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Research Article

Activation of Flucloxacillin-Specific CD8+ T-Cells With the Potential to Promote Hepatocyte Cytotoxicity in a Mouse Model

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ABSTRACT

There are currently no animal models of drug-induced liver injury (DILI) where the adaptive immune system has been shown to damage the liver. Thus, it is difficult to explore the mechanistic basis of the tissue injury. The aim of this study was to use C57BL/6 CD4+-deficient mice with a mutation in the αβ gene encoding for Major histocompatibilty complex (MHC) class II molecules to (1) develop a mouse model of flucloxacillin sensitization, (2) explore whether drug-specific CD8+ kill primary hepatocytes, and (3) analyze perturbations in liver integrity following oral exposure to flucloxacillin. CD8+ T-cells from lymph nodes of flucloxacillin-sensitized mice were stimulated to proliferate, secrete interferon (IFN-γ) and granzyme B, and induce hepatocyte apoptosis in a concentration-dependent manner following *ex vivo* stimulation. The T-cell response was antigen-specific; T-cells were not activated with other β-lactam antibiotics. Furthermore, T-cell responses only occurred in the presence of flucloxacillin-pulsed antigen presenting cells. In separate experiments, flucloxacillin-specific T-cells were induced to migrate to the mesenteric lymph nodes using retinoic acid, prior to administration of oral flucloxacillin, and analysis of plasma biomarkers of liver injury. Oral exposure to flucloxacillin resulted in mild elevations in alanine aminotransferase, liver, and gall bladder leukocyte infiltration and a marked swelling of the gall bladder. Thus, CD4+-deficient mice represent a promising model to study the role of the adaptive immune system in DILI.

Key words: flucloxacillin; immunogenicity; liver injury

Delayed-type drug hypersensitivity reactions are a serious complication in the clinic and an impediment to the drug development process. Although skin is the tissue usually targeted by the immune system, internal organs are also damaged, either in isolation or as part of a generalized hypersensitivity reaction (Warner *et al.*, 2012).

Flucloxacillin is a β-lactam antibiotic that is effective against β-lactamase-producing bacteria. Human exposure is associated with hypersensitivity reactions that target the skin, liver, and kidneys. Drug-responsive T-cells have been identified in peripheral blood of patients presenting with each form of adverse reaction (Mousavi *et al.*, 2011; Maria and Victorino, 1997; Spanou *et al.*, 2006). In patients with flucloxacillin-induced cholestatic liver injury, CD8+ T-cells are preferentially activated by drug antigens (Monshi *et al.*, 2013). Flucloxacillin-specific CD8+ clones express gut homing chemokine receptors and secrete IFN-γ and cytolytic molecules including Fas-L, perforin, and granzyme-B when activated. In agreement with genetic

association studies that identified human leukocyte antigen (HLA)-B*57:01 as risk factor (Daly *et al.*, 2009), the drug-specific T-cell response was MHC class I restricted. It should be emphasized however that not all patients with flucloxacillin-induced liver injury express B*57:01, and drug-specific, MHC-restricted T-cell responses are also detectable in B*57:01 negative individuals. Furthermore, CD8+ T-cells from HLA-B*57:01 positive and negative human donors can be primed with flucloxacillin through dendritic cell presentation of the drug-derived antigen to naïve T-cells (Monshi *et al.*, 2013) or repeated rounds of drug stimulation using blood mononuclear cells as antigen presenting cells (APC) (Wuillemin *et al.*, 2013). Wuillemin *et al.* (2014) have recently shown that flucloxacillin-specific T-cells kill cell lines transfected with the relevant HLA-class I molecules, which suggests that the adaptive immune system may participate directly in the liver injury seen in patients with flucloxacillin-induced liver injury. Detection of MHC-restricted flucloxacillin-specific T-cell responses in human patients with and without HLA-B*57:01 indicates that it should also be possible to prime experimental animals against the drug-derived antigen.

To stimulate an antigen-specific T-cell response in experimental animals, low molecular weight protein-reactive chemicals can be painted directly onto the shaved abdomen. This leads to the priming of antigen-specific CD8+ T-cells in the draining lymph nodes. On re-exposure to the chemical, antigen-specific CD8+ T-cells travel to the site of contact and cause tissue damage through the release of cytokines and cytolytic molecules (Akiba *et al.*, 2002; Kehren *et al.*, 1999; Traidl *et al.*, 2000). Using this approach, it has been possible to model amoxicillin-induced skin injury and explore the role of T-cells (Rozieres *et al.*, 2010). Importantly, effective sensitization to amoxicillin was dependent on the use of a CD4+-deficient C57BL/6 mouse strain with a mutation in the αβ gene encoding for MHC class II molecules.

Attempts to develop animal models of drug-induced liver injury (DILI) that involve the adaptive immune system have been largely unsuccessful (Ju and Reilly, 2012; Uetrecht and Naisbitt, 2013). This likely relates to difficulties in overcoming tolerance (most human patients do not develop liver injury following drug exposure) and lack of effective oral dosing strategies that induce drug antigen-specific T-cell responses. Hence, in this study, we have used the CD4+-deficient MHC class II- C57BL/6 mouse to explore (1) the immunogenicity of flucloxacillin, (2) whether flucloxacillin-responsive T-cells kill hepatocytes *ex vivo*, and (3) whether oral exposure to flucloxacillin alters normal liver integrity.

MATERIALS AND METHODS

Reagents. I.V. grade flucloxacillin sodium was used in all experiments. Medium for cell culture consisted of RPMI 1640 (Sigma, Dorset, UK) supplemented with 10% foetal bovine serum (Life Technologies, Paisley, UK), L-glutamine (2 mM; Sigma) and 2-mercaptoethanol (50 μM; Sigma). All other reagents including phytohaemagglutinin (PHA), Hanks balanced salt solution, and dimethylsulfoxide (DMSO) were from Sigma.

