## Title
Pattern Recognition Receptor-reactivity Screening of Liver Transplant Patients



## Journal
Annals of Surgery, Publish Ahead of Print(&NA;)

## ISSN
0003-4932

## Authors
Sosa, Rebecca A
Rossetti, Maura
Naini, Bita V
et al.

## Publication Date
2020-05-01

## DOI
10.1097/sla.0000000000003085

Peer reviewed

HHS Public Access

Author manuscript

*Ann Surg*. Author manuscript; available in PMC 2020 May 22.

# Pattern Recognition Receptor-Reactivity Screening of Liver Transplant Patients: Potential for Personalized and Precise Organ Matching to Reduce Risks of Ischemia-Reperfusion Injury

**Rebecca A. Sosa, PhD**[1], **Maura Rossetti, PhD**[1], **Bita V. Naini, MD**[1], **Victoria M. Groysberg, BS**[1], **Fady M. Kaldas, MD**[2], **Ronald W. Busuttil, MD, PhD**[2], **Yu-Ling Chang, PhD**[1], **David W. Gjertson, PhD**[1], **Jerzy W. Kupiec-Weglinski, MD, PhD**[1,2], and **Elaine F. Reed, PhD**[1,*]

[1]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, , Los Angeles, CA

[2]Division of Liver and Pancreas Transplantation, Department of Surgery, David Geffen School of Medicine at UCLA, , Los Angeles, CA

## Abstract

**Objective and background:** Pattern recognition receptors (PRRs) on immune and parenchymal cells can detect danger-associated molecular patterns (DAMPs) released from cells damaged during ischemia-reperfusion injury (IRI), in heart attack or stroke settings, but also as an unavoidable consequence of solid organ transplantation. Despite IRI being a significant clinical problem across all solid organ transplants, there are limited therapeutics and patient-specific diagnostics currently available.

**Methods:** We screened portal blood samples obtained from 67 human liver transplant recipients both pre- (portal vein sample; PV) and post- (liver flush; LF) reperfusion for their ability to activate a panel of PRRs, and analyzed this reactivity in relation to biopsy-proven IRI.

**Results:** PV samples from IRI+ OLT patients (n=35) decreased activation of hTLR4- and hTLR9-transfected cells, whereas PV from IRI- patients (n=32) primarily increased hTLR7 and hNOD2 activation. LF samples from OLT-IRI patients significantly increased activation of hTLR4 and hTLR9 over IRI- LF. Additionally, the change from baseline reactivity to hTLR4/9/NOD2 was significantly higher in IRI+ than IRI- OLT patients.

**Conclusion:** These results demonstrate that TLR4/7/9 and NOD2 are involved in either promoting or attenuating hepatic IRI, and suggest a diagnostic test screening of portal blood for reactivity to these PRRs might prove useful for prediction and/or therapeutic intervention in OLT patients before transplantation.

## MINI-ABSTRACT

[*]**Correspondence:** Elaine F. Reed, 1000 Veteran Ave. Los Angeles, CA 90095 United States, Tel: 310-794-4943, Fax: 310-206-3216, ereed@mednet.ucla.edu.

SUPPLEMENTAL DIGITAL CONTENT
Sosa_Supplemental_Data_1.docx

CONFLICTS OF INTEREST
The authors of this manuscript have no conflicts of interest to disclose as described by the Annals of Surgery.

Pattern recognition receptors (PRRs) detect danger-associated molecular patterns (DAMPs) released from cells damaged by ischemia-reperfusion injury (IRI) during orthotopic liver transplantation (OLT). Analysis of OLT portal blood shows biopsy-proven IRI+ patients have signficiantly lower reactivity to TLR4/7/9/NOD2 pre-reperfusion and higher TLR4/9 post-reperfusion than IRI- counterparts.

**Keywords**

liver transplantation; ischemia-reperfusion injury; sterile inflammation; pattern recognition receptors; damage-associated molecular patterns; innate immunity; risk score

## INTRODUCTION

Pattern recognition receptors (PRRs) are germline-encoded antigen recognition receptors for innate immunity, which recognize broad classes of molecular structures called pathogen-associated molecular patterns (PAMPs) common to groups of microorganisms[1]. PRRs can also recognize a variety of components from damaged cells termed damage-associated molecular patterns (DAMPs) to promote sterile inflammation.[2] The hepatocellular damage occurring following donor liver removal, storage and transplant termed ischemia-reperfusion injury (IRI), can be a major contributor to primary allograft non-function or late dysfunction in orthotopic liver transplantation (OLT). Indeed, tissue IRI leads to a higher incidence of acute and chronic rejection, and contributes to the acute shortage of organs available for transplantation.[3] Therefore, minimizing the adverse consequences of IRI should improve not only transplantation outcomes, but also increase the number of transplantable patients. DAMPs/PRRs may be a key driver of hepatocellular injury in OLT-IRI, thereby allowing us to manipulate this molecular signaling system for therapeutic purposes.

Dependency of adaptive immune responses on components of the innate immune system has lead to the hypothesis that allograft rejection may be initiated by injury sustained during the transplant process. PRRs play an important role in the activation of innate immunity, and in controlling adaptive host immune responses.[4] IRI upregulates expression of endothelial adhesion molecules, as well as production of inflammatory cytokines and chemokines that are likely mediated by PRRs. We previously assessed longitudinal cytokine signatures in OLT recipient blood obtained pre-, intra- and post-reperfusion and found that the IRI-mediated innate-adaptive immune switch occurs when the recipient's portal blood is reperfused through the donor organ.[5]

The growing number of PRRs includes the most extensively studied family of toll-like receptors (TLRs),[6, 7], as well as AIM-2-like receptors (ALRs)[8–10], C-type lectin receptors (CLRs), NOD-like receptors (NLRs),[11, 12] and RIG-I-like receptors (RLRs)[13], that each respond to their own class of PAMP ligands. However, small molecule agonists that can act as DAMPs for these PRRs are largely undescribed, especially in the context of IRI. Many intracellular components have been noted to become liberated in isolated or complexed forms during IRI processes, including histones, heat-shock proteins, amphoterin (also termed high-mobility group box protein-1; HMGB1), DNA, rRNA, miRNAs, haem and/or ATP, making DAMP analysis difficult in OLT patients. In the absence of a comprehensive

list of potential DAMPs to look for in patient blood samples, we decided to take the approach of investigating PRR reactivity, to first identify specific PRRs that are related to IRI, providing key information to aid in narrowing the ongoing investigation regarding which DAMPs might be present that are capable of acting through IRI-modulated pathways.

Here, we examined the two key patient portal blood samples taken either pre- or post-reperfusion (pre-reperfusion portal vein blood = PV; post-reperfusion liver flush = LF) through the allograft in their ability to activate PRRs that could potentially mediate the switch from innate to adaptive immune signaling during OLT we previously identified.[5] We show that patients classified as IRI+ by histopathology have significantly lower activation of TLR4 and TLR9 by PV coupled with higher activation of TLR4 and TLR9 by LF than IRI- recipients, whose activation of TLR7 and NOD2 was increased by PV. These data identify specific PRRs involved in the outcome of OLT with regards to IR-stress, providing therapeutic targets for attenuating IRI, as well as important mechanistic insight into the key players responsible for mediating the switch from innate to adaptive immune responses in OLT patients.

## MATERIALS AND METHODS

### Study design, sample collection and histopathological IRI scoring

Adult primary orthotopic liver transplant (OLT) recipients were recruited between May 10, 2013 and March 27, 2016 (Table 1). All studies described were reviewed and approved by the UCLA Institutional Research Board. Patients were provided informed consent prior to their participation in the study. Routine standard of care and immunosuppressive therapy was administered as specified by UCLA liver transplant protocols. Study data were collected and managed using REDCap electronic data capture tools hosted at UCLA.[14] Organs were perfused with 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 preservation solution to the removal of the liver from cold storage. Intra-operative portal blood was collected from the recipient portal vein prior to reperfusion (PV) and as it was first flushed through the vena cava of the donor liver during reperfusion (LF). Protocol Tru-Cut needle biopsies were taken from the left lobe intra-operatively after complete revascularization of the allograft (two hours post-reperfusion) prior to surgical closing of the abdomen and graded for IRI as previously described.[5] For correlation between demographic data and IRI, student's T-test was used for continuous variables, and Fisher's exact test was used for categorical variables.

