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J Allergy Clin Immunol. Author manuscript; available in PMC 2023 September 27.

Published in final edited form as:

J Allergy Clin Immunol. 2023 August ; 152(2): 486–499.e7. doi:10.1016/j.jaci.2023.03.029.

Development of mouse models with restricted HLA-B*57:01 presentation for the study of flucloxacillin-driven T-cell activation and tolerance in liver injury

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Abstract

Background: Flucloxacillin (FLX)-induced liver injury is immune-mediated and highly associated to HLA-B*57:01 expression. Host factors leading to drug-induced liver injury are not yet well understood.

Objective: Characterize *in vivo* immune mechanisms determining the development of $CD8^+$ T cells reactive to FLX in animals expressing the risk human leukocyte antigen (HLA) allotype.

Methods: HLA-B*57:01 transgenic mice (Tg) or Tg strains with H2-K^bD^b knockout (Tg/KO) or H2-K^bD^b/PD-1 double knockout (Tg/DKO) were treated with drug and/or anti-CD4 antibody. Drug-induced liver injury was evaluated on the basis of liver enzyme and histologic changes at day 10 of treatment. FLX-reactive $CD8^+$ T cells were characterized *in vitro* by release of effector molecules on drug restimulation, gene expression, and flow cytometry analysis, and functionality tested for hepatic cytotoxicity.

Results: $CD8^+$ T-cell responses to FLX in Tg were dependent on both HLA and mouse major histocompatibility complex I presentation and *in vivo* priming. Eliminating H2-K^bD^b in Tg/KO to allow exclusive presentation of FLX by HLA resulted in a less robust drug-specific $CD8^+$T-cell response unless $CD4^+$ cells, including regulatory T cells, were depleted. Treatment of Tg/KO with anti-CD4 antibody and FLX led to subclinical liver inflammation associated with an increase in $PD1^+CD8^+$ T cells in the lymphoid organs and liver. Impaired PD-1 expression in Tg/DKO led to liver histopathologic and transcriptional alterations but without hepatic enzyme elevations. Moreover, effector lymphocytes accumulated in the liver and showed FLX-dependent hepatic cytotoxicity *in vitro* when tolerogenic liver cells were depleted.

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Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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Conclusions: In our *in vivo* models, FLX primes $CD8^+$ T cells to recognize drug presented by HLA-B*57:01 and murine major histocompatibility complex I. HLA-B*57:01–dependent $CD8^+$ T-cell reaction to FLX is limited by the presence of $CD4^+$ cells, presumably regulatory T cells, and PD-1 expression. Tolerogenic hepatic cells limit clinical disease through PD-L1 or additional unexplored mechanisms.

Keywords

Flucloxacillin; HLA-B*57:01 transgenic mice; drug-induced liver injury; tolerance

Adverse reactions to antibiotics are primarily immune-mediated events that occur at significant frequency and can have severe health consequences to the affected patients.[1,2] Understanding the mechanisms of adverse reactions to antibiotics, therefore, is critical for public health management to ensure appropriate antibiotic usage and to avoid withdrawal of effective products under development because of side effects.[3] In particular, flucloxacillin (FLX), a narrow-spectrum β-lactam antibiotic prescribed to combat gram-positive bacterial infections (ie, Staphylococcus) causes cholestatic hepatitis resulting in liver inflammation and injury.[4–6] Genome-wide association studies established a significant linkage between human leukocyte antigen (HLA) alleles B*57:01 and B*57:03, and FLX–drug-induced liver injury (FLX-DILI), with 83% of FLX-DILI cases carrying the B*57 alleles.[7,8] The frequency of FLX-DILI varies between 1 to 11 cases for every 10,000 FLX-treated patients depending on treatment length, sex, and age.[9] The idiosyncratic nature of its clinical manifestations suggests that other host and environmental factors might play a role in controlling liver injury in addition to HLA.

The detection of liver-infiltrating, FLX-reactive $CD8^+$ T lymphocytes in patients with DILI supports the idea that liver pathogenesis is immune mediated.[4,10,11] Although 2 models have been proposed to explain FLX presentation by HLA-B*57:01,[12,13] the hepatotoxic mechanism of FLX-reactive T cells is not yet clearly understood. Studies with human peripheral blood mononuclear cells (PBMCs) demonstrated that FLX-reactive $CD8^+$ and $CD4^+$ T cells could be enriched from both drug-naive HLA-B*57:01^+ and HLA-B*57:01^- healthy subjects when cultured *in vitro* with the drug.[14–17] Also, drug-reactive $CD8^+$ T cells were enriched in PBMCs from HLA-B*57:01^+ patients experiencing liver injury,[15,16] but not PBMCs from HLA-B*57:01^- individuals. Surprisingly, the high frequency of drug-reactive cells measured *in vitro* contrasts with the low clinical incidence of FLX-DILI, implicating *in vivo* tolerogenic mechanisms in the control of the disease onset.