Animals and sensitization protocol. Wild-type C57BL/6 mice were used in initial experiments to optimize (1) sensitization protocols against the model contact allergen dinitrochlorobenzene and flucloxacillin, and (2) the *ex vivo* analysis of antigen-specific T-cell responses. Dinitrophenol hapten-specific proliferation and IFN-γ release were detected with lymph node cells from sensitized mice; however, sensitization of mice with flucloxacillin was not observed. Thus, C57BL/6 mice with a mutation in the αβ gene encoding for MHC class II molecules (8–20 weeks of age; Charles River, Kent, UK) were used in all subsequent experiments. To deplete residual regulatory CD4+ T-cells, which is critical for the induction of sensitization against mild to moderate chemical allergens (Rozieres *et al.*, 2010; Vocanson *et al.*, 2009), mice were administered with a rat anti-mouse CD4 antibody (100 μg per mouse (100 μl ip in phosphate buffered saline; GK 1.5 BioXCell, West Lebanon, New Hampshire) on day 0 (prior to drug exposure) and on day 7. The absence of CD4+ positive cells was confirmed by flow cytometry (data not shown). Mice were sensitised on days 1–3 through painting with flucloxacillin (50 μl, 1 g/ml; in 70% DMSO) on a shaved area at the abdomen (approximately 3 cm^2 surface area). Preliminary experiments with lower doses of flucloxacillin did not induce detectable antigen-specific T-cell responses. Vehicle mice were painted with 70% DMSO alone. Experiments were conducted in groups of 3 mice and all procedures were conducted on at least 3 separate occasions in accordance with criteria outlined in the license granted under the Animals (Scientific Procedures) Act 1986. Draining lymph node cells were removed on day 8 for *ex vivo* experiments.

Cell isolation. For T-cell assays, responder cells were isolated from inguinal lymph nodes. Single cell preparations were prepared by maceration through 100 μm nylon filters (BD Biosciences, California). Cells were washed in Hanks balanced salt solution and suspended in culture medium prior to use. CD11c positive cells were isolated from mesenteric lymph nodes from lymph nodes using positive selection with CD11c magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Surrey, UK). These cells are referred to as (APC) throughout the manuscript.

Analysis of CD8+ T-cell proliferation and IFN-γ and granzyme B release. Inguinal lymph node cells (1.25×10^5 cells/well; 200 μl total volume, in U-bottomed 96 well plates) were cultured for 5 days at 5% CO_2 and 37°C with flucloxacillin (0.25–1 mg/ml) in the presence or absence of additional CD11c+ APC (2.5×10^4). In certain experiments, CD11c+ APC were removed from the inguinal lymph nodes via magnetic isolation, prior to *ex vivo* incubation with flucloxacillin. Cultures without flucloxacillin served as a negative control.

Proliferation was measured by the addition of [^{3}H]thymidine (0.25 μCi/well; Moravek, Brea, California) for the last 16 h of culture. Plates were then harvested and incorporated [^{3}H]thymidine counted. Supernatants were collected and analyzed for IFN-γ secretion using a mouse IFN-γ ELISA kit (R&D Systems Inc, Minneapolis, Minnesota). Quantification of IFN-γ-secreting cells per draining lymph node was determined using ELISpot. In the ELIspot experiments, lymph node cells (2.5×10^5 cells/well) were incubated with CD11c + APC (2.5×10^4 cells/well) and flucloxacillin for 48 h (37°C; 5% CO_2) in MultiscreenHTS filter plates (Millipore, Watford, UK). Plates were processed for IFNγ secreting cells according to the kit instructions (BD Biosciences, Oxford, UK).

In separate experiments, lymph node cells were incubated with carboxyfluorescein succinimidyl ester (CFSE; 5 mM; 0.5 μl; Ebioscience, Hatfield, UK) for 5 min prior to washing and incubation with flucloxacillin and CD11c + APC. Throughout a 5-day culture period proliferating CD8 + T-cells were analyzed using a FACSCanto II flow cytometer (BD Biosciences). A minimum of 2×10^5 events were counted and the data were analyzed with

Cyflogic. Cells exposed to 5 μg/ml PHA served as a positive control.

To measure the cytolytic activity of flucloxacillin-responsive T-cells, draining lymph node cells from flucloxacillin-sensitized and control mice were analyzed for granzyme B release by ELIspot. Briefly, lymph node cells (1.25×10^5 cells/well) were incubated with CD11c + APC (2.5×10^4 cells/well) and flucloxacillin in precoated ELISpot plates. Cells were incubated for 48h (37°C; 5% CO_2) after which wells were developed according to the kit instructions (R&D Systems, UK).

Antigen specificity and mechanism of CD8+ T-cell activation. To measure antigen specificity, lymph node cells from flucloxacillin-sensitized mice were cultured with CD11c+ APC and either flucloxacillin, amoxicillin, or piperacillin (0.5–2 mM for each drug). After 5 days, proliferation was measured through incorporation of [^{3}H]thymidine. IFN-γ secretion was measured in culture supernatant using ELISA.

Flucloxacillin (0.5–2 mM) was also incubated with CD11c+ APC alone for 16 h. After repeated washing with drug-free medium, the pulsed APC were cultured with lymph node cells from sensitized mice and proliferation and IFN-γ secretion were measured as described above. Lymph node cells from drug naive mice served as a control.

Hepatocyte isolation and culture for analysis of immune-mediated killing. CD4-deficient C57BL/6 mice were anesthetised with sodium pentobarbital and once the foot reflex had ablated, were surgically opened to expose the hepatic portal vein and cannulated. The liver was then flushed with wash buffer (Hanks, 900 mM $NaHCO_3$, HEPES) for 4 min at 12 ml/min and then with digestion buffer (Wash buffer, 5 mM $CaCl_2$, 0.5 mg/ml collagenase D, 0.07 mg/ml trypsin inhibitor from soybean) at 12 ml/min until the liver began to soften and show striations. The liver was then eviscerated, the capsule removed through use of sharp forceps, and the organ placed in a Petri dish. Incubation buffer (Wash buffer, 0.1 mg/ml DNAse) was added to just cover the liver and hepatocytes were softly shaken out. The cell suspension was poured through a 100-μm nylon filter to remove large cell clumps, then centrifuged (60 g, 3 min) and washed prior to analysis of viability using trypan blue dye exclusion.

Lymph node cells from flucloxacillin-sensitized mice were cultured with CD11c+ APC and flucloxacillin as outlined above for 24 h. The activated cells were then washed repeatedly to remove unbound drug and transferred to white flat bottomed 96 well plates containing viable hepatocytes (2.5×10^3/well). Cell viability, cytotoxicity, and apoptosis were measured using an ApoTox-Glo kit (Promega, UK). The kit simultaneously measures 2 protease activities; one a marker of viability, the other a marker of cytotoxicity. The assay also uses a luminogenic caspase 3/7 substrate to measure caspase activity.