### Pattern Recognition Receptor (PRR) Activation Screening Assay

Human (h)TLR-2/−3/−4/−5/−7/−8/−9/Null, NOD-1/−2, and Dectin1b-specific HEK-Blue™ reporter cells (InvivoGen, San Diego, CA), were grown, maintained and utilized as specified by the manufacturer (InvivoGen, San Diego, CA). To establish response ratio curves of natural ligands (PAMPs) of each PRR we used positive controls (Table 2) according to the manufacturer's instructions (InvivoGen, San Diego, CA). Polymixin B (InvivoGen, San Diego, CA) was used in all experiments to eliminate interference from endogenous LPS. (See Supplemental Methods for further description).

# RESULTS

## OLT recipient and donor characteristics

To assess pattern recognition receptor (PRR) reactivity of OLT-IRI, we analyzed portal blood samples from 67 OLT recipients. Recipients were scored for IRI by histopathology using intra-operative post-reperfusion biopsies of the liver allograft as previously described.[5] Thirty-five patients were graded as IRI+ (51%) and 32 as IRI- (49%). Intra-operative blood was collected from the portal vein of the recipient before (PV) and after (liver flush, LF) donor organ reperfusion. The demographic data and clinical parameters of the recipients and donors, as well as outcome data for OLT recipients within the first year post-transplant are shown in Table 1. There was no correlation between IRI status and any of the parameters, with the exception of an increased risk amongst paired liver/kidney recipients. Similar to our previous study[5], clinical liver function tests were most informative in the beginning and end of the first week post-transplant (Fig. S1). Of the OLT patients who required a for-cause biopsy within the first year, IRI+ recipients were more commonly determined to have signs of ACR, AMR or chronic IRI, whereas IRI- patients were mostly HCV+ with few histopathological features at biopsy.

## OLT-IRI+ recipient portal blood differentially activates PRR-transfected cell lines according to patient IRI status

We cultured patient PV or LF samples with each of 10 commercially available human HEK-Blue™ PRR-transfected cell lines (hTLR½/¾/5/7/8/9, hNOD½, or hDectin1b). To establish biological relevance of our findings, we measured ligand sensitivity of canonical PAMPs as positive controls for each cell line (Table 2) to first determine the $EC_{max}$ (Fig. 1, solid lines) and $EC_{50}$ (Fig. 1, dashed lines) response ratios of positive controls, and media as negative control (Fig. 1, dotted lines), then compared patient response ratios to those values (Fig. 1, grey open and filled circles). Responses were significantly increased when stimulating all cell lines with LF compared to PV samples except hNOD2. Intriguingly, responses to hTLR4 were significantly increased in IRI+ patients by LF, yet significantly decreased by PV compared to that achieved with samples from IRI- patients. Reactivity to hTLR7 was increased in IRI- over IRI+ patients when stimulating with LF, whereas reactivity to hTLR9 was increased in IRI+ patients compared to IRI- cohort at this time point. Despite hNOD2 not having any significant changes with respect to time, IRI- patients had significantly higher responses to this cell line by PV than IRI+.

Recipients of paired liver-kidney transplants (n=11) correlated to IRI independently of their PRR-reactivity results (Table 1). Therefore, we analyzed the subgroup of 56 liver-only recipients and found no difference in PRR reactivity that contradicted our findings in the expanded cohort (Fig. S2).

## Unsupervised hierarchical clustering identifies PRRs that are similarly activated by OLT patient's blood samples

We used unsupervised hierarchical clustering to first identify pre- and post-reperfusion patterns of patient reactivity to 10 PRRs (Fig. S3). The algorithm clustered the samples by similarity of PRR reactivity. The samples clustered first by time point, then by patient IRI

status. Two main groups were identified by clustering, the first included response ratios for TLR4 and TLR9, which were highest in IRI+ patient's post-reperfusion LF samples, and lowest in IRI+ PV. The second included PRRs which were highest in IRI- patient LF, and lowest in IRI+ patient's PV, including TLR3, TLR7, TLR5, Dectin1b, TLR8, NOD1, TLR2, NOD2.

## Response ratios obtained from PRR-transfected cell lines can categorize OLT patients as negative or positive for activation of specific PRRs

We next employed a discriminant scoring system to assign positive and negative PRR-reactivity scores to each patient sample based on their stimulation of each cell line as compared to responses obtained by controls (Fig. 2). Patient samples with response ratios below the positive control PAMP ligand's $EC_{50}$ (dashed line) were considered negative (no shading) for stimulating that cell line, with those falling below the response of media alone (dotted line) scoring a '0' and those falling between the value obtained by media (dotted line) and the PAMP's $EC_{50}$ (dashed line) assigned a score of '1'. Patient samples with response ratios above the positive control PAMP ligand's $EC_{50}$ (dashed line) were considered positive (grey shading) for stimulating that cell line, with responses between the PAMP's $EC_{50}$ (dashed line) and $EC_{max}$ (solid line) assigned a score of '2' (light grey shading) and samples that stimulated a cell line higher than the PAMP's $EC_{max}$ (solid line) value scored a '3' (dark grey shading). A larger percentage of IRI+ patients received positive scores for TLR4 and TLR9 in LF as opposed to negative scores in PV, whereas scores for TLR7 and NOD2 were positive in a larger percentage of IRI- patients.

A representative IRI- patient is shown in Fig. 3A, with panel reactivity in the positive range (dark shaded areas as determined in Fig. 2) for PV hTLR7 and hNOD2, which associated with IRI- patient status (Fig. 1). Additionally, this patient is negative for hTLR4 activation by both PV and LF. It can also be noted that the individual IRI- patient shown (Fig. 3A), while overall representative of the IRI- population, also had positive reactivity to hTLR3, hTLR7, hTLR8, and hNOD1 cells by LF.

A representative IRI+ patient is shown in Fig. 3B, with mean panel reactivity in the negative range for both hTLR4 and hTLR9 by PV yet positive by LF. Low LF hTLR7 and NOD2 response ratios further implicate an increased risk of IRI occurring in this patient. Interestingly, 15/32 IRI+ patients (47%) with positive response ratios for hTLR4 scored a '3' in LF, indicating that their responses post-reperfusion are even higher than the $EC_{max}$ for LPS stimulation in this cell line. Coupled with the overall low activation of TLR4 at PV, this sudden shift to a very high activation level of TLR4 reasonably implicates a shock to the system of IRI+ OLT recipients that IRI- recipients are not experiencing.

## Change over baseline reactivity for TLR4/9 and NOD2 are increased in IRI+ OLT recipients

As we found that both negative PV TLR4/9 and positive LF TLR4/9 reactivity correlated with patient IRI status, we next determined if this change over a patient's own baseline was statistically significant (Fig. S4). Indeed, analyses showed very sharp slopes for IRI+ versus IRI- patient's paired PRR response ratios (Fig. S4) for many cell lines, and calculating the

delta revealed this was the case for hNOD2- as well as both hTLR4- and hTLR9-transfected cells (Fig. 4).

### IRI risk is increased when patient is negative for TLR4 reactivity by PV and positive by LF

We then performed a classification and regression tree (CART) analysis to detect the PRR reactivity data with the greatest contribution to discriminate IRI (Fig. 5). The algorithm was limited to include only TLR4 and TLR7 results as a correlation matrix determined these two PRRs to have very high similarity to TLR9 and NOD2 respectively. The CART algorithm had sensitivity of 80%, specificity of 78.13%, positive predictive value of 80%, and negative predictive value of 78.13% with 79.1% overall correctly classified.

