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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · FEBRUARY 2014

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Mild Isoniazid-Induced Liver Injury in Humans Is Associated with an Increase in Th17 Cells and T Cells Producing IL-10

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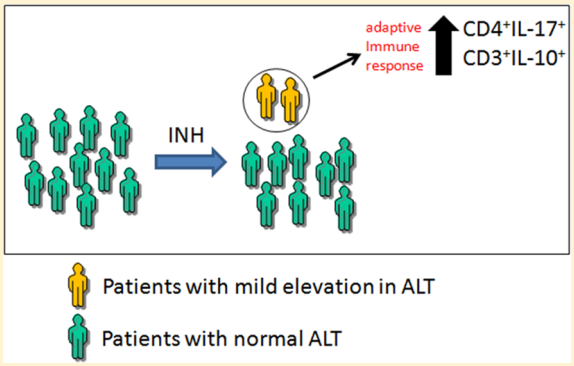
Supporting Information

ABSTRACT: Isoniazid (INH) remains a mainstay for the treatment of tuberculosis despite the fact that it can cause liver failure. The mechanism of INH-induced liver injury remains controversial. It had been proposed that the mechanism involves metabolic idiosyncrasy based on the observations that liver injury is not usually associated with fever, rash, or prompt increase in alanine aminotransferase (ALT) upon rechallenge. In the present study, we found that patients who were treated with INH because of a positive tuberculosis (TB) skin test and developed a small increase in ALT had an increase in Th17 cells as well as T cells that produce interleukin (IL)-10, which suggests stimulation of an adaptive immune response. Th17 cells are considered inflammatory and could be involved in causing the liver injury. IL-10 is considered anti-inflammatory and could be the reason that more serious liver injury did not occur. These changes were not observed in patients who did not have an increase in ALT. These are the first data to show a change in the T cell profile in patients with mild INH-induced liver injury; however, it is difficult to determine whether these changes were the cause or the result of the liver injury. Nevertheless, together with other studies, the data suggest that INH-induced liver injury is immune-mediated, with mild injury resulting in immune tolerance.



INTRODUCTION

Isoniazid (INH) remains a mainstay for the treatment of tuberculosis despite the fact that it is associated with a relatively high incidence of liver injury and even liver failure.[1−3] Classic studies performed several decades ago suggested that the hepatotoxic effects of INH are caused by bioactivation of the N-acetylhydrazine metabolite of INH.[4−6] However, this conclusion was based on an acute rat model of INH-induced liver injury with characteristics very different from the liver injury that occurs in humans. Recently, we demonstrated that INH itself can be bioactivated and bind covalently to the liver proteins of mice in vivo and to human liver microsomes in vitro.[7] INH binding to hepatic proteins in rats is significantly less than in mice;[7] therefore, the previous conclusions were probably based on the wrong type of liver injury in the wrong species.[8,9]

It was speculated that the mechanism of liver injury could be an immune-mediated reaction because it had characteristics similar to other idiosyncratic drug reactions that are immune-mediated.[2] In addition, there were cases in which patients had a rapid onset of liver injury upon rechallenge with INH as well as cases associated with fever, rash, and an eosinophilic infiltrate in the liver.[2] INH can induce an immune response because it frequently causes a fever and rash or anti-nuclear antibodies independent of liver injury, and occasionally it provokes an autoimmune reaction similar to lupus.[3,4] However, in most cases of INH-induced liver injury, especially mild injury, it is not associated with allergic features, and there is a lack of rapid onset on rechallenge.[2,10] Unsuccessful attempts to detect anti-INH antibodies also provided an argument against an immune-mediated mechanism for INH-induced liver injury, leading to use of the term metabolic idiosyncrasy.[10−12] The term metabolic idiosyncrasy has been used to differentiate it from immune idiosyncrasy; however, although slow acetylators are at slightly increased risk of INH-induced liver injury,[13] this does not explain the idiosyncratic nature of the reaction. Furthermore, Warrington found that patients with mild INH-induced liver injury had a positive lymphocyte transformation test (LTT) when their cells were incubated with INH or INH-modified proteins.[14,15] This indicates that these patients had memory T cells that recognize INH. More recently, we have shown that most patients with INH-induced liver failure have anti-INH antibodies and/or anticytochrome P450 antibodies.[16] As we have previously discussed,[3] this suggests that INH-induced liver injury is immune-mediated, and the term metabolic idiosyncrasy is incorrect or at least misleading.

