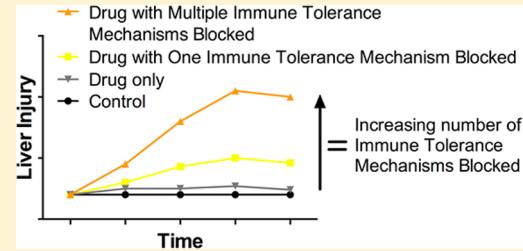


The Combination of Anti-CTLA-4 and PD1^{–/–} Mice Unmasks the Potential of Isoniazid and Nevirapine To Cause Liver Injury

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ABSTRACT: Our laboratory recently reported what we believe is the first valid animal model of idiosyncratic drug-induced liver injury (IDILI) by treating PD1^{–/–} mice with an anti-CTLA-4 antibody and amodiaquine (AQ). PD1 and CTLA-4 are important immune checkpoint receptors that are involved in inducing immune tolerance. This model was able to produce significant liver injury that looks very similar to the liver injury seen in humans. Although this model was shown to work with AQ, the question becomes whether blocking immune tolerance would unmask the potential of other drugs to cause IDILI. In this study, we tested isoniazid and nevirapine, both drugs with significant histories of causing IDILI in humans even though they do not cause significant injury in animals with doses that result in therapeutic blood levels. Both drugs in combination with these immune checkpoint inhibitors caused mild but significant delayed onset liver injury, which is similar to the mild injury that they can cause in humans. INH-induced liver injury in this model was associated with an increase in NK cells, while NVP-induced liver injury was associated with a greater increase in CD8 T cells. Although the liver injury caused by these drugs in this model was mild, these results suggest that impairing immune tolerance may be a general method for unmasking the potential of drugs to cause IDILI and therefore provide a screening tool for drug development.



INTRODUCTION

Idiosyncratic drug-induced liver injury (IDILI) can result in acute liver failure and either liver transplantation or death. However, more often patients experience only mild liver injury that resolves despite continued treatment; this resolution is termed adaptation.¹ A growing body of evidence suggests that IDILI, even mild injury, is immune-mediated, and therefore, this adaptation must represent immune tolerance. Our lab recently reported an animal model of amodiaquine (AQ)-induced IDILI that utilized PD1^{–/–} mice and an anti-CTLA-4 antibody.² PD1 and CTLA-4 are important immune checkpoint receptors that are involved in inducing immune tolerance.³ Targeting and blocking these immune checkpoints allowed for an increased immune response to AQ and resulted in liver injury. This may be the first animal model to correctly mimic human IDILI because it shows similar characteristics such as delayed onset and histology demonstrating a mononuclear inflammatory infiltrate with piecemeal necrosis.⁴ Although this appears to be a good model of IDILI involving AQ, it raises the question of whether this may be a general method for unmasking the potential of other drugs to cause IDILI.

Isoniazid (INH) remains a first-line drug for the treatment of tuberculosis, even though it can cause liver failure and is associated with mild IDILI in up to 20% of treated patients.⁵ Although the mechanism of INH-induced liver injury has been controversial, there is a growing body of evidence that most IDILI is immune-mediated. Specifically, T-cells from patients with mild INH-induced liver injury proliferate upon being incubated with INH-modified proteins, while T-cells from patients with severe INH-induced liver injury also proliferated upon being incubated with INH alone.^{6,7} In addition, most

patients with INH-induced liver injury also have antibodies against either INH-modified proteins or the cytochromes P450s that bioactivate INH.⁸ The classic animal model of INH-induced liver injury involved the wrong type of toxicity in the wrong species. Specifically, it involved acute toxicity with large doses of the drug rather than delayed onset toxicity with smaller doses, and the species utilized was the rat; the bioactivation of INH in mice appears to be much more like that in humans.^{9–11} Previous attempts in our lab to develop an animal model of INH-induced liver injury failed. Treatment of C57BL/6 mice with INH at reasonable doses led to no evidence of liver injury, and treatment with INH of mice that had been immunized with INH-modified hepatic proteins prevented the mild autoimmune liver injury that is induced by immunization with hepatic proteins.^{11,12}

Nevirapine (NVP) is a non-nucleoside reverse transcriptase inhibitor used for the treatment of HIV-1 infections. NVP is associated with a significant incidence of idiosyncratic skin rashes and/or liver toxicity.¹³ The incidence of NVP-induced ALT elevation is 8–18%; however, the incidence of liver failure is much lower.¹⁴ Evidence that suggests NVP IDILI is immune mediated includes a delayed onset and a higher incidence in individuals with high CD4 T-cell counts.¹⁵ Previous experiments in our lab with NVP-treated mice showed a very small increase in ALT that resolved, but no other evidence of liver injury.¹⁶