## DISCUSSION

Liver IRI is characterized by an innate immune-driven sterile inflammatory response that results from initial hepatocellular damage due to the interruption of oxygen supply to the donor allograft, followed by a rapid inflammatory response upon reperfusion with recipient blood. The resultant damaging effects of these events have a profound influence on the post-operative outcome. Although it is now widely appreciated that PRRs from the innate immune system can detect DAMPs released from damaged cells, the role DAMPs/PRRs play in mediating downstream immune responses in OLT-IRI remains unclear. Additionally, it has been difficult to assess a patient's individual reactivity to the cells expressing these receptors. Here, we examined the reactivity of 67 OLT recipients to a panel of 10 PRRs using commercially available HEK-Blue™ transfected cell lines. We show PV obtained from IRI+ patients has a significantly decreased ability to activate hTLR4 and hTLR9 cells, whereas it takes on an increased ability to activate these same cells by LF obtained at reperfusion immediately following its contact with the donor allograft. On the other hand, IRI- PV had an increased ability to activate hTLR7 and/or hNOD2 cells compared to IRI+. Together, our data reveals negative TLR4/TLR9 reactivity by PV, especially when coupled with positive TLR4/9 reactivity by LF, is a major risk factor for IRI in OLT. Further, negative TLR7/NOD2 reactivity by PV may indicate reduced risk for developing complications due to IRI in OLT.

DAMPs are intracellularly-sequestered molecules that remain unrecognized by the immune system under physiological conditions. Under tissue injury, these molecules are either actively secreted by stressed immune cells or passively released into the extracellular environment from dying cells or supporting damaged extracellular matrix.[15] Although initially beneficial, innate immunity-mediated protective inflammatory repair processes can become pathogenic. Inflammation in IRI is characterized by a storm of cytokines, chemokines, and growth factors in addition to potential DAMPs released by different cell types which could exacerbate tissue injury in acute and reparative phases. DAMPs arising in the stressed allograft serve as endogenous ligands for PRRs on cells of the innate immune system such as donor- or recipient-derived dendritic cells (DCs) and donor-derived vascular cells thereby activating them.[16] The activation of PRR-bearing DCs following sensing of DAMPs promotes their ability to elicit an adaptive immune response.[17–20] Of note, T cells can express PRRs, directly affecting adaptive immune responses via a non-antigen-specific

mechanism.[21] Although the PAMP LPS does not affect T cell cytokine secretion or proliferation profiles, it does influence other aspects of T cell physiology such as adhesion and migration.[22] Therefore it will be interesting to determine both direct/indirect effects of PRR reactivity increases in OLT patients on their T cell populations, particularly TLR4/7/9 and NOD2, which we have shown here to be important for modulating IRI immune cascade.

One of the most investigated DAMPs, High Mobility Group Box-1 (HMGB1) protein can exacerbate hepatic injury through activation of PRRs. Though TLR2 and TLR4 were initially believed to be the major mediators for HMGB1, murine studies have eliminated a role for TLR2 in hepatic IRI,[23] and TLR9 has recently been identified as a signaling partner with differential downstream effects.[24] We found positive LF reactivity to both TLR4/9 in our cohort of OLT-IRI recipients, yet low reactivity by PV, making the release of HMGB1 from the donor allograft an attractive candidate for regulating IRI. Further, it is possible that IRI- patients have a low-level of HMGB1 already present in their PV that induces a type of tolerance for mitigating the reactivity to hTLR4/9 in their subsequent LF since we saw no significant change over baseline for reactivity to these PRRs in these individuals.

We recognize the limitations of using HEK293 cells for PRR-signaling studies, which rely on activation via a reporter system rather than studying the endogenous gene(s) lying in its normal context.[25] Additionally, it was not possible to test a single patient's blood on all cell lines in the same well, therefore we do not know the complex interactions, competition or cross-talk that may occur when a particular mix of PRRs are present in a donor organ upon initial contact with recipient blood. For example, the IRI- population also had positive reactivity to hTLR3, hTLR7, hTLR8, and hNOD1 cells by LF. These could potentially alter downstream responses irrespective of the contribution of IRI that nevertheless may become important in their future outcome. Further investigation with larger cohorts are necessary to uncover common mechanisms, as this will likely prove highly beneficial in achieving the goal towards personalized treatment options for solid organ transplant recipients. Nevertheless, we also champion the value of using transfected cell lines, especially commercially available cell lines with vigorous quality control. To further support the biological relevance of our findings, we employed a rigorous scientific approach to determining PRR-reactivity for OLT patient plasma samples in the context of PRR-reactivity of canonical PAMP ligands for each cell line. Since LPS sensitivity requires the measurement of the $EC_{50}$ of LPS rather than the magnitude of response at individual concentrations, we chose to analyze our data in the context of the $EC_{50}$ for each PAMP of each PRR tested. High levels of LPS have long been appreciated to drive Th1 adaptive immune response, whereas low levels remarkably may cause a Th2 response,[26] but this phenomenon has not been tested for other PAMP-PRR pairs. Therefore, our description of OLT recipient portal blood responses as compared to $EC_{50}$ and $EC_{max}$ responses for canonical PAMP ligands may continue to provide useful information as these PAMP's functions are further characterized at various concentrations.

In this study, we obtained portal venous blood just before and after the reperfusion maneuver to enable the direct comparison of recipient blood before and after contact with the donor tissue; however, we recognize the perceived limitation of obtaining samples pre-transplant in this manner. We have demonstrated here (Fig. S1) and previously[5], clinical liver function

tests (LFT) values obtained in the first week can provide information regarding the existence of IRI in lieu of a biopsy, which remains the current gold-standard of IRI diagnosis. Nevertheless, due to the complexity of liver function, the use of clinical LFTs as a measure to predict outcome following liver surgery is widely regarded as inadequate at best[27], and even in our closely related studies the specific parameters indicating risk differ based on the specific cohort evaluated, making it impossible to assign a concrete threshold or time point of any particular LFT as the one true test for determining and/or alleviating OLT-IRI complications. Yet portal blood is more likely to have DAMPs in abundance than the peripheral blood used to obtain LFT values, which is unlikely to see increases in these molecules that are typically processed by the liver anyway. Novel non-invasive methods to obtain portal blood, such as the use of endoscopic ultrasound-guided portal venous access,[28] have already sparked interest in other fields[29] and therefore this or an alternate procedure could be adopted in pre-transplant patient screening as well. Due to the clinical, immunological and genetic heterogeneity that exists among donors and recipients, future immunosuppression protocols should be individualized to optimize outcome for each transplant patient. Since IRI+ patients were negative on screening of their PV blood on hTLR4/9 cells and higher on hTLR7/hNOD2 compared to IRI- counterparts, screening of OLT recipient's portal blood before transplantation might provide a much needed diagnostic strategy to reduce IRI and improve OLT outcomes, with positive reactivity to TLR4/9 indicating increased risk and positive reactivity to TLR7/NOD2 in the PV sample predicting low risk to IR-damage. In this way, PRR reactivity screening of pre-transplant patient portal blood, particularly for TLR4/7/9/NOD2, has the potential to provide personal IRI risk assessment for OLT recipients. Additionally, our present findings support a novel concept that either low-level pre-conditioning with exogenous recombinant TLR4/9 ligands or anti-TLR4/9 therapy during transport utilizing ex-vivo normothermic machine perfusion (NMP) may be beneficial in preventing this suddenly intense innate immune response, thereby attenuating risk of IRI even in patients with high potential for TLR4/9 reactivity post-reperfusion between their portal blood and a potential donor organ. NMP has been found successful in improving physiological function during pre-clinical trials on animal models[30–33], and currently four NMP devices have been approved for early phase clinical trials[34–39]; however, modulating downstream immunological function has yet to be fully addressed. Antibiotics have already been standardized as a component of the perfusate, therefore the addition of more specific anti-inflammatory mediators such as those that attenuate PRR signaling pathways is already feasible. Our data here provides the first IRI-specific innate-to-adpative immune pathways to be mechanistically involved in the IRI process which could be targeted therapeutically for improved OLT outcomes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

This work was supported by the NIH Ruth L. Kirschstein National Research Service Award T32CA009120 (RAS), NIH L60 MD011903 (RAS), NIH PO1 AI120944 (JWKW and EFR), and UL1TR001881 (UCLA Clinical and Translational Science Institute). We would like to thank Gregg Kunder, Antony Aziz, and the liver transplant team who go above and beyond to collect patient samples for this project. The authors hereby express their thanks for all

of the organ donors and their families, for giving the gift of life and the gift of knowledge, by their generous donation.

