

Increased expression of programmed cell death ligand 1 and galectin 9 in transplant recipients who achieved tolerance after immunosuppression withdrawal.

(免疫抑制剤中止後免疫寛容を達成した肝移植後レシピエントにおける programmed cell death ligand 1 と galectin 9 の高発現)

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Increased Expressions of Programmed Death Ligand 1 and Galectin 9 in Transplant Recipients Who Achieved Tolerance After Immunosuppression Withdrawal

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Programmed death 1 (PD1)/its ligand PD-L1 concomitant with T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3)/its ligand galectin 9 (Gal-9) and the forkhead box P3 (FOXP3) might be involved in tolerance after liver transplantation (LT). Liver biopsies from 38 tolerant, 19 nontolerant (including 16 samples that triggered reintroduction of immunosuppression [IS] and 19 samples after IS reintroduction), and 38 control LT patients were studied. The expressions of PD1, PD-L1, Gal-9, and FOXP3 were determined by immunohistochemical and immunofluorescence (IF) staining. The success period of IS withdrawal was calculated using Kaplan-Meier curve analysis. Tolerant and control patients exhibited higher PD-L1, Gal-9, and FOXP3 levels than nontolerant patients at the moment of triggering IS reintroduction. High expressions of PD-L1 and Gal-9 were associated with prolonged success of tolerance (83.3% versus 36.7% [$P < 0.01$] and 73.1% versus 42.9% [$P = 0.03$]). A strong correlation between PD-L1 and Gal-9 expression levels was detected (Spearman $r = 0.73$; $P \leq 0.001$), and IF demonstrated colocalization of PD-L1 and Gal-9 in the cytoplasm of hepatocytes. In conclusion, the present study demonstrated that increased expressions of PD-L1 and Gal-9 were associated with sustained tolerance after IS withdrawal in pediatric liver transplantation.

Liver Transplantation 0 1–12 2021 AASLD.

Received August 21, 2021; accepted October 6, 2021.

Immunosuppression (IS) has been extensively studied for the maintenance of durable graft survival and the prevention of allograft rejection after liver

transplantation (LT). However, the conventional IS regimen induces substantial long-term mortality and morbidity, such as cardiovascular disease, infection, and de novo neoplasm.^(1–4) Therefore, IS withdrawal (ISW) represents the ideal goal after LT, which allows patients to avoid the deleterious adverse effects. Given the tolerogenic characteristics of the liver, a clinical trial of ISW in LT recipients at Kyoto University Hospital identified a proportion of 38.1% patients as having

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; CC, cell counts; ELISA, enzyme-linked immunosorbent assay; FFPE, formalin-

successful ISW.⁽⁵⁾ Other institutions emphasized that ISW can be safely achieved with long-lasting normal graft function and with a successful feasibility of 31% to 63%.⁽⁶⁻¹⁰⁾ In these studies, the main endpoint of ISW was “tolerance,” defined by the absence of rejection accompanied by a normal liver function test and normal histological findings after IS discontinuation. However, its underlying mechanisms remain unclear and identification of a validated biomarker for tolerance is urgently required to identify candidates for ISW.

Programmed death 1 (PD1) is a member of the CD28 family and promotes self-tolerance by inhibiting the proliferation of cytotoxic T cells.⁽¹¹⁻¹³⁾ Its ligand PD-L1, which belongs to the B7 family, has features of a costimulatory molecule.⁽¹¹⁻¹³⁾ Numerous studies have identified the role of PD-L1 in negatively regulating T cell functions to maintain posttransplantation tolerance.^(14,15) Conversely, recent case reports acknowledged a significantly higher PD-L1 signal in graft biopsies from LT patients with recurrent hepatocellular carcinoma (HCC), who suffered fatal allograft rejection after inducing PD1 inhibitors.^(16,17) In contrast, patients without expressions of PD-L1 did not suffer from graft rejection after using PD1 inhibitors.^(18,19) Given the high rates of irreversible allograft

rejection in patients with elevated PD-L1 signals who were treated with PD1 inhibitors, the interaction between PD1 and PD-L1 is supposed to be blocked and then trigger T cell immune response, resulting in graft rejection. Thus, we hypothesize that the overexpression of PD-L1 represents a protective shield from graft rejection and therefore induces tolerance.

Galectin 9 (Gal-9), a ligand of T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), was recently considered a novel predictor of rejection after LT⁽²⁰⁾ and was proven to have a close relationship with PD-L1 expression in predicting the survival of patients with HCC.⁽²¹⁾ The trigger of TIM-3/Gal-9 coupled with targeting of the PD1/PD-L1 pathway resulted in synergistic restoration of T cell function and was more powerful in managing tumor growth than targeting either signaling pathway alone.^(22,23) In addition, a higher presence of forkhead box P3 (FOXP3) was correlated with better transplant outcomes.^(24,25) Indeed, FOXP3 bone marrow mesenchymal stem cells were proven to promote liver allograft tolerance, whereas the use of anti-CD25, a blockade of FOXP3, led to early graft loss after LT.⁽²⁶⁾ Based on the aforementioned data, we provide a new perspective in the management of IS regimens in LT patients by examining the expression levels of PD-L1, Gal-9, FOXP3, and their correlations.

fixed, paraffin-embedded; FOXP3, forkhead box P3; Gal-9, galectin 9; HCC, hepatocellular carcinoma; IF, immunofluorescence; IHC, immunohistochemistry; IQR, interquartile range; IS, immunosuppression; ISW, immunosuppression withdrawal; LT, liver transplantation; mRNA, messenger RNA; PD1, programmed death 1; PD-L1, programmed death ligand 1; PT, prothrombin time; RU, relative units; sGal-9, soluble galectin 9; sPD-L1, soluble programmed death ligand 1; TIM-3, T cell immunoglobulin and mucin domain-containing 3; Treg, regulatory T cell.

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This work was supported by The Japan Society for the Promotion of Science (JSPS), Grants-in-Aid for Scientific Research (KAKENHI) Grant 20K09006.

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DOI 10.1002/lt.26336

Potential conflict of interest: Nothing to report.

Patients and Methods

PATIENT POPULATION

From October 1990 to December 2016, 1902 cases of living donor LT were performed at Kyoto University Hospital. The patients began the ISW protocol when they satisfied the following requirements: younger than 18 years old, more than 2 years after LT, normal graft function, and no episodes of rejection or fibrosis for more than 1 year. Tolerance was defined as normal liver biopsy and a normal liver function test for more than 1 year after ISW.^(5,27) In contrast, nontolerance was demonstrated by biopsy-proven fibrosis or rejection (using the Banff criteria⁽²⁸⁾) and an elevated liver biochemistry. IS and ISW protocols have been described in detail elsewhere.⁽²⁹⁾ Indeed, 41 patients satisfied the criteria for tolerance at least once and were referred to as “tolerant patients” in this study. In 19 patients, ISW was attempted, but the criteria for tolerance were never satisfied, and they were referred to as “nontolerant patients.” A control group consisting of pediatric patients without an attempt at ISW who were maintained with a standard dose of IS

with normal liver function and liver histology was chosen to be matched to tolerant and nontolerant patients by sex, proportion of primary liver disease, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin. Details about the study population are presented in the flowchart of our approach (Fig. 1).

LIVER SPECIMENS

Specimens of liver tissues were available in 38 tolerant, 19 nontolerant, and 38 control LT patients. Liver biopsies of tolerant and control patients were assigned to group A and group C, respectively. In tolerant patients, liver biopsy samples from the time in which they first satisfied the criteria for tolerance were analyzed. In nontolerant patients, we examined 2 biopsy samples, including 1 that showed fibrosis or rejection-triggering IS reuse (group B1) and the other at a time after IS reuse when the patient was stable (group B2). Liver biopsy specimens were fixed in formalin and embedded in paraffin for routine histological diagnosis.

SERUM SAMPLES

Beginning in July 2020, we prospectively collected blood samples from patients who belonged to our liver

biopsy cohort (including tolerant, nontolerant, and control patients) during their routine follow-up examinations at Kyoto University Hospital. They were immediately immersed in melting ice and allowed to clot for 1 hour at room temperature before centrifugation at 1400g for 15 minutes to remove cells and debris. The serum layer was isolated and aliquoted into several sterile polypropylene tubes. All samples were stored at -80°C in a monitored freezer within 2 hours of withdrawal and were thawed only once prior to use.

IMMUNOHISTOCHEMICAL STAINING

Formalin-fixed, paraffin-embedded (FFPE) liver specimens were incubated with the primary antibodies mouse monoclonal anti-PD1 (Abcam, Cambridge, UK), rabbit monoclonal anti-PD-L1 (Abcam, Cambridge, UK, Novus Biologicals, USA), rabbit monoclonal anti-Gal-9 (Abcam, Cambridge, UK), mouse monoclonal anti-FOXP3 (Abcam, Cambridge, UK), rabbit monoclonal anti-CD4 (Rabbit Abcam, Cambridge, UK), and rabbit polyclonal anti-CD8 (Rabbit Abcam, Cambridge, UK). Detailed information on the staining reagents and antibodies is outlined in Supporting Table 1.

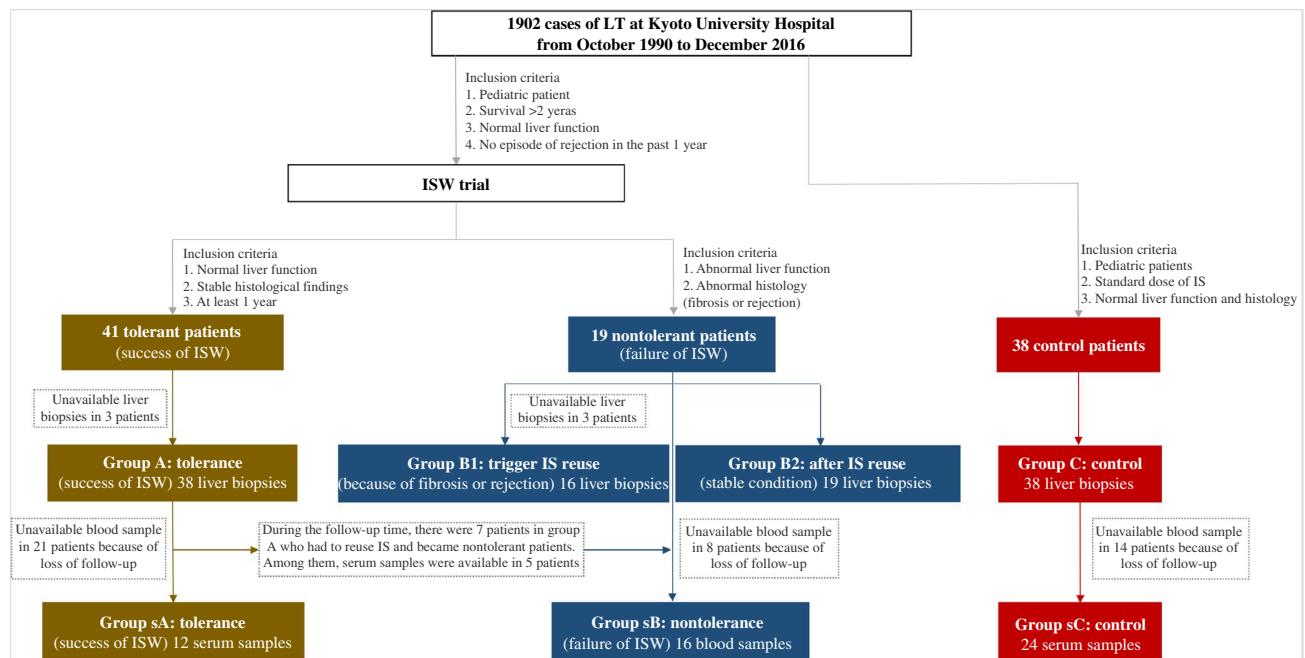


FIG. 1. Flowchart of study.

COMPUTATIONAL IMMUNOHISTOCHEMISTRY QUANTIFICATION

The PD-L1 and Gal-9 expression levels and the number of FOXP3 cells, infiltrating CD4⁺ T cells, and infiltrating CD8⁺ T cells were analyzed and calculated by ImageJ software (National Institutes of Health, Bethesda, MD; <http://imagej.nih.gov/ij/>).

IMMUNOFLUORESCENCE STAINING

FFPE liver sections that were 4- μ m-thick were stained with rat monoclonal anti-PD-L1 and rabbit monoclonal anti-Gal-9 for the detection of the colocalization of PD-L1 and Gal-9 expressions via immunofluorescence (IF).

ENZYME-LINKED IMMUNOSORBENT ASSAY

Serum levels of soluble PD-L1 (sPD-L1) and soluble Gal-9 (sGal-9) were measured using a human PD-L1 enzyme-linked immunosorbent assay (ELISA) kit 28-8 (Human PD-L1 ELISA kit [28-8] [Abcam, Cambridge, UK]) and human Gal-9 Quantikine ELISA kit (R&D Systems, Minneapolis, US). Further detailed protocols are described in the Supplemental Materials and Methods.

ETHICS APPROVAL

This study was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine (approval code R2422), and all procedures were conducted in accordance with the 2013 Declaration of Helsinki. Signed informed consent was obtained from all patients for the use of their blood and tissue in our research.

STATISTICAL ANALYSIS

Continuous variables are represented as the median (interquartile range [IQR]), and categorical variables are represented as numbers and percentages. The distribution of PD-L1 expression level, Gal-9 expression level, FOXP3 cells, CD4 cells, and CD8 cells according to groups was expressed using box-and-whisker plots with medians and IQRs. Spearman's correlation coefficients were used to evaluate the correlations between each parameter at a significance level of $P < 0.05$. The Mann-Whitney U test

was used to compare 2 groups, and the Kruskal-Wallis test was applied for multiple comparisons.

Among the patients maintained without IS at the time of biopsy, the ISW success period was calculated using Kaplan-Meier curve analysis with different thresholds and combinations of the 3 immune proteins. IS reuse as a result of abnormal liver function or abnormal histological findings (fibrosis or rejection) was considered an event. Overall tolerance was defined as the time from ISW to IS reuse or last follow-up. A P value < 0.05 (2-tailed) was considered statistically significant. All statistical analyses were performed using JMP Pro 14.0 (SAS Institute Inc., Cary, NC).

Results

PATIENT CHARACTERISTICS OF THE LIVER BIOPSY COHORT

The liver biopsy cohort, which was derived from 95 patients, consisted of 38 tolerant samples (group A), 16 samples with abnormal histology that triggered immunosuppression (IS) reuse (group B1), 19 samples after IS reuse (group B2) in nontolerant patients, and 38 control samples (group C). There were 31 males and 64 females. The median age at LT was 1.9 years (IQR, 0.8–6.1 years). Biliary atresia was the predominant cause of the primary liver disease (76.3%). The interval between LT and ISW was 7.9 years (IQR, 3.5–11 years) in the tolerant group and 7.6 years (IQR, 5.4–9.7 years) in the nontolerant group. The interval between ISW and liver biopsy in the tolerant group (9.6 years; IQR, 4.7–12.6 years) was comparable with that in the nontolerant group (6.8 years; IQR, 3.5–10 years). The interval between ISW and IS reuse in the tolerant group (7.6 years; IQR, 3.6–11.7 years) was comparable with that in the nontolerant group (7.6 years; IQR, 3.5–10 years). There were no significant differences in sex, AST, ALT, or total bilirubin among groups A, B1, B2, and C. The characteristics of the patients are listed in Supporting Table 2.

