Peer Review File

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Comment 1: In this work, the authors list and discuss many of the unknowns surrounding the pathogenesis of idiosyncratic, drug-induced liver injury (IDILI). Although essentially all of what is discussed in the manuscript has been discussed in works previously published by the two authors, compiling a listing of knowledge gaps could be of interest to some readers in the field. There is some unevenness in the treatment of various topics that are discussed. The need to use RUCAM in diagnosing IDILI has been made in previous publications by Dr. Teschke; it seems a bit overemphasized in an article about unresolved mechanistic issues. Similarly, there is an overemphasis on the immune system as a cause of IDILI to the exclusion of other possible mechanisms. Table 1 provides an excellent summary of the points made by the authors. Specific comments follow.

Response 1: Thank you for this information regarding our invited review article.

Comment 2: P2: The abstract reveals an assumption about the pathogenesis of IDILI, ie, that an adaptive immune response underlies essentially all IDILI. Although there is evidence of adaptive immune system involvement for some drugs, this hypothesis is far from proven for the vast majority of drugs that cause IDILI. This narrowness unfortunately limits the discussion presented in the review.

Response 2: This is a recurring issue in comments so it will be addressed here in detail. There are multiple lines of evidence that most IDILI is immune mediated, specifically by the adaptive immune system. They include:

1. HLA associations are strong evidence of an immune mediated mechanism. With the drugs that cause IDILI for which a sufficient number of cases were available for genetic testing, the general association that was found was an HLA association. There were also some general immune-related genes that were associated with an increased risk such as missense variant of PTPN22, which is also associated with various autoimmune diseases, and this was independent of the drug that caused the IDILI (Cirulli ET, Nicoletti P, Abramson K et al. A Missense Variant in PTPN22 is a Risk Factor for Drug-induced Liver Injury. Gastroenterology. 2019;156:1707-1716.e2.). Ann Daly also looked for an association with BSEP genes but found no association; however, as far as we know that has not been published. However, even if BSEP inhibition is involved in the mechanism of some IDILI, it does not mean that it is not immune mediated; some type of cell stress is likely necessary to induce an immune response. There have been few other significant genetic associations. One is an association between the risk of valproic acid IDILI and a mitochondrial DNA

polymerase. It is clear that valproic acid IDILI involves mitochondria, although it still may have an immune component, and the characteristics of valproic acid IDILI are significantly different from other IDILI. There is a weak association between isoniazid IDILI and the N-acetyltransferase gene, but given that 50% of most populations have the slow acetylator phenotype, that does not explain the idiosyncratic nature of isoniazid IDILI. In fact, the only case that I know of in which there was sufficient cases to genotype, and no HLA association was found is isoniazid. But there is independent evidence that isoniazid IDILI is immune mediated. Specifically, an increase in ALT during isoniazid treatment is associated with an increase in Th17 cells and T cells that produce IL-10 (Metushi IG, Zhu X, Chen X, Gardam MA, Uetrecht J. Mild isoniazid-induced liver injury in humans is associated with an increase in th17 cells and T cells producing IL-10. Chem Res Toxicol. 2014;27:683-689.), patients with isoniazid-induced liver failure have antibodies against isoniazid-modified proteins (Metushi IG, Sanders C, Lee WM, Uetrecht J. Detection of anti-isoniazid and anti-cytochrome P450 antibodies in patients with isoniazid-induced liver failure. Hepatology. 2014;59:1084-1093.), and patients with isoniazid IDILI have a positive lymphocyte transformation test (Warrington RJ, Tse KS, Gorski BA, Schwenk R, Sehon AH. Evaluation of isoniazid-associated hepatitis by immunological tests. Clin Exp Immunol. 1978;32:97-104.). The histology of isoniazid-induced liver failure also We have studied the covalent binding of isoniazid. The reactive metabolite of isoniazid binds to lysines, and on an immunoblot, it appears that all proteins are modified. Therefore, there are a very large number of possible isoniazid-modified proteins, and even a larger number of isoniazid-modified peptides. Thus, there should be one HLA that can “recognize” one of these drug-modified peptides, and it would be surprising if there were a strong HLA association.

2. The histology of IDILI is characteristic of an immune reaction similar to viral hepatitis, with a mononuclear infiltrate of T cells, especially CD8 T cells (although with fewer NK cells than viral hepatitis) and often with eosinophils. Although injury can cause the infiltration of leukocytes, acute injury is associated with a response of neutrophils, and more chronic injury is associated with macrophages. But the histology if hepatocellular IDILI is dominated by CD8 T cells. The function of CD8 T cells is to kill virus-infected or malignant cells that express abnormal antigens, not tissue repair. The histology of IDILI caused by drugs without a known HLA association is basically the same as the histology of IDILI with a known HLA association. This suggests that the basic mechanism is the same.

