bilirubin at baseline was 8.3 ± 3.4 mg/dL (range, 3-17 mg/dL); bilirubin levels at all time points are shown in Table 2. Forty-one percent of participants had a liver transplant by 360 days post-HPE (median time to transplant, 149.5 days post-HPE), and 1 individual died after the transplant. As detailed in the recently published PRIME study,⁽²³⁾ there was no significant increase in the proportion of IVIg participants with a serum total bilirubin <1.5 mg/dL at 90, 180, or 360 days post-HPE compared to a historical placebo-arm group (Table 2). Survival with the native liver in the IVIg participants showed no significant benefit over that of the historical

placebo-arm participants, with a difference at 360 days of -11.9% .⁽²³⁾ There were 17 participants (59%) in the Good Outcome group and 12 participants (41%) in the Poor Outcome group.

ACTIVATED NK CELLS, DECREASED Tregs, AND HIGH IL-8 AND NEUTROPHIL ACTIVATION PRODUCTS CORRELATE WITH CHANGE IN SERUM BILIRUBIN

Quantification of immune markers was performed at baseline and at 60, 90, 180, and 360 days post-HPE. Statistical analyses focused on changes in immune marker values relative to changes in serum total bilirubin in the first 90 days post-HPE based on the fact that this time period overlaps with the time frame of IVIg infusions. Furthermore, 90 days post-HPE has been shown to be a useful time point to analyze the strength of a biomarker (e.g., bilirubin) in predicting short-term outcome in BA.⁽³²⁾ Spearman correlation coefficient analyses revealed that the percentage of NK cells co-expressing activation markers HLA-DR and CD38 (HLADR⁺CD38⁺NK cells) as well as NK cell CD69 (activation marker) and HLA-DR levels from baseline to 90 days post-HPE positively correlated with a change in bilirubin over the same period (Fig. 1). In contrast, the change in the percentage of anti-inflammatory Tregs (CD3⁺4⁺25⁺Foxp3^{hi}CD127^{low}) from baseline to 90 days post-HPE negatively correlated with a change in serum bilirubin.

IL-8 (chemokine [C-X-C motif] ligand 8 [CXCL8]) is a chemokine that recruits neutrophils

TABLE 1. PATIENT COHORT⁽²³⁾

Baseline demographics (n = 29)	Value
Age at HPE, days (mean \pm SD)	60 \pm 19
Age at HPE, n (%)	
\leq 30 days	2 (7%)
>30 to \leq 45 days	5 (17%)
>45 to \leq 60 days	7 (24%)
>60 to \leq 90 days	14 (48%)
>90 to \leq 120 days	1 (3%)
Female, n (%)	18 (62%)
Race, n (%)	
White/Caucasian	22 (76%)
Black/African American	3 (10%)
Asian	2 (7%)
Refused/not reported	2 (7%)
Ethnicity, n (%)	
Hispanic	9 (31%)
Non-Hispanic	20 (69%)

TABLE 2. PATIENT OUTCOMES

	Baseline	Day 60	Day 90	Day 180	Day 360
Total bilirubin, mg/dL					
n	29	26	27	21	10
Mean (SD)	8.3 (3.4)	6.0 (7.5)	6.0 (7.0)	3.7 (6.4)	2.1 (3.9)
Median (IQR)	7.7 (6, 10)	3.3 (0.9, 9.7)	2.3 (0.6, 12.5)	0.9 (0.3, 2.4)	0.65 (0.3, 2.0)
Minimum, maximum	3, 17	0.4, 34.9	0.2, 21.4	0.2, 25.4	0.18, 12.9
Patient outcomes over time (n = 29)					
Transplant/deaths, n (%)	0 (0%)	2 (6.9%)	5 (17.2%)	9 (31.0%)	12 (41.4%)
Alive with native liver:					
Bilirubin <1.5 mg/dL, n	0	9	11	14	7
Bilirubin ≥ 1.5 mg/dL, n	29	16	13	5	3