CD4 T cells are an important cell type of the adaptive immune system that provide help to antigen-presenting cells and CD8 cells to initiate an immune response. CD4 T cells are divided

Received: January 12, 2014

   dx.doi.org/10.1021/tx500013z | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

Table 1. Demographic Data for Study Patients[a]

	gender	country of origin	age	year enrolled in study	concomitant disease	other medication
1	M	Philippines	56	2010		vitamins
2	F	Vietnam	47	2010		
3	M	Iraq	43	2010	hypertension	Lisinopril
4	F	South Korea	25	2011		multivitamin
5	M	China	24	2011		
6	M	Ghana	54	2011	hypertension, high cholesterol	Altace, Lipitor, Lipidil, ASA
7	F	Moldova	28	2011		birth control pill
8	M	Jordan	59	2011	diabetes Type 2, asthma, COPD, osteoporosis	Ventolin Flovent
9	M	Canada	20	2011		topical acne product (name unknown)
10	F	Philippines	22	2011		
11	M	Canada	19	2011		vitamin C, D
12	F	Philippines	44	2011	asthma	Advair 250
13	F	Philippines	48	2012		protein powder, vegetable powder
14	M	Chile	30	2013		
15	M	India	33	2013	acid reflux	acid reflux med
16	F	Nepal	39	2013		

[a]Part of this data has been previously shown in our recent publication.[16] M, male; F, female.

into various subtypes depending on the cytokine secretion pattern, which can lead to different types of immune responses such as Th1 cells, which secrete IFN-γ; Th2 cells, which secrete IL-4, IL-5, and IL-13; the classical T regulatory (T_{reg}) cells, which are characterized as $CD4^+CD25^+FoxP3^+$ and secrete IL-10/TGF-β; and Th17 cells, which secrete IL-17 and have been implicated in a variety of autoimmune diseases and liver injury.[17] We have proposed that mild cases of liver injury resolve with immune tolerance, and this may eliminate memory T cells.[3] This might also prevent a rapid response on re-challenge; only severe cases with failure of immune tolerance would exhibit the hallmark feature of immune memory.[3] In this article, we re-examine whether INH-induced liver injury is associated with an immune response by phenotyping leukocytes in patients who receive prophylactic INH because of a positive tuberculosis skin test.

PATIENTS AND METHODS

Human Subjects. After approval of the protocol by the University of Toronto Health Sciences Research Ethics Board, a total of 35 patients undergoing prophylaxis with INH because of a positive TB skin test were recruited by the Toronto Western Hospital (Toronto, ON) between June 2010 and August 2013. None of the patients had risk factors for hepatitis B, HIV infection, or any other condition that would cause liver injury, and all patients had a normal ALT. Their demographic characteristics and concomitant drugs are presented in Table 1. After obtaining informed consent, blood was drawn into heparinized tubes before the initiation of INH therapy, and patients were followed prospectively until they finished INH therapy. Liver function tests for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and bilirubin were measured by the clinical laboratory at Toronto Western Hospital. None of the 35 patients developed severe hepatotoxicity; six patients developed a small increase in alanine aminotransferase (ALT, 47−144 U/L).

PBMC Isolation and Flow Cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (GE Healthcare, Cooksville, ON) density gradient centrifugation. Briefly, blood (10 mL) was collected from patients into a heparinized tube and mixed with 10 mL of phosphate buffered saline (PBS, pH 7.4). The mixture was slowly overlaid on top of 15 mL of Ficoll and spun at 600g for 30 min; centrifuge deceleration was set to its lowest. The PBMCs were collected from the interface and washed twice with fresh PBS. One million cells/stain were aliquoted for flow cytometry.

