Effects of immunization and checkpoint inhibition on amodiaquine-induced liver injury

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ABSTRACT
If idiosyncratic drug-induced liver injury (IDILI) is immune-mediated, it is possible that an individual’s prior exposure to antigens may affect their susceptibility to IDILI. An individual’s repertoire of memory immune cells is shaped by every past exposure to antigens. Subsequent drug-induced adverse drug reactions may therefore involve an immune cell’s cross-reactivity between a prior antigen and resulting drug-modified proteins. Therefore in this experiment, mice were immunized with amodiaquine (AQ)-modified hepatic proteins to mimic a previous exposure; treated with a RIBI adjuvant and anti-CD40 antibodies to stimulate an immune response; and, treated with anti-PD1 and anti-CTLA-4 antibodies prior to AQ treatment in order to overcome immune tolerance. This treatment led to greater liver injury than treatment with AQ alone. However, the mice did not develop serious liver injury. PD1<sup>−/−</sup> mice were then immunized and treated with AQ and anti-CTLA-4 antibodies so that immune tolerance would be impaired, both during immunization and also during AQ treatment. However, even this did not result in liver failure, and the liver injury was not significantly increased relative to un-immunized PD1<sup>−/−</sup> mice treated with anti-CTLA-4 and AQ. From these results we conclude that, although previous antigen exposure may affect the risk of IDILI, it appears that a very strong stimulus is required, and impairing immune tolerance remains the most effective method for producing an animal model of IDILI.

Introduction
Clinical evidence suggests that most idiosyncratic drug-induced liver injury (IDILI) is immune-mediated (Uetrecht & Naisbitt 2013). Amodiaquine (AQ), which causes idiosyncratic drug-induced liver injury in humans, was found to cause mild delayed-onset immune-mediated liver injury in mice that resolves despite continued treatment with the drug (Metushi et al. 2014). In general, drugs that can cause serious IDILI more often result in mild injury that resolves despite continued treatment. Therefore, this AQ model appears to mimic the milder form of IDILI. We previously tried to increase AQ-induced liver injury in mice by immunizing them with AQ-modified hepatic proteins prior to treatment with AQ. However, this immunization actually protected mice from the expected mild liver injury and resulted in a marked increase of tolerogenic immune cells into the liver (Mak & Uetrecht 2015a). These results demonstrate the degree to which the dominant response in the liver is immune tolerance.

Recently, a valid animal model of AQ IDILI was developed using PD1<sup>−/−</sup> mice and an anti-CTLA-4 antibody. PD1 and CTLA-4 are important immune checkpoint receptors involved in inducing immune tolerance. This model is characterized by significant liver injury, liver dysfunction, and liver histology that resemble clinical IDILI (Metushi et al. 2015; Mak & Uetrecht 2015b). Although the PD1<sup>−/−</sup>/anti-CTLA-4 model has shown great promise as a model for IDILI, it is unlikely that patients who develop such injury have the same degree of impaired immune tolerance as this impaired immune tolerance model. Why then do some patients develop serious IDILI? One possible explanation is that these patients have been exposed to some virus or other antigen that strongly stimulates the immune system and happens to cross-react with the drug-derived immunogen. This priming of the immune system might shift the balance of the immune response away from immune tolerance and lead to a much stronger anti-drug immune response and liver injury.

We do not know what such a cross-reacting antigen might be, but if this hypothesis is true, it might be possible to produce drug-induced liver injury without impairment of immune tolerance during drug treatment if we impaire immune tolerance during immunization with drug-modified hepatic proteins. Therefore, in this experiment, mice were immunized with AQ-modified hepatic proteins along with a RIBI adjuvant and a stimulatory anti-CD40 antibody to boost the immune response. The mice were also treated with anti-PD1 and anti-CTLA-4 antibodies only during the immunization period in order to overcome immune tolerance.