If inhibiting immune tolerance unmasks the potential for other drugs to cause IDILI, it could lead to a general animal

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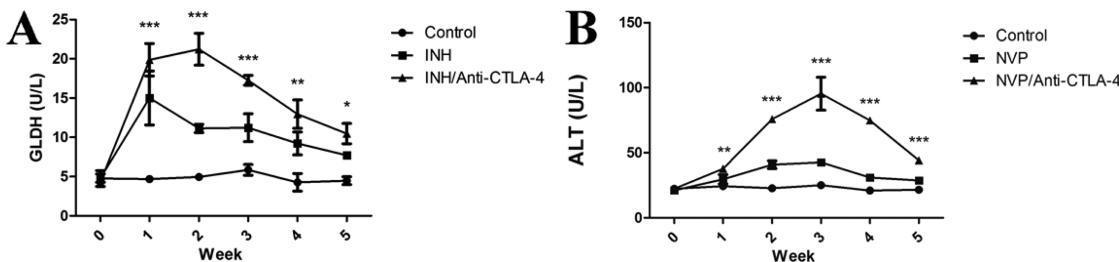


Figure 1. Liver injury as measured by GLDH and ALT levels in serum. (A) GLDH levels in mice treated with INH. (B) ALT levels in mice treated with NVP. Values represent the mean \pm SE. Analyzed for statistical significance by two-way ANOVA. A p of <0.05 was considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

model of IDILI. As a test of this hypothesis, PD1 $^{−/−}$ mice were treated with INH or NVP along with anti-CTLA-4, and liver enzymes were quantified and hepatic leukocytes phenotyped.

MATERIALS AND METHODS

Animals and Drug Treatments. Female PD1 $^{−/−}$ mice between 10 and 12 weeks of age were housed as previously described.² INH and NVP were thoroughly mixed with rodent meal at concentrations of 0.2% (w/w) and 0.3% (w/w), respectively, and then provided to the mice in small jars *ad libitum*. The INH dose was \sim 300 mg/kg/day and results in blood levels of $4.5 \pm 0.9 \mu\text{g}/\text{mL}$, which is similar to the therapeutic C_{\max} of INH in humans.¹⁷ The NVP dose was \sim 950 mg/kg/day, which is the same dose used in previous experiments.¹⁶ Mice metabolize nevirapine very rapidly, and this dose, although larger than the dose that we use for rats, leads to serum levels that are difficult to measure; the level of hepatic protein binding is lower than in rats.¹⁶ Mice were split into three groups per drug and four mice per group: a control PD1 $^{−/−}$ group (Control), a group treated with either INH or NVP only (INH or NVP), and a group treated with anti-CTLA-4 (clone 9D9; Bristol-Myers Squibb, Redwood City, CA) and INH or NVP (INH/anti-CTLA-4 or NVP/anti-CTLA-4). The anti-CTLA-4 antibody (250 μg) was administered 3 and 1 days before the start of drug treatment and then weekly to sustain CTLA-4 inhibition. All animal protocols used in this study were approved by the University of Toronto Animal Care Committee and conducted in an animal facility accredited by the Canadian Council on Animal Care.

GLDH and ALT. Blood and the resulting serum were collected as previously described.¹⁸ INH reacts with and depletes pyridoxal 5'-phosphate [a cofactor in the alanine aminotransferase (ALT) assay]; therefore, glutamate dehydrogenase (GLDH, Randox, Crumlin, U.K.) levels were measured using a modified protocol¹⁷ to determine INH-induced liver injury. Serum ALT levels (Thermo Scientific, Middle-town, VA) were measured to determine NVP-induced liver injury as described by the manufacturer.

Western Blotting. INH and NVP covalent binding to proteins was assessed by Western blotting using previously produced INH and NVP antibodies.^{17,16} The Western blotting procedure was performed as previously described.¹⁸

Histology. Liver and spleen samples were extracted and placed in a 10% neutral buffered formalin solution (Sigma, Ottawa, ON) overnight. The samples were paraffin-embedded, sectioned to 4 μm , and then stained with H&E (Department of Pathology at the Hospital for Sick Children, Toronto, ON).

Isolation of Mononuclear Cells and Flow Cytometry. Mononuclear cells were isolated from livers and spleens, stained with antibodies, and then phenotyped by flow cytometry by a previously described method.¹⁹ Mononuclear cells were stained for macrophages (M1 and M2), myeloid-derived suppressor cells (MDSC), CD8 T-cells, CD4 T-cells, Th17 cells, Treg cells, NK cells, NKT cells, B-cells, and memory T-cells.