Early mouse models developed to study FLX-induced liver injury[18,19] did not address the role of HLA during transient DILI due to the lack of HLA expression in murine cells. The generation of transgenic mice expressing HLA-B*57:01 has been instrumental in helping us gain knowledge on the immune response triggered by abacavir (ABC)[20–23] and on the immunogenicity of FLX-haptenated peptides or human serum albumin.[24,25] Although FLX can be presented by non–HLA-B*57:01 molecules,[14,15,18,19] we chose to focus on HLA-B*57:01 using Tg mice to elucidate the mechanisms of drug presentation and subsequent immune activation by the human risk allele with a higher association with FLX-DILI. We confirmed that murine MHC class I (mMHC-I) alleles, in addition to HLA-B*57:01,

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can prime FLX-reactive T cells. We subsequently studied the role of HLA-B*57:01 in FLX-specific presentation to T cells in recently developed mouse strains exclusively expressing HLA-B*57:01. We demonstrated that the robustness of $CD8^+$ T-cell responses to the drug *in vivo* was dependent on the absence of $CD4^+$ T cells (including regulatory T [Treg] cells) and programmed cell death 1 (PD-1) expression. Infiltrates of drug-reactive cells in livers of HLA-transgenic animals lacking mMHC-I and PD1, and treated with anti-CD4 antibody (aCD4Ab) and FLX, were associated with histopathologic changes, although insufficient to cause DILI. Drug-specific infiltrating cells, however, were cytotoxic to HLA-positive hepatocytes *in vitro*, suggesting that liver tolerance rather than clonal deletion may prevent liver injury *in vivo*.

METHODS

Mice

HLA-B*57:01/H2-D^d (a3) transgenic (Tg) and HLA-B*57:01 Tg/H2-K^bD^b knockout mice (Tg/KO) were generated as previously described.[20,24] HLA-B*57:01 Tg/H2-K^bD^b KO/PD-1 KO mice (Tg/DKO) were generated by backcrossing the Tg/KO to $Pdcd1^{tm1.1Shr}$/J mice (The Jackson Laboratory, Bar Harbor, Maine). Wild-type mice (WT) were negative for the transgene and expressed intact mMHC-I. Experimental mice were female unless otherwise indicated, and were older than 12 weeks. Mice were bred and housed under specific-pathogen–free conditions in the Association for Assessment and Accreditation of Laboratory Animal Care International (aka AAALAC)-accredited animal facility of the US Food and Drug Administration (FDA)'s Division of Veterinary Medicine (Silver Spring, Md) in compliance with the Institutional Animal Care and Use Committee regulations of the FDA (protocol 2017–63).

In vitro cell culture assays

In vitro T-cell responses to drug were measured in cultures of purified $CD8^+$ T lymphocytes from spleen or lymph nodes (LNs) or total splenocytes as described in this article's Methods section in the Online Repository at www.jacionline.org.

In vivo drug sensitization/gavage treatment

Retinoic acid ([RA] MP Biomedicals, Santa Ana, Calif); 100 μg in =0 μL of 100% dimethyl sulfoxide ([DMSO] Sigma, St Louis, Mo) with or without FLX (50 mg in 100 μL of 70% of DMSO; Altamedics Gmbh, Cologne, Nordrhein-Westfalen, Germany) were topically administered on days 1–3 (sensitization). FLX (2.3 mg/250 μL of water for injection) was provided by oral gavage on days 8–9. aCD4Ab (a-CD4 Ab, clone GK1.5; BioXcell, Lebanon, NH) was injected intraperitoneally at 0.25 mg per dose on days −3, 1, 4, and 7. Preparation of reagents is described in the Methods section in the Online Repository.

Blood cell phenotype analysis and expression of HLA and H2-K^b molecules

Blood was collected into MiniCollect Z Serum Separator tubes (Greiner Bio-One, Frickenhausen, Germany) and analyzed by flow cytometry on red blood cell lysis (ammonium–chloride–potassium lysing buffer [Gibco, Thermo Fisher Scientific, Waltham, Mass]).[20]

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Liver enzyme levels in serum

Blood was collected at the time of euthanasia by cardiac puncture into MiniCollect tubes as recommended. Alanine transaminase (ALT) levels were measured using commercially available assay kits (BioAssay Systems, Hayward, Calif).

Histology

Liver sections were submerged into 10% neutral buffered formalin, processed into paraffin blocks, and sectioned at 5 μm onto positively charged slides. Hematoxylin and eosin (H&E) staining was performed by Histoserv (Germantown, Md).