Materials and Methods

Animals and drug treatments
Female C57BL/6 mice (8–10-wk-of-age) were purchased from Charles River Laboratories (Montreal, QC, Canada). Female PD1<sup>−/−</sup> mice between 10–12-wk-of-age were bred and housed as described in Metushi et al. (2015). Amodiaquine (AQ) (Ipcas Laboratories Ltd., Mumbai, India) was thoroughly mixed with rodent meal (2018 Teklad Global 18% Protein Diet, Envigo; Mississauga, ON, Canada) at a concentration of 0.2% (w/w) then
provided in small jars *ad libitum*. This AQ dose has been shown to produce serum concentrations of 250–300 ng/ml in female mice, which is within the range of 80–800 ng/ml seen in humans (Metushi et al. 2014).

Mice were split into six groups (Table 1); a control C57BL/6 mouse group (Control, *n* = 7); a group treated with AQ only (AQ, *n* = 4); a group immunized with AQ-modified proteins, treated with a RIBI adjuvant (Sigma, ON, Canada), anti-CD40, anti-PD1, and anti-CTLA-4 antibodies (mAb clone FGK4.5, clone RMP1–14 and clone 9D9, respectively; Bio-X-cell, West Lebanon, NH) during the immunization period, then treated with AQ (S9/AQ, *n* = 7); a group immunized with AQ-modified proteins, treated with a RIBI adjuvant, anti-CD40, anti-PD1, and anti-CTLA-4 antibodies during the immunization period, without AQ treatment (S9, *n* = 4); a PD1/−/-- mouse group immunized with AQ-modified proteins and a RIBI adjuvant, treated with anti-CD40 during the immunization period, treated with anti-CTLA-4 throughout the experiment and treated with AQ (PD1/S9/AQ/anti-CTLA-4, *n* = 3); and, a PD1/−/-- group treated with anti-CTLA-4 and AQ (PD1/AQ/anti-CTLA-4, *n* = 3). The timeline of treatments is described in Figure 1. Anti-CD40 (50 µg) was administered SC once a week during the immunization period. A dose of 250 µg of anti-PD1 and anti-CTLA-4 antibody was administered IP 3 and 1 days before immunization and then weekly to sustain PD1 and CTLA-4 blockade.

All protocols used here were approved by University of Toronto Animal Care Committee and conducted in an animal facility accredited by the Canadian Council on Animal Care.

Table 1. Mouse groups and their treatments.

| Group Name | Genotype | Treatments | *n* |
|------------|----------|------------|-----|
| Control    | Wildtype C57BL/6 mice | No treatment | 7   |
| AQ         | Wildtype C57BL/6 mice | AQ | 4   |
| S9/AQ      | Wildtype C57BL/6 mice | Immunization with AQ-modified proteins + RIBI adjuvant + Anti-CD40 + Anti-PD1 + Anti-CTLA-4 + AQ | 7   |
| S9         | Wildtype C57BL/6 mice | Immunization with AQ-modified proteins + RIBI adjuvant + Anti-CD40 + Anti-PD1 + Anti-CTLA-4 + AQ | 4   |
| PDI/S9/AQ/anti-CTLA-4 | PD1/−/-- | Immunization with AQ-modified proteins + RIBI adjuvant + Anti-CD40 + Anti-PD1 + Anti-CTLA-4 + AQ | 3   |
| PDI/AQ/anti-CTLA-4 | PD1/−/-- | Anti-CTLA-4 + AQ | 3   |

Figure 1. Timeline of treatments. (A) Untreated control mice. (B) C57BL/6 mice treated with AQ. (C) S9/AQ; C57BL/6 mice immunized with AQ-modified proteins, treated with a RIBI adjuvant, anti-CD40, anti-PD1 and anti-CTLA-4 antibodies during the immunization period, then treated with AQ. (D) S9; C57BL/6 mice immunized with AQ-modified proteins, treated with a RIBI adjuvant, anti-CD40, anti-PD1 and anti-CTLA-4 antibodies during the immunization period, without AQ treatment. (E) PD1/S9/AQ/anti-CTLA-4; PD1/−/-- mice immunized with AQ-modified proteins, treated with a RIBI adjuvant and a anti-CD40 antibody during the immunization period, treated with anti-CTLA-4 throughout the experiment and treated with AQ. (F) PD1/AQ/anti-CTLA-4; PD1/−/-- mice treated with anti-CTLA-4 and AQ.
**Drug-modified proteins and immunization**