3. The general characteristics of IDILI are typical of an immune mediated reaction and very similar to the characteristics of other types of idiosyncratic drug reactions,

many of which such as toxic epidermal necrolysis and drug reaction with eosinophilia and systemic symptoms that include liver injury and are clearly immune mediated.

4. The animal model in which inhibition of immune tolerance unmasks the ability of drugs to cause liver injury is further evidence. As mentioned below, it is not surprising that the injury is not more severe given that it presumably requires specific MHC and T cell receptors to induce the maximal immune response response. Even though the injury is not as severe as injury in humans, it has the same characteristics, i.e. delay in onset and similar hepatic histology. Most patients that develop IDILI also only have mild injury, it is only a much smaller number of patients who develop liver failure.

5. In vitro, we see that the supernatant from hepatocyte spheroids incubated with drugs that cause IDILI activate inflammasomes in THP-1 macrophages (Kato R, Uetrecht J. Supernatant from Hepatocyte Cultures with Drugs That Cause Idiosyncratic Liver Injury Activates Macrophage Inflammasomes. Chem Res Toxicol. 2017;30:1327-1332. Kato R, Ijiri Y, Hayashi T, Uetrecht J. The 2-Hydroxyiminostilbene Metabolite of Carbamazepine or the Supernatant from Incubation of Hepatocytes with Carbamazepine Activates Inflammasomes: Implications for Carbamazepine-Induced Hypersensitivity Reactions. Drug Metab Dispos. 2019;47:1093-1096. Mak A, Kato R, Weston K, Hayes A, Uetrecht J. Editor's Highlight: An Impaired Immune Tolerance Animal Model Distinguishes the Potential of Troglitazone/Pioglitazone and Tolcapone/Entacapone to Cause IDILI. Toxicol Sci. 2018;161:412-420.). We would never accept in vitro studies to prove the mechanism of IDILI in humans, but it is consistent with the hypothesis.

6. Although it has not been published yet, in mice we see a marked early and transient immune response to drugs such as nevirapine even though these are wild-type mice, and they do not develop liver injury. The immune response includes an infiltration of inflammatory Ly6C monocytes in the spleen, a decrease in the number of B cells in the spleen and peripheral circulation, and an increase in INF gamma in the liver.

We think the reason that most scientists believe that most IDILI is immune mediated is because the evidence is very strong. However, biological systems are very complex, and it is likely that there are exceptions, which is the reason for the adjective most, not all. As our arguments in favor of immune responses were broadly included in the initially submitted text, there is no need to expand on them now, which would represent an overemphasis.

Comment 3: P4, lines 97-99: Reference to a musical will be meaningless to most readers without some elaboration.

Response 3: Most scientists and physicians are music enthusiasts, play an instrument, go to concerts, and are familiar with the music of Charles Ives and his musical, so further elaboration is not really needed. Those few not familiar with the association can google and expand their view.

Comment 4: P4, lines 109-112: Providing details about how the literature searching was done is laudable. However, the description of the PubMed search and the clause "search terms were used alone or in combination" does not give the reader a complete idea of what was done. For example, certainly some of the terms like "exosomes" and "innate immune system" were not used alone. Perhaps details of the search should be included as an appendix.
Response 4: We have added additional details and think more is not warranted, even as an appendix.

Comment 5: P5, lines 145-146: One could argue with the assumption that "Idiosyncratic DILI mechanistic steps are best analyzed using patients…instead of experimental models…." By the time a doctor sees an IDILI patient, the initial steps in the pathogenesis are long passed. This makes establishing cause and effect of initiating mechanisms in human patients difficult, if not impossible.
Response 5: The addressed time gap is difficult to reconcile, requiring speeding up. No text modification is required.
With the exception of some recent promising animal models, there have not been good methods to test mechanistic hypotheses. In vitro studies simply cannot be trusted to reproduce the complex and idiosyncratic nature of IDILI, and should only be used for evidence if a direct link can be made with what happens in patients. The drug concentrations should also be reasonable; not 100XCmax as is often used for such studies. Any model must have characteristics very similar to IDILI in patients if they are likely to involve the same mechanism. We were able to study the immune response in patients who had a mild elevation of ALT when treated with isoniazid. As mentioned later, an innate immune response is necessary to produce an adaptive immune response, and given that most patients produce the reactive metabolite that is likely responsible for initiating an immune response, it is likely that most patients have an innate immune response to the drug that can be studied in patients being started on the drug. This is certainly true of clozapine and we have evidence that it is also true for other drugs such as nevirapine as mentioned.

Comment 6: P6, lines 160-161: The authors should explain what they mean by "misconducted studies."