Gene expression analysis

Liver biopsy samples were immediately submerged in TRIzol (Invitrogen; Thermo Fisher Scientific, Waltham, Mass) on excision, flash-frozen, and stored at −80°C until processing. Sample processing was conducted as previously described.[20]

Flow cytometry

Immune cell subset analysis by intracellular and/or surface marker staining[20] was performed using antibodies as described in the Methods section in the Online Repository. Intracellular staining was performed using the BD Cytofix/Cytoperm Plus kit (BD Biosciences, Franklin Lakes, NJ) as per the manufacturer's instructions.

Isolation of primary hepatocyte

Mouse hepatocytes were isolated using either collagenase type I digestion (Sigma-Aldrich, St Louis, Mo) or enzymes from the mouse liver dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Details of both protocols are provided in in the Methods section in the Online Repository.

Isolation of nonparenchymal cells and liver leukocytes

Nonparenchymal cell (NPC) isolation was performed upon perfusing the liver with 5–10 mL of 1 × PBS using a peristaltic pump. NPC suspensions were obtained with the mouse liver dissociation kit (Miltenyi Biotec) in an Octo Dissociator with Heaters as per the manufacturer's instructions. Liver leukocytes were subsequently pelleted in 37.5% Percoll (Cytiva, Marlborough, Mass) solution, washed, and resuspended in DMEM-10 (Gibco).

Cytotoxicity assay

Primary hepatocytes and liver leukocytes or NPC cultures with or without FLX were prepared and maintained in an xCELLigence real-time cell analysis platform (Agilent Technologies, Santa Clara, Calif) as indicated in the Methods section in the Online Repository. Cytolysis was calculated as follows: % Cytolysis = [(Normalized Cell $\text{Index}_{\text{no effector}}$ − Normalized Cell $\text{Index}_{\text{effector}}$)/Normalized Cell $\text{Index}_{\text{no effector}}$)] × 100.

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RESULTS

FLX-reactive CD8$^+$ T cells are enriched in spleen of FLX-treated Tg animals

Splenic CD8$^+$ T cells from drug-naive Tg and WT animals were cultured in the presence of FLX or ABC (used as positive control) (Fig 1, A). As reported earlier,[20] ABC-treated T cells produced high levels of IFN-γ and granzyme B (GZMB) within the first cycle of stimulation, unlike those treated with FLX, that required 2–3 cycles. Cells from WT mice were not reactive to drug stimulation for 28 days. We then asked whether *in vivo* priming of the mice with drug could enhance detection of FLX-reactive cells *in vitro*. Cells from mice treated orally with the human-equivalent dose of FLX did not respond to drug reexposure *in vitro* (data not shown). Thus, we adopted a RA skin sensitization/oral gavage strategy to administer FLX, which was previously used to generate drug-reactive cells in an MHC-II KO, CD4$^+$ T-cell–depleted, non-HLA–expressing mice.[18] This treatment strategy promotes hapten formation, T-cell activation, and migration to the gut–liver axis. Priming Tg animals with FLX *in vivo* accelerated secretion of IFN-γ by splenic CD8$^+$ T cells when restimulated with drug *in vitro*. Increased IFN-γ was measurable starting at day 5 of culture as opposed to at day 12 for cultures of cells from drug-naive animals (Fig 1, B). The addition of FLX to cocultures improved the magnitude of released IFN-γ by T cells compared to cultures where FLX was washed off after pulsing the feeder cells (see Fig E1 in the Online Repository at www.jacionline.org). FLX-reactive T cells exhibited morphologic changes associated with cell activation (Fig 1, C). These data indicate that oral administration of FLX after skin sensitization elicits a systemic drug-reactive CD8$^+$ T-cell response in immunocompetent Tg mice.

Generation of FLX-reactive CD8$^+$ T cells is driven by both HLA-B*57:01 and mMHC-I

T-cell reactivity to FLX has been shown in mice lacking HLA expression,[18] suggesting that mMHC-I plays a role in FLX presentation. As such, the Tg strain used here has the potential to present drug via both HLA-B*57:01 and mMHC-I. We isolated CD8$^+$ T cells from the LNs (Fig 2, A) and spleens (Fig 2, B) of untreated Tg or FLX-primed Tg and WT animals. Within 2 days of coculture with drug-pulsed feeder cells, LN CD8$^+$ T cells from drug-primed Tg mice stimulated with 250 μg/mL of FLX showed low but significantly increased levels of IFN-γ in the culture supernatant compared to those from untreated Tg or drug-treated WT mice (Fig 2, A, left). By day 4–5, IFN-γ was detected in cultures of FLX-treated WT animals, although at significantly lower yields than in cultures of the Tg counterparts (Fig 2, A, center and right). The time delay in T-cell activation was also observed in splenocytes (Fig 2, B).