AQ-modified hepatic proteins were produced and verified as described in Mak and Uetrecht (2015a); here, the S9 protein fraction was used compared to the S100 protein fraction in that previous experiment in order to incorporate CYP450s into the protein fraction. RIBI adjuvant (Sigma) was used as described by the manufacturer. Mice in the immunization groups were immunized with AQ-modified proteins and stimulated by RIBI adjuvant and anti-CD40 antibody once a week for 3 wk. These mice were allowed to rest untreated for 2 wk to allow the effects of immunization on CYP450 levels to subside. Following the 2-wk rest, all mice to be treated with AQ were given ad libitum access to 0.2% (w/w) AQ in food.

**ALT**

Blood was collected weekly as previously described (Mak & Uetrecht 2015a). Serum ALT levels were measured to determine AQ-induced liver injury using an Infinity™ ALT kit (TR71121; Thermo Scientific, Middleton, VA). L-Alanine and 2-oxoglutarate reagents in the kit were converted to pyruvate and L-glutamate by ALT present in the serum sample. The kinetic breakdown of pyruvate to L-lactate by LDH in the kit was then measured spectrophotometrically to determine ALT levels.

**Histology**

Liver and spleen samples were extracted and placed in 10% neutral buffered-formalin solution (Sigma). The samples were paraffin-embedded, sectioned to 4-µm, stained with H&E, and then scanned (CFIBCR Histology/Microscopy Core Unit; ON, Canada).

**Isolation of mononuclear cells and flow cytometry**

Mononuclear cells were isolated from livers and spleens, stained with antibodies, and then phenotyped by flow cytometry (Mak & Uetrecht 2015b). The following antibodies were used in this experiment: PerCP-Cy5.5-conjugated anti-F4/80 (PerCP-Cy5.5-anti-F4/80), APC-anti-CD11b, Alexa 488-anti-iNOS, eFluor 450-anti-IL-12, Alexa 700-anti-IL-10 and APC-eFluor 780-anti-Gr1, eFluor 450-anti-CD3e, FITC-anti-CD4, PE-Cy7-anti-CD62L, PE-ant Foxp3, eFluor 560-conjugated Fixable Viability Dye (eBioscience, San Diego, CA), PE-anti-Arg1 (R&D, Minneapolis, MN), and APC-Cy7-anti-CD8a (BD Biosciences, San Jose, CA).

Mononuclear cells were stained for macrophages (M1 and M2), myeloid-derived suppressor cells (MDSC), CD8 T-cells, CD4 T-cells, T117 cells, regulatory T (Treg)-cells, NK cells, NKT cells, B-cells, and memory T-cells. Macrophages were first characterized as CD11b^+ F4/80^+ and then further characterized as M1 (CD11b^+ F4/80^+ and iNOS^+ or IL-12^+) or M2 (CD11b^+ F4/80^+ and Arg1^+ or IL-10^+). MDSCs were characterized as CD11b^+ Gr1^+ CD4^+ CD8^+ CD3^+ CD8^+ T-cells as CD3^+ CD8^+ T117 cells as CD4^+ IL-17^+, Treg cells as CD4^+ FoxP3^+, NK cells as NK1.1^- CD3^- B-cells as CD45R^-, and memory T-cells as CD4^- CD62L^+. All cells were analyzed using a BD LSR II: 3 laser analyzer (BD Biosciences, San Jose, CA) and system-associated software.

**Statistical analysis**

Mean ± SEM values were calculated for each experimental group. Statistical analysis were performed using Prism software (GraphPad, San Diego, CA). Data were analyzed using a two-way or one-way analysis of variance (ANOVA). A p value <0.05 was considered significant (**p < 0.01, ***p < 0.001).

**Results**

**Addition of immune checkpoint inhibitors during the immunization period significantly increased AQ-induced liver injury**

Serum ALT levels were measured weekly to assess AQ-induced liver injury. There were no significant elevations in ALT in the Control, AQ, S9/AQ or S9 groups during the immunization period or rest period. During the drug treatment period, mice treated with AQ alone displayed the previously described delayed onset rise in ALT that resolves despite continued treatment (Figure 2). Mice in the S9/AQ group also displayed a delayed onset rise in ALT once AQ treatment began, and the peak ALT levels in this group were significantly greater than the peak ALT levels in the mice treated with AQ alone (Figure 2). However, the mice in the S9/AQ group also appeared to be recovering from the liver injury despite continued treatment.