SOURCE OF FUNDING

This work was supported by the NIH Ruth L. Kirschstein National Research Service Award T32CA009120 (RAS), NIH L60 MD011903 (RAS), NIH PO1 AI120944 (JWKW and EFR), and UL1TR001881 (UCLA Clinical and Translational Science Institute).

## REFERENCES

1. Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 1989; 54 Pt 1:1–13.
2. Tolerance Matzinger P., danger, and the extended family. Annu Rev Immunol 1994; 12:991–1045. [PubMed: 8011301]
3. Zhai Y, Busuttil RW, Kupiec-Weglinski JW. Liver Ischemia and Reperfusion Injury: New Insights Into Mechanisms of Innate – Adaptive Immune-Mediated Tissue Inflammation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2011; 11(8):1563–1569.
4. Medzhitov R, Janeway CA Jr. Innate immunity: impact on the adaptive immune response. Curr Opin Immunol 1997; 9(1):4–9. [PubMed: 9039775]
5. Sosa RA, Zarrinpar A, Rossetti M, et al. Early cytokine signatures of ischemia/reperfusion injury in human orthotopic liver transplantation. JCI Insight 2016; 1(20):e89679.
6. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006; 124(4): 783–801. [PubMed: 16497588]
7. Medzhitov R, Janeway C Jr. Innate immunity. N Engl J Med 2000; 343(5):338–44. [PubMed: 10922424]
8. Unterholzner L The interferon response to intracellular DNA: why so many receptors? Immunobiology 2013; 218(11):1312–21. [PubMed: 23962476]
9. Danilchanka O, Mekalanos JJ. Cyclic dinucleotides and the innate immune response. Cell 2013; 154(5):962–70. [PubMed: 23993090]
10. Barber GN. Cytoplasmic DNA innate immune pathways. Immunol Rev 2011; 243(1):99–108. [PubMed: 21884170]
11. Kersse K, Bertrand MJ, Lamkanfi M, et al. NOD-like receptors and the innate immune system: coping with danger, damage and death. Cytokine Growth Factor Rev 2011; 22(5–6):257–76. [PubMed: 21996492]
12. Clarke TB, Weiser JN. Intracellular sensors of extracellular bacteria. Immunol Rev 2011; 243(1): 9–25. [PubMed: 21884164]
13. Loo YM, Gale M Jr. Immune signaling by RIG-I-like receptors. Immunity 2011; 34(5):680–92. [PubMed: 21616437]
14. Harris PA, Taylor R, Thielke R, et al. Research electronic data capture (REDCap)--a metadata-driven methodology and workflow process for providing translational research informatics support. J Biomed Inform 2009; 42(2):377–81. [PubMed: 18929686]
15. Medzhitov R Inflammation 2010: New Adventures of an Old Flame. Cell 2010; 140(6):771–776. [PubMed: 20303867]
16. Land W Allograft injury mediated by reactive oxygen species: from conserved proteins of Drosophila to acute and chronic rejection of human transplants. Part III: interaction of (oxidative) stress-induced heat shock proteins with toll-like receptor-bearing cells of innate immunity and its consequences for the development of acute and chronic allograft rejection. Transplantation Reviews; 17(2):67–86.
17. Land WM. Postischemic reperfusion injury to allografts: its impact on T-cell alloactivation via upregulation of dendritic cell–mediated stimulation, co-stimulation, and adhesion. Current Opinion in Organ Transplantation 1999; 4(2).
18. Gallucci S, Matzinger P. Danger signals: SOS to the immune system. Curr Opin Immunol 2001; 13(1):114–9. [PubMed: 11154927]

19. Matzinger P The danger model: a renewed sense of self. Science 2002; 296(5566):301–5. [PubMed: 11951032]
20. Land WG. The role of postischemic reperfusion injury and other nonantigen-dependent inflammatory pathways in transplantation. Transplantation 2005; 79(5):505–14. [PubMed: 15753838]
21. Kaczanowska S, Joseph AM, Davila E. TLR agonists: our best frenemy in cancer immunotherapy. J Leukoc Biol 2013; 93(6):847–63. [PubMed: 23475577]
22. Zanin-Zhorov A, Tal-Lapidot G, Cahalon L, et al. Cutting Edge: T Cells Respond to Lipopolysaccharide Innately via TLR4 Signaling. The Journal of Immunology 2007; 179(1):41–44. [PubMed: 17579019]
23. Shen XD, Ke B, Zhai Y, et al. Absence of toll-like receptor 4 (TLR4) signaling in the donor organ reduces ischemia and reperfusion injury in a murine liver transplantation model. Liver Transpl 2007; 13(10):1435–43. [PubMed: 17902130]
24. Yanai H, Taniguchi T. Nucleic acid sensing and beyond: virtues and vices of high-mobility group box 1. Journal of Internal Medicine 2014; 276(5):444–453. [PubMed: 25041239]
25. Du X, Poltorak A, Silva M, et al. Analysis of Tlr4-mediated LPS signal transduction in macrophages by mutational modification of the receptor. Blood Cells Mol Dis 1999; 25(5–6):328–38. [PubMed: 10660480]
26. Eisenbarth SC, Piggott DA, Huleatt JW, et al. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med 2002; 196(12):1645–51. [PubMed: 12486107]
27. Hoekstra LT, de Graaf W, Nibourg GAA, et al. Physiological and Biochemical Basis of Clinical Liver Function Tests: A Review. Annals of Surgery 2013; 257(1):27–36. [PubMed: 22836216]
28. Garg R, Rustagi T. Endoscopic Ultrasound-guided Portal Venous Access: Diagnostic and Therapeutic Implications. J Clin Gastroenterol 2017; 51(8):677–682. [PubMed: 28742731]
29. Chapman CG, Waxman I. Portal-vein blood samples as a new diagnostic entity for pancreatic cancer. Expert Review of Gastroenterology & Hepatology 2016; 10(6):665–667. [PubMed: 27077275]
30. Sutton ME, op den Dries S, Karimian N, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. PLoS One 2014; 9(11):e110642.
31. op den Dries S, Karimian N, Sutton ME, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am J Transplant 2013; 13(5):1327–35. [PubMed: 23463950]
32. Yska MJ, Buis CI, Monbaliu D, et al. The role of bile salt toxicity in the pathogenesis of bile duct injury after non-heart-beating porcine liver transplantation. Transplantation 2008; 85(11):1625–31. [PubMed: 18551070]
33. Graham JA, Guarrera JV. “Resuscitation” of marginal liver allografts for transplantation with machine perfusion technology. J Hepatol 2014; 61(2):418–31. [PubMed: 24768755]
34. Angelico R, Perera MTPR, Ravikumar R, et al. Normothermic Machine Perfusion of Deceased Donor Liver Grafts Is Associated With Improved Postreperfusion Hemodynamics. Transplantation Direct 2016; 2(9):e97.
35. Vogel T, Brockmann JG, Friend PJ. Ex-vivo normothermic liver perfusion: an update. Curr Opin Organ Transplant 2010; 15(2):167–72. [PubMed: 20186059]
36. Mergental H, Roll GR. Normothermic machine perfusion of the liver. Clinical Liver Disease 2017; 10(4):97–99. [PubMed: 31186895]
37. Ravikumar R, Jassem W, Mergental H, et al. Liver Transplantation After Ex Vivo Normothermic Machine Preservation: A Phase 1 (First-in-Man) Clinical Trial. Am J Transplant 2016; 16(6): 1779–87. [PubMed: 26752191]
38. Jochmans I, Akhtar MZ, Nasralla D, et al. Past, Present, and Future of Dynamic Kidney and Liver Preservation and Resuscitation. Am J Transplant 2016; 16(9):2545–55. [PubMed: 26946212]
39. Minor T, Efferz P, Fox M, et al. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant 2013; 13(6):1450–60. [PubMed: 23617781]