These results, however, did not determine whether the bulk of the response observed in Tg is driven by mMHC-I or HLA. To answer this question, LN (Fig 2, C) and splenic (Fig 2, D) CD8$^+$ T cells from FLX-primed Tg were cocultured with drug-pulsed autologous or WT feeders, with or without FLX. At day 2, low levels of IFN-γ were detected in cocultures of CD8$^+$ T cells with autologous feeders, but higher than with WT feeders. At day 4, though, IFN-γ yields became comparable, confirming that although mMHC-I could support the T-cell reactivity to FLX, the presence of the transgene allowed cells to react earlier (day 2) to *in vitro* restimulation. To further assess the role of the HLA in this response, we

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blocked antigen presentation with anti–HLA-B/C or anti–H2-K^b/H2-D^b antibodies. IFN-γ production was slightly decreased by anti–HLA-B/C antibody, although not to statistically significant levels, in contrast to the absolute interference by anti–H2-K^b/H2-D^b antibody (Fig 2, E). These data confirm the ability of mMHC-I to present FLX to $CD8^+$ T cells, although the early $CD8^+$ T-cell activation may be HLA dependent in Tg mice. Because FLX presentation by HLA-B*57:01 was masked by mMHC-I, we opted to repeat the experiments in a new mouse strain derived from backcrossing Tg with H2-K^bD^b KO mice (Tg/KO).[24]

Lack of H2-K^bD^b Tg/KO results in lower numbers of systemic $CD8^+$ T cells and weaker drug-specific responses

Splenic $CD8^+$ T cells from FLX-treated Tg/KO animals cocultured with drug-pulsed autologous feeders and FLX did not secrete detectable IFN-γ until day 7 (Fig 3, A). In general, cells from female mice released higher levels of IFN-γ than those from males; indeed, 67% of all female samples had ≥1000 pg/mL IFN-γ compared to 17% of all male samples. Addition of anti-HLA antibody in the culture reduced cytokine production by at least 50% in all the animals at some time point (Fig 3, B), indicating a role of HLA in FLX presentation to $CD8^+$ T cells. More pronounced effects occurred in cultures with higher IFN-γ production, and slightly different kinetics were observed for each individual animal.

Of note, drug-naive Tg/KO animals had approximately 3 times less total splenic $CD8^+$ T cells than Tg, while $CD4^+$ T-cell and natural killer cell levels were comparable (Fig 3, C). Low frequency of $CD8^+$ T cells could be associated with the lack of mMHC-I expression[26] (Fig 3, D). In addition, although HLA expression was enhanced 0.5–3-fold in Tg/KO versus Tg cells (Fig 3, E), the Tg/KO HLA mean fluorescence intensity (MFI) was 4–8.5 times lower than the H2-K^b MFI on Tg or WT cells (Fig 3, F). The difference in the MFI of class I molecules could be attributed to the density of HLA versus H2-K^b molecules on the cell surface in different strains. Although FLX-modified epitopes are presented exclusively by HLA in Tg/KO, the lower expression of overall class I molecules together with the lower frequencyof $CD8^+$ T cells observed in Tg/KO may limit the number of FLX-primed clones in circulation, resulting in a weaker/delayed IFN-γ response after *in vitro* restimulation of the cells.

Depletion of $CD4^+$ T cells, including Treg cells, induces mild liver inflammation and enrichment of infiltrating drug-reactive PD-1^+CD8^+ T cells

In humans, FLX-induced liver injury manifests with hepatic enzyme elevation because of liver damage.[4,7] Although FLX-DILI has been linked to HLA-B*57:01, its frequency is very low,[7–9] which is thought to be in part due to robust immunoregulatory mechanisms. Tg/KO treated with FLX did not show signs of liver inflammation or disease (Fig 4) despite developing FLX-reactive $CD8^+$ T cells by day 10 of treatment. In an attempt to break hepatic tolerance to FLX, we depleted the animals of $CD4^+$ T cells (including Treg cells) before and during drug treatment. Anti-CD4 antibody in combination with FLX did not lead to either ALT elevation (Fig 4, A) or hepatic histologic change (Fig 4, B). However, gene expression analysis revealed mild liver inflammation, characterized by increased transcription of several inflammatory genes, including T-cell markers and effector molecules *(Cd8, Infg, Gzmb1, Nfatc2, Pd1)*, calgranulin *S100a9*, antigen-presenting

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cell markers *(Xcr1, Cd11b, Cd38)*, chemokines and receptors *(Cxcr2, Cxcl10)*, oxidative stress pathways *(Nos2)*, apoptotic molecules *(Fas)*, and *Akrb10* (Fig 4, C). Although the expression of these genes was elevated in liver of animals treated with either aCD4Ab alone or in combination with drug, the magnitude of the inflammatory response was amplified in mice treated with FLX.