Oral exposure of sensitized mice to flucloxacillin and assessment of liver integrity. Mice were sensitized against flucloxacillin as described above (topical application; 1 g/ml, 50 μl) and retinoic acid (applied to the same site; 0.1 mg, 50 μl). Control mice received vehicle and retinoic acid, but not flucloxacillin. In the first experiment, oral flucloxacillin dosing (2.3 mg/day) was initiated on day 7 for 2 days. Forty eight hours after dosing blood was collected and serum alanine aminotransferase (ALT), levels were determined using the ALT liquid stable reagent-based kinetic assay (Thermo, Waltham, Massachusetts). In a second experiment, oral dosing was continued for 10 days and readouts were recorded on days 0, 2, 4, and 10 of oral dosing. Readouts included: gross examination of the liver and gall bladder, analysis of blood serum liver damage biomarkers ALT, HMGB1, mir-122, GLDH, total keratin-18, ALP, and total bilirubin levels using validated methods described elsewhere (Antoine *et al.*, 2009, 2013) and histological examination of the liver. For this purpose, liver slices were excised, fixed in 10% neutral buffered formalin and routinely paraffin wax-embedded. Sections (3–5 μm) were prepared, stained with hematoxylin and eosin and histologically examined by a veterinary pathologist (AK) in a blinded fashion.

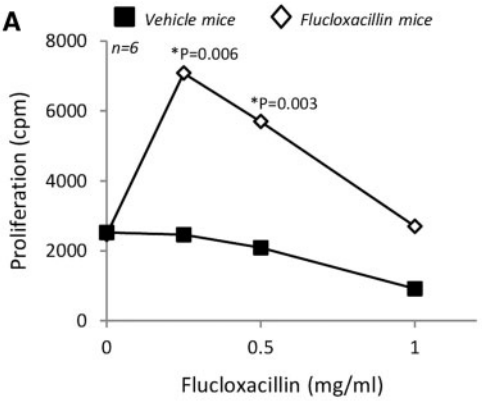
Statistics. Statistical analysis was performed using the students *t*-test. *P* values of less than 0.1 are shown with *P* values of less than 0.05 being considered significant (SigmaPlot, Systat Software Inc, version 12.5).

RESULTS

Priming of CD8+ T-cells to flucloxacillin in CD4+ T-cell-deficient mice. Mice depleted of residual CD4+ T-cells were sensitised on days 1–3 by the application of flucloxacillin to the shaved abdomen. Priming of CD8+ T-cells from draining lymph nodes was assessed *ex vivo* through analysis of proliferation and IFN-γ secretion. A significant increase in proliferation was observed when lymph node cells from flucloxacillin-sensitized mice were cultured in the presence of the drug (0.25–0.5 mg/ml; Fig. 1A). The dose-dependent proliferative response of cells from 6 flucloxacillin-treated mice is shown in the figure. Flow cytometry was used to quantify the number of CD8+ T-cells that were proliferating in response to flucloxacillin. Upon CFSE staining, 5–11% of lymph node cells were found to be activated by the drug (Fig. 1B). In agreement with the [^{3}H]thymidine data, a maximal response was observed with 0.25–0.5 mg/ml flucloxacillin. Lymph node cells from mice treated with the vehicle control were not stimulated to proliferate *ex vivo* with the drug (Fig. 1A).

APCs are required for flucloxacillin-specific CD8+ T-cell activation. To investigate the role of APC in the detection of the drug-specific T-cell response *ex vivo*, CD11c+ cells were depleted from lymph node cells. Alternatively, additional CD11c+ cells were added to lymph node cell cultures. When the CD11c+ cells were removed, flucloxacillin-specific proliferation and IFN-γ secretion were not detected (Fig. 2A). When the CD11c+ cells were not depleted, flucloxacillin-specific proliferative responses were observed; however, IFN-γ secretion into the culture supernatant was not significant (Fig. 2B). Finally, when CD11c+ cells were isolated from non-draining lymph nodes and added to the assay, significant increases in flucloxacillin-specific proliferation and IFN-γ secretion were seen (Fig. 2C). Similar results were obtained in repeat experiments when an IFN-γ ELISpot was used as a readout (results not shown).

Flucloxacillin hapten-specific activation of CD8+ T-cells. APC pulsing experiments can be used to discriminate between hapten-specific T-cell responses and T-cell responses to the parent drug, which presumably binds non-covalently to MHC prior to activating T-cells (Monshi *et al.*, 2013; Wuillemin *et al.*, 2013). APC pulsed with flucloxacillin for 16 h, followed by repeated washing to remove unbound drug, stimulated CD8+ T-cells from sensitized mice to proliferate and secrete IFN-γ (Fig. 3). In contrast, T-cells from vehicle-treated control mice were not activated with the flucloxacillin-pulsed APC.



B

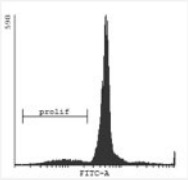
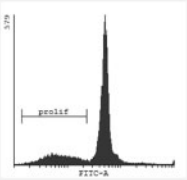
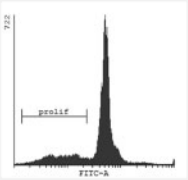
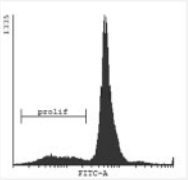
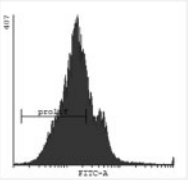
Condition	No drug	Flucloxacillin (mg/ml)			PHA
		0.25	0.5	1.0	
CFSE labelled lymph node cells					
% dividing CD8+ T-cells	11.77%	22.93%	21.97%	16.73%	69.78%

FIG. 1. Sensitization of mice against flucloxacillin. *Ex vivo* activation of draining lymph node cells from 6 sensitized mice with flucloxacillin. The skin of mice was exposed to flucloxacillin (1 g/ml, 50 μl) or vehicle alone on days 1–3. On day 8, the draining lymph node cells were removed, macerated to a single cell suspension and incubated (1.25×10^5 cells/well; 200 μl total volume, in U-bottomed 96 well plates) in triplicate with flucloxacillin (0.5–1 mg/ml). Cells were cultured for 5 days after which proliferation was measured through [^{3}H]thymidine incorporation. The data were analyzed using the students *t*-test. A, Combined data from 6 mice; B, CFSE-labelled cells were incubated for 5 days with flucloxacillin. The cells were harvested, labelled with an anti-CD8 antibody and the number of dividing CD8+ cells analyzed by flow cytometry.