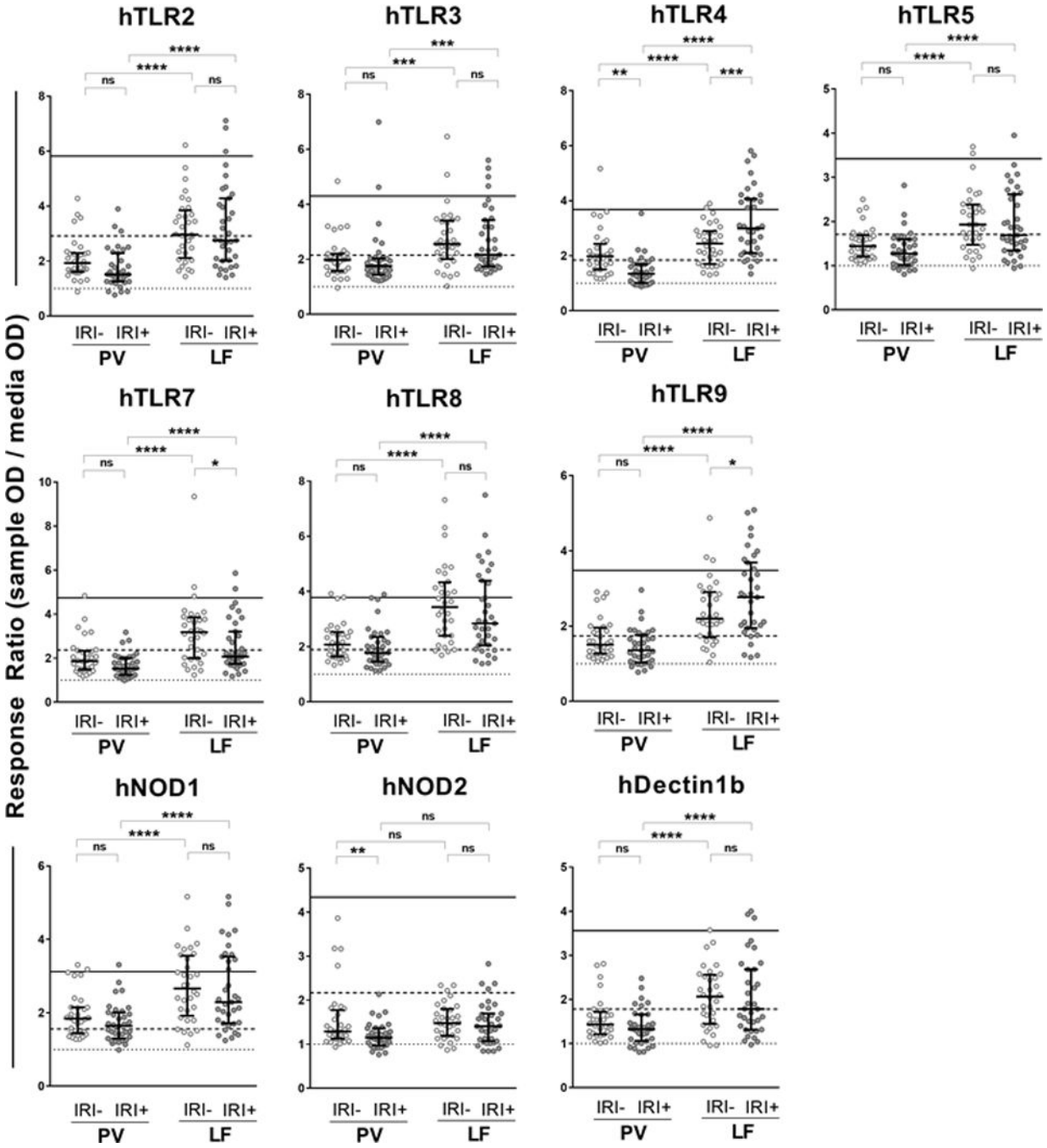


**Figure 1 –. Orthotopic liver transplant recipient portal blood differ in response ratios obtained from hTLR4 and hTLR7 PRR-transfected cell lines.**

Patient plasma samples obtained from the portal vein pre-reperfusion (portal vein; PV) or just after being flushed through the liver during reperfusion (liver flush; LF) were tested for reactivity to pattern recognition receptors (PRRs) using a panel of 10 human (h) HEK-Blue™ transfected cell lines (hTLR2, hTLR3, hTLR4, hTLR5, hTLR7, hTLR8, hTLR9, hNOD1, hNOD2, and hDectin1b). Shown are mean response ratios obtained for PV (open circles) and LF (closed circles) samples for each patient (n=67) with reference lines added for $EC_{max}$ (solid line) and $EC_{50}$ (dashed line) response ratios of canonical PAMP specific to

PRR tested (Table 2), or media control (dotted line; response ratio set to 1) in each transfected cell line as indicated over three independent experiments. Data are presented as scatter plots with error bars indicating median values with inner fences reaching 1.5 times the interquartile range for IRI+ or IRI- groups. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 by two-way ANOVA with Sidak's multiple comparisons test was used to determine differences amongst time and/or IRI status.