$CD8^+$ T cells were enriched in the liver of animals treated with aCD4Ab 1 FLX, especially PD-1^+CD8^+ T cells, averaging 34% of the total $CD8^+$ T-cell subset compared to 18% in the aCD4Ab control group or 5% within the untreated mice (Fig 4, D, top).

A higher frequency of PD-1^+CD8^+ T cells was also detected in the spleens and LNs of aCD4Ab + FLX–treated animals (Fig 4, E and F), indicating that the effect of FLX on $CD8^+$ T-cell activation was systemic. Anti–CD4Ab + FLX treatment slightly increased the number of splenic macrophages (Fig 4, E). Although the frequency of conventional dendritic cell (DC) subsets was not altered by any of the treatments (data not shown), depletion of $CD4^+$ cells had an impact on expression of DC maturation markers, such as CD86. This occurred in lymphoid organs but not in liver (Fig 4, D–F), implicating the lack of DC maturation in maintaining suboptimal T-cell activation in the liver.

Interference with PD-1 expression increases liver inflammation and FLX-reactive $CD8^+$ T cells without breaking liver tolerance

We hypothesize thatPD-1expression by FLX-reactive$CD8^+$ T cells of Tg/KO may prevent the expansion of the effector immune response, especially in the liver, where resident cells such as liver sinusoidal endothelial cells (LSECs) and Kupffer cells (KCs) express high levels of programmed cell death ligand 1 (PD-L1).[27,28] Therefore, we generated a double-knockout mouse strain lacking PD-1 expression (Tg/DKO) from the Tg/KO. PD-L1 expression was evaluated on liver cells (see Fig E2 in the Online Repository at www.jacionline.org). Drug-naive Tg/DKO animals had higher levels of circulating $CD8^+$ T cells than Tg/KO animals ($P = .0408$) (Fig 5, A) and inflammation (see Fig E3 in the Online Repository). Splenocytes isolated from aCD4Ab + FLX–treated Tg/DKO animals, unlike those from similarly treated Tg/KO, were enriched in IFN-γ^+CD8^+ and $GZMB^+CD8^+$ T cells (Fig 5, B and C). These results were supported by early (day 2) measurable levels of IFN-γ in Tg/DKO splenocyte cultures supplemented with the drug (Fig 5, D). Animals that received FLX had enlarged gallbladders compared to the other control groups (Fig 5, E). The liver parenchyma of several Tg/DKO from the aCD4Ab + FLX group was inflamed, as evidenced by the presence of cell swelling, hepatocyte apoptosis, and hepatocyte dropout near the central vein endothelium, consistent with zone 3 inflammation, in addition to periportal inflammation with portal vein endotheliitis (Fig 5, F, and see Fig E4 in the Online Repository). Lymphoid foci and microgranulomas were more abundant compared to the aCD4Ab + FLX–treated Tg/KO (Fig 4, D) and Tg/DKO control groups (Fig 5, F). ALT levels remained unchanged at day 10 of drug treatment (Fig 5, G). Histologic changes correlated with an increase in RNA expression of genes related to immune response and inflammation, especially *Tnfa, Il1b, Il-6* and *Cxcr*2 (Fig 5, H), which have been associated with cholestasis in previous studies.[29,30]

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The frequency of $CD8^+$ T cells with an effector/effector memory phenotype ($CD44^+CD62L^-$) was increased in the spleen of Tg/DKO animals treated with aCD4Ab + FLX compared to control groups (Fig 6, A). $CD44^+CD62L^-CD8^+$ T cells were also found elevated in the liver of aCD4Ab- and aCD4Ab + FLX–treated animals (Fig 6, B). The combination of aCD4Ab + FLX led to an increase of macrophages in spleen but not liver without affecting $CD11b^+$ DC levels (Fig 6, C and D), mimicking our previous observations in Tg/KO. Similarly, the expression levels of CD86 were elevated on $CD11b^+$ DC isolated from the spleen but not liver of aCD4Ab + FLX animals (Fig 6, E and F). Collectively these results show that although PD-1 plays a role in limiting the expansion of FLX-reactive cells in Tg/KO, PD-1 is not a critical factor in maintaining liver tolerance *in vivo*, as shown in Tg/DKO.