Flucloxacillin-specific CD8+ T-cells show limited reactivity against other β-lactam antibiotics. Lymph node cells from flucloxacillin-sensitized mice were cultured in the presence of the β-lactam antibiotics amoxicillin and piperacillin to assess antigen specificity and possible cross reactivity. Cells from all 3 sensitized mice were found to proliferative *ex vivo* when activated with flucloxacillin (Fig. 4). In contrast, proliferative responses to amoxicillin and piperacillin were only detected in 1 out of 3 mice and in each case; the magnitude of the response was approximately 50% lower than that seen with flucloxacillin. Each of the drugs failed to induce proliferation of lymph node cells from vehicle control mice (Fig. 4; bottom right panel).

Lymph node cells from flucloxacillin-sensitized mice release granzyme B following drug stimulation and induce T-cell-mediated increases in hepatocyte apoptosis. IFN-γ and granzyme B secretion from flucloxacillin-specific CD8+ T-cells were analyzed using ELIspot. Dose-dependent increases in the number of spot forming units were observed in both assays when lymph node cells were cultured *ex vivo* with flucloxacillin and APC (granzyme B, Fig. 5A; IFN-γ, Fig. 5B), These data clearly highlight the potential cytotoxic capacity of flucloxacillin-specific CD8+ T-cells. Hence, in subsequent experiments, lymph node cells from flucloxacillin-sensitized and control mice were cultured with flucloxacillin and APC for 24 h, thoroughly washed to remove unbound drug, and added to fresh hepatocytes to measure T-cell-mediated killing. T-cells from flucloxacillin-sensitized mice caused a significant increase in hepatocyte cytotoxicity and apoptosis when activated *ex vivo* in the presence of antigen presenting cells pulsed with flucloxacillin. In contrast, apoptosis and cytotoxicity was not detected in similar experiments with T-cells from vehicle control mice (Fig. 5C).

Interestingly, a significant increase in hepatocyte apoptosis was also observed when unstimulated T-cells from the flucloxacillin-sensitized mice were cocultured with hepatocytes (Fig. 5C; data points on the Y axis). This indicates that T-cells are isolated from lymph nodes of flucloxacillin-sensitized mice, but not vehicle control mice, in a partly activated form. No significant increase in cytotoxicity or apoptosis was observed when hepatocytes and T-cells were cultured separately with flucloxacillin.

Figure 5D shows data from 3 individual mice where increased hepatocyte apoptosis with unstimulated T-cells from flucloxacillin-sensitized mice is enhanced following drug

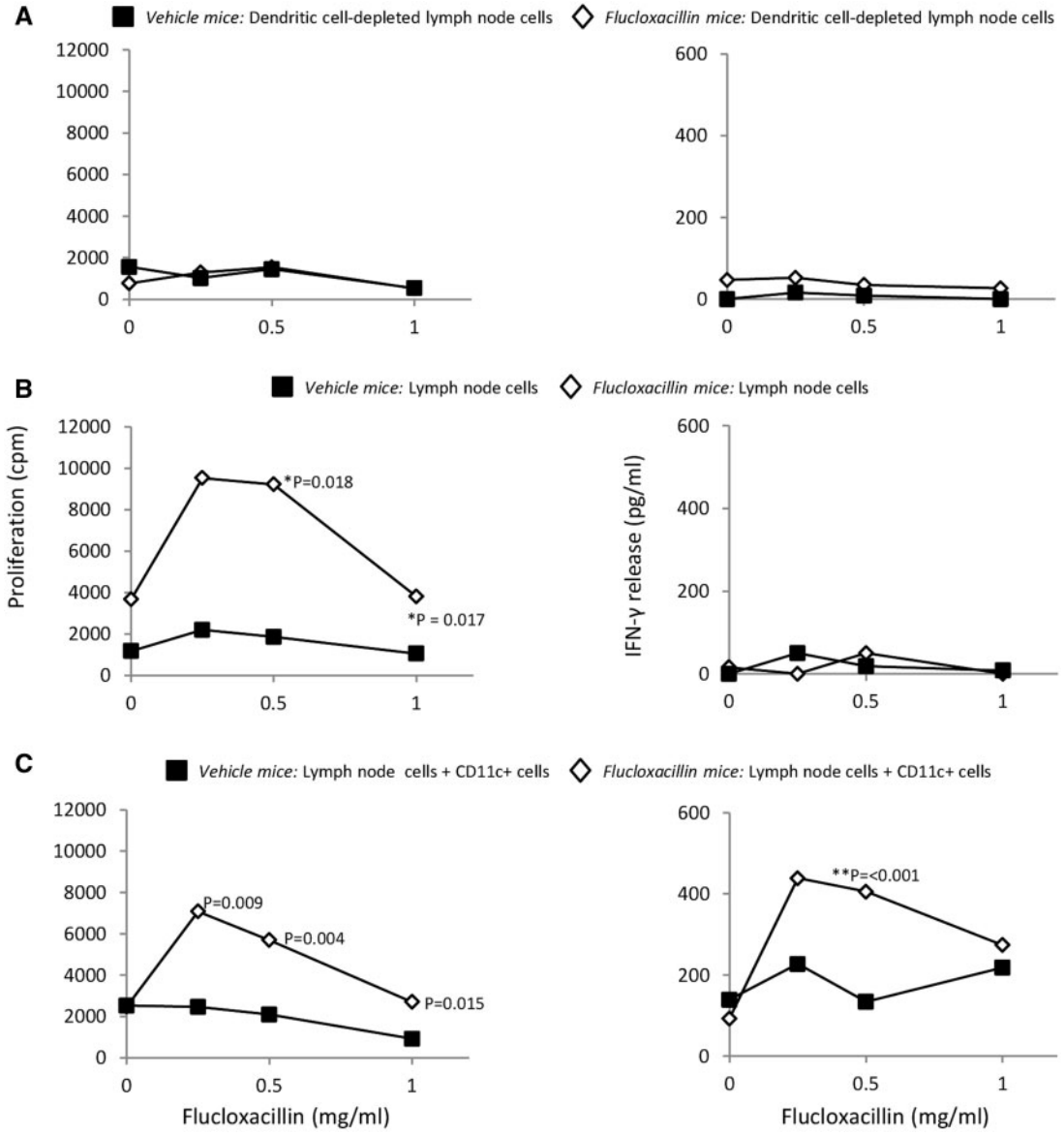


FIG. 2. Antigen presenting cells are required for the activation of flucloxacillin-specific CD8+ T-cells. Draining lymph node cells were removed from flucloxacillin-sensitized mice, macerated to a single cell suspension. A, CD11c cells were removed using antibody-coated magnetic beads. B, Cells were not treated. C, CD11c-positive cells were isolated from mesenteric lymph nodes of naïve mice and added to the T-cell assay (2.5×10^4/well). Cells from A–C (1.25×10^5 cells/well; 200 µl total volume, in U-bottomed 96 well plates) were incubated in triplicate with flucloxacillin (0.5–1 mg/ml) for 5 days after which proliferation was measured through [^{3}H]thymidine incorporation. The data were analyzed using the students *t*-test ($n = 3$).

stimulation *ex vivo*. Three mice are shown to illustrate the difference in response seen with individual mice.