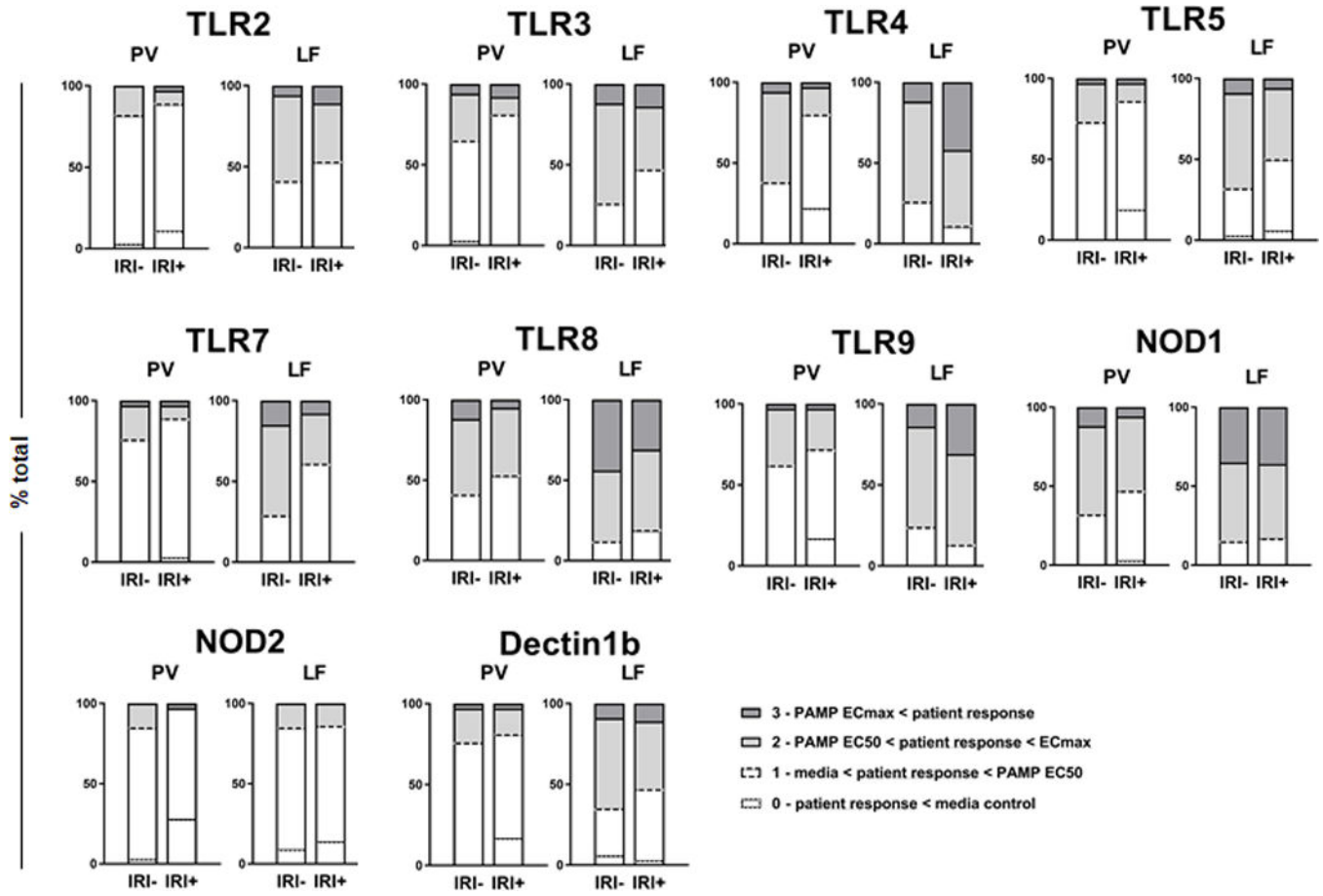


**Figure 2 –. Relationship between PRR activation and response ratios obtained from PRR-transfected cell lines**

Response ratios obtained from 10 PRR-tranfected cell lines (HEK-Blue™; (hTLR2, hTLR3, hTLR4, hTLR5, hTLR7, hTLR8, hTLR9, hNOD1, hNOD2, and hDectin1b) co-cultured with OLT patient (n=67) portal blood samples obtained pre-transplant (portal vein; PV) and post-transplant (liver flush; LF) were assigned a semi-quantitative score based on $EC_{max}$ (solid line) and $EC_{50}$ (dashed line) response ratios of canonical PAMP specific to PRR cell line tested, or media control (dotted line; response ratio set to 1) in each transfected cell line over three independent experiments. Patient samples with response ratios higher than the $EC_{50}$ of respective PAMP are positive for activating that PRR (grey shading), with dark grey indicating values higher than the $EC_{max}$ of the related PAMP and light grey indicating response ratio between $EC_{50}$ and $EC_{max}$. Patient samples with response ratios lower than the $EC_{50}$ of respective PAMP are negative for activating that PRR (no shading). Shown are percent of total IRI+/− population receiving each score at each time point.

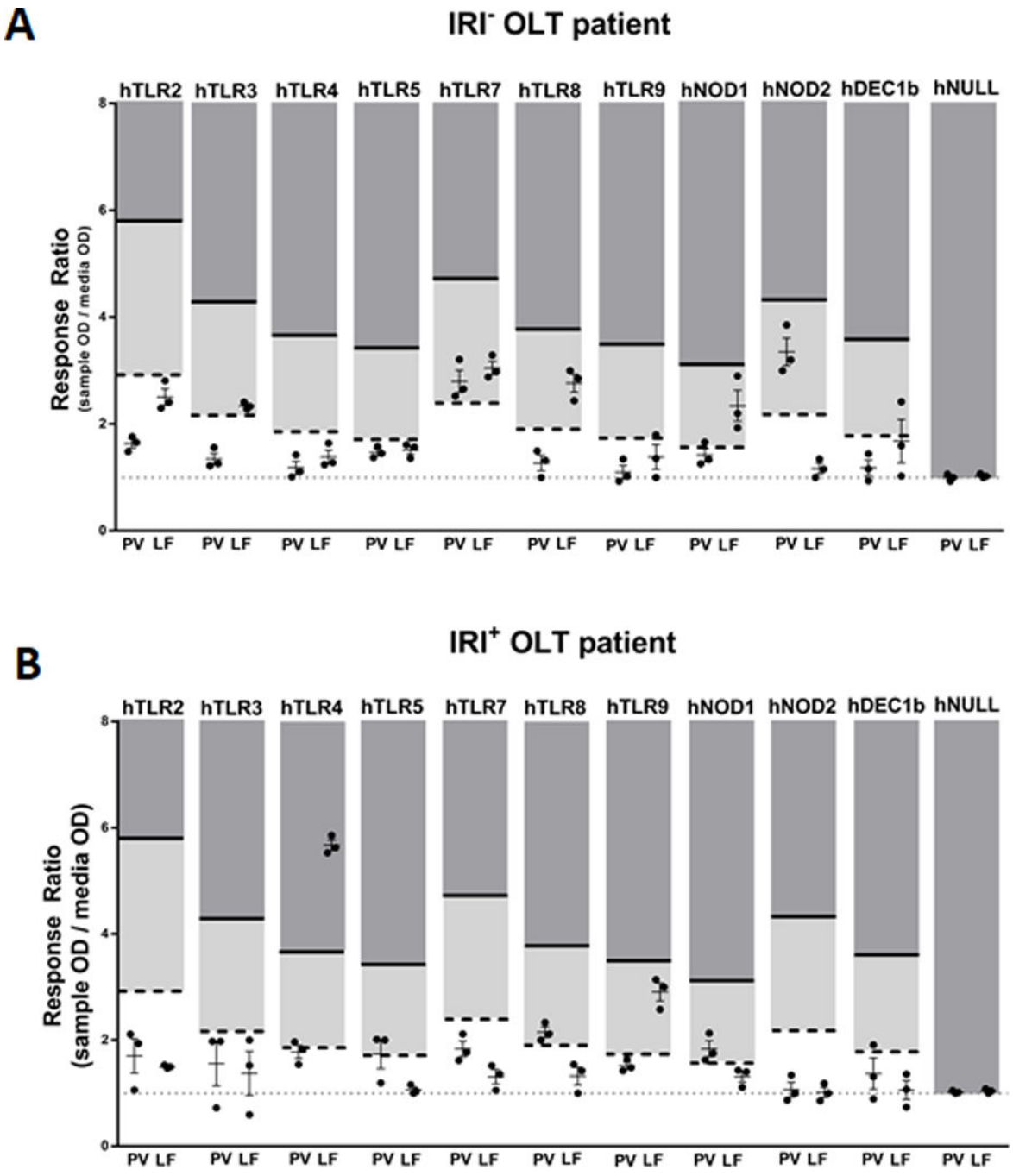


**Figure 3 –. PRR reactivity panel screening results for representative IRI+/− OLT patients**

Prototypical patient's results for OLT patients who are representative of the (A) IRI- or (B) IRI+ PRR reactivity profile. Shown are mean response ratios obtained ± SEM for PV and LF samples from a single representative (A) IRI- or (B) IRI+ OLT patient with reference lines added for $EC_{max}$ (solid line) and $EC_{50}$ (dashed line) response ratios of canonical PAMP specific to PRR tested (Table 2), or media control (dotted line; response ratio set to 1) in each transfected cell line as indicated over three independent experiments.