Response 6: Done L162-175.

Comment 7: P 7, lines 185ff: The implication that IDILI must be immune mediated because liver injury is manifested differently among individuals is specious. There are many other possible explanations for individual differences in response, eg, polymorphisms in transporters or drug metabolizing enzymes, for example. The authors also conflate differences in types of injury within the liver with differences in target organs from SARS-COV2. One could make the same argument for acetaminophen toxicity, ie, in some patients, kidney injury is more predominant than liver injury. The implication here that IDILI must be immune-based because liver injury responses differ among individuals is misleading.
Response 7: The acetaminophen analogy is not appropriate; although there are certainly interindividual differences in responses to virtually any drug, they are not the type of differences seen with IDILI. If you give virtually any person or any mouse a very large dose of acetaminophen, they will develop toxicity in both liver and kidney if they live long enough. In contrast, with IDILI, no matter what dose of most drugs that cause IDILI, the patients will not develop typical IDILI; they may very well die, but they will not develop IDILI. We indicated "Such variations in individual responses are common, especially when the immune system is involved.", so we acknowledge that there are other sources of interindividual variation. As mentioned, when we talk to Ann Daly, she is just not finding hardly any other significant genetic risk factors for IDILI. And even though such risk factors undoubtedly exist, they do not preclude an immune mechanism. For example, if a patient has high activity of a specific cytochrome P450 that is responsible for forming the reactive metabolite of a drug, and that is a risk factor, it does not preclude an immune mechanism. But the fact is no such strong genetic factors have been found.

Comment 8: P7, line 205-p8, line 206: What is the evidence that exosomes that carry drug modified proteins are "most important for the mechanism of idiosyncratic DILI"? In the next sentence, the fact that "exosomes did not lead to activation of the antigen presenting cells" could mean that they are not involved in the hepatotoxicity, having nothing to do with leading to immune tolerance. This statement serves to promulgate the unsupported assumption that all IDILI is immune mediated.
Response 8: Text was clarified L219-224.

Comment 9: P8, line 225: What do the authors mean by "valid" data? Have invalid data been published? If so, the authors should elaborate.

Response 9: Thank you. Sentence received clarification L240-244.

Comment 10: P9, lines 225-272: This critique of the EMA's letter of support for microRNA as a diagnostic biomarker, while of potential interest in other contexts, is off topic for this review and should be deleted.
Response 10: Our critique is not off the topic but clarifies relevant issues of the dilemma EMA created.

Comment 11: P10, line 283: Please correct "und".
Response 11: Thank you. Done L298.

Comment 12: P11, lines 291-294: This argument about inflammatory bowel disease is a weak one. Animal models of IDILI suggest that a bout of inflammation caused by acute LPS exposure might be necessary to evoke an hepatotoxic response. Chronically high levels of LPS in IBD patients could lead to endotoxin tolerance.
Response 12: The LPS animal model bears no relationship to what happens in humans. It is very similar to acute LPS toxicity that is, in some cases, increased by a drug, and the histology is totally different from the histology of IDILI in humans, i.e. it is characterized by an infiltration of neutrophils not the mononuclear infiltrate that is observed in clinical IDILI. We have tried to produce animal models of IDILI with characteristics similar to IDILI in humans using agents such as LPS, and it did not work. You cannot infer the mechanism of IDILI in humans from an animal model in which the characteristics are completely different from those of clinical IDILI. The clinical observation that, in general, inflammatory conditions such as ulcerative colitis are not a significant risk factor for IDILI is much more important than an invalid animal model. The livers of such patients would be exposed to very large amounts of LPS and other inflammatory molecules. How can such observations be discounted?

Comment 13: P11, lines 297-299: "Macrophages and monocytes play a critical role in the control of immune responses. Therefore, it seems plausible that they would play an important role in the mechanism of idiosyncratic DILI." These two statements do not follow logically. Moreover, statements like this again reveal the implicit bias that all IDILI results from an immune response, which has not been proven. The fact that it has not been proven should be a focus of an article entitled "unresolved basic issues."
Response 13: It is known that macrophages and monocytes play a critical role in control of immune responses – that is a true statement. It is also true, as stated

earlier, that we consider the evidence that most IDILI is immune mediated is very strong. We did not say, and would never say, all. It may very well be that there are other mechanisms; however, the strongest evidence we have is for immune mechanisms. It would be less appropriate to concentrate on mechanisms for which we have much less evidence.