EXPRESSIONS OF PD1, PD-L1, GAL-9, AND FOXP3 IN LIVER SPECIMENS

In liver allografts, the expression of PD1 was hardly detectable (Supporting Fig. 1A). Increased PD-L1 and Gal-9 expressions were identified in biopsies from group A (ie, in tolerant patients). Their expressions were localized in the parenchymal regions, where PD-L1

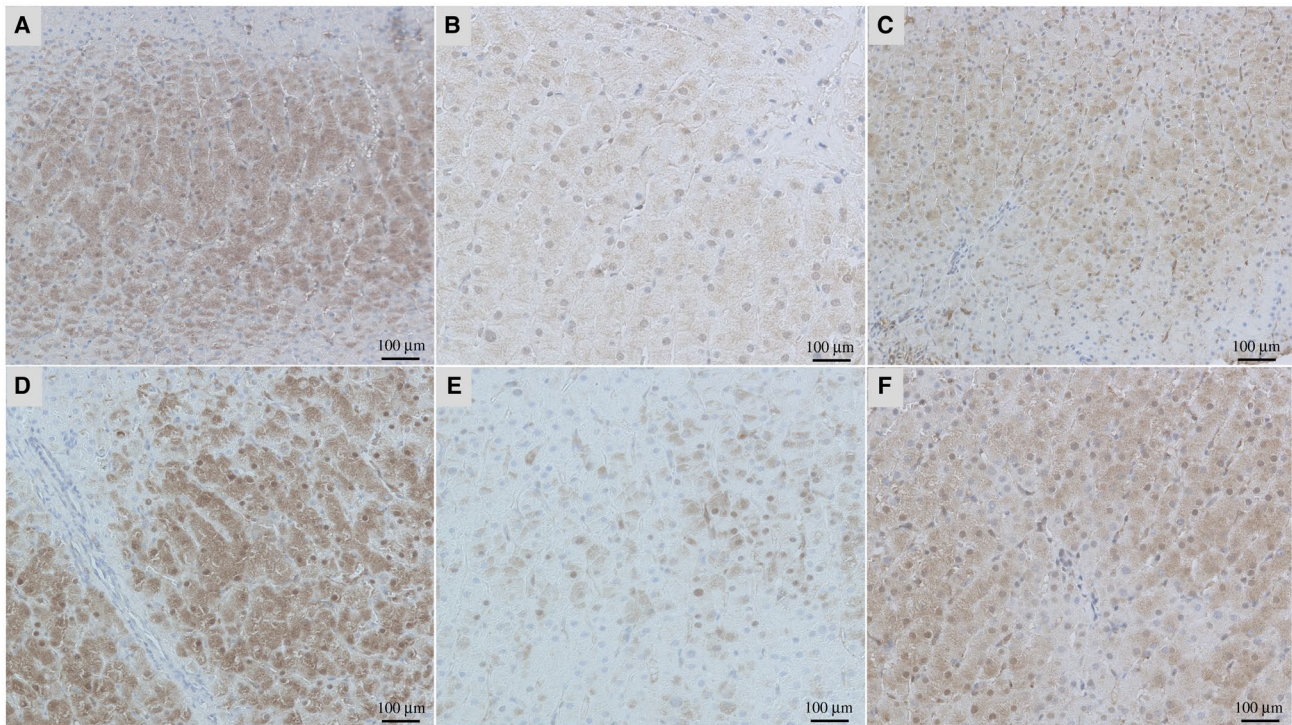


FIG. 2. Representative immunohistochemistry staining of PD-L1 and Gal-9 in FFPE human liver graft biopsy. Enhanced expressions of PD-L1 and Gal-9 were detected on hepatocytes in (A,D) tolerant patients who successfully withdrew IS and (C,F) in control patients, whereas (B,E) low expressions of PD-L1 and Gal-9 were observed in nontolerant patients. Sections were stained with anti-PD-L1 antibody (clone 28-8) at 4 µg/mL and anti-Gal-9 antibody (clone ab69630) at 4 µg/mL. Original magnification $\times 200$; scale bars, 100 µm.

(Fig. 2A) and Gal-9 (Fig. 2D) were predominantly observed in the cytoplasm of hepatocytes. Elevated PD-L1 and Gal-9 expressions were also observed in group C (ie, in control patients; Fig. 2C,F). The absence or low levels of PD-L1 and Gal-9 expressions were observed in biopsy specimens from nontolerant patients in groups B1 and B2 (Fig. 2B,E). In addition, FOXP3 expression was localized in the lymphocytes and was abundantly detected in tolerant patient biopsies (Supporting Fig. 1B). The specificity of immunostaining was validated with appropriate negative and positive control experiments (Supporting Figs. 2 and 3).

The intensity of PD-L1 staining was quantified (Supporting Fig. 4) and showed higher PD-L1 levels in group A than in group B1 and group B2: median 20.8 relative units (RU; IQR, 16.2–26 RU) versus median 14.3 RU (IQR, 7.9–17.9 RU; $P < 0.001$) and median 12 RU (IQR, 8.2–20.4 RU; $P = 0.01$; Fig. 3A). In addition, the PD-L1 level in group A was comparable with that in group C (Fig. 3A). Similar results were found for Gal-9 expression, with significantly higher expressions of Gal-9 in group A

than in group B1 and group B2: median 11.8 RU (IQR, 5.6–18.9 RU) versus median 5.2 RU (IQR, 3.5–9.3 RU; $P < 0.01$) versus median 7 RU (IQR, 3.7–14.1 RU; $P = 0.04$). There was no significant difference in Gal-9 levels between group A and group C (Fig. 3B). Quantification of FOXP3 staining (Supporting Fig. 5) showed a significantly higher number of FOXP3+ regulatory T cell (Treg) cells in group A than in group B1 (median 4 RU [IQR, 0.8–11.8 RU] versus median 0 RU [IQR, 0–2.5 RU]; $P = 0.02$). The FOXP3+ Treg cells were comparable between group A, group B2, and group C.

CORRELATION AND COLOCALIZATION OF PD-L1 AND GAL-9 EXPRESSIONS

A significant but weak correlation between PD-L1 and FOXP3 levels (Spearman $r = 0.24$; $P = 0.02$) and between Gal-9 expression levels and FOXP3 (Spearman $r = 0.32$; $P < 0.01$) was identified. We detected a strong correlation between PD-L1 and Gal-9 expression levels (Spearman

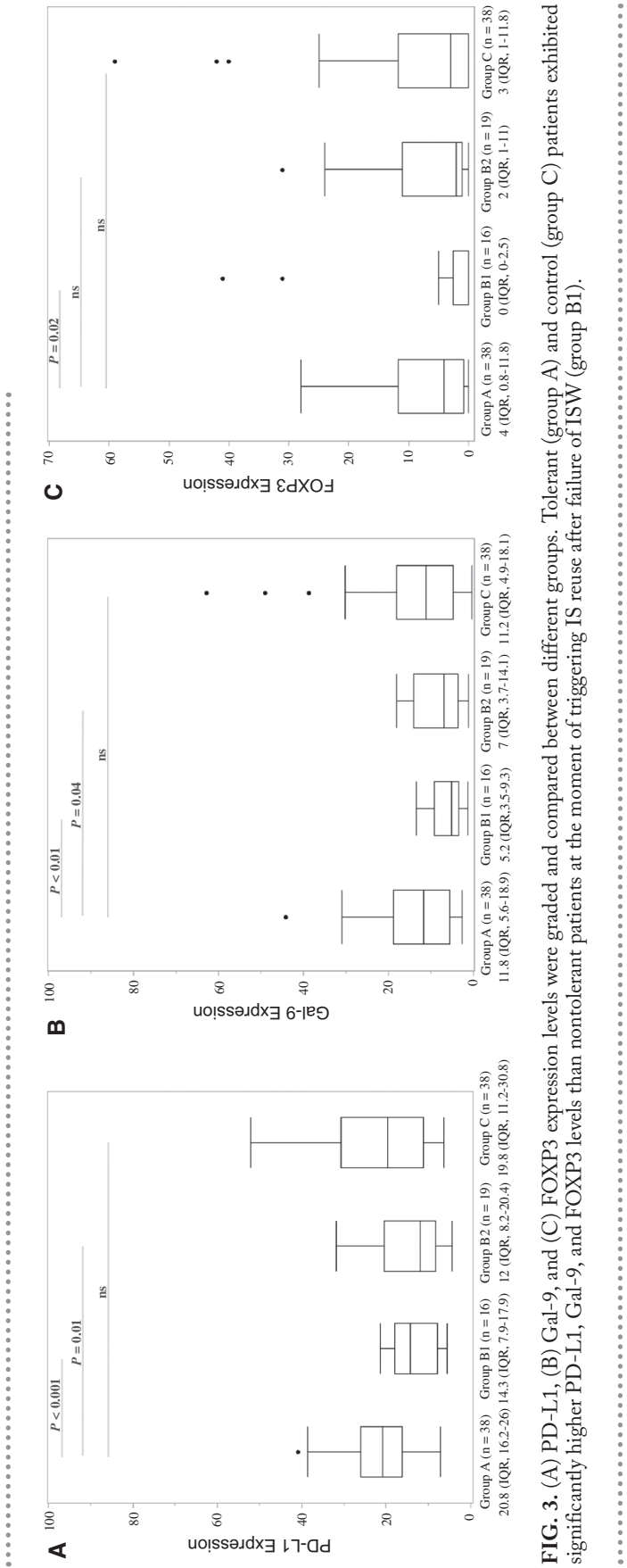


FIG. 3. (A) PD-L1, (B) Gal-9, and (C) FOXP3 expression levels were graded and compared between different groups. Tolerant (group A) and control (group C) patients exhibited significantly higher PD-L1, Gal-9, and FOXP3 levels than nontolerant patients at the moment of triggering IS reuse after failure of ISW (group B1).

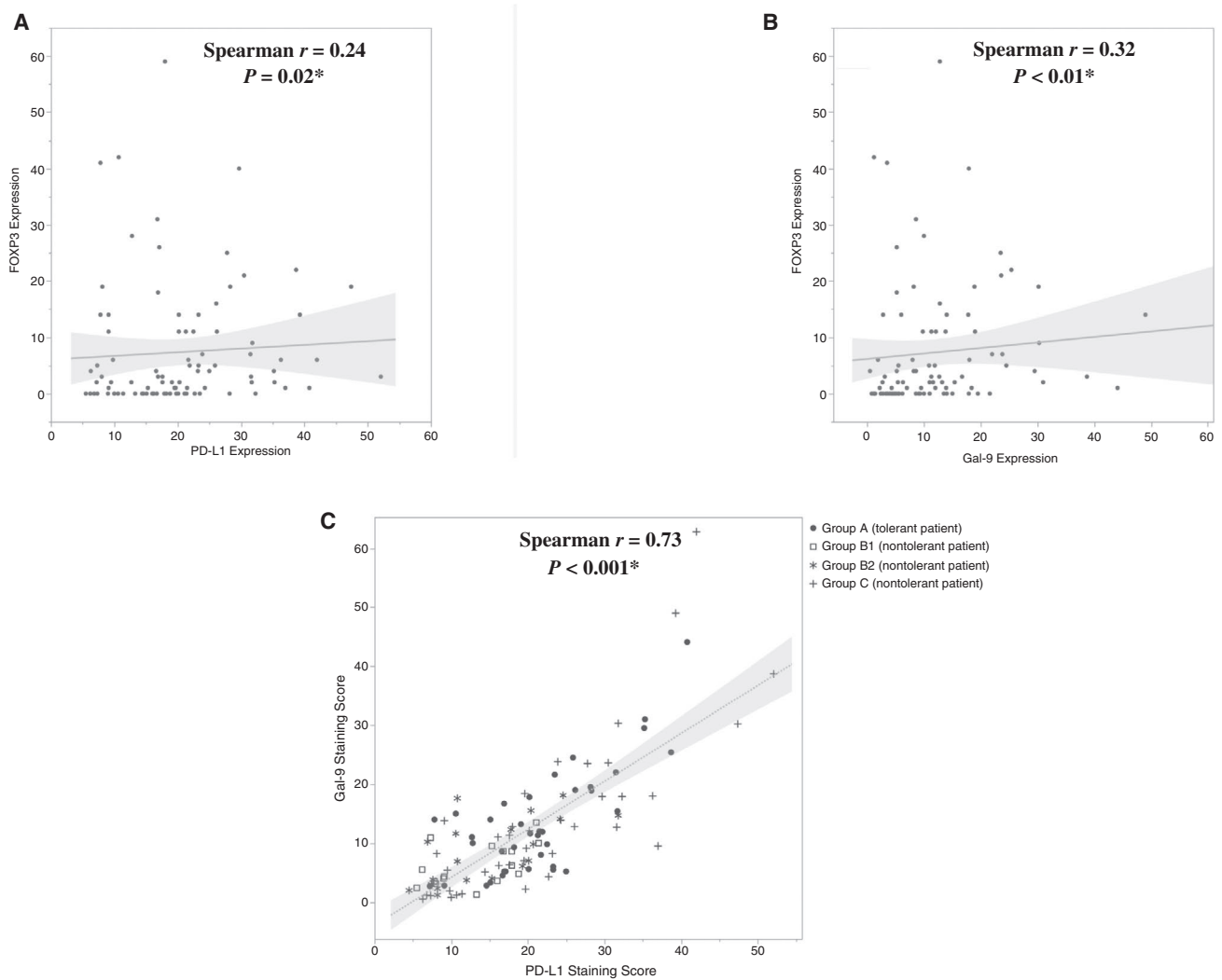


FIG. 4. (A) Weak correlation between PD-L1 and FOXP3 with an r correlation coefficient of 0.24 and (B) between Gal-9 and FOXP3 with an r correlation coefficient of 0.32 were found. (C) In contrast, a significant strong correlation between PD-L1 and Gal-9 was found with an r coefficient of 0.73.

$r = 0.73$; $P \leq 0.001$; Fig. 4). IF for PD-L1 and Gal-9 showed a similar staining pattern as immunohistochemistry (IHC) with cytoplasm exhibition. The coexpression of PD-L1 and Gal-9 in hepatocytes demonstrated a positive signal in the cytoplasm (Supporting Fig. 6).