In vitro FLX-reactive $CD8^+$ T cells hepatotoxicity is dependent on KCs and LSECs

To address whether FLX-reactive cells play a role in hepatotoxicity, we examined the cytotoxicity of purified leukocytes from perfused livers of aCD4Ab + FLX–treated mice on primary hepatocytes. Effector cells from both Tg/KO and Tg/DKO treated with aCD4Ab + FLX ($E^{aCD4Ab+FLX}$) quickly killed hepatocyte targets of aCD4Ab + FLX–treated mice ($T^{aCD4Ab+FLX}$) on restimulation with drug *in vitro* (Fig 7, A and B, left). The cytotoxicity of the $E^{aCD4Ab+FLX}$ cells was drug concentration dependent, and although its intensity varied among independent experiments, drug specificity was consistent compared to unstimulated cells in both Tg/DKO and Tg/KO. $E^{aCD4Ab+FLX}$ from Tg/DKO animals showed low levels of hepatotoxicity without restimulation. Unlike $E^{aCD4Ab+FLX}$ from Tg/KO, those from Tg/DKO were cytotoxic to hepatocytes of drug-naive mice (T^{Unt}) showing enhanced effector function when soluble FLX was added to the cultures (Fig 7, A, right). The lack of HLA expression on hepatocytes impaired $E^{aCD4Ab+FLX}$ cytotoxicity (Fig 7, B, right).

Liver leukocyte samples did not include KCs ($F4/80^+Clec4f^+$) and LSECs ($CD45^-CD146^+$) (Fig 7, C), in contrast to suspensions of bulk NPC analyzed before submitting the samples to Percoll gradient separation (Fig 7, D). Bulk NPC from aCD4Ab + FLX–treated Tg/DKO animals, unlike liver leukocytes, were unable to kill hepatocyte targets (Fig 7, E).

These data, taken together, indicate that *in vivo* FLX-primed and expanded $CD8^+$ T cells are not deleted in the liver and can kill hepatocytes from aCD4Ab + FLX–treated mice depending on the presence of KCs and LSECs. Lack of PD-1 expression predisposes some effector cells to be cytotoxic to drug-naive targets when drug is in solution. Additional studies are needed to understand the *in vivo* mechanisms responsible for the control exerted by KCs and LSECs on DILI onset.

DISCUSSION

A number of drugs have been described to interact with different HLA alleles and lead to hypersensitivity reactions.[31] The development of HLA transgenic animal models is critical to elucidate the mechanisms driving immune-mediated drug hypersensitivity reactions (including FLX-DILI). Here, we characterize FLX immune reactivity in existing and novel HLA-B*57:01 Tg strains and discuss tolerogenic mechanisms that may contribute to restrict drug-responsive $CD8^+$ T cells.

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$CD8^+$ T cells obtained from drug-naive Tg animals reacted to FLX after 2–3 cycles of *in vitro* stimulation demonstrating comparable kinetics to those reported in PBMC cultures from drug-naive healthy HLA-B*57:01^+ human donors.[14,17] Compared to ABC, murine cell activation with FLX was delayed and less robust, suggesting a more oligoclonal immune response by FLX. FLX primed T cells *in vivo*, as evidenced by rapid IFN-γ release to drug reexposure *in vitro*. This was analogous to PBMCs from FLX-treated patients with liver injury that respond to drug when restimulated *in vitro*, as opposed to cells from drug-tolerant or drug-naive subjects.[15]

As shown, splenic and LN $CD8^+$ T cells from FLX-treated HLA-B*57:01^- mice (WT) also produced IFN-γ in culture. This was not unexpected; FLX-reactive $CD8^+$ T cells were previously reported from drug-treated mice not expressing the human risk allele[18] and from PBMC of HLA-B*57:01^- human donors prescribed FLX.[15] Interestingly, however, cells from WT mice showed a delayed IFN-γ release of at least 48 hours compared to cells from Tg mice, which could be attributed to differences in the mechanisms by which class I molecules present the drug to T cells.[32] While Tg T cells showed a rapid response to soluble drug, as observed in HLA-B*57:01^+ PBMCs from healthy donors and DILI patients,[14–16] the delayed response of WT T cells may rely on drug–haptenated peptide formation dependent on intracellular antigen processing, similar to what was reported for HLA-B*57:01^- PBMCs.[14] Differences in the IFN-γ levels between Tg and WT cells at day 5 of culture (Fig 2, A and B) may be related to an additive effect of drug presentation by HLA and mMHC-I in Tg mice.

To focus on exclusive presentation of FLX epitopes by HLA-B*57:01,we adopted the Tg/KO strain. The weaker drug response observed in Tg/KO mice compared to Tg mice could be attributed to fewer drug-specific $CD8^+$ T cells due to lower peripheral $CD8^+$ T-cell levels in H2-K^bD^bKO mice,[26,33,34] and a diminished density of total class I molecules. However, studies showing that TCR-Vβ diversity as well as the rate of thymic generation/egress of $CD8^+$ T cells are not affected by the lack of H2-K^bD^b [26,34] would suggest that the low numbers of circulating FLX-reactive $CD8^+$ T cells in Tg/KO are due to an impairment in the cell maintenance rather than their generation. Factors limiting the expansion of FLX-reactive $CD8^+$ T-cell response could be the absence of FLX-reactive WT (HLA^-) T cells acting as helper cells and/or the presence of $CD4^+$ Treg cells.