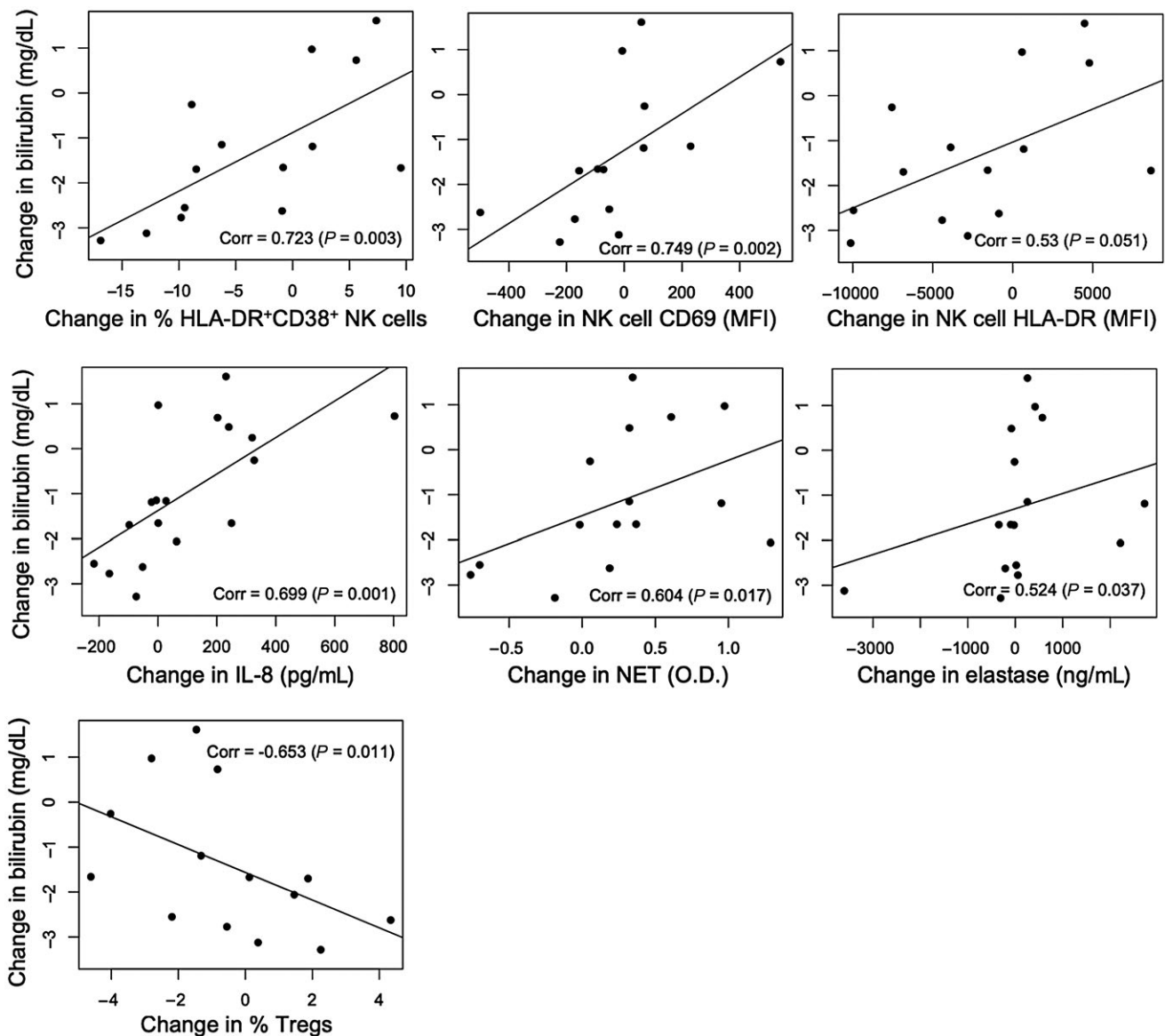


FIG. 1. Spearman correlation of change in immune markers with change in bilirubin from baseline to 90 days post-HPE. Shown here are significant positive correlations of change in bilirubin with change in activated NK cells, IL-8, and neutrophil byproducts and a negative correlation of change in bilirubin with change in Tregs.

and other leukocytes to sites of tissue injury. IL-8 signals the initiation of the oxidative burst in activated neutrophils, resulting in increased production of reactive oxygen species and proteolytic enzymes, such as elastase and MPO.⁽³³⁾ Furthermore, a byproduct of neutrophil activation is the formation of NETs that have been shown to have both antimicrobial properties as well as the potential for stimulating inflammatory and autoimmune responses.⁽³⁴⁻³⁷⁾ The change in IL-8, elastase, and NETs from baseline to day 90

post-HPE positively correlated with a change in bilirubin (Table 3; $P < 0.05$). Individual patient data of the changes in a specific immune marker with the change in bilirubin at 90 days post-HPE are displayed in Fig. 1; these data are fitted with a regression line to show the linear trends over time. Descriptive statistics of the immune markers that were found to correlate with outcome are shown in Supporting Table S1. Descriptive statistics of baseline values of all immune markers analyzed are shown in Supporting Table S2.