HIGH EXPRESSION OF PD-L1 OR GAL-9 WAS ASSOCIATED WITH PROLONGED SUCCESS OF TOLERANCE

Kaplan-Meier curve analysis was performed to evaluate the role of these proteins in predicting the

sustainability of tolerance. The cutoff value to distinguish between high and low expression proteins was based on the IHC staining median value. Patients with high expressions of PD-L1 (83.3% versus 36.7%; $P < 0.01$) and high expressions of Gal-9 (73.1% versus 42.9%; $P < 0.01$) were significantly associated with sustained tolerance (Fig. 5A,B), whereas the chance of tolerance in patients with higher expressions of FOXP3 was close to being statistically significant (72% versus 44.8%; $P = 0.06$; Fig. 5C). When summing the staining score of PD-L1 and Gal-9, the results from Kaplan-Meier curve analysis emphasized that patients with high (PD-L1 + Gal-9)

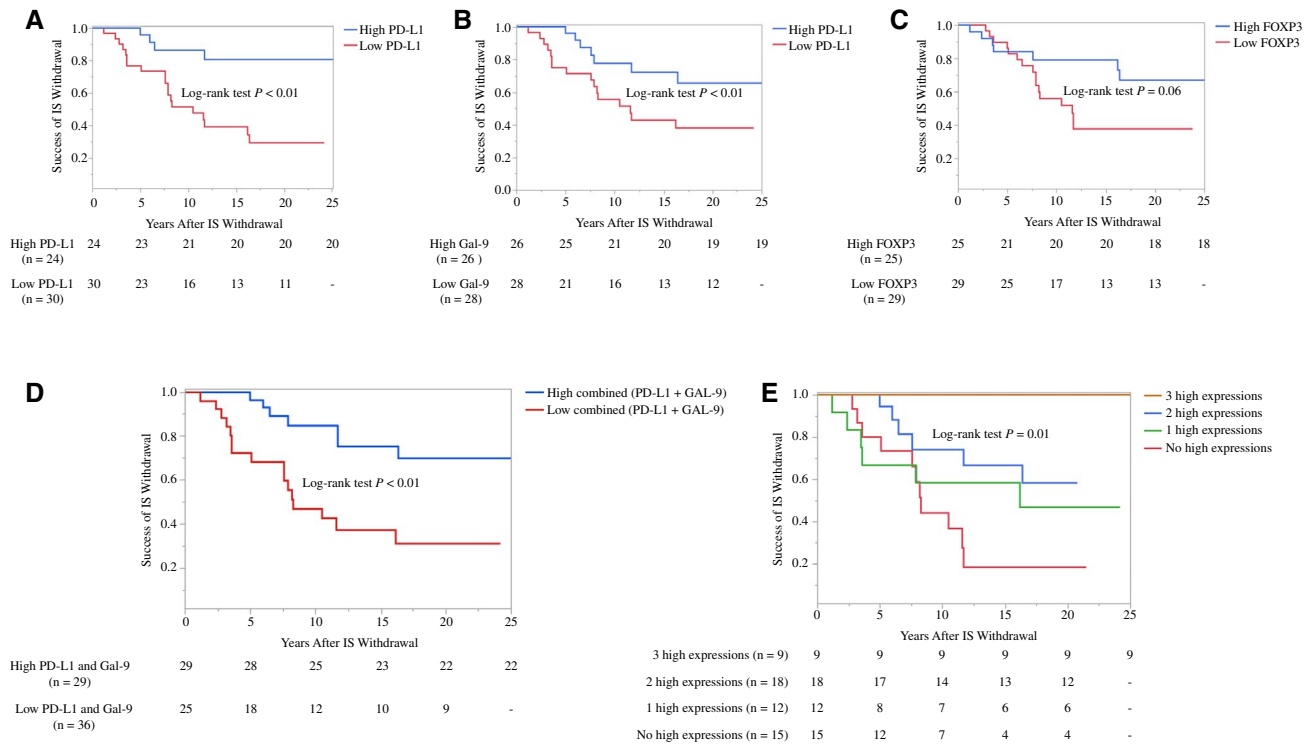


FIG. 5. Relationship between PD-L1, Gal-9, and FOXP3 expressions and the success rate of the ISW rate. Kaplan-Meier curve graphs showed the success rate of ISW rate in patients with (A) high versus low PD-L1 expression, (B) high versus low Gal-9 expression, (C) high versus low FOXP3 expression, and (D) high combined (PD-L1 + Gal-9) versus low combined (PD-L1 + Gal-9) expression in liver biopsy tissue. The combination of PD-L1, Gal-9, and FOXP3 expressions improves the prognostic value of these immune markers in predicting the success of IS withdrawal. (E) Kaplan-Meier curve graphs showed that the combination of high PD-L1, high Gal-9, and high FOXP3 expressions in liver biopsy tissue yielded a 100% success rate of IS withdrawal.

expressions increased the success of ISW compared with that of patients with low expressions (PD-L1 – Gal-9; 69.7% versus 31%; $P < 0.01$; Fig. 5D). The receiver operating characteristics analysis determined that the optimal cutoff values of the staining score of PD-L1, Gal-9, and combined (PD-L1 + Gal-9) for the success of ISW were 18.2, 4.5, and 21.2, respectively (area under the curve [AUC] = 0.76, sensitivity = 71%, specificity = 71.4%; AUC = 0.69, sensitivity = 93.6%, specificity = 38.1%; AUC = 0.75, sensitivity = 93.6%, specificity = 47.6%, respectively). In the subgroup analysis focusing on tolerant patients, higher expressions of PD-L1 represented a predictive factor of prolonged success of tolerance with a trend toward significance (90.9% versus 68.6%; $P = 0.09$; Supporting Fig. 7A), whereas there was no difference between high and low expressions of Gal-9 in tolerant patients (82.6% versus 80%; $P = 0.83$; Supporting Fig. 7B). Interestingly, Kaplan-Meier curve data

showed that 100% of patients who had a combination of high PD-L1, high Gal-9, and high FOXP3 in liver grafts achieved tolerance (Fig. 5E).

EXPRESSIONS OF CD4 AND CD8 IN LIVER SPECIMENS

IHC demonstrated that infiltrating CD4+ T and CD8+ T cells were dominantly present in the periportal area (Supporting Fig. 1C,D). The infiltrating CD8+ T cells were significantly more abundant in group B1 than in groups A, B2, and C (57 cell counts [CC; IQR, 41.1–99.1 CC] per field versus 13.3 CC [IQR, 7.1–24.4 CC], 11 CC [IQR, 7.8–20 CC], and 12.3 CC [IQR, 6.2–37.3 CC]; $P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively), whereas the number of infiltrating CD4+ T cells was comparable between these 4 groups (14.6 CC [IQR, 9.5–42 CC] versus 13.7 CC [IQR, 8.8–31.3 CC], 10.7 CC [IQR, 9–19

CC], and 13 CC [IQR, 2.9-27.7 CC]; $P = 0.84$, $P = 0.28$, and $P = 0.46$, respectively; Supporting Fig. 8).

PATIENT CHARACTERISTICS OF THE SERUM COHORT

Serum samples were obtained from 12 tolerant patients (group sA), 16 nontolerant patients (group sB), and 24 control patients (group sC). Apart from age, there were no significant differences between patients in these 3 groups by sex, distribution of primary liver disease, graft-to-recipient weight ratio, interval between LT and ISW, interval between ISW and serum collection, white blood cells, red blood cells, hemoglobin, platelets, prothrombin time (PT), international normalized ratio of PT, AST, ALT, total bilirubin, albumin, gamma-glutamyltransferase, alkaline phosphatase, or lactate dehydrogenase (Supporting Table 3). All patients were in stable condition without abnormal signs or symptoms during their regular health check-up.

SOLUBLE FORMS OF PD-L1 AND GAL-9 AND THEIR CORRELATION WITH LIVER EXPRESSION

The level of sPD-L1 in group sA, measured in duplicate, was comparable with that in group sB and group sC (60.4 pg/mL [IQR, 50.3-67.1 pg/mL] versus 56.9 pg/mL [IQR, 50-64.4 pg/mL] and 41.8 pg/mL [IQR, 22.5-80.5 pg/mL]; $P = 0.44$ and $P = 0.22$; Supporting Fig. 9A). A similar finding was also detected for the level of sGal-9, with comparable levels between group sA, group sB, and group sC (3.6 ng/mL [IQR, 2.9-4.3 ng/mL], 3.6 ng/mL [IQR, 3.2-4 ng/mL], and 3.6 ng/mL [IQR, 2.9-4.3 ng/mL]; $P = 0.86$ and $P = 0.96$; Supporting Fig. 9B). There was no correlation between sPD-L1 and sGal-9 levels in the patients' serum samples (Spearman $r = 0.29$; $P = 0.13$; Supporting Fig. 9C). To determine a link between liver tissue expression and peripheral blood levels, we examined the correlation between the level of sPD-L1 (as well as sGal-9) and the expression of PD-L1 (as well as Gal-9) in liver tissue. No statistical association was observed between the level of PD-L1 or Gal-9 in liver tissue and their soluble forms in the corresponding serum (Spearman $r = 0.11$ [$P = 0.58$]; Spearman $r = -0.09$ [$P = 0.66$]; Supporting Fig. 10A,B).

Discussion

Achievement of immune tolerance in LT recipients is ideal because the adverse effects of IS complicate the management of the patients and can sometimes be life threatening. There is an unsolved question regarding the detection of candidates who can safely achieve the tolerance after ISW. Although numerous studies have proven the involvement of immune proteins in regulating liver graft tolerance, a robust marker to predict the success of safe IS cessation is lacking. In this study, we explored the clinical value of PD-L1, Gal-9, and FOXP3 expressions in liver biopsy tissue and their soluble forms in serum among tolerant and nontolerant patients. Our results indicated that a high expression of PD-L1 or Gal-9 was associated with prolonged success of tolerance, and a high correlation between these 2 proteins was shown with colocalization in the cytoplasm of hepatocytes.

Irreversible rejection after induction of nivolumab, an anti-PD1 inhibitor, in LT patients with abundant PD-L1 expression in liver biopsy implicated the role of this immune checkpoint protein in the pathogenesis of rejection.^(16,17) The PD1/PD-L1 pathway is considered as the key component in modulating peripheral immune regulation, and its blockade leads to the restoration of specific T cells that are likely associated with rejection. Thus, we proposed that overexpression of PD-L1 in liver grafts will establish a protective mechanism against the upregulation of T cells. As shown in Fig. 2B, PD-L1 was dominantly expressed in the cytoplasm of hepatocytes, and there was no membrane staining. Despite numerous findings of PD-L1 membranous signaling in liver tissue,⁽³⁰⁻³²⁾ cytoplasmic exhibition of PD-L1 has been reported in liver allografts⁽³³⁾ and HCC tissue.⁽²¹⁾ The cytoplasmic manifestation of the PD-L1 signal might be explained as an intracellular stock of PD-L1, and it will be transported to the cell surface upon immunological contact.⁽³⁴⁾ Also, the cytoplasmic PD-L1 might be synthesized in the membrane of hepatocytes and then translocates onto the cytoplasm as the response to regulatory immune cells. The mechanism of cytoplasmic PD-L1 interacting with PD1 remains unclear and should be studied in the future. Our analysis showed that patients with higher PD-L1 signals had significantly greater sustained tolerance (Fig. 5A). Particularly, in the tolerant group, patients with high PD-L1 staining were more likely to achieve prolonged tolerance success, whereas those

with low PD-L1 signaling seemed to suffer IS reintroduction (Supporting Fig. 7A). In a mouse model, Morita et al. demonstrated that PD-L1 knockout mice had a significantly lower rate of graft survival than wild-type mice and blockade of the PD1/PD-L1 pathway using an anti-PD-L1 antibody promoted the antidonor immune responses that resulted in early graft loss.⁽³⁵⁾ Regarding human research, DeLeon et al. revealed that, after using PD1 inhibitors, patients with a history of LT who lacked PD-L1 hepatic expression did not suffer rejection, whereas those with abundant PD-L1 signaling in graft tissue developed rejection.⁽¹⁸⁾ Despite the small sample size, the aforementioned studies implied a predictive role of liver graft PD-L1 expression in selecting the appropriate candidates for the indication of PD1 inhibitors. This implication reasonably supports the results of our study, especially the essential role of PD-L1 in the prognosis of tolerance after ISW.

The prognosis of Gal-9 expression in liver tissue has not yet been examined in predicting tolerance. A previous publication identified the predictive role of exosome-derived Gal-9 in patients who suffered rejection after LT.⁽²⁰⁾ In our study, hepatic Gal-9 expression was significantly higher in tolerant patients than in nontolerant patients. Kaplan-Meier curve analysis showed that recipients with high Gal-9 expression likely achieve sustained tolerance. Furthermore, an interesting finding was a strong correlation between intrahepatic PD-L1 and Gal-9 expressions and their colocalization in the cytoplasm of hepatocytes, which suggests their mutual role in regulating the immune system. These ligands belong to the immune checkpoint protein family, and among them, PD-L1 has been demonstrated to have a crucial impact on tolerance in numerous transplantation models. Therefore, the identification of Gal-9 as another biomarker for tolerance will hopefully enhance its application in the acceptance of liver allografts, and the coexpression of these 2 proteins represents a promising biomarker of tolerance. In addition, Pons et al. postulated that the increase in FOXP3 expression in peripheral blood and its elevated messenger RNA (mRNA) expression are associated with tolerance during IS cessation.⁽²⁵⁾ In line with previously published results,^(26,36) we describe here the immunological role of FOXP3 by detecting higher hepatic expression in patients who developed tolerance. Although the FOXP3 signal approached the borderline of significance via univariate analysis, it could be considered a hopeful biomarker for predicting tolerance.

It seems undeniable that a single biomarker is unable to manifest all of the alterations in the immune system before and after ISW. PD-L1 or Gal-9 staining alone might not reflect the overall immune condition. Thus, a panel of multiple immune proteins is required to accurately reflect the immunological background of each individual. In conjunction with the significant result of each immune protein, we herein suggest that investigating PD-L1 and Gal-9 expressions on biopsy specimens after ISW may be useful to predict sustainability of tolerance and therefore to adjust the intensity of follow-up. Because our population sample size was limited, these pilot data required further research in a larger population with a prospective design.

Previous studies have discovered the availability of sPD-L1 and sGal-9 and their similar function to conventional PD-L1 and Gal-9.⁽³⁷⁻³⁹⁾ These circulating proteins might derive from the proteolytic cleavage of the membrane-bound form or from the translation of alternative spliced mRNA.⁽³⁷⁾ Given the remarkable role of PD-L1 and Gal-9 in liver biopsy, we investigated its circulating form in the serum. In this context, exploration of these molecules in the peripheral bloodstream, which is more convenient and less invasive than routine liver biopsy, might potentially replace to the liver tissue expression and act as a promising noninvasive biomarker to predict individual immune status in LT patients. Unfortunately, there were no such differences in the level of the soluble form between tolerant, nontolerant, and control patients. The difference in timing of liver and serum sample collection might have resulted in the lack of association between PD-L1 and Gal-9 serum levels and their hepatic expression levels. Further studies could focus on the modification of the expression of these soluble expressions before and after ISW to investigate their kinetic differences.