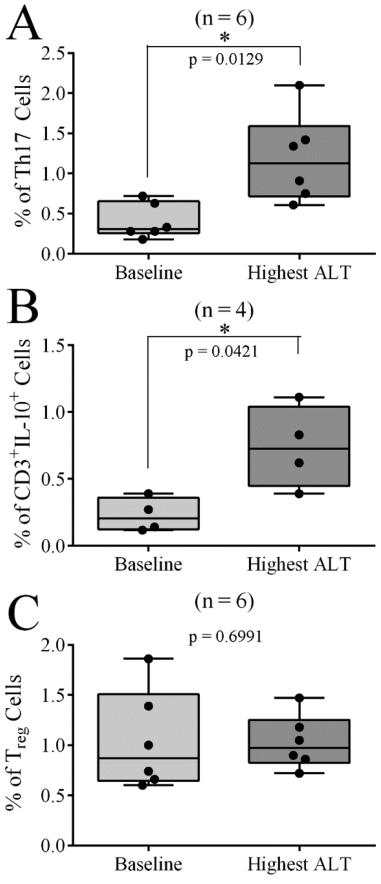


Figure 1. Changes in T cell phenotype relative to baseline when there was an increase in ALT. (A) Th17 cell percentages were plotted at baseline and at the time of highest ALT. (B) Same procedure as panel A was used for $CD3^+IL\text{-}10^+$ cells, but for patient 4, the cell percentages at revisit after 77 days were used as baseline because baseline measurements were not available for $CD3^+IL\text{-}10^+$ cells. (C) T_{reg} cell percentages were also plotted at baseline and at the time of highest ALT. Data are plotted as box plots (min/max) showing medians; *, $p < 0.05$ was considered significant.

Before staining with antibody, nonspecific binding was blocked by incubating the cells for 20 min with 20 μL/10^6 cells of human Fc

dx.doi.org/10.1021/tx500013z | Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