TABLE 3. SPEARMAN CORRELATION BETWEEN CHANGE IN IMMUNE MARKER AND CHANGE IN BILIRUBIN FROM BASELINE TO 90 DAYS POST-HPE AND 180 DAYS POST-HPE

	Change From Baseline to Day 90			Change From Baseline to Day 180		
	Median (IQR)	Correlation With Bilirubin Change	P Value	Median (IQR)	Correlation With Bilirubin Change	P Value
Total bilirubin	-1.66 (-2.55, 0.25)			-1.96 (-2.85, -1.14)		
% HLADR ⁺ CD38 ⁺ NK	-0.92 (-9.49, 1.75)	0.723	0.003	-2.67 (-10.39, 8)	0.198	0.497
NK cells: CD69 MFI	-52 (-156, 59)	0.749	0.002	-43.5 (-124, 46)	0.704	0.005
NK cells: HLADR MFI	-1,567 (-6,839, 682)	0.530	0.051	-1,793 (-5,474, 2,069)	0.316	0.292
% Tregs	-0.69 (-2.19, 1.46)	-0.653	0.011	0.43 (-1.18, 1.82)	-0.716	0.004
IL-8 (pg/mL)	-27.7 (-51.8, 240.6)	0.699	0.001	-5.2 (-71.2, 39.6)	0.583	0.018
NET (O.D.)	0.32 (-0.02, 0.61)	0.604	0.0172	0.13 (-0.55, 0.51)	0	1.00
Elastase (ng/mL)	1.44 (-152.9, 337.5)	0.524	0.037	109.83 (-99.3, 247.12)	0.602	0.029

Only four cytokines were consistently detectable in the sera at all time points (IL-4, IL-6, IL-8, and TNF- α).

NK cell CD69 expression, Treg, IL-8, and elastase correlations with change in bilirubin persisted at 180 days post-HPE. It should be noted that these analyses did not include 2 patients who received a transplant before 90 days and 8 patients who received a transplant before 180 days. It is possible that the correlation may have been stronger if these patients had been included. The other immune markers analyzed did not correlate with a change in bilirubin at 90 or 180 days post-HPE (data not shown). In summary, NK cell activation, Treg deficiency, and high levels of IL-8 were associated with increases in elastase and NETs and correlated with rising bilirubin. This suggests that these immune pathways may contribute to bile duct injury in BA.

ACTIVATED NK CELLS AND IL-8 ASSOCIATED WITH NEED FOR LIVER TRANSPLANT

We then asked the question as to whether or not the immune biomarkers that correlated with the change in bilirubin levels would also be associated with the risk for liver transplant/death by 360 days post-HPE. All immune markers were analyzed over time based on the patient outcome group. The Good Outcome group had serum bilirubin <1.5 mg/dL and no liver transplant/death, and the Poor Outcome group had serum bilirubin \geq 1.5 mg/dL or liver transplant/death. This revealed trends in changes over time, especially in the first 90 days post-HPE; however, there was no significant difference in the actual value of the immune marker at each time point between

groups (Fig. 2). Cox modeling for the risk of transplant/death at 360 days post-HPE was performed, demonstrating the hazard ratios (HRs) for the risk of transplant/death in relation to the immune biomarker change from baseline to 60 days post-HPE. This analysis revealed that an increase in the percentage of HLA-DR⁺CD38⁺ NK cells (HR, 1.15; 95% confidence interval [CI], 1.00-1.32; $P = 0.055$) and in plasma IL-8 levels (HR, 1.71; 95% CI, 1.24-2.36; $P = 0.001$) at 60 days post-HPE was significantly associated with an increased risk of transplant/death by 360 days post-HPE (Table 4). In addition, Cox modeling with the actual IL-8 level at 60 and 90 days post-HPE as a predictor also indicated that IL-8 was the strongest marker associated with outcome. The actual IL-8 level at 60 and 90 days post-HPE predicted survival with the native liver at 360 days, after adjusting for baseline level (60 days: HR, 1.57; 95% CI, 1.12-2.21; $P = 0.009$; 90 days: HR, 1.59; 95% CI, 1.03-2.47; $P = 0.037$). Logistic regression modeling revealed trends toward a marginal significance in the percentage of Treg change from baseline to 60 days post-HPE in the Good Outcome group (odds ratio [OR], 0.31; 95% CI, 0.09-1.12; $P = 0.074$) and a change from baseline to 90 days post-HPE in NK cell CD69 expression (OR, 7.87; 95% CI, 0.69-90.04; $P = 0.097$).