Previous studies have also identified some predictive factors of successful tolerance. A longer period of enrollment in ISW since LT was proven to be associated with the probability of being tolerant.^(6,8) Indeed, Benítez et al. revealed that the rate of successful ISW was 79% for patients who were recruited after more than 10.6 years, whereas it dropped to 38% for those who had an interval of 5.7 years.⁽⁶⁾ These findings are in contrast to the study by Lin et al.,⁽⁹⁾ which determined a shorter period between LT and ISW in tolerant patients. The inconsistency might be explained by the differences in the enrolled population. Although the 2 headmost studies focused on adult LT, the latter study

recruited pediatric patients with more naive immunological characteristics. In contrast, we did not reveal any difference in time since LT to ISW between tolerant and nontolerant patients. Although they have the same target population as pediatric patients, variation in ethnicity and IS and ISW protocols might produce this difference. Lower stimulation lymphocyte reactivity was identified to predict tolerance.⁽⁸⁾ In support of this statement, lower CD8 CC in the tolerant group were also found in our study. The higher CD8 CC exhibited an excessive immune response in LT patients who could not achieve tolerance. In addition, male sex and older age at transplantation were also demonstrated to have a close relationship with tolerance.^(6,8) Although the reason for the imbalance in the distribution of sex in the tolerant group is unclear, the immunosenescence in older LT recipients seems to be involved in successful ISW. In our study, although the age at LT in the tolerant group was higher than that in the nontolerant group, the difference was not statistically significant. Restriction in sample size and immunological disparity between adult and pediatric populations might contribute to the discrepancy.

Our research has limitations that need to be acknowledged. Although the present study is based on a unique cohort of LT patients who underwent an ISW trial at Kyoto University Hospital, which remains a restrictive protocol, the limited sample size in the investigation of liver biopsy and serum as a result of loss of follow-up statistically induces an inadequate interpretation of our results to a certain extent. In addition, the impact of IS regimen on the evaluation of these immune proteins is hardly inescapable. In addition, it would be more interesting and even more convincing if we could reach the patient's peripheral blood at the beginning of the ISW trial to eliminate the bias introduced by the cause-effect relation. Also, quantification of protein expression levels is generally best performed by Western blot analysis, but unfortunately in our study, frozen liver tissue samples were not available for Western blot analysis. The only feasible quantification experiment that we could perform was immunohistochemical staining. Therefore, as the first study concentrated on the coordination of various immune markers in tolerance, rather than in rejection, our preliminary data would hopefully be initial evidence to encourage and introduce further prospective clinical trials, such as investigations of PD-L1 and Gal-9 on liver biopsy after ISW with larger sample sizes.

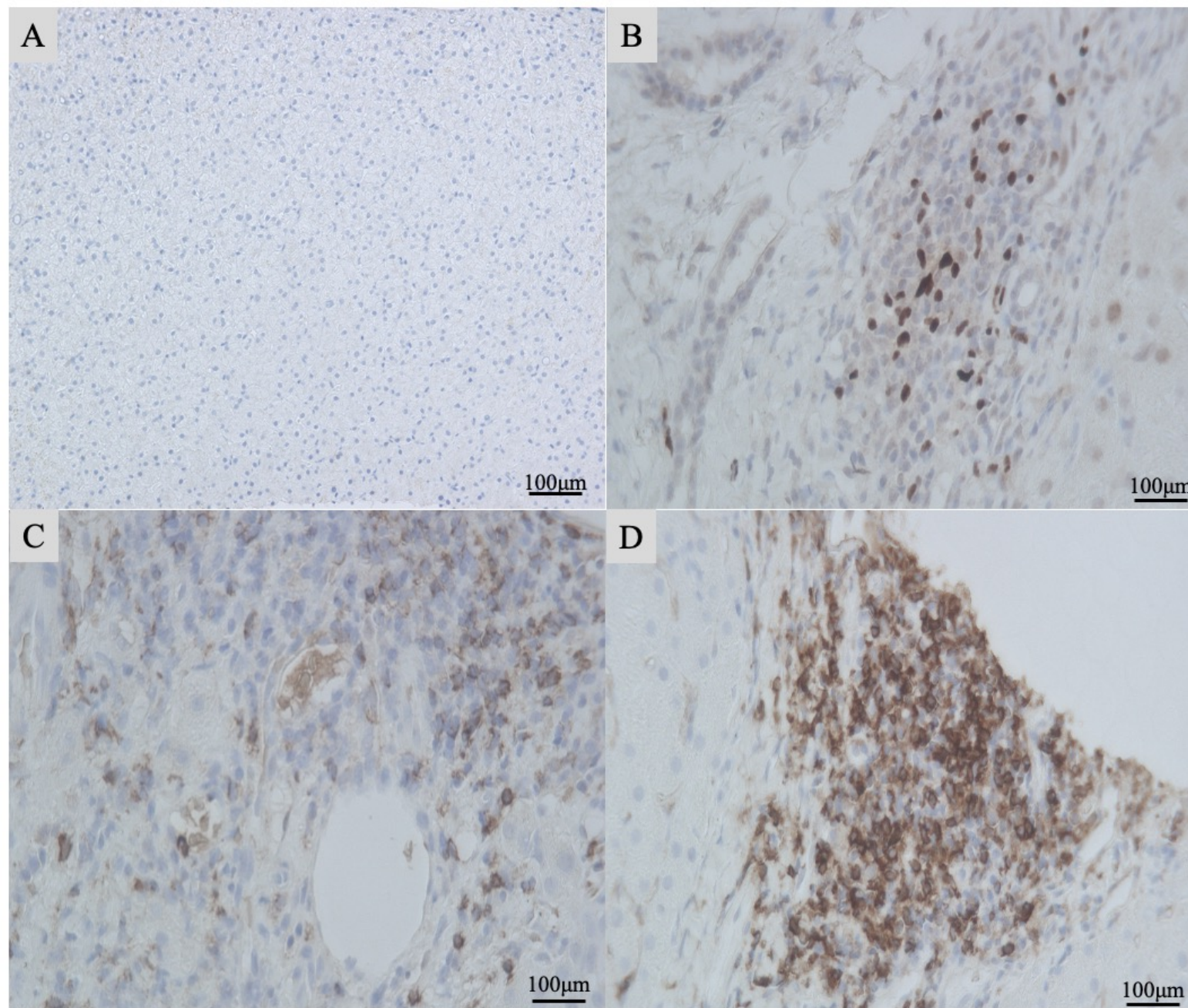
In conclusion, we demonstrated that the coexpression of PD-L1 and Gal-9 remains a promising biomarker for the prediction of tolerance in human LT. Evaluation of PD-L1 and Gal-9 in liver biopsy samples may provide further information about the stability of the tolerance. Future prospective trials are necessary to validate our results.

Acknowledgments: The authors are grateful to the members of the Transplantation Counseling Office in Kyoto University Hospital for arranging all liver transplantations.

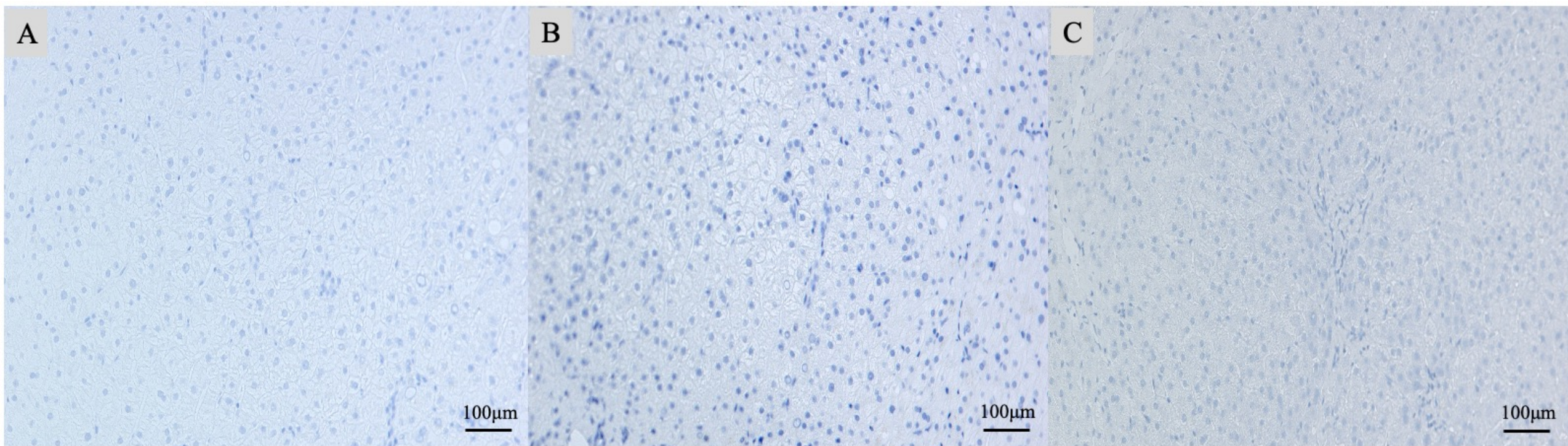
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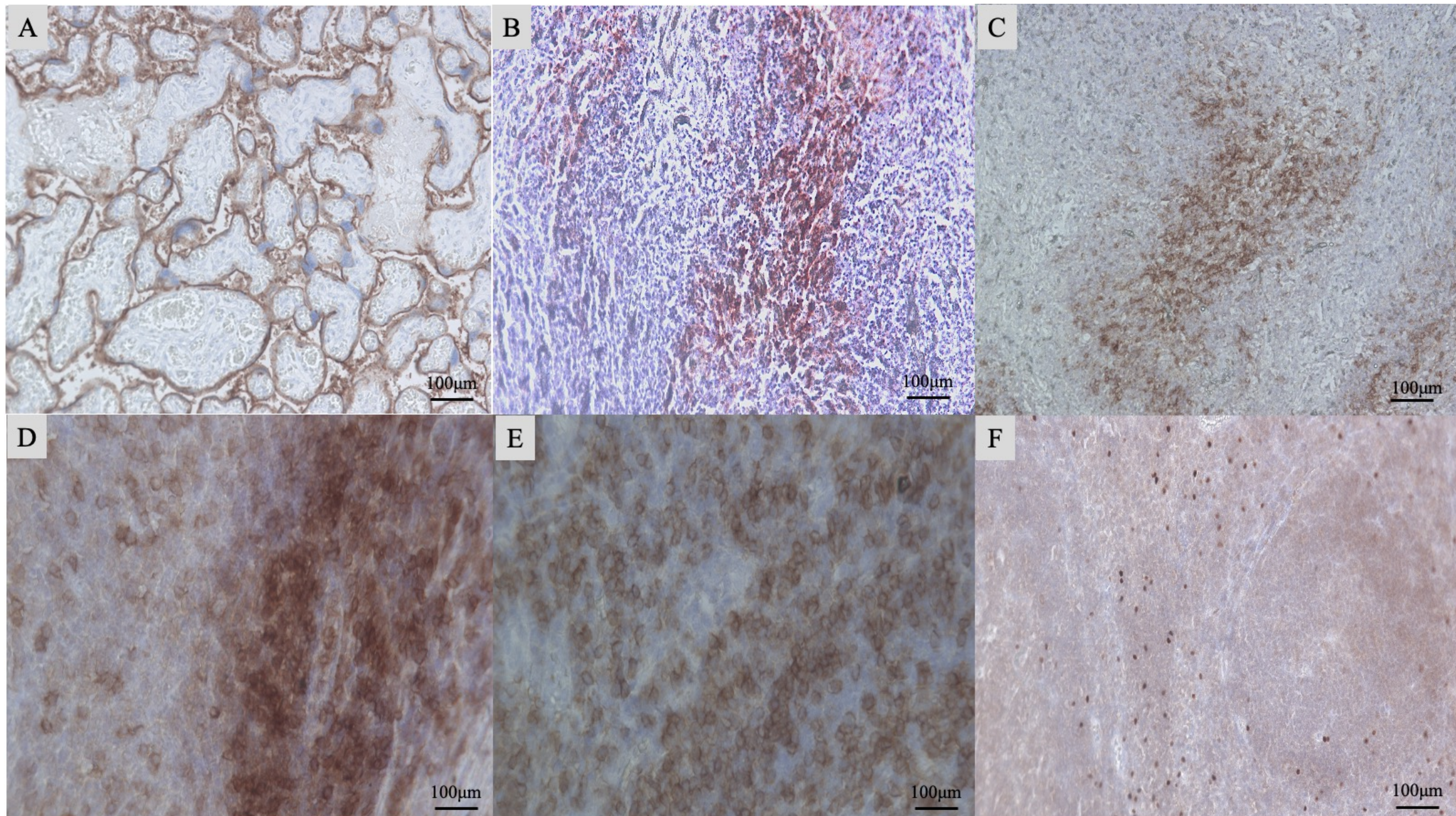
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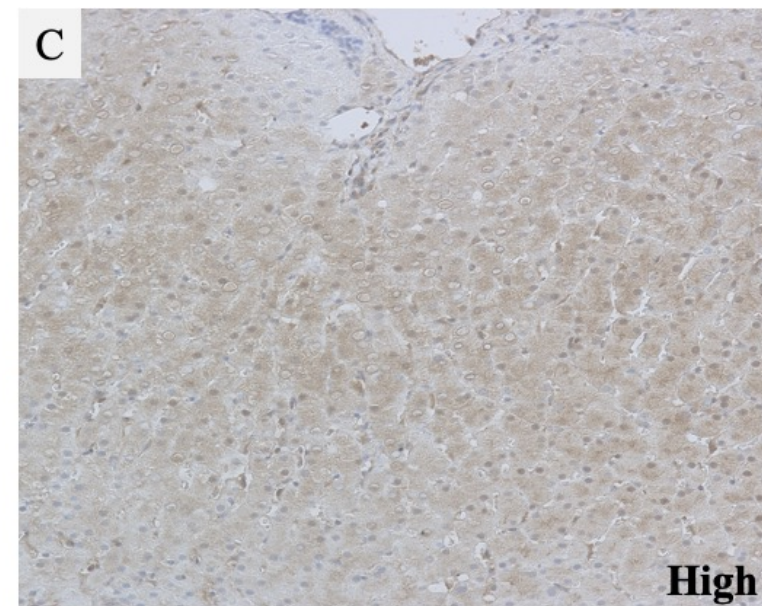
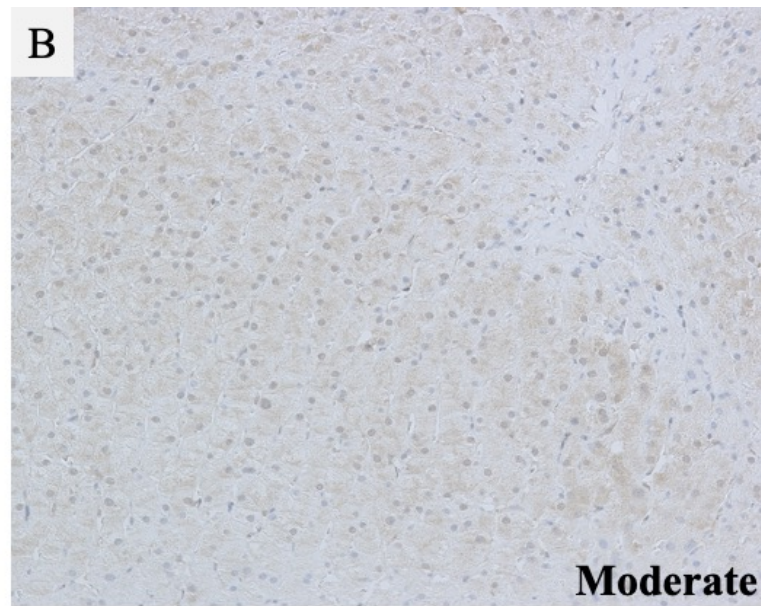
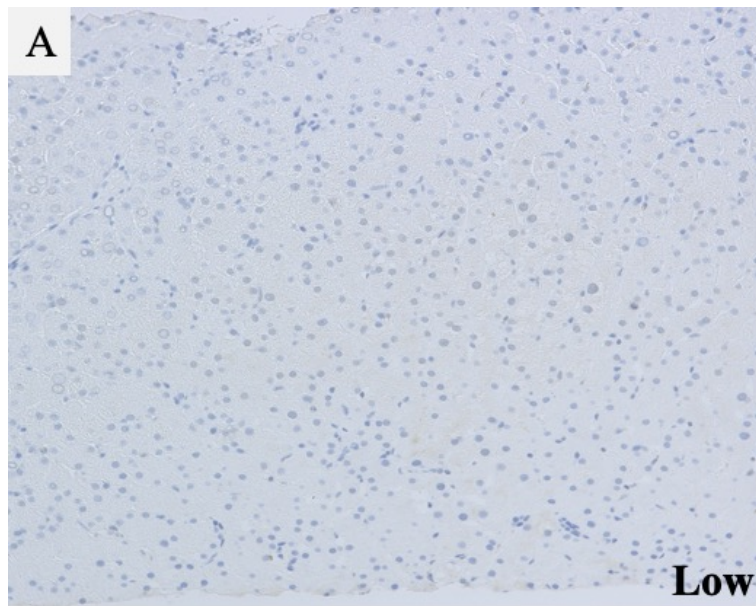
Supporting Figure 1