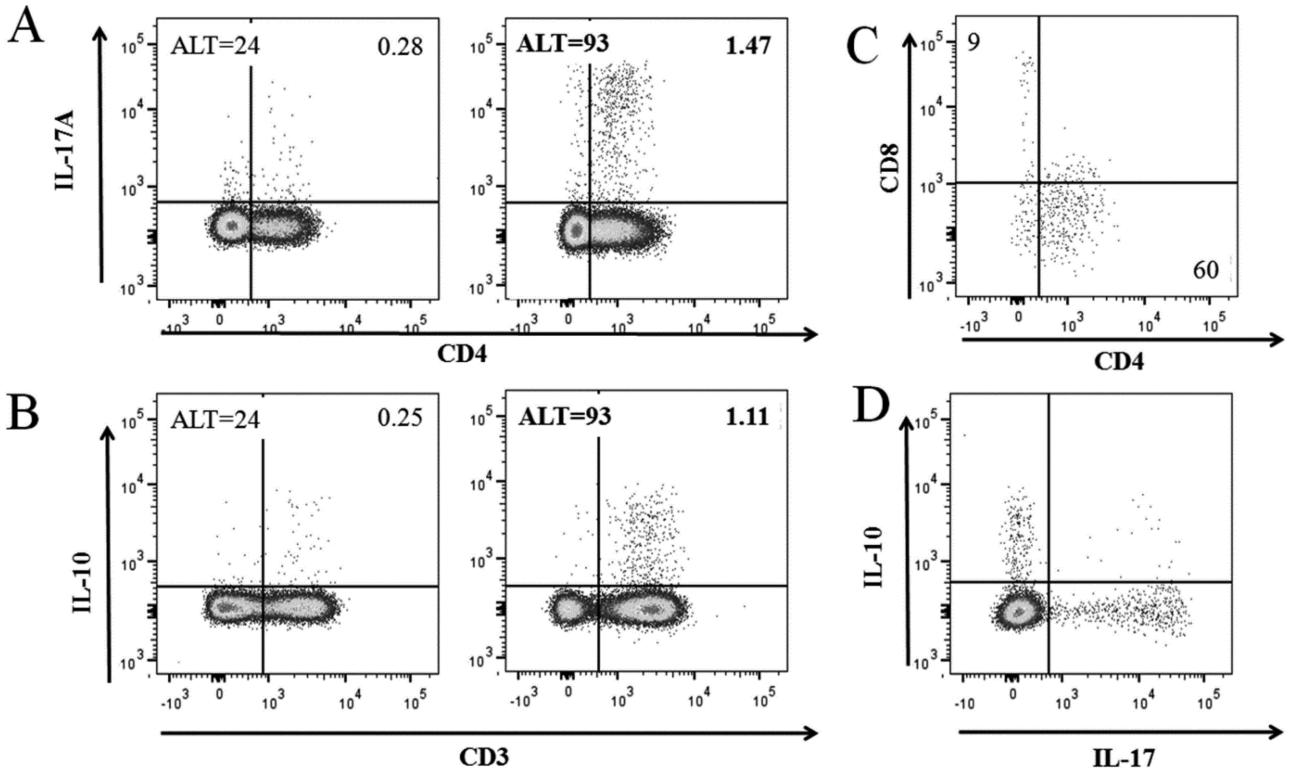


Figure 2. Representative lymphocyte phenotyping in patient 16. (A) Th17 cells at baseline (ALT = 18) and when there was a small increase in ALT (ALT = 93). (B) IL-10-producing cells in the same patient. (C) Percentage of $CD4^+$ vs $CD8^+$ cells from the $CD3^+IL\text{-}10^+$ panel when the ALT = 93. (D) Distribution of IL-10 and IL-17 (when ALT = 93) by first gating on $CD4^+$ cells.

binding receptor inhibitor (eBioscience, San Diego, CA). A total of 50 000 events were collected for each sample by gating on the lymphocytes, cell doublets were gated out, and live cells were used for analysis by using eFlour 506 viability dye (eBioscience) and gating on the negative population. The data was collected using a BD FACSCanto II flow cytometer and analyzed using FlowJo software (Tree Star, Ashland, OR).

The protocol used to stain Foxp3 was the one-step protocol for intracellular proteins adapted from eBioscience and using the Foxp3 fixation/permeabilization working solution. Cells were first surface stained for CD3-APC/CY7, CD4-eFluor@450, CD8-APC, and CD25-Alex Flour 488 followed by washing and staining with viability dye. Then, cells were fixed by using the fixation/permeabilization working solution (eBioscience) for 30 min at 4 °C and stained for Foxp3-PE using the 1× permeabilization buffer for 30 min at 4 °C. Cells were washed twice with 1× permeabilization buffer and resuspended in flow cytometry staining buffer for analysis.

For flow cytometry, 10^6 cells/mL were suspended for 5 h in culture medium, which contained 10% heat-inactivated fetal bovine serum in RPMI 1640 medium (Life Technologies, Burlington, ON), 50 ng of phorbol myristate acetate/mL (Sigma, Oakville, ON), 750 ng/mL of ionomycin (Sigma), and golgi stop/golgi plug (BD Biosciences, Mississauga, ON), which contains monesin and brefelding A solutions to be used as protein transport inhibitors during cell stimulation. The procedure for staining intracellular antigens was adapted from the two-step protocol for intracellular proteins (eBioscience). Briefly, cells were surface stained for CD3-APC/CY7, CD4-eFluor@450, CD8-APC followed by washing and staining with the viability dye. Then, cells were washed, fixed, and resuspended in flow cytometry staining buffer (eBioscience) and incubated overnight at 4 °C. The next day, intracellular staining was performed by first preincubating cells for 30 min with 1× permeabilization buffer at 4 °C and reincubating cells with anti-human IL-17A-FITC (eBioscience), IL-10-PE, IL-4-Alexa Fluor 488, IFN-γ-PerCP-CY5.5, and human Fc binding receptor inhibitor for 1 h in 1× permeabilization buffer at 4 °C. Cells were washed twice with 1× permeabilization buffer twice and resuspended in flow cytometry staining buffer for analysis. This procedure is similar to the one previously used for mice.[18] Supporting Information Figure 1 illustrates the various colors and the methods for cellular phenotyping. For patient 3, the analysis of IL-10 the cell percentages at revisit after 77 days was used as baseline because baseline measurements were not available for $CD3^+IL\text{-}10^+$ cells.

Luminex. Serum cytokines were analyzed using Bio-Rad's Bio-Plex Pro human cytokine 27 plex: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF. Five samples from patients who received INH but did not have an increase in ALT and five samples from patients who received INH and had a mild increase in ALT were analyzed.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Data was analyzed using the Mann−Whitney U test. A p value < 0.05 was considered significant (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

RESULTS

Flow cytometry was used to phenotype the peripheral lymphocytes of patients treated with INH. Out of a total of 35 patients who enrolled in the study, the samples of only 16 patients were suitable for analysis. Four patients with high basal ALT were excluded from the study, and another 15 patients either withdrew shortly after being enrolled in the study or were lost to follow up by the clinic. Demographic data for each patient is shown in Table 1. A total of six patients developed a small increase in ALT during INH treatment, with the time to onset of ALT being approximately 93 ± 19 days (mean ± SE), and the average maximal ALT activity was 78 ± 15 U/L (mean ± SE). We looked for a Th1 ($CD4^+IFN\text{-}\gamma^+$) or Th2 ($CD4^+IL\text{-}4^+$) immune response in patients treated with INH; however, we did not observe any differences between patients who had a

dx.doi.org/10.1021/tx500013z | Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