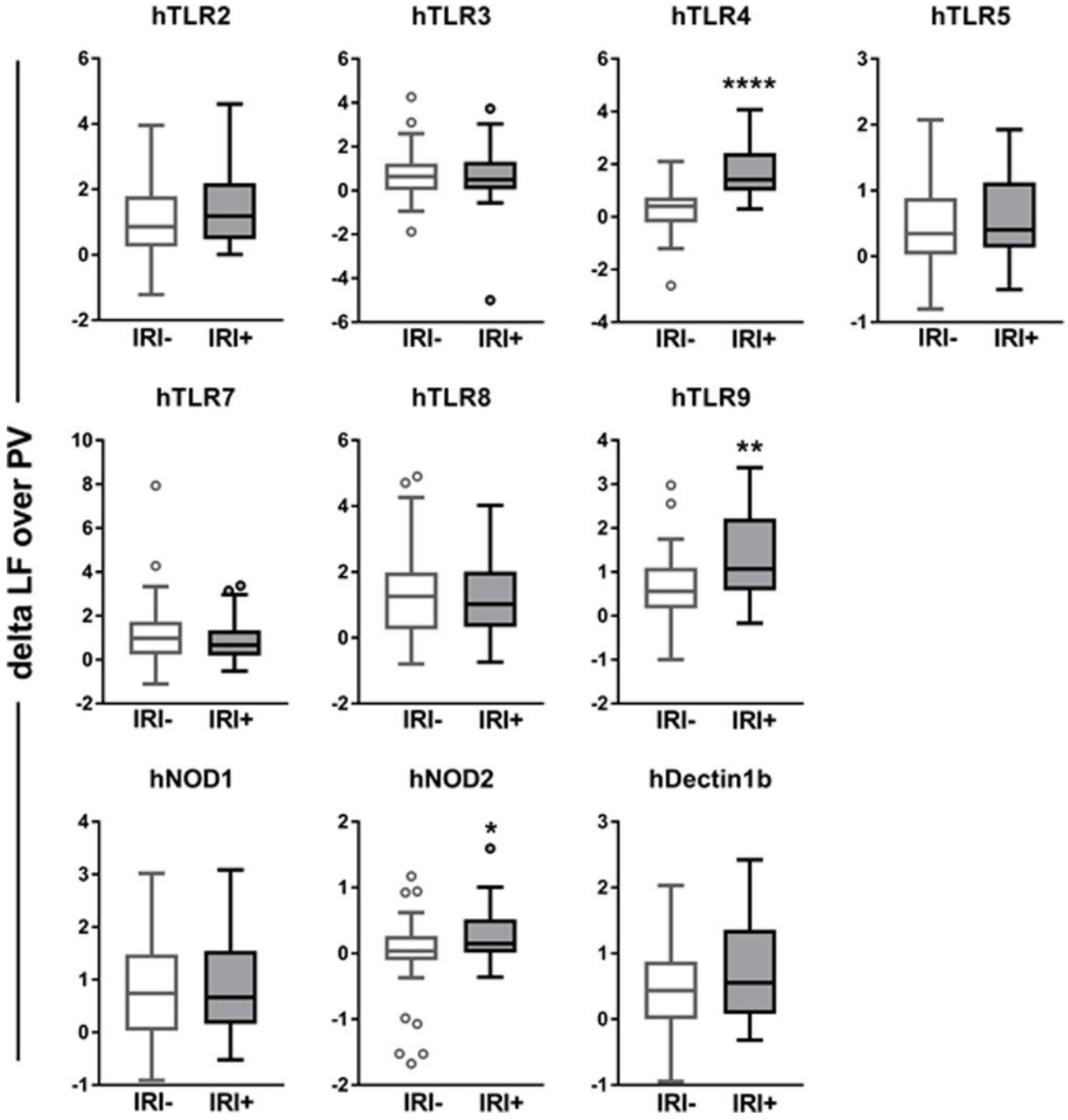


**Figure 4 –. Change over baseline for TLR4/9 and NOD2 is increased for IRI+ OLT patients**

Patient plasma samples obtained from the portal vein pre-reperfusion (portal vein; PV) or just after being flushed through the liver during reperfusion (liver flush; LF) were tested for reactivity to pattern recognition receptors (PRRs) using a panel of 10 human HEK-Blue™ transfected cell lines (hTLR2, hTLR3, hTLR4, hTLR5, hTLR7, hTLR8, hTLR9, hNOD1, hNOD2, and hDectin1b). Delta of mean post-reperfusion LF over mean baseline PV was determined for each patient, and data are presented as Tukey box-and-whisker plots in which 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 group. *P<0.05; **P<0.01; ****P<0.0001 by two-way ANOVA with Tukey's multiple comparisons test.

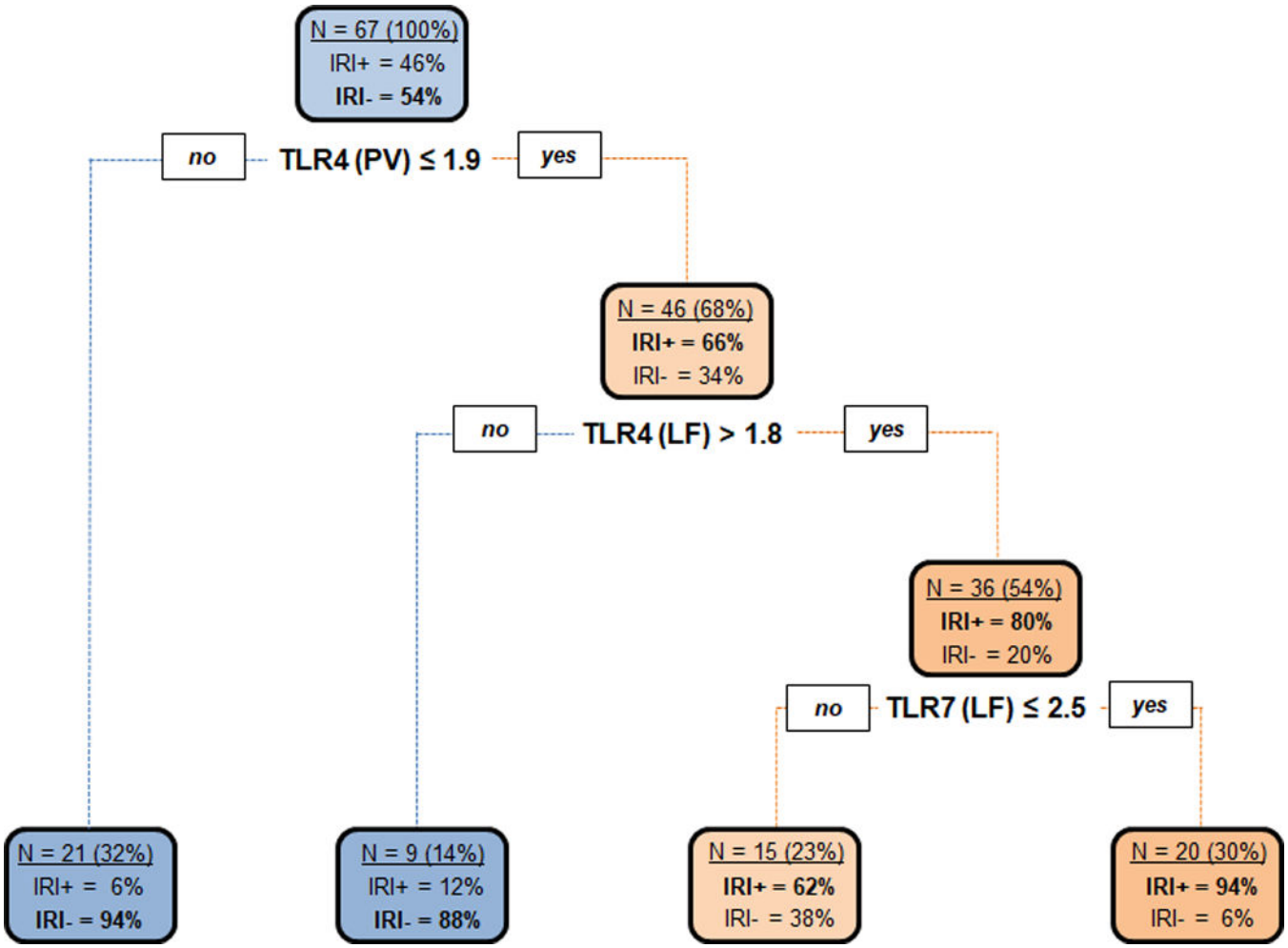


**Figure 5 –. PRR screening of patient portal blood before and after reperfusion through allograft for TLR4/7 reactivity predicts increased risk for IRI during OLT.**