Discussion

This study provides comprehensive immunophenotyping in BA, identifying significant associations of specific immune markers with short-term outcomes.

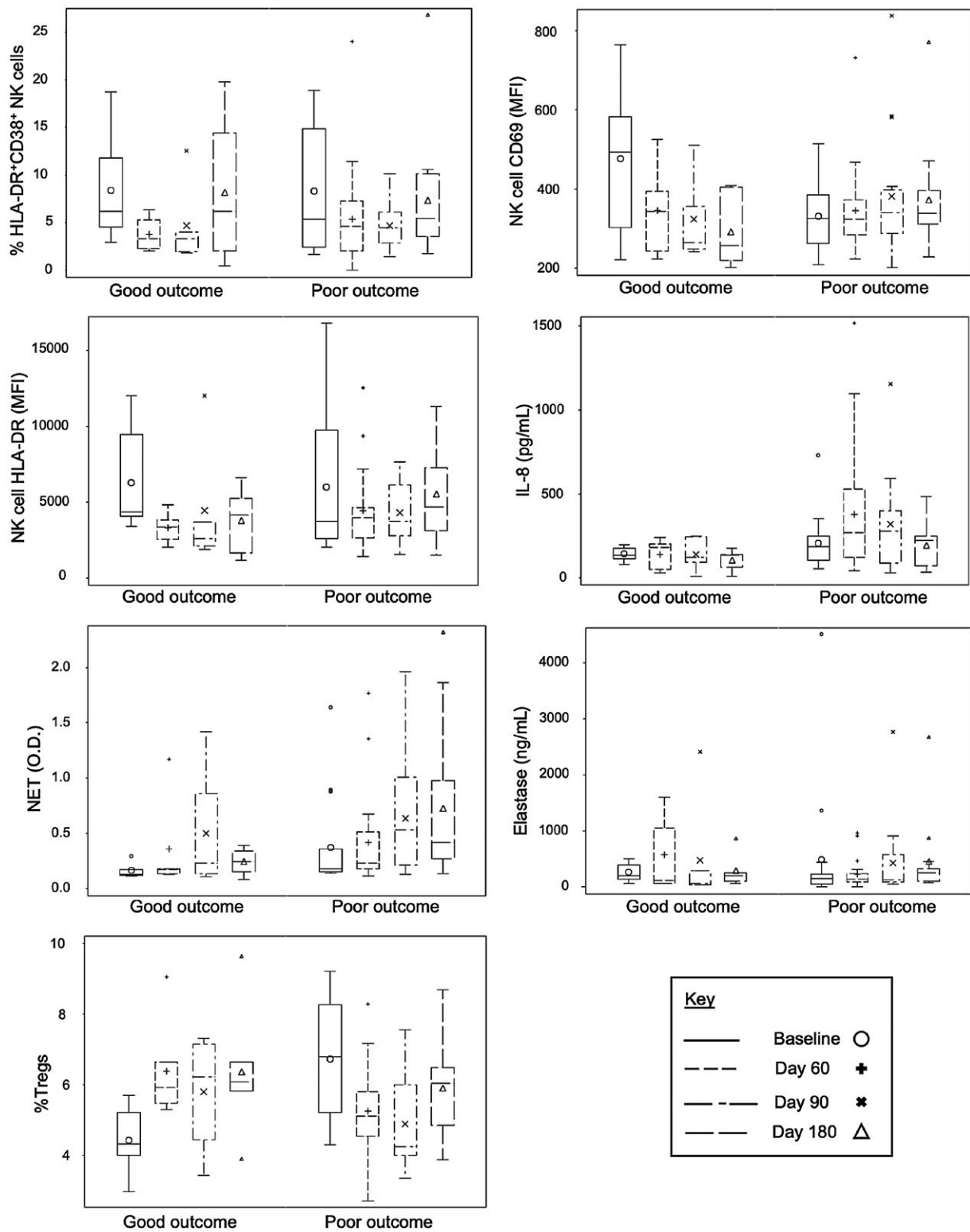


FIG. 2. Immune marker levels over time in Good Outcome and Poor Outcome groups. Box plots of specific immune markers in the Good Outcome group (bilirubin <1.5 mg/dL and no liver transplant/death; n = 17) and the Poor Outcome group (bilirubin ≥1.5 mg/dL or liver transplant/death; n = 12) over time. Shown is the mean (symbol), median (dash), IQR (box), 1.5 × IQR (whiskers).