Treg cells play a key role in the maintenance of T-cell homeostasis, immune tolerance, and T-cell–mediated autoimmunity prevention.[35–37] Depletion of $CD4^+$ T cells, including Treg cells, as applied previously in other murine models of drug hypersensitivity[18,20,22] led to mild inflammation that was insufficient to trigger hepatocyte injury as measured by ALT release or histopathology. This was in contrast to a nontransgenic mouse model of FLX in which ALTs were transiently elevated but drug presentation and T-cell activation by the murine MHC-I was not addressed.[18] In the Tg/KO, treatment with aCD4Ab + FLX increased $PD1^+$ $CD8^+$ T cells both in peripheral lymphatic organs and liver, consistent with TCR engagement and T-cell priming by drug antigens. Lack of $CD4^+$ non-Treg cells did not seem to compromise the generation of $CD8^+$ T cells reactive to drug. Although splenic and LN DCs presenting drug epitopes and expressing costimulatory molecules could contribute to the activation of peripheral $CD8^+$ T cells, liver-infiltrating $CD8^+$ T cells

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encountered an environment of low costimulation and high immune suppression and were not responsive (Fig 4, D, and Fig E2). Drug-reactive cells activated in lymphoid organs may reach the liver sinusoids from the periphery and get arrested in the epithelium of the vessels or infiltrate the tissue if the organ is under stress or inflammation.[28] There, $CD8^+$ T cells will encounter resident cells such as KCs, LSECs, and hepatocytes, biased to enforcing tolerance by the secretion of anti-inflammatory cytokines and expression of inhibitory PD-L1 molecules.[27,28,38,39] Additionally, $CD8^+$ T cells will end up tolerized by LSECs presenting antigen in an MHC-I–restricted manner through PD-L1 but not CD80/86 upregulation.[27] Thus, in the liver, the constitutive expression of PD-L1 on resident cells could account for the decreased effector function of infiltrating $PD1^+CD8^+$ T cells.

Abrogation of PD-1 expression in mice, together with CTLA-4 blocking, has been shown to sustain amodiaquine liver inflammation transiently for several weeks.[40] To evaluate the role of PD-1 in FLX tolerance, we administered drug to aCD4Ab-treated Tg/DKO. Mice showed liver parenchymal alterations comparable to those described in the amodiaquine mouse model and in liver sections of patients with FLX hypersensitivity that are characterized by influx of leukocytic cells, especially $CD8^+$ T cells and macrophages, with some hepatocyte damage, evidenced by the presence of apoptotic cells.[4,11,40] Additionally, gallbladder enlargement by FLX indicated possible alterations in bile acid metabolism, although cholestasis was not supported by histology or liver enzyme elevations. The higher levels of effector/effector memory $CD44^+CD62L^-CD8^+$ T cells (T_{EM}) in Tg/DKO were attributed to lymphocyte dysregulation due to the lack of PD-1 molecules.[41,42] Liver injury with ALT elevations was not evident despite increased splenic and hepatic T_{EM}, indicating that liver-infiltrating leukocytes were still tolerized. Differences in liver damage between our animal model and what happens in FLX-DILI patients may be attributed to factors such as differences in drug bioavailability, antibiotic metabolism, and adduct formation leading to higher concentration of target drug neoantigens to stimulate T cells in humans; levels of preexisting drug-reactive T cells, perhaps from previous antibiotic priming; bacterial innate signaling from ongoing infection that activates antigen-presenting cell for T-cell priming; immune-inhibitor pathways such as checkpoint molecules and Treg cell function; and/or the duration of drug treatment.

Liver leukocytes isolated from aCD4Ab + FLX–treated Tg/DKO and Tg/KO animals showed hepatocyte killing *in vitro* when depleted from KCs and LSECs, confirming the coexistence of multiple tolerizing mechanisms exerted by these hepatic resident cells on FLX-reactive $CD8^+$ T cells *in vivo*. Although hepatocytes can prime naive $CD8^+$ T cells *in vitro* by acting as nonprofessional antigen-presenting cells,[43] the rapid cytotoxic response of effector cells from aCD4Ab + FLX–primed animals to drug restimulation, as well as the lack of toxicity to target cells not expressing class I molecules, suggests drug-epitope presentation by hepatocytes to *in vivo* drug-primed effectors.