Statistical Analysis. Mean \pm SEM values were calculated for each experimental group. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data were

analyzed using a two-way analysis of variance (ANOVA) or a one-way ANOVA. A p value of <0.05 was considered significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

RESULTS

Treatment of PD1 $^{−/−}$ Mice with INH or NVP Along with Anti-CTLA-4 Resulted in Mild but Significant Liver Injury. PD1 $^{−/−}$ mice treated with anti-CTLA-4 along with INH or NVP had significantly increased levels of GLDH or ALT, respectively, compared to the control (Figure 1). However, the injury resolved with either drug despite continued treatment. After treatment for 5 weeks, when the level of GLDH or ALT had returned to almost normal, liver histology from mice treated with anti-CTLA-4 along with INH or NVP showed no significant abnormalities (Figure 2). Liver protein fractions from INH- and NVP-treated mice run on a Western blot and stained with the anti-INH and anti-NVP

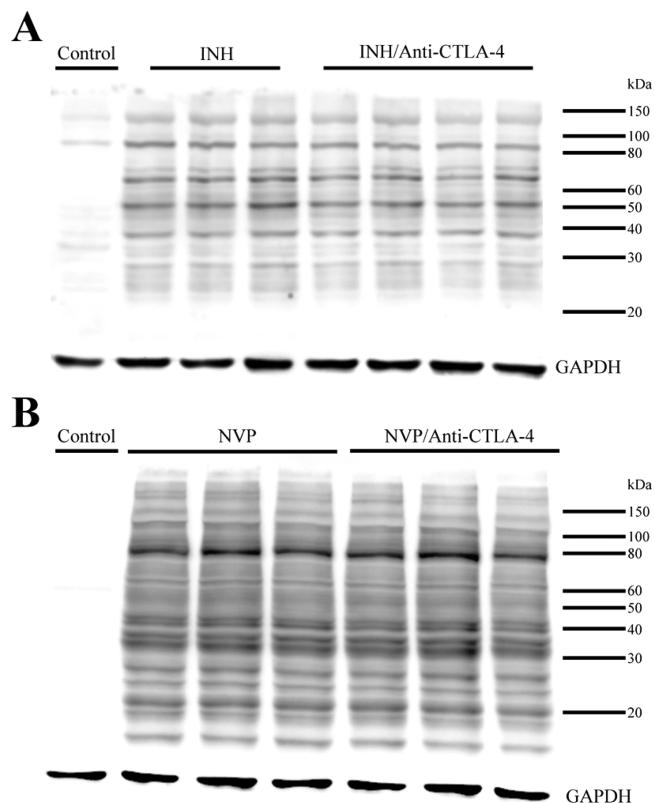


Figure 2. Comparison of (A) isoniazid or (B) nevirapine covalent binding in the liver of mice treated with isoniazid or nevirapine alone, or in combination with anti-CTLA-4.

antibodies, respectively, showed significant covalent binding to hepatic proteins (Figure 3). However, there was no significant difference in the amount of drug-modified hepatic proteins between drug alone and drug along with anti-CTLA-4.