Oral exposure of sensitized mice to flucloxacillin causes mild transient rises in ALT levels and a dilation of the gall bladder, but no significant histological changes in the liver. Previous studies have shown that systemic administration of flucloxacillin to rats yields protein adducts in liver cytosol, detectable by western blotting (Carey and van Pelt, 2005). Thus, in our final experiments we studied the effect of oral drug exposure on the liver of flucloxacillin-sensitized and vehicle control mice. The sensitization protocol was modified slightly to include retinoic acid, which induces the gut homing chemokine receptor expression and the migration of T-cells. In the first experiment, flucloxacillin-sensitized and control mice were administered oral flucloxacillin for 2 days, killed and ALT levels measured. A 3–6-fold increase in ALT levels was detected in flucloxacillin-sensitized mice in comparison to control mice (Fig. 6A). Thus, a second experiment was conducted with analyses performed 2, 4, and 10 days after commencing oral dosing. ALT levels rose steadily in flucloxacillin-sensitized mice through the first 4 days of oral dosing before subsiding at day 10 (Fig. 6B). Other markers of liver injury were not raised in either treatment group (Supplementary Table 1). Somewhat surprisingly, the gross examination revealed dilation of the gall bladder, similar to that seen with bile duct obstruction, in sensitized mice 2 days after commencing oral dosing (Fig. 6C). There was no gall bladder dilation in vehicle control mice during the first 2 days of oral dosing; however, dilation similar to the flucloxacillin-sensitized mice was observed on day 10 in all mice. The histological examination of liver parenchyma and gall bladder of animals in both treatment groups did not identify any significant treatment-related changes. In none

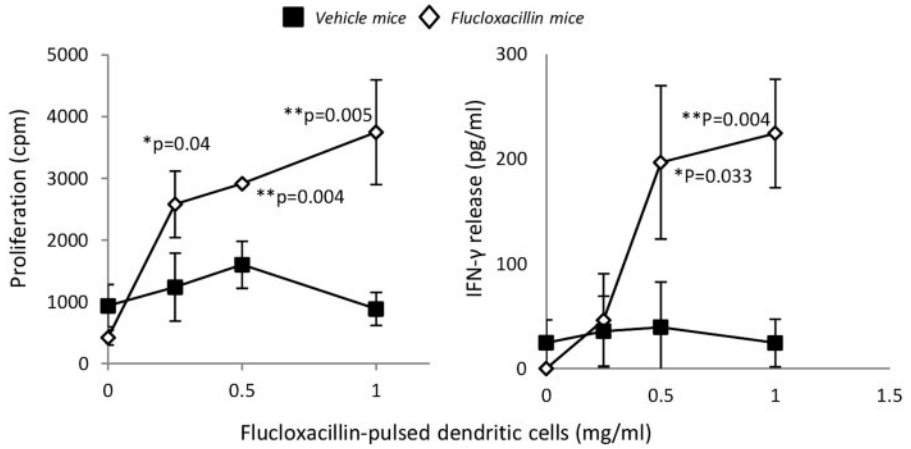


FIG. 3. Flucloxacillin-specific T-cells are activated with hapten-modified antigen presenting cells. Lymph node cells were isolated from flucloxacillin-sensitized and vehicle control mice and incubated (1.25×10^5 cells/well; 200 µl total volume, in U-bottomed 96 well plates) with APC (2.5×10^4/well) pulsed with flucloxacillin (0.25–1 mg/ml) for 24 h. After 5 days incubation, cells were analyzed for proliferation by [^{3}H]thymidine incorporation and IFN-γ secretion by ELISA ($n = 3$). The data were analyzed using the students *t*-test.

of the groups was there a substantial inflammatory infiltrate, though in some flucloxacillin treatment groups, animals were found to exhibit slight multifocal portal mononuclear (lymphocytes, macrophages) infiltration. In the group of sensitized mice examined 10 days after commencing oral flucloxacillin, such an infiltration was seen in 3 of the 4 animals (results not shown). Also, the immunohistological demonstration of apoptotic hepatocytes by staining for cleaved caspase-3 did not reveal a significant increase in hepatocyte apoptosis in any treatment group at any time point.

In the gall bladders, a variable degree (slight to moderate) of intra- and/or subepithelial leukocyte infiltration, either mononuclear (lymphocytes, macrophages) or mixed (with neutrophils) was observed in both flucloxacillin-sensitized and vehicle control groups exposed to oral flucloxacillin. In none of the animals was there histological evidence of an impaired bile flow, because bile accumulation was neither seen within biliary ductules (bile plugs) nor within hepatocytes.

DISCUSSION

Flucloxacillin exposure is associated with cholestatic liver injury in human patients. The delayed onset of clinical symptoms and identification of HLA-B*57:01 as an important risk factor are indicative of an immune pathogenesis. Accordingly, we have isolated flucloxacillin-responsive T-cells from patients with DILI (Monshi *et al.*, 2013). The majority of T-cells were CD8+ and secreted high levels of IFN-γ and cytolytic molecules following drug exposure, in an MHC class I-restricted manner. Importantly, these T-cell responses were detectable in DILI patients with and without the risk allele HLA-B*57:01. This study aimed to (1) develop a mouse model of flucloxacillin-sensitization, (2) explore whether drug-specific CD8+ kill primary hepatocytes and (3) analyze perturbations in liver integrity following oral exposure to flucloxacillin in sensitized and nonsensitized mice. Our studies utilized C57BL/6 mice with a mutation in the Aβ gene encoding for MHC class II molecules that become sensitized to weak chemical allergens including the β-lactam antibiotic amoxicillin following epicutaneous application (Vocanson *et al.*, 2006, 2009). This strain was selected to study the CD8+ T-cell response that predominates in human patients with flucloxacillin-induced liver injury. CD4+ T-cells displaying regulatory function are present in low numbers in these mice (Kish *et al.*, 2007); however, they were depleted using an anti-CD4 antibody prior to drug exposure.