Table 2. Liver Function Tests and Cell Phenotyping in Patients That Had Mild DILI during INH Prophylaxis

			percentage of cell types		
patient ID	days on INH	ALT (U/L)/AST (U/L)/ ALP (U/L)/bilirubin (μmol/L)	$CD4^+IL\text{-}17^+$	$CD3^+IL\text{-}10^+$	T_{reg}
1	0	34/21/100/10	0.72		1.39
	51	44[a]/31/82/11	1.67		1.46
	93	144[a]/63[a]/102/11	1.34		0.91
2	0	14/18/58/8	0.63		1.0
	28	15/22/51/9	0.96		1.34
	56	30/26/54/7	0.98		1.31
	98	32/26/51/15	0.91		1.59
	140	46[a]/36[a]/54/11	0.81		1.35
	182	57[a]/44[a]/57/14	1.74		1.23
	231	73[a]/52[a]/52/10	2.1		0.82
3	0	23/19/85/6	0.18		0.66
	77	15/18/69/8	0.38	0.12	0.94
	108	39/35[a]/68/7	0.27	0.12	0.99
	156	58[a]/48[a]/69/7	0.3	0.24	0.85
	205	59[a]/49[a]/66/9	0.61	0.34	1.18
	247	49[a]/38[a]/63/8	0.72	0.69	1.16
9	0	15/20/72/13	0.28	0.46	1.86
	28	18/20/66/5	0.35	0.56	2.29
	49	25/25/61/8	0.28	0.23	1.38
	77	47[a]/36[a]/65/12	0.91	0.8	1.05
	113	39/30/59/10	0.38	0.61	0.86
	153	36/30/57/9	0.32	0.25	0.74
11	0	22/17/81/13	0.33	0.14	0.61
	28	23/22/69/12	0.28	0.21	0.67
	64	52[a]/28/61/8	0.75	0.62	0.91
	104	34/23/68/10	0.64	0.66	1.0
	208	22/17/54/11	0.67	0.56	1.26
16	0	24/18/73/6	0.28	0.25	0.74
	28	33/22/71/6	0.35	0.36	0.86
	56	41/32/61/7	0.71	1.01	1.39
	98	93[a]/54[a]/78/8	1.42	1.11	0.98
	146	93[a]/64[a]/79/7	1.25	1.29	0.82
	202	77[a]/49[a]/68/10	0.57	0.74	0.86

[a]Indicates abnormal liver function tests as reported by the Toronto Western Hospital.

mild increase in ALT or no increase in ALT versus baseline (data not shown). There was an increase in Th17 cells (defined as $CD4^+IL\text{-}17A^+$) in patients who had an increase in ALT (Figure 1A). In addition, the increase in Th17 cells was associated with an increase in T cells with detectable intracellular IL-10 levels ($CD3^+IL\text{-}10^+$, Figure 1B). An example is shown in Figure 2, where patient 16 who had a small increase in ALT also had an increase in Th17 cells (Figure 2A) and $CD3^+IL\text{-}10^+$ cells (Figure 2B). Most of the cells that produced IL-10 were $CD4^+$ (Figure 2C), and the $IL\text{-}17^+$ and $IL\text{-}10^+$ cells were from different $CD4^+$ cell populations (Figure 2D). We looked for changes in the percentage of T_{reg} cells (defined as $CD4^+CD25^+Foxp3^+$), but we did not observe any differences (Figure 1C). Liver function tests and cell percentages for the six patients who had a small increase in ALT are shown in Table 2, and individual flow cytometry phenotyping data from all of the patients can be found in Supporting Information Table 1. In four patients (2, 9, 11, and 16), there was a correlation between the percentage of Th17 cells and ALT (Figure 3). Patient 13 had an increase in Th17 cells but no increase in ALT after 1 month; however, this patient developed a skin rash and discontinued treatment (Table S1). We measured serum IL-17 and IL-10 in five patients who had an increase in ALT and an increase in Th17 cells by flow cytometry and compared them with five patients who received INH but did not have a significant increase in ALT. No differences were observed between the two groups (Figure S2).