Supporting Figure 2



Supporting Figure 3

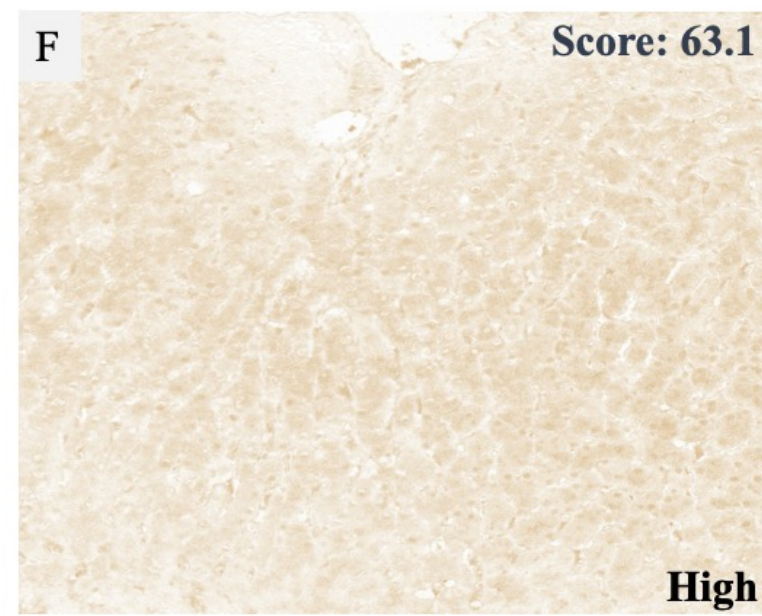
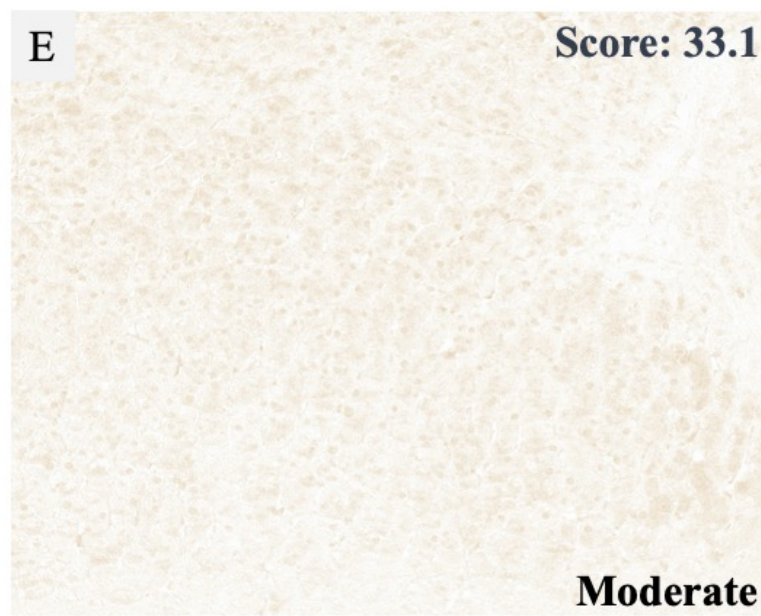
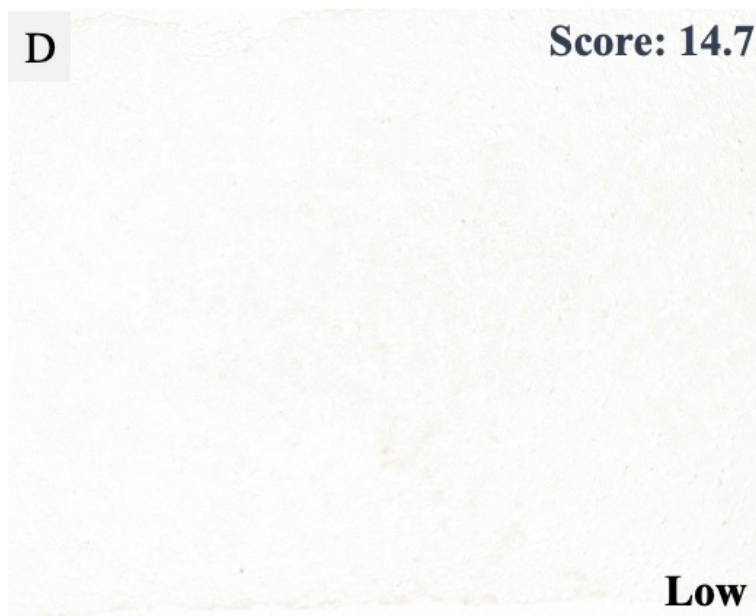