CD8+ T-cells from lymph node cells of sensitized mice, but not vehicle exposed controls, were stimulated to proliferate *ex vivo* with flucloxacillin. The T-cell response was dependent on the presence of APC as depletion of CD11c+ cells from lymph nodes of sensitized mice completely abrogated the *ex vivo* drug-specific proliferative response, while proliferation and IFN-γ secretion was detected when the lymph node cells were cultured with flucloxacillin and additional CD11c+ cells. A similar pattern of activity has been reported with human antigen-specific T-cell clones, with certain T-cells requiring extra antigen presentation to promote the secretion of cytokines (Hecht *et al.*, 1983). Because APC were required to activate T-cells with flucloxacillin, pulsing experiments were conducted to show that APC transfer the flucloxacillin hapten to T-cells.

Flucloxacillin, like other β-lactam antibiotics contain a penicilloyl structure and bind covalently to specific lysine residues on protein to form adducts that might activate T-cells (Monshi *et al.*, 2013; Nhim *et al.*, 2013; Whitaker *et al.*, 2011). Hence, we utilized 2 drugs, amoxicillin and piperacillin, which are both associated with T-cell-mediated hypersensitivity reactions in humans, to study antigen specificity. These studies are important as human patients are exposed to multiple β-lactam antibiotics and experimental data describe a degree of T-cell cross reactivity between drugs such as flucloxacillin, penicillin G, amoxicillin, and ampicillin (Mauri-Hellweg *et al.*, 1996). In the mouse model, flucloxacillin-responsive T-cells displayed limited reactivity with amoxicillin and piperacillin, indicating that the side-chain of flucloxacillin contributes to the specific MHC-drug-T-cell receptor binding interaction.

Animal models of DILI that involve the drug-specific activation of the adaptive immune system have been difficult to develop and/or reproduce and most end in failure (Ng *et al.*, 2012; Uetrecht and Naisbitt, 2013). As an example, halothane, which is metabolized in liver to a reactive intermediate, is thought to cause DILI in humans through a mechanism involving the adaptive immune reaction (Bird and Williams, 1989; Knight *et al.*, 1994). In experimental models halothane adducts are detectable in liver (Kenna *et al.*, 1993; Smith *et al.*, 1993), and drug exposure is associated with an innate immune response

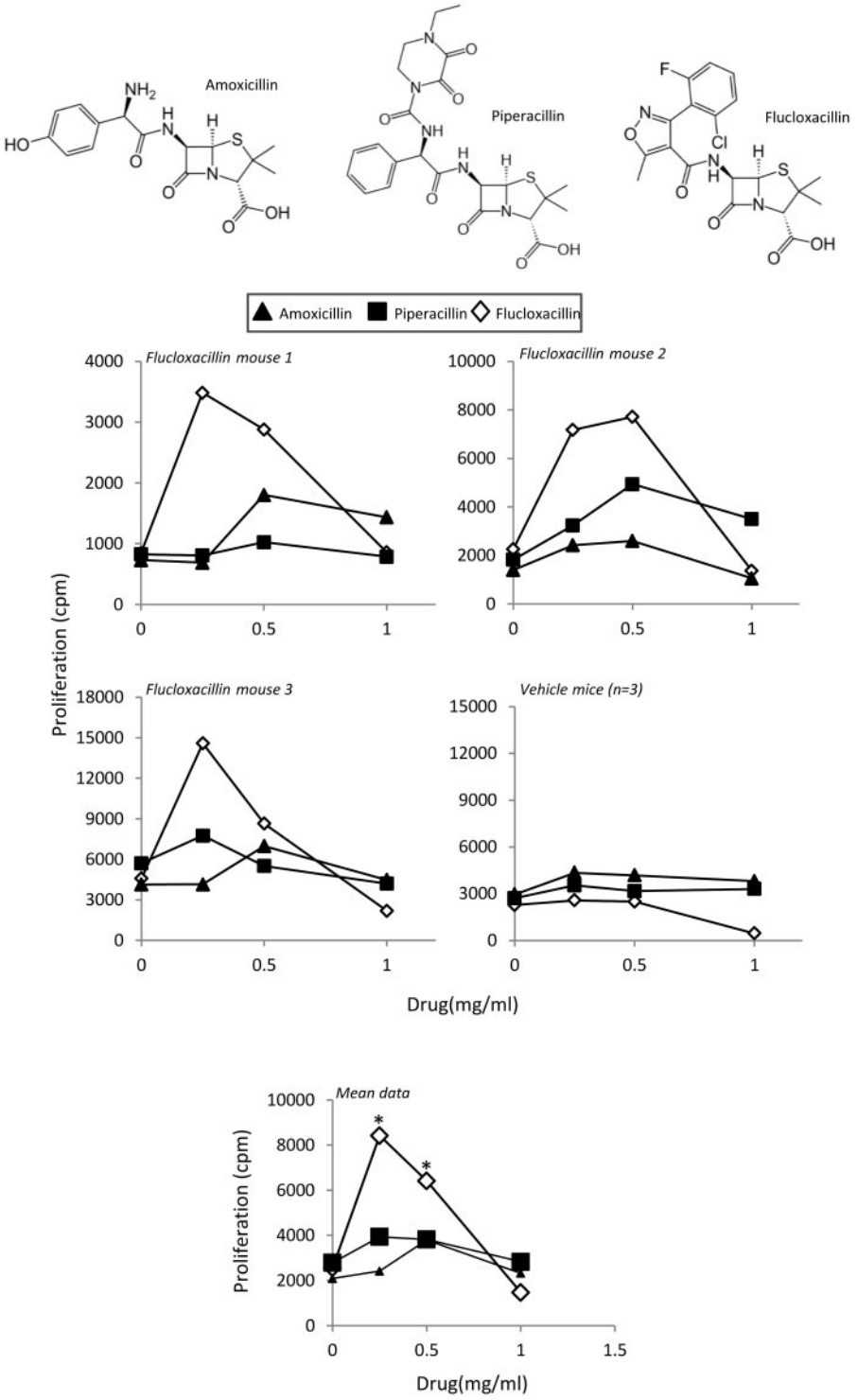


FIG. 4. Flucloxacillin-specific CD8+T-cells show limited responses to other β-lactam antibiotics. Lymph node cells from flucloxacillin sensitized and vehicle exposed mice were incubated (1.25×10^5 cells/well; 200 µl total volume, in U-bottomed 96 well plates) with antigen presenting cells (2.5×10^4/well) and flucloxacillin, piperacillin, or amoxicillin (0.25–1 mg/ml). After 5 days incubation, cells were analyzed for proliferation by [^{3}H]thymidine incorporation. The results show data from individual flucloxacillin-sensitized mice, mean data from the vehicle control group ($n = 3$) and mean data from the group-treated groups ($n = 3$; data were analyzed using the students *t*-test).

and a hepatic infiltration of immune cells such as eosinophils (Proctor *et al.*, 2013; You *et al.*, 2006). However, this type of damage is typical of the mild tissue injury seen in humans and not the severe life-threatening DILI that is thought to involve drug-specific T-cells. Thus, the next objective of our study was to investigate whether T-cells from flucloxacillin-sensitized mice kill primary hepatocytes.