TABLE 4. COX MODEL HRs FOR LIVER TRANSPLANT IN RELATION TO IMMUNE MARKER CHANGE FROM BASELINE TO 60 DAYS POST-HPE

Biomarker	HR* (95% CI)	P Value
% HLADR ⁺ CD38 ⁺ NK cells	1.15 (1.00, 1.32)	0.055
NK cells: CD69 (per 100 MFI)	1.4 (0.79, 2.47)	0.246
NK cells: HLADR (per 100 MFI)	1.004 (0.99, 1.02)	0.686
% Tregs	0.81 (0.58, 1.12)	0.196
IL-8 (pg/mL)	1.71 (1.24, 2.36)	0.001
NET (O.D.)	2.79 (0.6, 12.95)	0.191
Elastase (ng/mL)	1.00 (1.00, 1.00)	0.205

*Cox regression model was used, conditional on survival up to day 60.

Despite the anti-inflammatory effects of IVIg in the first 90 days post-HPE, activated NK cells, high IL-8 and neutrophil byproducts, and decreased Tregs directly correlated with a poor outcome based on rising bilirubin and/or need for liver transplant. A plausible mechanism as to how these immune cells interact with each other, resulting in worsening biliary disease, is outlined in Fig. 3. Previous investigations in patients with BA and in the rotavirus-induced mouse model of BA (murine BA) have shown decreased number and function of Tregs.⁽¹⁹⁻²²⁾ Here, we show for the first time that Treg deficiencies correlate with rising bilirubin levels and the need for liver transplant, suggesting that the lack of Treg control of inflammation results in

increased bile duct damage. Downstream effects of the diminished regulation of inflammation include activation of NK cells and increased production of IL-8. Similar to Tregs, there is abundant literature supporting the contribution of NK cells to biliary disease in both human and murine BA.^(5,18,19) Our study is the first to show that activated NK cells circulating in peripheral blood are associated with worse clinical outcome.

IL-8 is produced by multiple immune cells, including macrophages and NK cells.⁽³³⁾ The main function of IL-8 is to act as a chemokine and promote neutrophil activation and migration to sites of inflammation (i.e., liver). Our finding of elevated IL-8 as an immune marker for poor outcome is supported by studies showing that liver IL-8 levels correlated with liver inflammation, fibrosis, and portal hypertension in BA.⁽³⁸⁻⁴⁰⁾ Activated neutrophils produce potent byproducts that can directly or indirectly damage cells and include elastase, MPO, and NETs.⁽³⁴⁻³⁷⁾ IL-8-activated neutrophils have also been shown to promote fibrosis through activation of stellate cells. Activated hepatic stellate cells (myofibroblasts) are a major source of extracellular matrix production leading to progressive liver fibrosis (Fig. 3).^(41,42) There is a paucity of data on the role of neutrophils in bile duct injury in BA that warrants further research based on the findings that elastase and NETs correlated with rising bilirubin post-HPE. The small number of patients with BA analyzed in this study prohibits us