PD-L1 and Gal-9 staining score

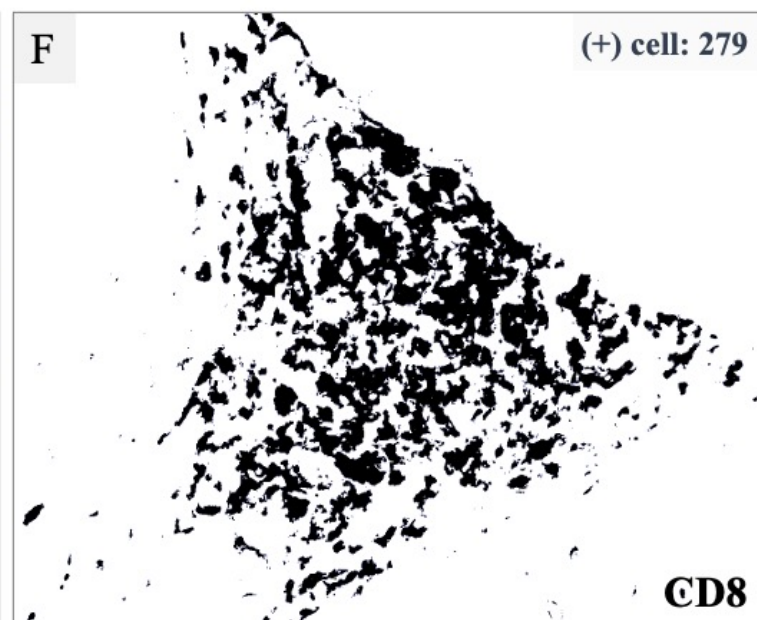
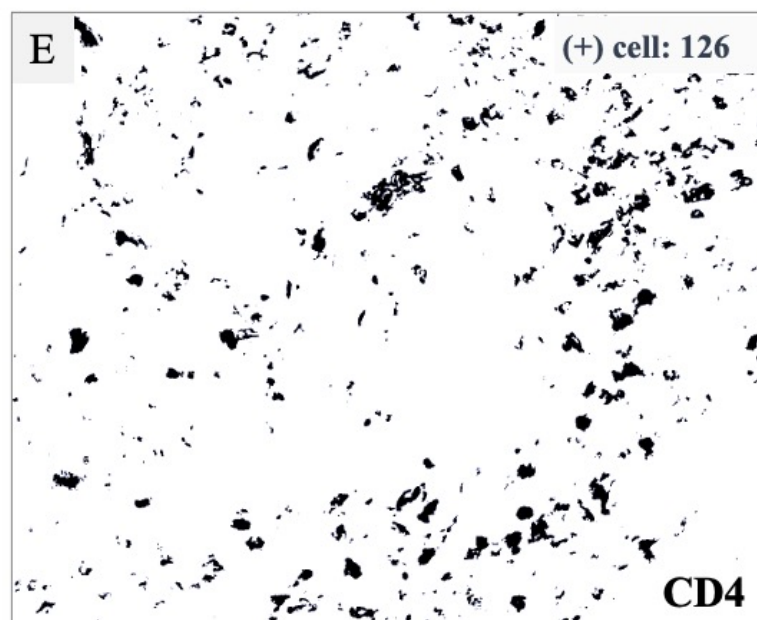
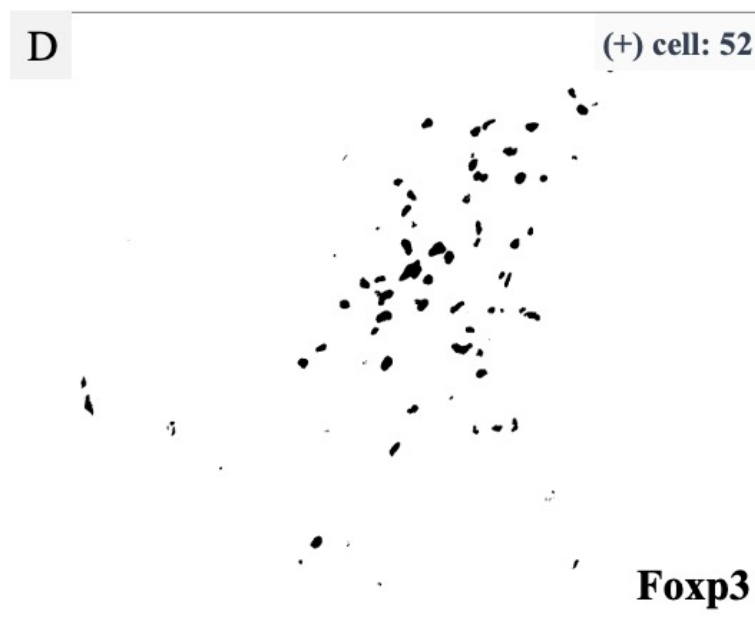
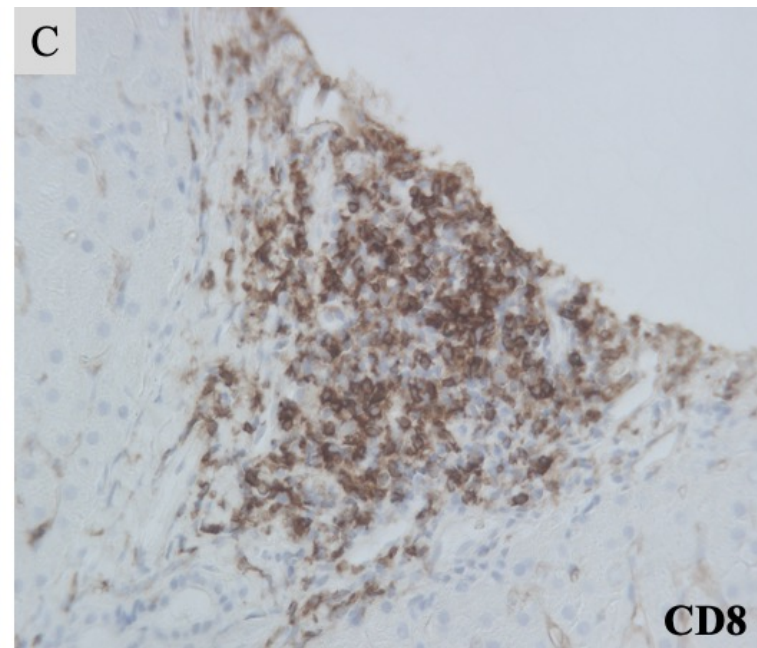
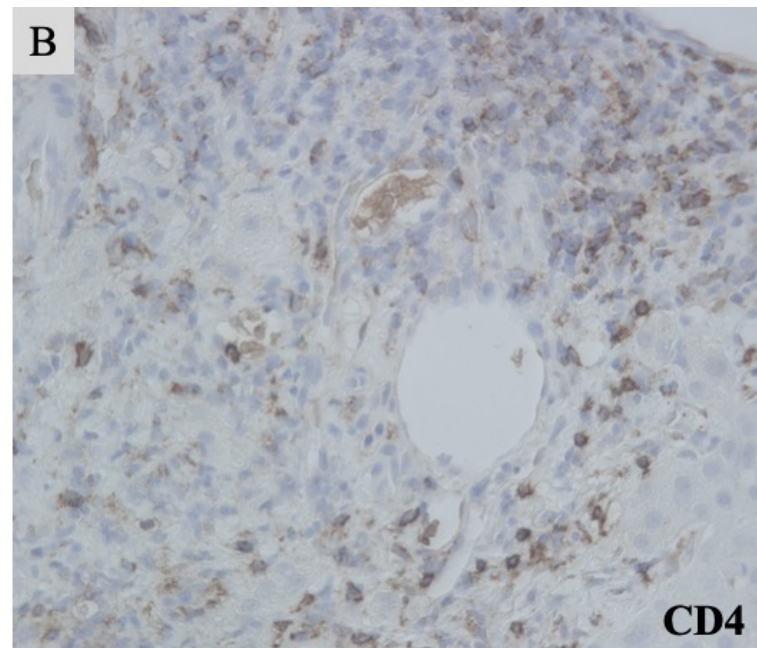
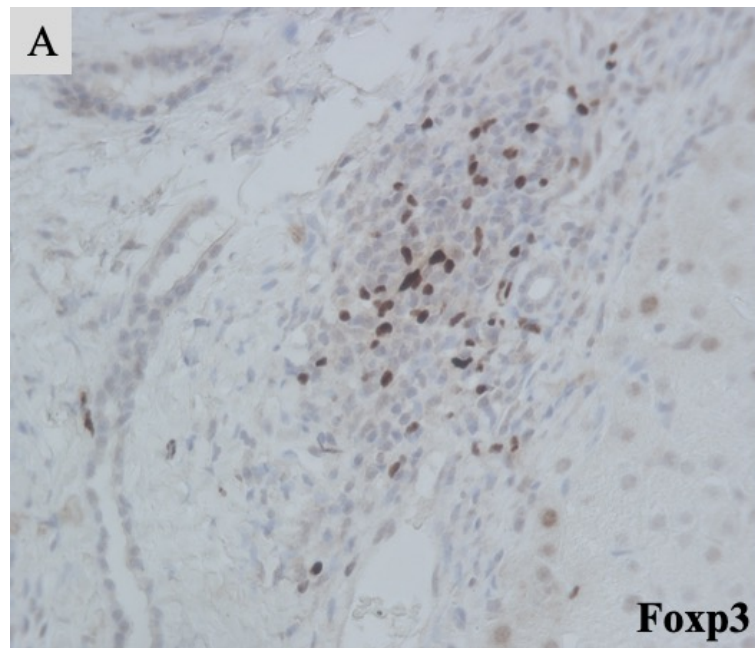
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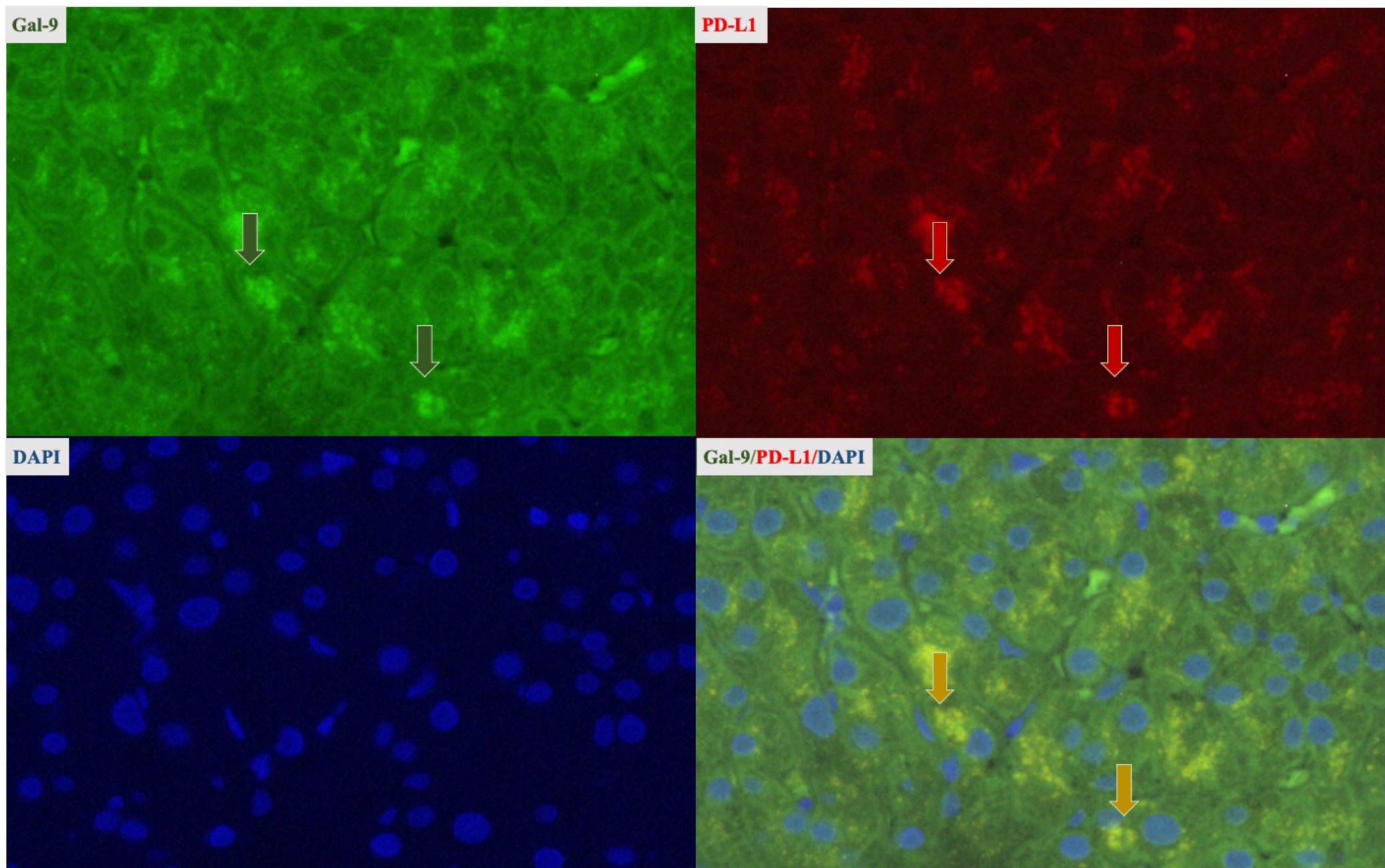
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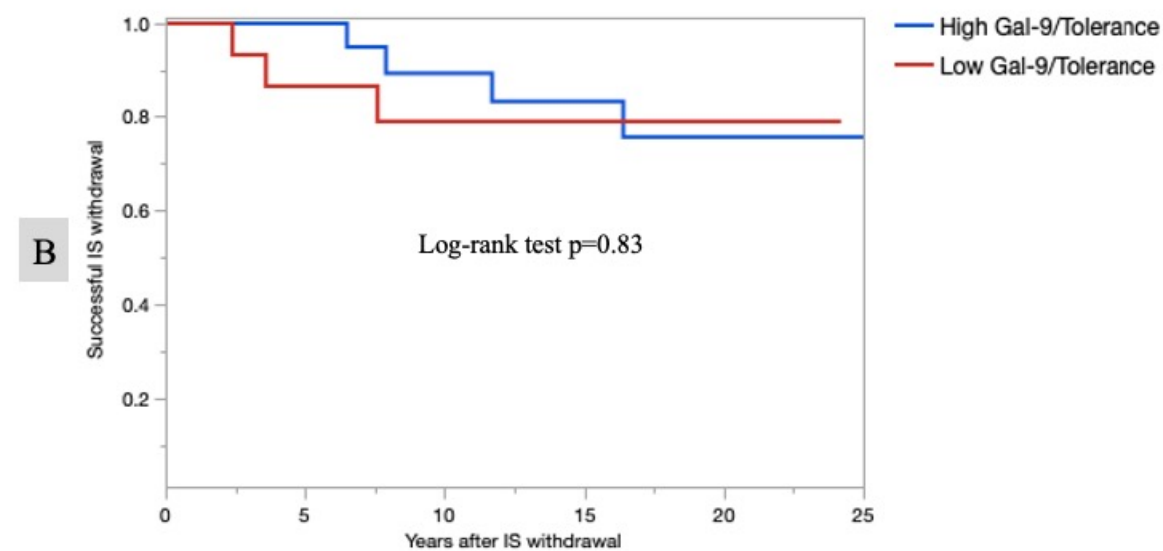
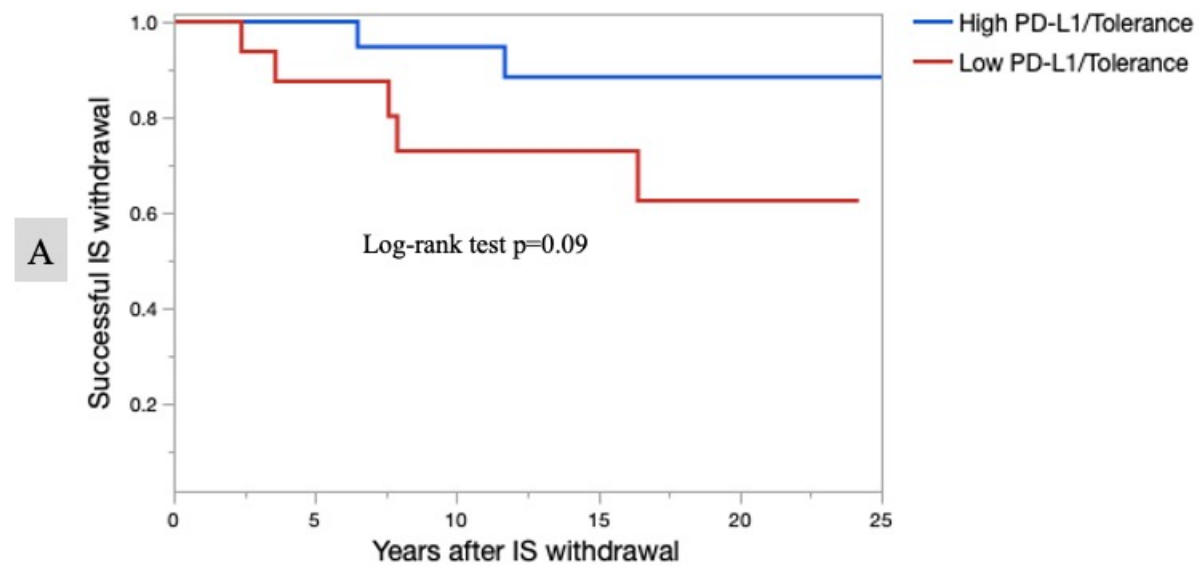
Supporting Figure 4



Supporting Figure 5



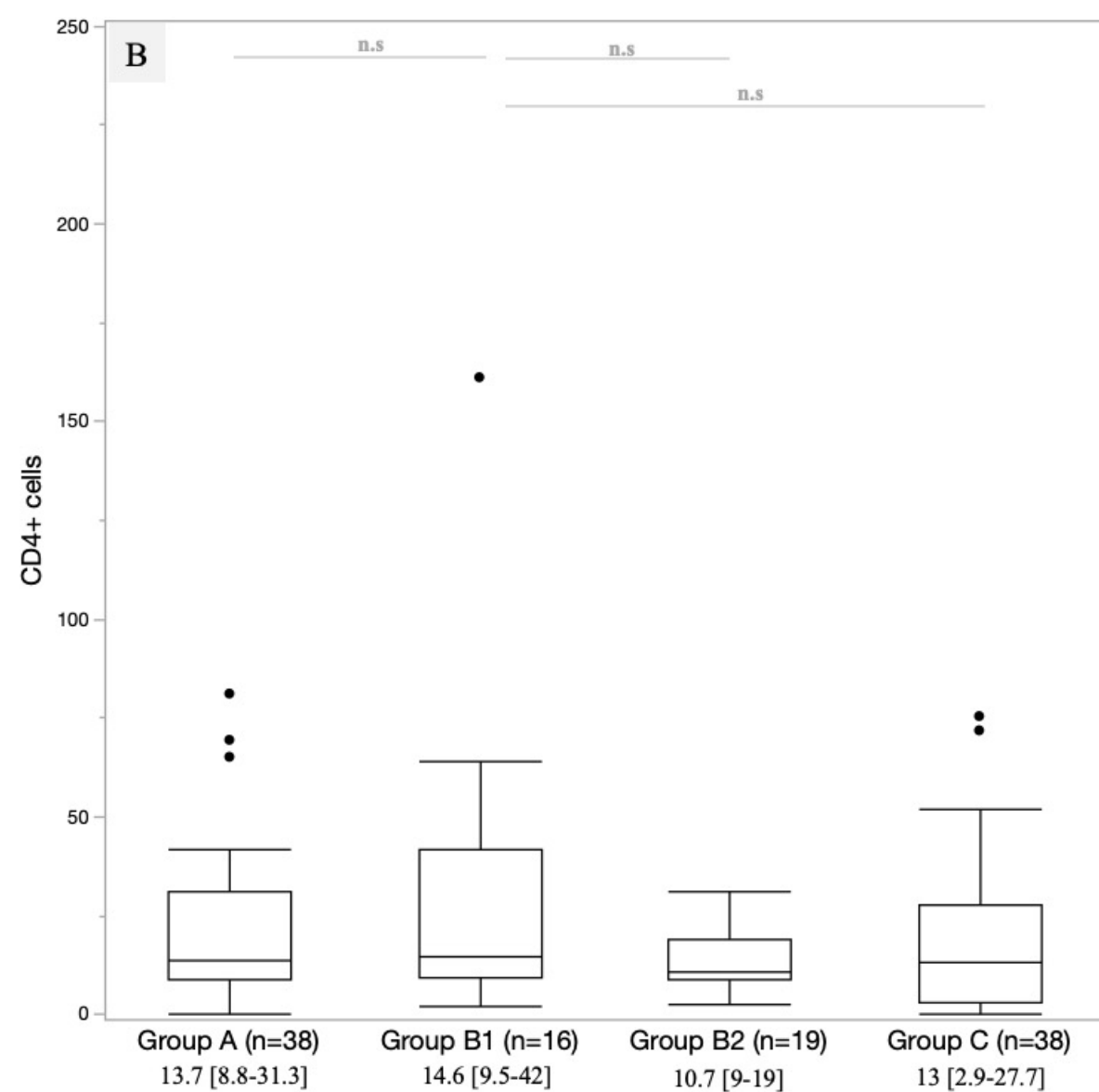
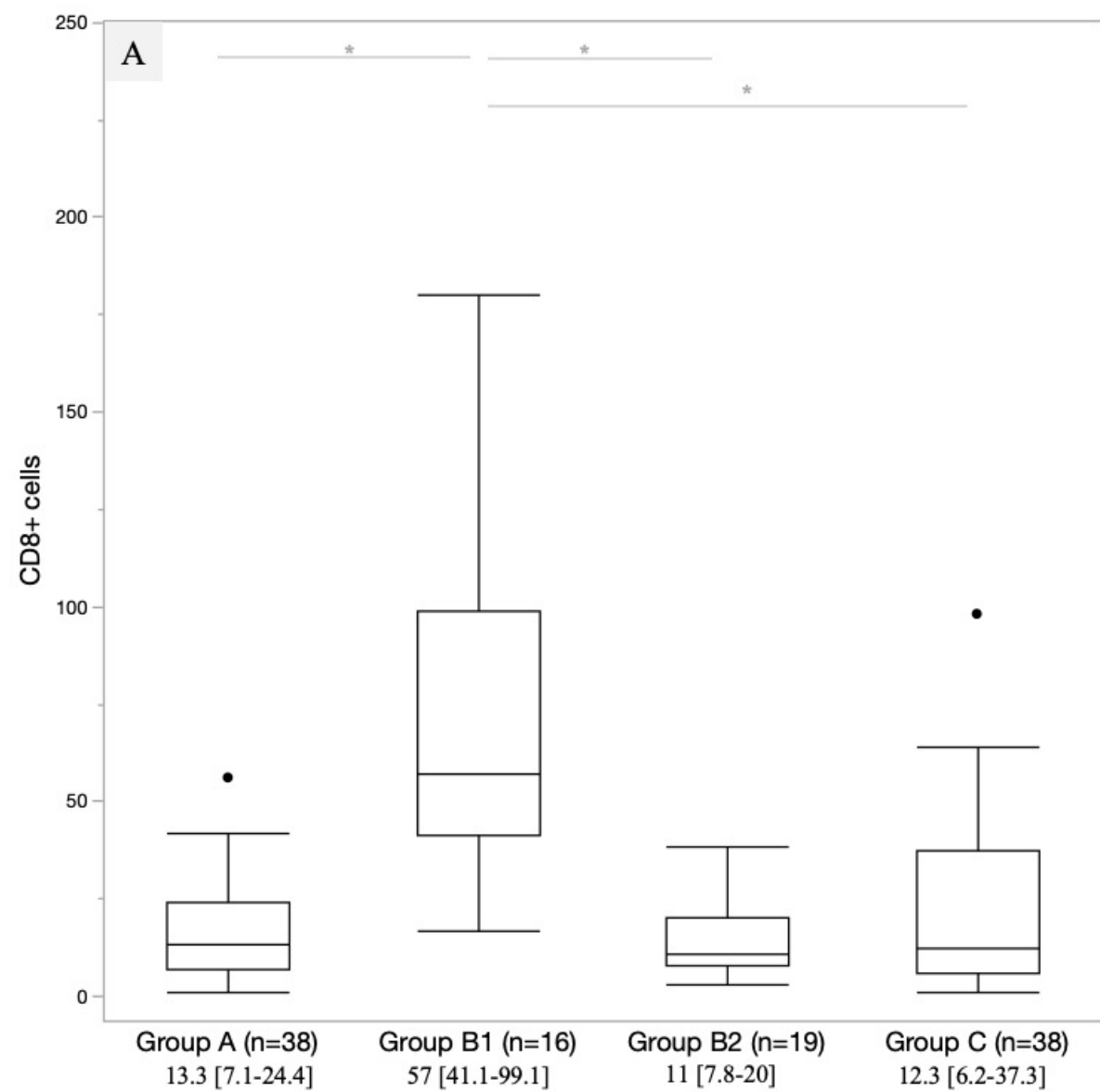
Supporting Figure 6