Patient plasma samples obtained from the portal vein pre-reperfusion (portal vein; PV) or just after being flushed through the liver during reperfusion (liver flush; LF) were tested for reactivity to pattern recognition receptors (PRRs) using a panel of 10 human HEK-Blue™ transfected cell lines (hTLR2, hTLR3, hTLR4, hTLR5, hTLR7, hTLR8, hTLR9, hNOD1, hNOD2, and hDectin1b). A classification and regression tree (CART) analysis was performed to detect the PRR reactivity data with the greatest contribution to discriminate IRI.

**Table 1 –**

**Recipient and Donor Demographics.**

ACR, acute cellular rejection; AIH, autoimmune hepatitis; ALF, acute liver failure; AMR, antibody-mediated rejection; DBD, deceased after brain death; DCD, deceased after cardiac death; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; MELD, model for end-stage liver disease; NAFLD/NASH, nonalcoholic fatty liver disease/nonalcoholic steatohepatitis; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; SD, standard deviation. *T test for continuous variables; Fisher exact test for categorical variables.

| **Recipient** | **Total (n=67)** | **IRI+ (n=35)** | **IRI- (n=32)** | **P-value*** |
|---|---|---|---|---|
| **Age, years [mean±SD]** | 56±10 | 55±10 | 58±10 | 0.15 |
| **Gender [n (%)]** | | | | 0.62 |
| Female | 25 (37) | 12 (34) | 13 (41) | |
| Male | 42 (63) | 23 (66) | 19 (59) | |
| **Race [n (%)]** | | | | 0.53 |
| Asian | 6 (9) | 4 (11) | 2(6) | |
| Black/African American | 4(6) | 2(6) | 2(6) | |
| White/Caucasian | 36 (54) | 16 (46) | 20 (63) | |
| Other | 21 (31) | 13 (37) | 8 (27) | |
| **Ethnicity [n (%)]** | | | | 022 |
| Hispanic/Latino | 29 (43) | 18 (51) | 11 (34) | |
| Non-Hispanic/Latino | 38 (57) | 17 (49) | 21 (66) | |
| **Liver Disease Etiology [n (%)]** | | | | 0.80 |
| alcoholic | 18 (27) | 12 (34) | 6 (19) | |
| HBV | 5 (7) | 3 (8) | 2 (6) | |
| HCV | 27 (40) | 13 (37) | 14 (44) | |
| NAFLD/NASH | 13 (19) | 7 (20) | 6 (19) | |
| AIH | 3 (4) | 2 (6) | 1 (3) | |
| PBC | 2 (3) | 0 (0) | 2 (6) | |
| PSC | 0 (0) | 0 (0) | 0 (0) | |
| ALF | 2 (3) | 1 (3) | 1 (3) | |
| other | 3 (4) | 2 (6) | 1 (3) | |
| **HCC [n (%)]** | 22 (33) | 9 (26) | 13 (41) | 0.30 |
| **MELD, at list** | 24±12 | 26±12 | 21 ±12 | 0.10 |
| **MELD, at transplant** | 35±6 | 36 ±6 | 33±8 | 0.13 |
| **ABO [n (%)]** | | | | >0.99 |
| identical | 60 (90) | 31 (89) | 29 (91) | |
| compatible | 7 (7) | 4 (11) | 3 (9) | |
| **Transplant(s) [n (%)]** | | | | 0.05 * |
| isolated liver | 56 (84) | 26 (74) | 30 (94) | |
| liver-kidney | 11 (16) | 9 (26) | 2 (6) | |
| **Outcome(s) within the first year [n (%)]** | | | | |

| Recipient | Total (n=67) | IRI+ (n=35) | IRI- (n=32) | P-value* |
|---|---|---|---|---|
| IRI | 15 (22) | 10 (29) | 5 (16) | 0.24 |
| HCV | 9 (13) | 3 (9) | 6 (19) | 0.29 |
| NASH | 2 (3) | 0 (0) | 2 (6) | 0.49 |
| ACR (or suspicious) | 5 (7) | 4 (11) | 1 (3) | 0.36 |
| AMR (or suspicious) | 4 (6) | 3 (8) | 1 (3) | 0.61 |
| >1 for-cause biopsy required | 6 (9) | 3 (8) | 3 (9) | >0.99 |
| >1 outcome at any for-cause biopsy | 8 (12) | 7 (20) | 1 (3) | 0.06 |
| **Donor** | | | | |
| **Age, years [mean±SD]** | 42±17 | 42±17 | 41 ±18 | 0.95 |
| **Gender [n (%)]** | | | | 0.46 |
| Female | 24 (36) | 11 (31) | 13 (41) | |
| Male | 43 (64) | 24 (69) | 19 (59) | |
| **Race [n (%)]** | | | | 0.24 |
| Asian | 7 (10) | 6 (17) | 1 (3) | |
| Black/African American | 5 (7) | 3 (9) | 2 (6) | |
| White/Caucasian | 34 (51) | 15 (43) | 19 (59) | |
| Other | 21 (31) | 11 (31) | 10 (31) | |
| **Ethnicity [n (%)]** | | | | >0.99 |
| Hispanic/Latino | 21 (31) | 11 (31) | 10 (31) | |
| Non-Hispanic/Latino | 46 (69) | 24 (69) | 22 (69) | |
| **Status** | | | | 0.10 |
| DBD | 64 (96) | 35 (100) | 29 (91) | |
| DCD | 3 (4) | 0 (0) | 3 (9) | |
| **Cold Ischemia, hours [meaniSD]** | 7.5±2.0 | 7.5±2.4 | 7.5±1.5 | 0.90 |

*T-test for continuous variables; Fisher's exact test for categorical variables

**Table 2 –**

Pathogen-associated Molecular Pattern (PAMP) ligands used as positive controls.

| Cell Line | Pos Control Agonist | Description | $EC_{max}$ | $EC_{50}$ |
|---|---|---|---|---|
| hTLR2 | Pam2Csk4 | Synthetic diaculated lipopeptide | 5.82 | 2.91 |
| hTLR3 | Poly(l:C) | Polyinosine-polycytidylic acid HMW | 4.30 | 2.15 |
| hTLR4 | LPS-EK Ultrapure | Lipopolysaccharide fromE. coli K12 | 3.68 | 1.84 |
| hTLR5 | FLA-ST Ultrapure | Flagellin from S. typhimurium | 3.42 | 1.71 |
| hTLR7 | Garquidimod | Imidazoquinoline compound | 4.74 | 2.37 |
| hTLR8 | ssRNA40/LyoVec | single-stranded GU-rich oligonucleotide complexed with LyoVec | 3.78 | 1.89 |
| hTLR9 | ODN 2006 (ODN 7909) | Class B CpG oligonucleotide | 3.48 | 1.74 |
| hN0D1 | iE-DAP | gamma-D-Glu-mDAP, a peptidoglycan-like molecule | 3.12 | 1.56 |
| hN0D2 | MDP | muramyl dipeptide, minimal bioactive peptidoglycan motif: L-D isomer | 4.34 | 2.17 |
| hDECTINIb | Zymosan Depleted | hot alkali-treated Zymosan | 3.56 | 1.78 |