In terms of histology, the spleens revealed no significant differences between the groups (data not shown) and liver histology was normal in the Control, AQ (Figure 3A), and S9 groups. Although no significant necrosis was seen in the livers of the S9/AQ mice, there was evidence of small focal mixed-cell infiltrates (Figure 3B).

Characterization of liver and spleen mononuclear cells was performed by flow cytometry to better understand the immune response involved. The results indicated there were no significant differences between any of the groups (data not shown).

**PD1^-/- mice immunized and then treated with AQ developed significantly increased liver injury compared to wild-type C57BL/6 Mice**

In a follow-up attempt to cause severe AQ-induced liver injury, the previously described impaired immune tolerance model consisting of PD1^-/- mice and anti-CTLA-4 was used instead of...
wild-type C57BL/6 mice. PD1<sup>−/−</sup> mice were immunized with AQ-modified proteins and treated with anti-CD40, anti-PD1, and anti-CTLA-4 antibodies, then treated with AQ (n = 3); PD1/S9/AQ/anti-CTLA-4 = PD1<sup>−/−</sup> mice immunized with AQ-modified proteins, treated with RIBI adjuvant and anti-CD40 antibody during immunization period, treated with anti-CTLA-4 throughout experiment and treated with AQ (n = 3). The ALT levels for the S9/AQ, PD1/S9/AQ/anti-CTLA-4, and PD1/AQ/anti-CTLA-4 groups were significantly greater than Control for Weeks 8–11. The ALT levels for the PD1/S9/AQ/anti-CTLA-4 and PD1/AQ/anti-CTLA-4 were never significantly different from each other. The stars represent significant differences between S9/AQ and PD1/S9/AQ/anti-CTLA-4 group. Values shown are means ± SE. Two-way ANOVA; ***p < 0.001.

Discussion

There is now strong evidence that suggests most IDILI is immune mediated and the majority of patients do not develop severe liver injury because the dominant response to drugs that cause IDILI is immune tolerance (Uetrecht & Naisbitt 2013). At this point in time, it is almost impossible to determine who will develop IDILI due to a drug. Genetically, some IDILI has a strong HLA association, but with the exception of abacavir, even if a patient has the HLA genotype associated with a high risk, they are still unlikely to develop IDILI if treated with the drug in question. Therefore factors other than genetic predisposition must be involved. Co-existing diseases are an attractive hypothesis to explain this discrepancy; however, with notable exceptions, preexisting liver disease and other inflammatory conditions do not appear to be major factors (Zimmerman 1999). The immune system is shaped by everything it has ever been exposed to, and although there are an almost limitless number of possible T-cell receptors, each individual has a limited number of T-cells. Therefore, previous exposure to antigens or pathogens may shift
the T-cell repertoire to cells that can cross react with drug-modified protein antigens, which could be an important but unpredictable risk factor. If memory T-cells from previous antigen exposures were a risk factor for IDILI, it would be expected that the time to onset would be short. However, if the number of memory cells is very low it may still take a long time for them to expand, and there are many clearly immune mediated adverse reactions that do not occur rapidly on re-challenge (Cho & Uetrecht 2017). Even unrelated antigens can affect subsequent response to an antigen. Specifically, there are many possible interactions between an antigen and a T-cell receptor, and it has been shown that exposure to one virus can markedly affect an animal’s response to a second virus even if the two viruses do not share any epitopes (Welsh & Selin 2002). This is referred to as heterologous immunity.

Unfortunately, it is not possible to predict which unrelated antigens might influence the subsequent immune response to a drug. As mentioned, we had previously found that immunization with AQ-modified hepatic proteins paradoxically protected animals from liver injury when subsequently challenged with AQ, and this was accompanied by a large increase in cells associated with immune tolerance. Multiple reports have also shown that inhibition of immune tolerance leads to animal models of IDILI with characteristics similar to IDILI that occurs in humans (Chakraborty et al. 2015; Metushi et al. 2015). However, patients who develop IDILI are unlikely to have this severe impairment.