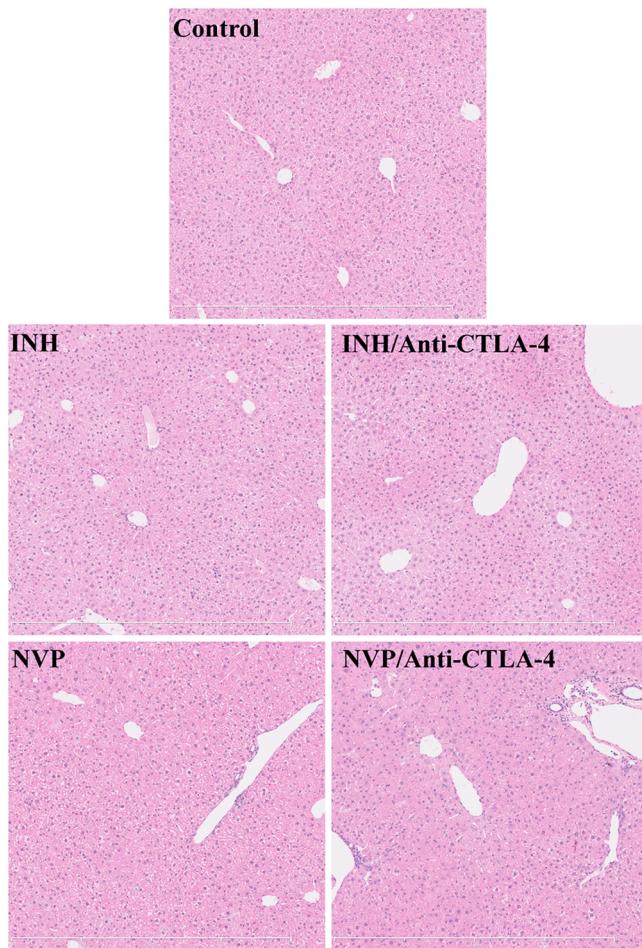


Figure 3. Representative H&E-stained liver sections of mice treated with isoniazid or nevirapine alone, or in combination with anti-CTLA-4.

INH Liver Injury Is Characterized by Hepatic NK Cell Infiltration, while NVP Liver Injury Is Characterized by CD8 T-Cell Infiltration. Flow cytometry was utilized to determine the immune cell changes in the liver and spleen. In the liver, the INH/anti-CTLA-4 group showed significantly increased percentages of CD11b+GR1+ cells [presumably myeloid-derived suppressor cells (MDSCs)], NK cells, and Th17 cells compared to the control and significantly decreased percentages of Treg cells compared to the control (Figure 4). There were no significant differences in the spleen of mice treated with INH (data not shown). The NVP/anti-CTLA-4 group had a significantly increased percentage of hepatic MDSCs, Th17 cells, CD8 T-cells, and splenic CD8 T-cells compared to the control and a significantly decreased percentage of hepatic NK cells compared to the control (Figure 4).

■ DISCUSSION

In general, preclinical animal testing of drugs fails to reveal their potential to cause idiosyncratic drug-induced liver failure. Although we think we developed the first animal model of

IDILI by inhibition of immune tolerance in AQ-treated animals, it was unclear whether this strategy would unmask the potential of other drugs to cause serious IDILI in humans. In this study, we investigated two additional drugs with relatively high risks of causing serious IDILI. Previous attempts to create an animal model of INH- or NVP-induced liver injury had failed; no significant liver injury was observed in previous studies in wild-type mice with these drugs alone.^{11,16} In contrast, PD1^{-/-} mice treated with anti-CTLA-4 and both INH or NVP induced liver injury that was not observed in wild-type animals in the absence of anti-CTLA-4. However, it was not as severe as that caused by AQ under the same conditions, and it resolved despite continued treatment with either drug (Figure 1). This pattern of injury is similar to the mild injury in humans, which is more common than liver failure. Liver histology from INH- or NVP-treated mice was performed to provide further evidence of liver injury; however, histology failed to reveal significant injury (Figure 2). This could be because the liver sections were collected at week 5 of treatment when the level of GLDH or ALT had almost returned to normal. Nevertheless, these results show that impairing immune tolerance can unmask the potential of drugs to cause liver injury. Additionally, the mechanisms of immune tolerance are highly redundant, and liver injury was more severe when anti-CTLA-4 antibodies were added to the injury caused in PD1^{-/-} mice (Figure 1). This provides further evidence that the liver injury caused by these drugs is immune-mediated.

Multiple lines of evidence suggest that most IDILI is immune-mediated, and therefore, flow cytometry was utilized to characterize the immune cell changes in the liver and spleen. NK cells appear to be the major cytotoxic cell upregulated in the liver by INH in this model (Figure 4). This is similar to NK cell-mediated mild AQ liver injury in wild-type mice.²⁰ When PD1^{-/-} mice were treated with AQ and anti-CTLA-4, there was significant liver dysfunction, and this was shown to be caused by hepatic CD8 T-cells.¹⁹ Therefore, it appears that NK cells can mediate mild liver injury, but unless the major immune response changes to CD8 T-cells, there is unlikely to be significant liver dysfunction. NVP-induced liver injury in this model shows a significantly increased percentage of hepatic CD8 T-cells and significantly decreased percentages of hepatic NK cells compared to the control (Figure 4). This pattern of immune cell changes is similar to that in PD1^{-/-} mice treated with AQ and anti-CTLA-4. However, AQ liver injury remains elevated throughout drug treatment, while NVP liver injury recovers despite continued drug treatment. Although CD8 T-cells are elevated in PD1^{-/-} mice treated with NVP and anti-CTLA-4, in this case blocking these two immune checkpoints does not appear to be enough to cause major liver injury. INH or NVP along with anti-CTLA-4 also appears to increase the percentage of hepatic MDSCs and Th17 cells (Figure 4). MDSCs are strong anti-inflammatory cells, and Th17 cells are strong pro-inflammatory cells involved in liver homeostasis.^{21,22} There appears to be many immune cell changes, both pro- and anti-inflammatory; however, at this point, the roles of each cell type involved in this liver injury are not well-understood.