Comment 14: P12, lines 319-323: The statement that "…consensus exists that the hepatic immune system is involved in DILI caused by many drugs'' is true. The statement that "compelling evidence exists that for most idiosyncratic DILI cases the hepatic immune system plays an prominent pathogenetic role" is not true. The first two sentences of this paragraph should be deleted. The cited reference (24) discusses mostly associative evidence of immune system involvement, but clear cause and effect evidence remains elusive, and the claim that MOST cases result from an immune mechanism is not well supported by evidence.
Response 14: The evidence is quite compelling. We could add even more. In contrast, there is a lack of persuasive clinical evidence for alternative mechanisms. However, biological systems are very complex and so we have added a statement that it is possible that some IDILI involves other mechanisms: L338-341.

Comment 15: P12, lines 337-338: "…an association with HLA genes is unclear if alternative causes have not been excluded…." This clause is not clear. Do you mean that some studies have shown HLA associations with certain drugs but that it is unclear in those studies that the liver injury in the cases was drug-induced?
Response 15: Yes, your interpretation is correct. Nevertheless, rewording was done L356-359.

Comment 16: P 13, line 363: Do you mean "…implicating participation of CYP in metabolism…"?
Response 16: Your interpretation is correct, but to be on the safe side rewording was done L382-383.

Comment 17: P14, lines 386-388: It seems that the coin is really 3-sided, with the third side being production of reactive, toxic drug metabolites.
Response 17: Well, the coin is 2-sided not 3-sided, no changes are needed.

Comment 18: P14, lines397-398: Is there evidence that drug metabolism is required for release of exosomes containing CYP, or does this occur in liver injuries generally, even when CYP aren't involved in the injury?

Response 18: Our statements are backed up by the provided references, other assumptions are speculative. No changes of the text are needed.

Comment 19: P17, lines 464-466: The liver injury in this model is very modest, unlike IDILI that is of clinical significance. Isn't this another "unresolved basic issue" with this and similar models?

Response 19: It is somewhat surprising that the model works at all because an adaptive immune response requires specific MHC and T cell receptors. However, the model very closely mimics the mild IDILI caused by drugs that is always more common that serious IDILI caused by the same drugs. The obvious, but untested, explanation is that there is a range of affinities of the MHC and T cell receptors for drug-modified peptides, and it requires very strong binding to produce the most severe liver injury. With inhibition of immune tolerance, we have tipped the balance so that even weaker binding produced a significant immune response and liver injury, but still not sufficient to produce liver failure. However, if you look at the histology with amodiaquine, it looks just like the histology of clinical IDILI; there really is major hepatic necrosis, which persists at least for a matter of several weeks. Even though the ALT is not in the same range as that for acute acetaminophen toxicity, because the injury persists for a long period of time, it represents the death of a large number of hepatocytes, and we also see an increase in bilirubin, so you could call this a "Hy's Law case".

Comment 20: P 17, lines 473-476: "It is likely that most humans and even many animals have an innate immune response to drugs that can cause idiosyncratic DILI, but without the required HLA and T cell receptors, no adaptive immune response leading to injury occurs." What is the basis for thinking that this scenario "is likely"? Change to "seems possible"?

Response 20: If covalent binding occurs in most patients and even most animals, that is likely to provoke an innate immune response. That is clearly true with clozapine in patients, and we are starting to see this in animals as mentioned with nevirapine, but we are just starting these experiments.

Comment 21: P18, line 488: There seems to be an abrupt transition here that departs in the remainder of the paragraph from the topic of this section (ie, nonparencymal cells). Lines 488-496 would seem better placed in the final conclusion section.

Response 21: Moving these lines with their references to the conclusion section is not practicable as references are usually not found in conclusions.

Comment 22: P19, line 524: Should read "…evidence exists…." Also, the claim that "sufficient evidence exist that the hepatic adaptive immune system mediates most of the liver injury cases…" is debatable as noted above. Although this is a commonly held opinion, the evidence for it is rather insufficient.
Response 22: See the list of evidence above.

Comment 23: P19, lines 534-535: Does losing regulatory support necessarily render a biomarker "outdated"?
Response 23: Yes, we think so.

Comment 24: P19, lines 536-537: This sentence is a bit awkward. "…a major part…are metabolized…." Suggest correcting the syntax in that part of the sentence and also dividing the sentence into two.
Response 24: Syntax was corrected L556-559.

Comment 25: P 20, line 545: "A better mechanistic understanding of the mechanisms…." Please rewrite.
Response 25: Sentence was rewritten, deleting "mechanistic". L565-566.

Comment 26: P 34, Fig. 1: This figure and the text that accompanies it above provides detail that is above what is devoted to other potential aspects of IDILI (eg, specific immune mediators and cell types) in this review. This is ok, but it does incorporate some unevenness to the discussion.
Response 26: Your ok is fine, no changes are needed.

Comment 27: P35ff, Table 1: This table has some spacing and punctuation issues, but it is an excellent summary of the discussion in the manuscript.
Response 27: Thank you, issues were corrected.