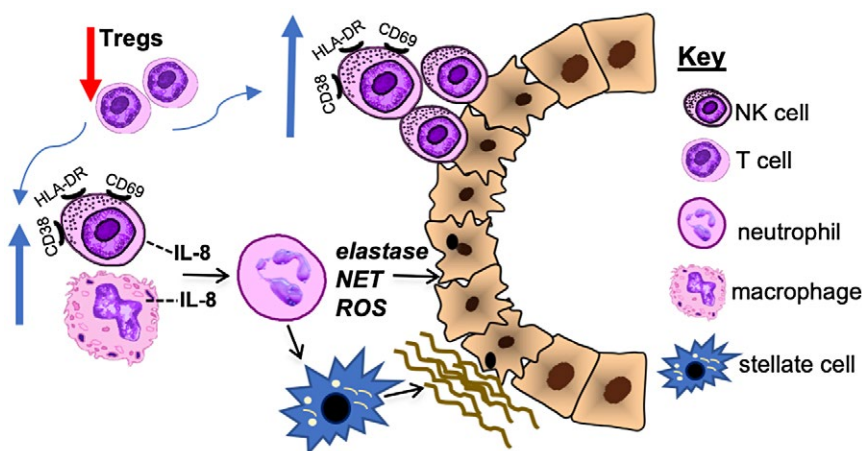


FIG. 3. Proposed contribution of immune markers to bile duct injury in BA. Decreased Tregs leads to increased NK cell activation that directly damages biliary epithelia. In addition, NK cells and macrophages produce IL-8, resulting in neutrophil activation and trafficking to the liver. Neutrophils release elastase and NETs that further damage epithelia as well as activate hepatic stellate cells, resulting in fibrogenesis. Abbreviation: ROS, reactive oxygen species.

from making any broad conclusions regarding the use of IL-8 as a biomarker of outcome in BA, and future studies should be appropriately powered in order to determine the accuracy of IL-8 as a biomarker of severity of disease. Currently, the only validated biomarker of outcome is the total bilirubin level at 3 months post-HPE.⁽³²⁾

There were several limitations to this study. Most importantly, immunophenotyping was performed only on patients with BA who had received IVIg within 60 days post-HPE. Whether or not IVIg had a direct effect on altering a patient's immunophenotype is unclear. However, based on the fact that there was no overall improvement in bilirubin at 90 days post-HPE or in survival with the native liver compared to historical controls,⁽²³⁾ it is doubtful that IVIg had any positive impact on disease progression or severity.

Second, because there were no control groups of other age-matched liver diseases or normal infants, we were unable to definitively answer the question of whether or not the observed BA immune marker levels differed from controls. However, a review of the literature identified multiple studies that reported levels of IL-8, elastase, and Tregs in healthy infants. Published literature on normal values of NK cell activation markers and NETs in infants was not found. The mean IL-8 level of normal infants in the first week of life is ~13-17 pg/mL (range, 0.4-50) and remains at this low level throughout childhood.⁽⁴³⁻⁴⁵⁾ These published normal IL-8 levels are much lower than the observed IL-8 levels in patients with BA at all time points (means ranged from 163 pg/mL to 328 pg/mL; see Supporting Table S1). The serum elastase levels of normal infants age 5 ± 0.65 months old was 64.1 ± 12.9 ng/mL.⁽⁴⁶⁾ In our patients with BA, the serum elastase mean ranged from 300 ng/mL to 435.5 ng/mL at all time points (Supporting Table S1). The almost 10-fold increase in IL-8 and elastase in patients with BA compared to historical normal controls warrants future research with appropriate comparison groups (age-matched, other liver diseases, healthy children). Treg quantities in normal infants are similar to adults by 1 week of age, and Treg levels reported in normal infants are similar to those described here in BA.⁽⁴⁷⁾ Despite similar levels of Tregs compared to historical controls, one could argue that Tregs should be higher than normal in the inflammatory state of BA; future research should focus on determining if Treg function is abnormal in BA.

Third, the immunophenotype in the peripheral blood may or may not reflect the inflammatory environment within the liver. Interestingly, published investigations on liver-specific inflammation in BA have described increased and activated NK cells,^(5,17-19) decreased Tregs,^(19,21,22,48) and increased IL-8^(39,40) in the liver and/or biliary remnant in human and murine BA, mirroring our findings in peripheral blood. This study provides further evidence that many of the immune pathways that are altered at the time of diagnosis persist post-HPE and may contribute to ongoing intrahepatic biliary injury and fibrosis. Inflammatory molecules associated with robust organ-specific inflammation can "spill over" into the peripheral blood, altering the immunophenotype.^(49,50) However, other immune markers identified as up-regulated in the liver of patients with BA, such as the Th1 cytokines^(4,6-9) IL-2, IFN- γ , and TNF- α , as well as IL-17,⁽⁵¹⁾ were undetectable/low in the serum of patients with BA. This may be related to the level of assay detection for these cytokines or an effect from the IVIg. Future studies should determine correlations of serum and liver immune profiles to understand the significance of these inflammatory pathways over time.

In summary, loss of adequate regulation of inflammation and exaggerated NK cell, IL-8, and neutrophil responses were associated with poor short-term outcomes in BA. Future research should focus on these immune pathways in patients with BA in the absence of IVIg treatment and in comparison to control groups in order to determine the specific contributions of these immune markers to bile duct injury and fibrosis. Determination of key immune markers associated with poor outcome is necessary in order to develop targets for immunotherapy.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1332/supinfo.