Flucloxacillin-treated T-cells from sensitized mice were found to secrete significantly higher levels of the cytolytic molecule granzyme B when compared with cells from vehicle

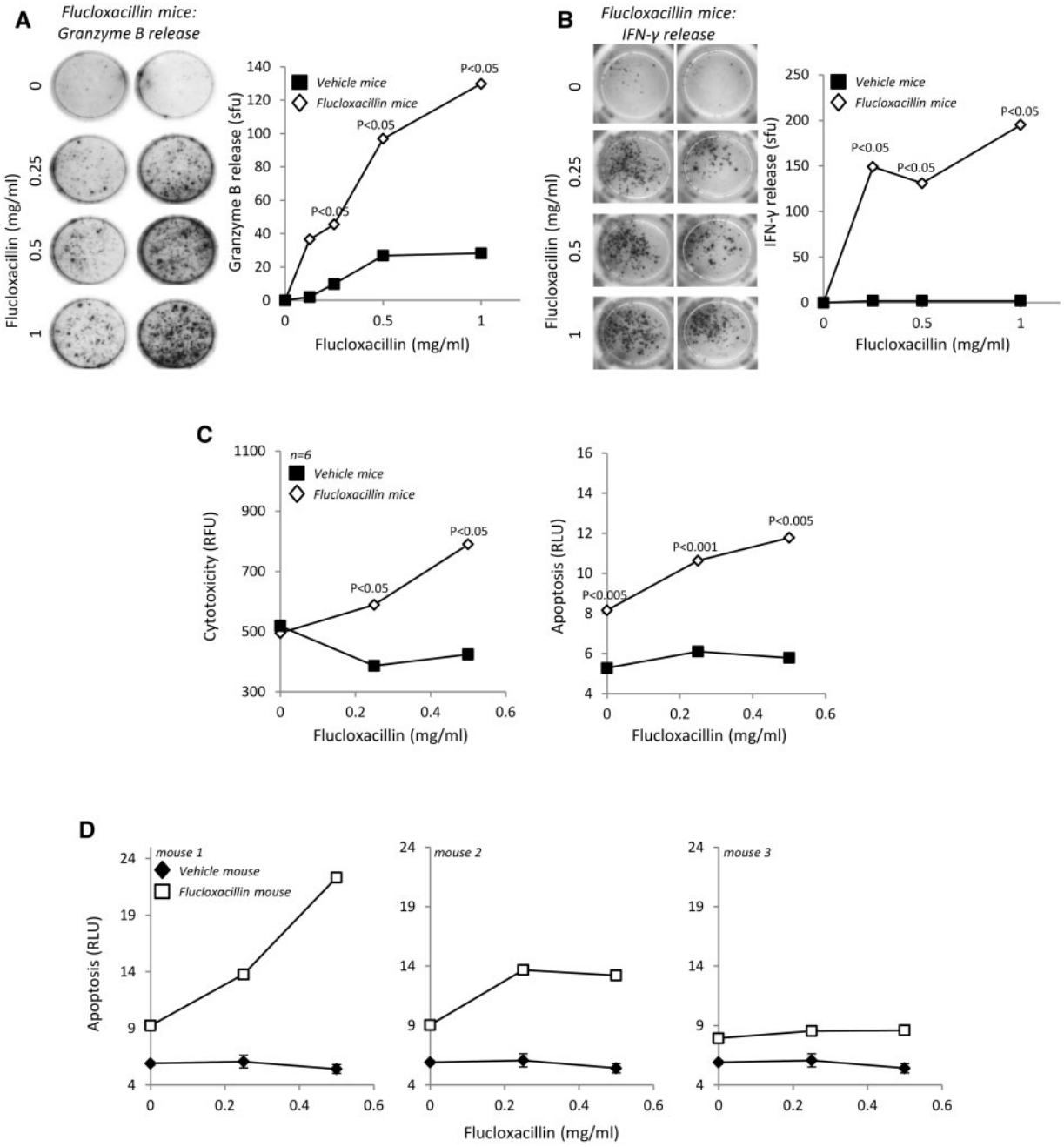


FIG. 5. Flucloxacillin-treated CD8+T-cells from sensitized mice secrete IFN-γ and granzyme B and induce hepatocyte apoptosis. A, B, Lymph node cells from flucloxacillin sensitized and vehicle exposed mice were cultured (1.25×10^5 cells/well; 200 µl total volume, in U-bottomed 96 well plates) with flucloxacillin (0.1–1 mg/ml) for 2 days. Granzyme B and IFN-γ release was measured by ELIspot ($n = 3$). Antigen presenting cells (2.5×10^4 cells/well) were cultured with draining lymph node cells (1.25×10^5 cells/well) in the presence and absence of flucloxacillin (0.25–0.5 mg/ml) for 24 h. The cells were then incubated with freshly isolated hepatocytes (2500 cells/well) for 4 h. The Promega APOTOX GLO kit was used to analyze cytotoxicity and apoptosis. C, Combined results from 6 mice; D, Results from 3 individual mice. Statistics were performed using the students T-test (RLU; relative light units; RFU, relative fluorescence units).

controls (Fig. 5). Significant increases in hepatocyte apoptosis was also observed using an ApoTox-Glo assay, which discriminates between apoptotic and necrotic cell death (Proasad *et al.*, 2012), when T-cells from sensitized mice were activated *ex vivo* with flucloxacillin then cultured with freshly isolated hepatocytes. Interestingly, hepatocyte apoptosis was observed with T-cells from sensitized mice (in the absence of *in vitro* drug stimulation) and the number of cells undergoing apoptosis increased further following activation *ex vivo*. In contrast, cytotoxicity and apoptosis was not seen with T-cells from control mice. These data show that flucloxacillin-specific CD8+ T-cells kill hepatocytes through the release of cytolytic mediators. Future studies should utilize mice lacking regulatory T-cells, but with otherwise normal CD4+ T-cells, to delineate the role these cells play in flucloxacillin-induced liver injury.