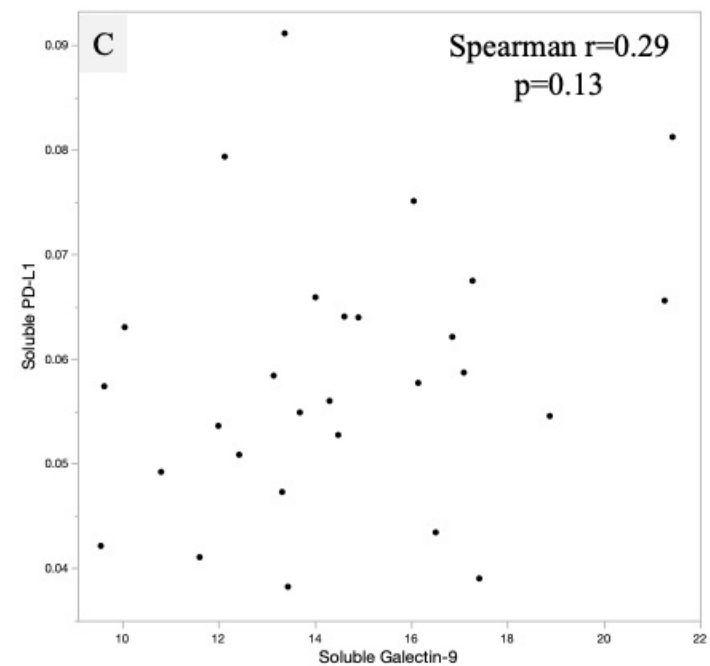
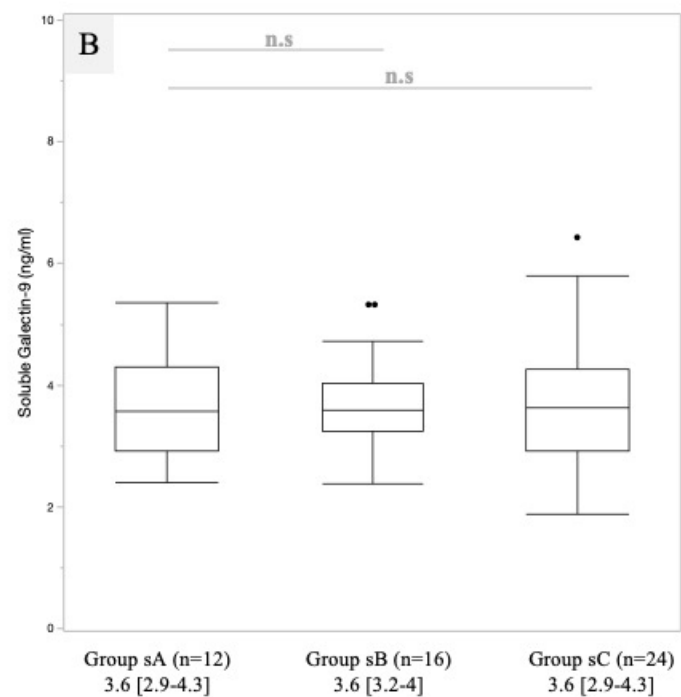
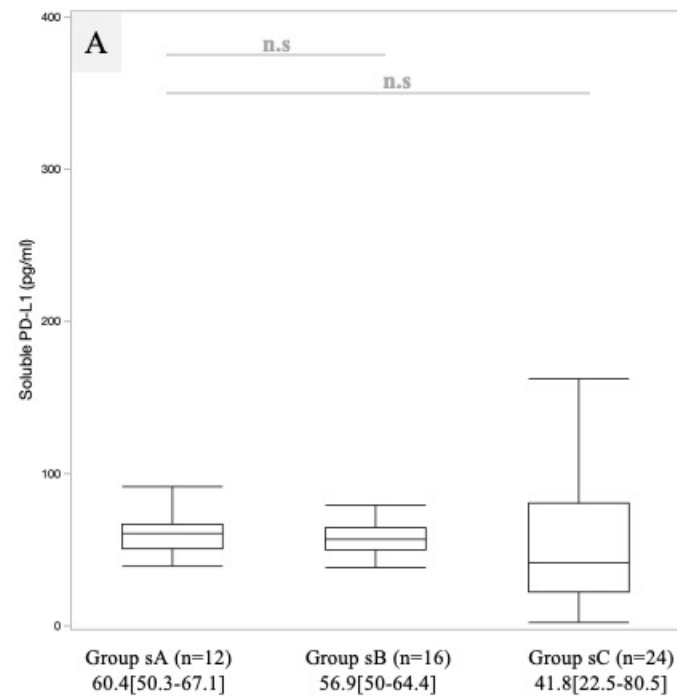
High PD-L1/Tolerance (n=22)	22	22	21	20	20	20
Low PD-L1/Tolerance (n=16)	16	14	12	11	11	-

High Gal-9/Tolerance (n=23)	23	23	21	20	19	19
Low Gal-9/Tolerance (n=15)	15	13	12	12	12	12

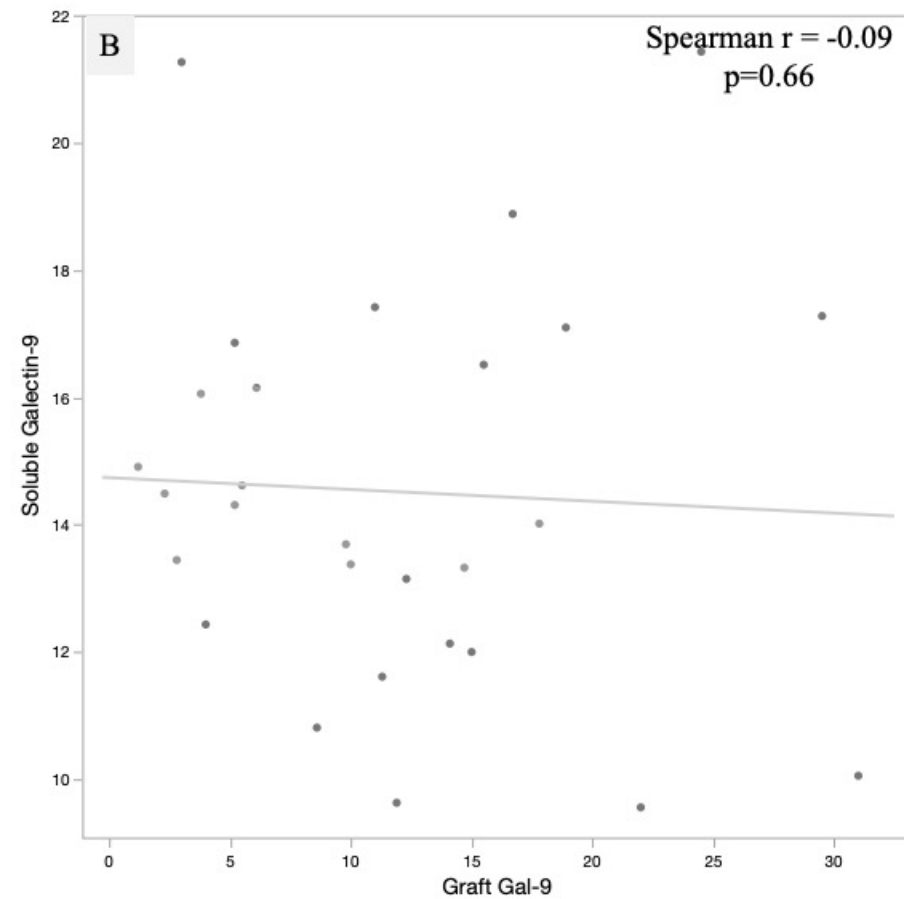
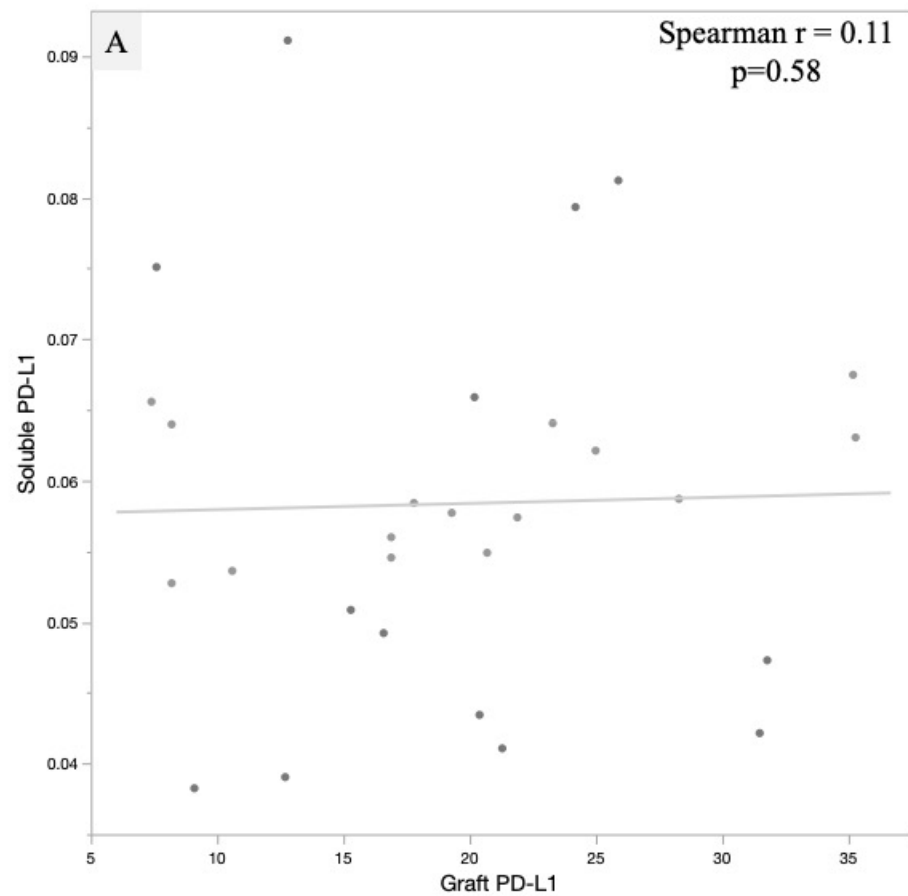
Supporting Figure 7



Supporting Figure 8



Supporting Figure 9



Supporting Figure 10

Table S1: List of antibodies and staining reagents

Antibodies, staining reagents and ELISA kit	Clone/Code	Species	Vendor	Working dilution / Range
Anti-PD-1	NAT105	Mouse	Abcam, Cambridge, UK	1:50
Anti-PD-L1	28-8	Rabbit	Abcam, Cambridge, UK	4 µg/mL
Anti-PD-L1	MIH5	Rat	Novus Biologicals, USA	1:100
Anti-Gal-9	ab69630	Rabbit	Abcam, Cambridge, UK	4 µg/mL
Anti-Foxp3	236A/E7	Mouse	Abcam, Cambridge, UK	10µg/mL
Anti-CD4	EPR6855	Rabbit	Abcam, Cambridge, UK	1:500
Anti-CD8	ab4055	Rabbit	Abcam, Cambridge, UK	1:200
Rabbit mAb IgG XP® Isotype Control	DA1E	Rabbit	Cell Signaling Technology, Danvers, MA	4 µg/mL
Dako Envision System HRP Labeled Polymer anti-mouse		Mouse	Dako North America Inc., Carpinteria, USA	Ready-to-use
Dako Envision System HRP Labeled Polymer anti-rabbit		Rabbit	Dako North America Inc., Carpinteria, USA	Ready-to-use
Goat anti-rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	A-11007		Thermo Fisher Scientific, Waltham, MA, USA	4 µg/mL
Goat anti-rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	A-11008		Thermo Fisher Scientific, Waltham, MA, USA	4 µg/mL
Protein Block Serum-Free	X0909		Dako North America Inc., Carpinteria, USA	Ready-to-use
Dako Real Peroxidase-Blocking Solution	S2023		Dako Denmark A/S, DK-2600, Denmark	Ready-to-use

DakoCytomation Target Retrieval solution Citrate pH6 (x10)	S2369		Dako Denmark A/S, DK-2600, Denmark				
Dako Liquid DAB + Substrate Chromogen System	K3468		Dako North America Inc., Carpinteria, USA				
Vectashield-DAPI mounting medium	Cat # H-1500- 10		Vector Laboratories, Burlingame, CA, USA				
Mayer's Hematoxylin solution			FUJIFILM Wako Pure Chemical Corporation, Japan				Ready-to-use
Human PD-L1 ELISA kit [28-8]	ab214656	Human	Abcam, Cambridge, UK				21.87-1400 pg/ml
Human Galectin-9 Quantikine ELISA kit	DGAL90	Human	R&D Systems				0.2-10 ng/ml

Table S2: Characteristics of the patients in the liver biopsy cohort.