DISCUSSION

In this study, we demonstrated that patients who underwent INH treatment because of positive TB test and had a small increase in ALT also had an increase in Th17 cells and T cells that produce IL-10 (Figure 1A,B). Although patients with active tuberculosis have been reported to have a reduction in Th17 cells, patients with latent tuberculosis were not different from healthy donors;[19] therefore, it is likely that the observed increase in Th17 cells was induced by INH. We did not observe any changes in the percentage of IL-4- or IFN-γ-producing T cells in patients who were treated with INH, with or without mild liver injury. This is consistent with the fact that IL-10 can inhibit Th1 and Th2 immune responses but not a Th17 immune response.[20] One patient had an increase in Th17 cells without an increase in ALT, but they did develop a skin rash, which is an indication of an immune-mediated reaction (patient 13, Table S1); therefore, INH-induced liver injury and rash may share a related mechanism that involves Th17 cells. Although there was a significant increase in circulating Th17 cells and $IL\text{-}10^+$ T cells, the absolute numbers are small, and there was no significant increase in serum IL-17 or IL-10. This is not

dx.doi.org/10.1021/tx500013z | Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

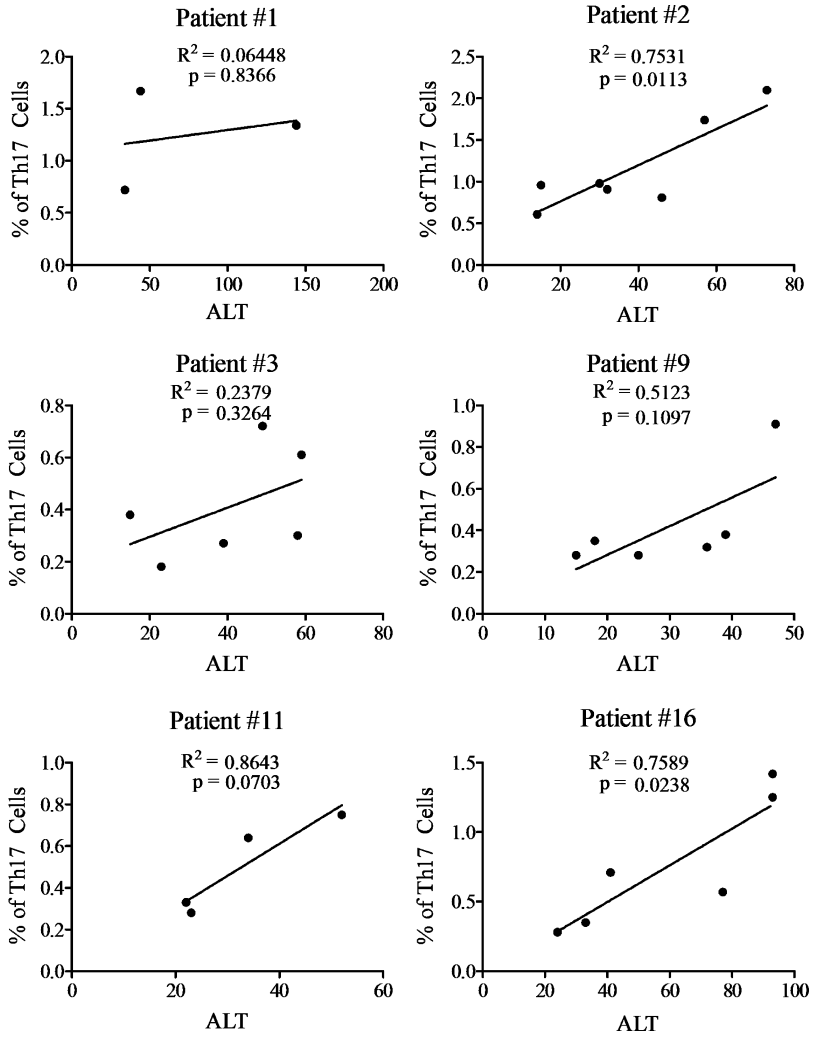


Figure 3. Linear regression analysis between ALT and the percent of Th17 cells in six patients that developed a mild increase in ALT.

surprising because this relatively weak immune response would be expected to be largely limited to the liver and associated lymph nodes/spleen.[21] Unfortunately, it is not possible to sample the liver and draining lymph nodes. In contrast, there is a marked increase in serum cytokines in cases of drug-induced liver failure.[22] Given the extensive liver damage and the fact that the samples were obtained late in the course of the injury, the changes in cytokines in liver failure are just as likely to be involved in the immune response to the injury as a cause of the injury.