Figure 5. Flow cytometry analysis of mononuclear leukocytes in liver. A representative zebra plot and average of three animals is displayed. Mononuclear cells with significant differences among the four treatment groups are displayed. (A) Changes in the percentage of hepatic MDSC. (B) Changes in the percentage of hepatic CD8+ T cells. (C) Changes in the percentage of hepatic Treg cells. (D) Changes in the percentage of hepatic NK cells. Control = C57BL/6 mice; S9/AQ = C57BL/6 mice immunized with AQ-modified proteins, treated with RIBI adjuvant, anti-CD40, anti-PD1, and anti-CTLA-4 antibodies, then treated with AQ; PD1/S9/AQ/anti-CTLA-4 = PD1-/- mice immunized with AQ-modified proteins, treated with RIBI adjuvant and anti-CD40 antibody during immunization period, treated with anti-CTLA-4 throughout experiment and treated with AQ; PD1/AQ/anti-CTLA-4 = PD1-/- mice treated with anti-CTLA-4 and AQ. Values shown are means ± SE. One-way ANOVA; *p < 0.05, **p < 0.01, or ***p < 0.001).
of immune tolerance, and therefore these models are more likely useful to understand the steps leading to IDILI, but not who will develop IDILI. In this experiment we added antibodies against the checkpoint inhibitors PD1 and CTLA-4 during the immunization to try to prevent the immune tolerance that was observed in the previous immunization experiment. This treatment led to an increase in the subsequent AQ-induced liver injury relative to AQ-induced liver injury without the prior immunization; however, it was still mild and resolved despite continued treatment with AQ. The RIBI adjuvant system is a stable oil-in-water emulsion made up of monophosphoryl lipid A (MPL) and synthetic trehalose dicorynomycolate (TDM) in squalene oil, TWEEN80 and water. MPL is a chemically modified form of a portion of bacterial LPS, while TDM is a lipid component of mycobacterial cord factor. In this experiment a RIBI adjuvant was chosen because it is less toxic than Freund's adjuvant, and both MPL and TDM are capable of inducing humoral and cell-mediated immune responses. An anti-CD40 antibody was used as an additional immunostimulant. It is, of course, possible that a different adjuvant or a different immunostimulant could have led to a greater T-cell response and subsequent liver injury.

Even when PD1<sup>−/−</sup> mice were treated instead of wild-type C57BL/6 mice, and anti-CTLA-4 was administered to the mice both during the immunization and AQ treatment, although this led to significant liver injury that did not resolve, it did not lead to liver failure and the injury was not significantly greater than without the prior immunization with AQ-modified liver proteins. Interestingly, the liver injury in the PD1/S9/AQ/anti-CTLA-4 group appeared to be associated with a significant infiltration of CD8<sup>+</sup> T-cells, probable MDSCs, and T<sub>reg</sub> cells, while the liver injury in the S9/AQ group appeared to be associated with an increase in NK cells. NK cells in the S9/AQ group were also elevated in the first experiment; however, this increase was not statistically significant. Nevertheless, it is difficult to compare between experiments because the first experiment involved 7 wk of AQ treatment, while the second experiment involved 6 wk of treatment. Ultimately, NK cells appear to be involved in the mild liver injury, while we had previously shown that depletion of CD8<sup>+</sup> T-cells prevented the more severe liver injury (Mak & Uetrecht 2015b).

Conclusions

We conclude that although prior exposure to antigens may influence the risk of IDILI, the immune response must be quite strong in order to have a significant effect. In these experiments, the animals that were immunized had a significant increase in T<sub>reg</sub> cells and MDSC, an indication that immune tolerance was still the dominant response. Viruses stimulate immune responses by several different mechanisms, and this may be what is required to overcome the dominant tolerogenic immune response in the liver. Therefore, impairing immune tolerance remains the more effective method to induce IDILI in animal models; unfortunately, there are many redundant mechanisms of immune tolerance.

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