Although INH and NVP in the PD1^{-/-}/anti-CTLA-4 model were unable to cause sustained liver injury, these results suggest that impairing immune tolerance may be a general method for unmasking the potential of drugs to cause IDILI and therefore useful as a screening tool in drug development. As mentioned, there are many redundant mechanisms of immune tolerance, and blocking additional mechanisms such as

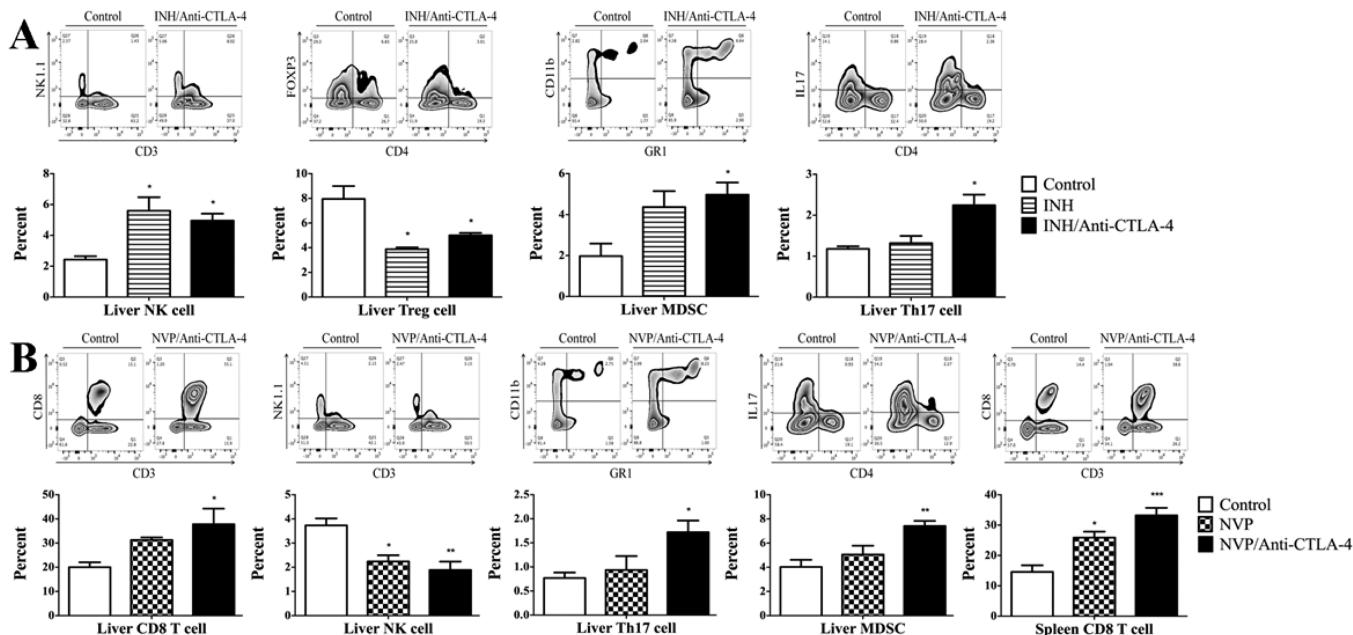


Figure 4. Immune cell changes in the liver. (A) Flow cytometry staining of hepatic mononuclear cells in mice treated with INH. (B) Flow cytometry staining of hepatic and splenic mononuclear cells in mice treated with NVP. Values represent the mean \pm SE. Analyzed for statistical significance by one-way ANOVA. A *p* of <0.05 was considered significant (**p* < 0.05; ***p* < 0.01).

depleting MDSCs and or regulatory T-cells may lead to even greater liver injury.²³ Further experiments with additional drugs and other methods of blocking immune tolerance will be required to determine the extent to which this strategy can be used to test drug candidates during drug development.

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Notes

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ABBREVIATIONS

INH, isoniazid; NVP, nevirapine; AQ, amodiaquine; PD1, programmed cell death 1; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; GLDH, glutamate dehydrogenase; ALT, alanine aminotransferase; IDILI, idiosyncratic drug-induced liver injury; NK cell, Natural Killer cell; MDSC, myeloid-derived suppressor cell; Th17, T-helper 17 cell; Treg, regulatory T-cell

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