	Tolerance group	Nontolerance group		Control group
	(n=38)	Trigger IS reuse (n=16)	After IS reuse (n=19)	(n=38)
Sex (male:female)	10:28	8:8	8:11	13:25
Age at LT (years)	1.7 (0.8–5.6)	0.8 (0.6–1.1)	0.8 (0.6–1.1)	4.5 (1–10.4)
Primary liver disease (BA:non-BA)	28:10	16:0	19:0	31:7
Recipient BMI (kg/m ²)	15.6 (14.7-17.1)	16.1 (15-17)	16.4 (15.3-17)	16.3 (15.4-18.1)
Graft weight (gram)	240 (220-275)	243 (215-288)	235 (210-280)	300 (245-370)
GRWR (%)	2.6 (1.4-3.6)	3.6 (2.9-3.9)	3.6 (2.9-3.9)	2 (1.3-2.6)
Donor age	32 (28.8-38.6)	32.4 (30.4-39.2)	33.1 (30.1-38.4)	35.9 (31.6-42.4)
Donor BMI (kg/m ²)	21.5 (19.8-23.4)	21 (19.7-24)	21.4 (19.7-23.3)	21.6 (20.3-23.9)
Interval between LT and liver biopsy (years)	16.7 (12.8-19.8)	13.2 (9.1-18.4)	20 (12.7-23.4)	13.9 (3.9-19.5)
Interval between LT and ISW (years)	7.9 (3.5-11)	7.6 (5.4-9.7)	-	-
Interval between ISW and liver biopsy (years)	9.6 (4.7-12.6)	6.8 (3.5-10)	13.2 (10.5-16.6)	
Interval between ISW and IS reuse (years)	7.6 (3.6-11.7)	-	7.6 (3.5-10)	-
AST (UI/L)	22.5 (18-28.5)	29 (23.5-34)	25 (20-31)	24.5 (20-29)
ALT (UI/L)	18 (15-26)	25.5 (17.5-34.5)	21 (18-25)	17 (14-26)
Total bilirubin (mg/dL)	0.75 (0.58-1)	0.6 (0.5-1.1)	0.6 (0.5-0.9)	0.6 (0.4-1)

Non-BA diseases included Alagille syndrome, Propionic acidemia, hepatoblastoma, fulminant hepatitis, cryptogenic liver fibrosis, Wilson's disease, Budd-Chiari syndrome, Byler's disease, Caroli's disease, Ornithine transcarbamylase deficiency, Congenital porto-systemic venovenous malformation/ portopulmonary hypertension

Table S3: Characteristics of the patients in the serum cohort.

	Tolerance group (n=12)	Nontolerance group (n=16)	Control group (n=24)
Sex (male:female)	3:9	4:12	9:15
Age at LT (years)	2.7 (0.9-5.1)	1.1 (0.7-3.9)	4.8 (1.5-10.7)
Primary liver disease (BA:non-BA)	7:5	15:1	20:4
GRWR (%)	2.6 (1.4-3)	3.3 (1.6-3.7)	1.9 (1.3-2.5)
Interval between LT and ISW (years)	8.1 (2.8-12)	6.9 (1.8-8.3)	
Interval between ISW and serum collection (years)	19 (13-20.8)	17 (13-20)	
WBC (x10 ³ /μl)	5.2 (4.3-6.5)	5 (4.1-6.5)	5.3 (4.5-7.1)
RBC (x10 ⁶ /μl)	4.5 (4.3-5)	4.7 (4.5-4.9)	4.6 (4.2-5.1)
Hb (g/dl)	13.8 (13-15.6)	13.3 (12.7-14.8)	12.4 (10.3-14.8)
PLT (x10 ³ /μl)	171 (132-202)	176 (140-246)	185 (137-233)
PT (s)	13.1 (12.2-13.9)	13.8 (13-14.7)	12.8 (13.6-14.3)
INR	1.01 (0.96-1.09)	1.05 (1.01-1.14)	1.05 (0.98-1.09)
AST (UI/L)	24 (21-27)	24 (19-34)	26 (18-32)
ALT (UI/L)	20 (16-39)	17 (13-28)	20 (13.5-26)
Total bilirubin (mg/dL)	0.7 (0.6-1.1)	0.7 (0.5-1.1)	0.6 (0.4-0.9)
Albumin (mg/dL)	4.2 (4.1-4.3)	4.1 (3.8-4.3)	4.1 (3.9-4.2)
GGT (U/L)	37 (17-77)	31 (13-87)	21 (14-46)
ALP (U/L)	117 (78-198)	206 (97-289)	217 (155-368)

LDH (U/L)

171 (157-194)

177 (154-211)

182 (162-226)

Non - BA disease included Alagille syndrome, Propionic acidemia, hepatoblastoma, fulminant hepatitis, cryptogenic liver fibrosis, Wilson's disease, Budd-Chiari syndrome, Byler's disease, Caroli's disease, Ornithine transcarbamylase deficiency, Congenital porto-systemic venovenous malformation/portopulmonary hypertension

Supplementary materials and methods

Immunohistochemical (IHC) staining

Four µm-thick sections were cut from formalin-fixed paraffin-embedded (FFPE) blocks. After deparaffinization with xylene and rehydration through graded alcohols, antigen retrieval was performed by heating the sections in a steamer for 45 minutes in DakoCytomation Target Retrieval solution Citrate pH 6 (x10), code S 2369 (Dako Denmark A/S, DK-2600, Denmark), and allowed to cool to room temperature for 120 minutes. Sections were then washed three times in phosphate-buffered saline (PBS). Afterwards, endogenous peroxidase activity was blocked by treatment with Dako Real Peroxidase-Blocking Solution, code S2023 (Dako Denmark A/S, DK-2600, Denmark) for 30 minutes at room temperature. Sections were then washed three times in PBS. After blocking nonspecific binding with Protein Block Serum-Free ready-to-use, code X0909, Dako North America Inc., Carpinteria, USA) for 10 minutes at room temperature, sections were incubated with primary antibodies mouse monoclonal anti-PD-1 (dilution 1:50, clone NAT105, Abcam, Cambridge, UK), rabbit monoclonal anti-PD-L1 (4 µg /mL, clone 28-8, Abcam, Cambridge, UK), rabbit monoclonal anti-galectin-9 (4 µg/mL, clone ab69630, Abcam, Cambridge, UK), mouse monoclonal anti-Foxp3 (10 µg /mL, clone 236A/E7, Abcam, Cambridge, UK), rabbit monoclonal anti-CD4 (dilution 1:500, clone EPR6855, Abcam, Cambridge, UK), rabbit polyclonal anti-CD8 (dilution 1:200, clone ab4055, Abcam, Cambridge, UK) antibodies in humidified chamber at 4°C overnight. Sections were then washed three times in PBS. Subsequently, Dako Envision System HRP Labeled Polymer anti-mouse secondary antibody (Dako North America Inc., Carpinteria, USA) (for PD-1 and Foxp3 staining) and Dako Envision System HRP Labeled Polymer anti-rabbit secondary antibody (Dako North America Inc., Carpinteria, USA) (for PD-L1, CD4, CD8 staining) were added to the sections and the slides were incubated at room temperature for 60 minutes. Sections were then washed three times in PBS. Later, signal visualization was performed with the Dako Liquid DAB + Substrate Chromogen System, code K3468 (Dako North America Inc., Carpinteria, USA) and slides were immediately washed under tap water after color development. Finally, all sections were counterstained with Mayer's hematoxylin solution (FUJIFILM Wako Pure Chemical Corporation, Japan) for 2 minutes, dehydrated in alcohol, cleared with xylene and mounted. Detailed information on the staining reagents and antibodies is outlined in Supplementary Table 1.

IHC validation

Reagent controls

For isotype control, sections were incubated with a nonimmune antibody (Ab) of the same isotype (rabbit (DA1E) mAb IgG) and at the same concentration (4µg/mL) as the primary antibody (anti-PD-L1 antibody), followed by incubation with secondary antibodies and detection reagents. Regarding the no primary antibody control, sections were incubated with the antibody diluent alone and without primary antibody, followed by incubation with secondary antibodies and detection reagents. In the endogenous tissue background control, sections were incubated with the antibody diluent alone and without either primary antibody or secondary antibodies, followed by incubation with detection reagents.

In fact, the negative result of the isotype control confirmed that the observed staining was not caused by nonspecific interactions of the antibody with the tissue (Supplementary Figure 1, panel A), and the negative result of the no primary antibody control verified that staining was produced from detection of the antigen by the primary antibody and not by the detection system or the specimen (Supplementary Figure 1, panel B). Furthermore, for antigen (tissue) controls, the negative result of endogenous tissue background control affirmed that there was no endogenous background (Supplementary Figure 1, panel C).

Positive control tissue

The human second-trimester placental villi tissue served as a positive control for anti-PD-L1 Ab with a strong staining at the microvillous membrane and a moderate staining in the cytoplasm of the villous syncytiotrophoblasts (Supplementary Figure 2, panel A). Regarding the remaining antibodies, the human tonsil tissue was used as a positive control tissue with a strong staining in the cell surface and cytoplasm in the germinal centers for anti-PD-1 Ab (panel B) and anti-Gal-9 Ab (panel C); a strong membranous staining for anti-CD4 Ab (panel D) and anti-CD8 Ab (panel E); and a strong nucleus pattern staining for anti-Foxp3 Ab (panel F). The result of these staining approved that the staining procedure is working and it also ensured the validation of any negative staining results with these antibodies.

Computational IHC quantification

The PD-L1 and Gal-9 expression levels and the number of Foxp3 + Treg cells, infiltrating CD4+ T cells and infiltrating CD8+ T cells were digitized by scanning the whole section of the slide with a fluorescence microscope (Keyence, Osaka, Japan; BZ-9000). Five microscopic fields at 20× magnification in the periportal area (for Foxp3, CD4 and CD8 staining) and in the parenchymal region (for PD-L1 and Gal-9 staining) were randomly selected from each tissue sample. The obtained images were analyzed by the ImageJ software (National Institutes of Health, Bethesda, MD, USA) (<http://imagej.nih.gov/ij/>).

The percentage of positive hepatocytes and the staining intensity constituted the PD-L1 and the Gal-9 staining scores. Cytoplasmic PD-L1 and Gal-9 signaling was considered as a positive PD-L1 and Gal-9 expression. Using ImageJ, brown-colored cells that signified positive PD-L1 and Gal-9 staining were digitally converted into black and white binary. The color intensity values were calculated based on a range from 0 to 255, wherein, 0 represents the darkest shade of the color and 255 represents the lightest shade of the color. Illustrated examples of PD-L1 expression quantification and Gal-9 expression quantification are presented in Supplementary Figure 3. Finally, for better illustration, the PD-L1 and Gal-9 staining score was represented as a subtraction of 255 to the color intensity value.

Regarding the number of infiltrating CD4+ T cells and infiltrating CD8+ T cells, cells with linear membranous staining were counted as positive, while the nuclear staining pattern was defined as Foxp3+ Treg cells positive staining. Positive cell counts were digitally measured by ImageJ imaging analysis software. Interpreted examples of Foxp3+ Treg staining CD4+ T cells and infiltrating CD8+ T cells counting were outlined in Supplementary Figure 4. The average staining score (for PD-L1) and the average positive cell count (for Foxp3+ Treg cells, infiltrating CD4+ T cells and infiltrating CD8+ T cells) of the five different random fields was expressed as a final result for each section.

Immunofluorescence (IF) staining

Immunofluorescent staining with 4µm FFPE liver sections was performed for the detection of the colocalization of PD-L1 and Gal-9 expression in liver transplant tissues. After deparaffinization with xylene and rehydration through graded alcohols, epitope retrieval was performed by treating the slides with DakoCytomation Target Retrieval solution Citrate pH 6 (x10), code S 2369 (Dako Denmark A/S, DK-2600, Denmark). After cooling down to room temperature, the sections were consecutively blocked with Dako Real Peroxidase-Blocking Solution, code S2023 (Dako

Denmark A/S, DK-2600, Denmark) for 30 minutes and then with Protein Block Serum-Free ready-to-use, code X0909, Dako North America Inc., Carpinteria, USA) for 10 minutes at room temperature. Primary antibody reaction with rat monoclonal anti-PD-L1 (dilution 1:100, clone MIH5, Novus Biologicals, USA) and rabbit monoclonal anti-galectin 9 (4µg/mL, clone ab69630, Abcam, Cambridge, UK) was performed overnight at 4°C. Sections were then washed three times in PBS. After washing three times with PBS for 5 minutes, secondary antibody reactions were performed with Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (dilution 4µg/mL, Cat A-11008, Thermo Fisher Scientific, Waltham, MA, USA) and Goat anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (dilution 4µg/mL, Cat A-11007, Thermo Fisher Scientific, Waltham, MA, USA) for 30 minutes at room temperature. Nuclei were mounted with Vectashield-DAPI mounting medium (Vector Laboratories, Burlingame, CA, Cat # H-1500-10).

Enzyme-linked immunosorbent assay (ELISA)

Serum level of soluble PD-L1 (sPD-L1) and soluble Gal-9 (sGal-9) were measuring using Human PD-L1 Elisa kit 28-8 (ab214565, Abcam, USA). and Human Galectin-9 Quantikine Elisa kit (Catalogue number DGAL90, R&D Systems, Minneapolis, MN, USA). All reagents were equilibrated to room temperature (18-25°C) before starting the experiment.

To quantify the sPD-L1 level, 50 µL standard or serum sample per well were added with 50 µL Antibody Cocktail 10X (3 mL of the Antibody Cocktail was made by combining and mixing 300 µL Human PD-L1 Capture Antibody 10X and 300 µL Human PD-L1 Detector Antibody (RabMAb® 600 µL +4oC clone 28-8) with 2.4 mL Antibody Diluent CPI. Plates were incubated at room temperature for 2 hours on a horizontal orbital microplate shaker set at 500 ± 50 rpm. After aspirating and washing each well three times with 350 µL 1X Wash Buffer PT, 100 µL TMB Development Solution was added and incubated for 10 minutes to develop color reaction and subsequently stopped by adding 100 µL Stop Solution. Serial dilutions of recombinant human PD-L1 (ab214565, Abcam, USA) were used to establish the standard curve.

Regarding sGalectin-9 quantification, a 100 µl 4-fold dilution of serum sample was obtained by adding 25 µl serum sample to 75 µl Calibrator Diluent RD6-35. To assay sGalectin-9, 100 µL Assay Diluent RD1-9 per well was added with 100 µl diluted patient sera and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm. After aspirating

and washing each well four times with Wash Buffer, 200 μ L of Human Galectin-9 Conjugate was added to each well and incubated for 2 hours at room temperature on the shaker. Aspiration and four times of washing were repeated as previously. Subsequently, 200 μ L of Substrate Solution was added to each well and incubated for 20 minutes at room temperature on the benchtop. Color reaction was stopped by adding 50 μ L of Stop Solution per well. A standard curve using recombinant Human Galectin-9 Standard (R&D Systems, Minneapolis, MN, USA) was also performed.

Optical density was read at 450 nm within 30 minutes and quantified using an ELISA plate reader equipped with specialized software (XXX). All samples were tested in duplicate and mean values were used for analysis. The intra-assay and inter-assay variations were below 20%. The lower detection limits for ELISA were 21.87 pg/mL for sPD-L1 and 200 pg/mL for sGal-9. The upper detection limits for ELISA were 1400 pg/mL for sPD-L1 and 10000 pg/mL for sGal-9. Patients with an sPD-L1 or sGal-9 level below background cut-off were assigned with a value of 0. The results correspond to means $\pm$ SEM.