To determine whether oral administration of flucloxacillin to sensitized mice results in perturbation of the liver integrity, mice were sensitized with flucloxacillin and retinoic acid before oral administration of flucloxacillin for 10 days. Retinoic acid increases expression of the chemokine receptor CCR9 and

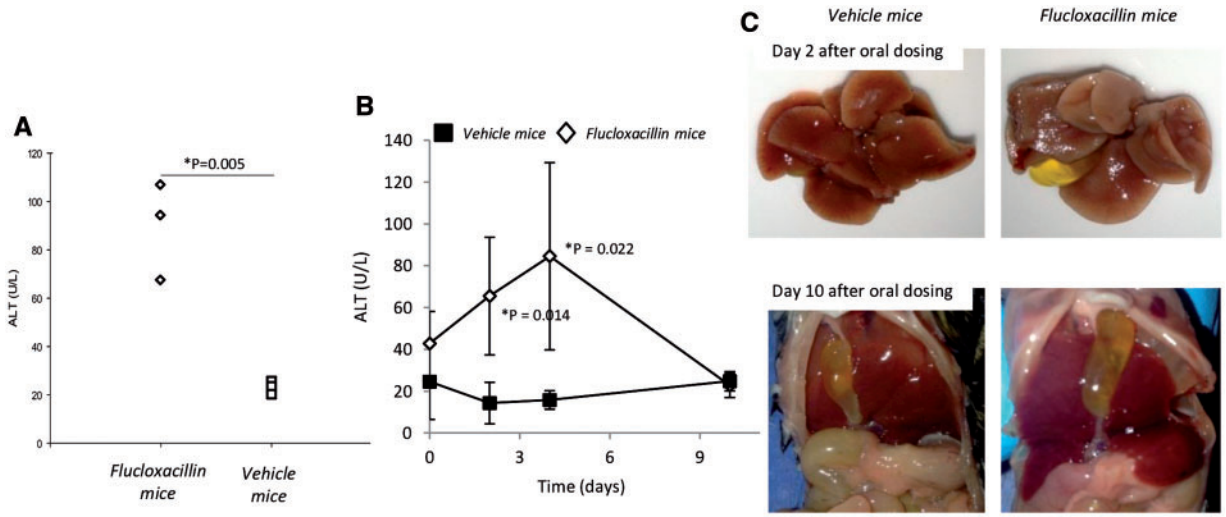


FIG. 6. Liver perturbation in flucloxacillin-treated mice. Mice were treated with flucloxacillin or vehicle by skin painting on 3 consecutive days. Twenty days later mice were exposed to oral flucloxacillin for 2 or 10 days. A, Experiment 1; alanine aminotransferase (ALT) levels after 2 day oral exposure to flucloxacillin. B, Experiment 2; ALT levels after 10 day oral exposure to flucloxacillin. C, Photographs of representative livers showing swollen gall bladders on days 2 and 10 of oral dosing in the flucloxacillin sensitized and vehicle control groups.

imprints gut-homing characteristics on activated T-cells indirectly through modulation of dendritic cell function (Eksteen *et al.*, 2009; Neumann *et al.*, 2012). In some instances of liver disease, endothelial cells have been shown to produce CCL25, the chemokine specific for CCR9 on gut-homing T-cells (Eksteen *et al.*, 2004). A mild elevation in serum ALT levels was observed in 2 separate experiments on days 2–4 after initiating oral dosing with flucloxacillin in sensitized mice. Similar findings were not seen with mice exposed to oral flucloxacillin without prior sensitization (Fig. 6). Importantly, the concentration of flucloxacillin used in these experiments was 10-fold lower than that used by Takai *et al.* (2014) to detect similar raises in ALT in an acute model of flucloxacillin-induced liver injury and are within the range of doses used in humans. Other markers of liver injury including HMGB1, mir-122, GLDH, total keratin-18, ALP, and total bilirubin were not raised in either treatment group. Furthermore, evidence of apoptotic hepatocytes was not observed through analysis of secreted cleaved keratin-18 and immunohistological staining for cleaved caspase-3. Thus, additional dose-ranging and/or chronic exposure studies are required to optimize the *in vivo* model. Somewhat surprisingly, a large swelling of the gall bladder was observed in flucloxacillin-sensitized mice after 2 days of oral dosing, while gall bladder dilation was seen in both sensitized and vehicle control mice after 10 days of oral flucloxacillin. These data suggest that long-term oral flucloxacillin exposure induces sensitization and the unwanted gall bladder response; however, it is also possible that the gall bladder swelling does not involve the adaptive immune system and this should be investigated further. In the gall bladders, a variable intra- and/or subepithelial leukocyte infiltration was observed in both flucloxacillin-sensitized and vehicle control groups exposed to oral flucloxacillin in the absence of other significant treatment-related changes. Interestingly, flucloxacillin-induced liver injury often manifests in humans as cholestasis with painless jaundice. In several cases of flucloxacillin-induced liver injury, biopsy specimens reveal that bile ducts are reduced in size and number, which lead Eckstein and colleagues to propose that flucloxacillin should be listed amongst the causes of vanishing bile duct syndrome (Eckstein *et al.*, 1993). Hepatobiliary injury that results in an enlargement of the gall bladder, similar to that seen on our experiments, can also be induced in experimental animals through bile duct ligation and administration of the toxic bile acid lithocholic acid (Woolbright *et al.*, 2013, 2014) and these models have been used extensively to study the mechanisms of cholestasis, the direct toxic effects of bile acids and the immune cell infiltrates that occur as a consequence of tissue injury.

In conclusion, the CD4-deficient C57BL/6 mouse seems to represent an interesting model for further studies to investigate the relationship between drug distribution, activation of the adaptive immune system, and the development of liver injury. In future experiments, we plan to measure (1) T-cell migration following cutaneous exposure to flucloxacillin and/or retinoic acid (2) the phenotype and function of liver infiltrating T-cells, and (3) the flucloxacillin-specific T-cell response in mice exposed to oral flucloxacillin.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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