Overall, this study provides additional evidence that INH-induced liver injury involves an adaptive immune response. The increase in Th17 cells is interesting because this cell type has been implicated in other types of liver disease.[17] Th17 cells have been shown to produce key cytokines such as IL-17, IL-6, TNF-α, and IL-22, which are important for immune responses against pathogens.[23] The increase in IL-10 may be part of the immune response that prevents more serious liver injury. IL-10 can inhibit a variety of innate and adaptive immune responses through the production of the repressor cytokine SOCS3; however, it does not generally inhibit IL-17 production.[20] We postulated that Th17 cells would mediate cell injury and macrophages would be involved in injury repair. However, acute acetaminophen-induced liver injury in mice, which is presumably not mediated by the adaptive immune system, is associated with an increase in Th17 cells just 2 h after treatment.[18] In addition, the most recent study of idiosyncratic drug-induced liver injury histology suggests that $CD8^+$ T cells are responsible for the injury.[24] This raises the question as to whether the role of Th17 cells in this liver injury is pathogenic or a response to the injury.[24] The delay in onset suggests that the injury does not involve direct cytotoxicity. In general, the increase in the Th17 and IL-10^+ T cells appeared to be coincident with the increase in ALT, but two patients (patients 2 and 3, Table 2) had an increase in ALT before the increase in Th17 cells, which suggests an immune response to the liver injury. This is consistent with IL-10^+ T cells being involved in immune tolerance that limits the injury. A significant limitation is that we can only sample the peripheral blood, and the cells actually mediating the injury are likely to be localized in the liver. In addition, the immune response is likely to evolve over time so that the cells involved in injury are likely to appear earlier than those involved in resolving the injury. Given the difficulty in studying these rare reactions, until we can develop a valid animal model of idiosyncratic drug-induced liver injury, it is unlikely that this question can be definitively answered.

There are other data that support an immunological basis for INH-induced liver injury. We have shown that INH itself can be bioactivated and bind covalently to rodent livers in vivo and to human liver microsomes in vitro.[7,9] This is consistent with

dx.doi.org/10.1021/tx500013z | Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

Warrington's findings in which patients with mild liver injury had a positive lymphocyte transformation test when the lymphocytes were stimulated with INH-modified protein but not with INH itself, whereas more severe cases of liver injury also had a positive LTT to INH.[14,15] Maria and Victorino also reported a positive LTT test with cells from a patient with INH-induced hepatotoxicity.[25] INH also commonly induces the production of autoantibodies, sometimes resulting in a lupus-like syndrome,[26] and more recently, we found that most patients with INH-induced liver failure had anti-INH and anti-cytochrome P450 antibodies.[16] In conclusion, these data, together with previous investigations, suggest that INH-induced hepatotoxicity is immune-mediated, not metabolic idiosyncrasy.

■ ASSOCIATED CONTENT

Supporting Information

Flow cytometry dot plots illustrating the method for cellular phenotyping, serum IL-17 and IL-10 in patients receiving INH, and ALT activity and percentage of Th17/T_{reg}/$CD3^+IL\text{-}10^+$ cells in patients receiving prophylactic INH. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

‖These authors contributed equally to this manuscript.

Funding

This work was supported by the Canadian Institutes of Health Research (CIHR).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Jack Uetrecht holds the Canadian Chair in Adverse Drug Reactions. Imir G. Metushi is a trainee of the Drug Safety and Effectiveness Cross Disciplinary Training Program, which is funded by CIHR. We thank Judith Lang and Andrea Moore for recruiting patients at the Toronto Western Hospital for this study.

■ ABBREVIATIONS

ALT, alanine aminotransferase; CYP, cytochrome P450; IL-10, interleukin 10; IL-17, interleukin 17; INH, isoniazid; LTT, lymphocyte transformation test; PBMCs, peripheral blood mononuclear cells; Th17 cells, T helper 17 cells; T_{reg} cells, regulatory T cells

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