A limitation of drug hypersensitivity animal models is the absence of baseline disease, such as bacterial infection for which FLX is used to treat patients. Considering a role of danger signals in the development of immune-mediated adverse drug reactions,[44] low inflammation due to the absence of pathogen-associated and danger-associated molecular patterns (aka PAMP and DAMP) could restrict the upregulation of costimulatory molecules on DC to

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enhance optimal drug-epitope presentation to the T cells and further activation. Although *in vivo* FLX-primed cells from both Tg/KO strains were able to quickly kill hepatocytes from drug-treated animals when restimulated *in vitro*, presumably through recognition of drug epitopes presented by target cells, only Tg/DKO effectors exerted cytotoxicity on drug-naïve targets. Early cytotoxicity *in vitro* may occur by TCR activation by noncovalently bound drug to HLA or TCR, which is labile and reversible and thus likely less stable than drug-haptenated peptides.[11,45] Suboptimal TCR stimulation in the absence of costimulation or/and proinflammatory cytokines may lead to T-cell tolerance or anergy.[46,47] Unlike Tg/KO, Tg/DKO had higher bystander liver inflammation associated with lack of PD-1 expression,[41,48] which was enhanced by the depletion of $CD4^+$ T cells. This, together with the propensity of PD-1 KO T cells to adopt a T_{EM} phenotype in lymphopenic environments characterized by rapid activation on restimulation,[42] could explain that $CD44^+CD62L^-$ cells from aCD4Ab + FLX–treated Tg/DKO recognizing noncovalently bound drug complexes in a proinflammatory environment (mimicking, perhaps, that derived from PAMP and DAMP in infections) can quickly kill hepatocytes despite being suboptimally activated through the TCR.

Of note, our study does not directly address the mechanisms by which FLX causes DILI in non–HLA-B*57:01, which occurs in approximately 17% of FLX-DILI cases. However, we intentionally chose the drug and HLA pair with the highest chances to result in FLX-DILI to generate a preclinical model with consistent liver inflammation. Determining whether the immune mechanisms we report that lead to FLX-DILI are exclusive for HLA-B*57:01 or common to other HLAs will require follow-up studies.

In summary, several key elements appear necessary to induce FLX-specific $CD8^+$ T-cell responses in mice even when these are only targeted to HLA-B*57:01, the allele associated with FLX-DILI. Depletion of $CD4^+$ T cells, presumably Treg cells, is necessary for T-cell priming, activation, and influx into liver. Lack of Treg cells could lead to DC activation and enhanced expression of costimulatory surface molecules including CD80 and CD86 in spleen and LN antigen-presenting cells,[20] but not in the liver. This suggests that part of the suppressive environment is acting to limit DC activation, possibly stabilizing these cells in a tolerizing state. The increase in $PD\text{-}1^+CD8^+$ T cells in the livers of aCD4Ab + FLX–treated Tg/KO suggests that these cells are recruited to the liver after peripheral activation in the lymphoid tissues and that PD-1 expression may limit T-cell function. Depletion of Treg cells may support T-cell responses to drug in the gut–lymphoid axis through activation of DCs and lack of IL-2 consumption. This most likely occurs during primary T-cell responses to FLX-modified liver self-antigens in draining LN and gut lymphoid tissue. T cells, once activated in a RA-rich environment that imprints gut–liver homing, can migrate back to the liver, where they are either tolerized or deleted. The recovery of cytotoxic activity from hepatic $CD8^+$ T cells on primary hepatocytes *in vitro* demonstrates that T cells are not depleted but instead suppressed in the liver environment, which includes KC- and LSEC-mediated mechanisms. PD-1 engagement on T cells by PD-L1 on NPC and possibly other checkpoint molecules limit $CD8^+$ T-cell–mediated activation and killing of hepatocytes, as suggested by the suppression of T-cell cytotoxicity by KCs and/or LSECs in a PD-1–deleted background.

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We believe that the knowledge gained with murine strains that express only HLA-B*57:01 will be useful to unravel host cell immune response and tolerance mechanisms to other drugs with potential to cause HLA-mediated DILI. Ongoing studies of these models are focused on manipulating host pathways that control hepatic tolerance resulting in sustained FLX-DILI to elucidate the clinical effects exerted by drug-reactive T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by the US Food and Drug Administration (FDA) Intramural Research Program, and in part by a Postgraduate Research Fellowship Award to K.K.S., S.S., and H.A. from the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the US Department of Energy and the FDA.

We thank Janet Woodcock, former director of the Center for Drug Evaluation and Research (CDER, FDA), for her support of this project. We also wish to thank the personnel of the Division of Veterinary Services (FDA) for care of the mice. We thank David Hone, Weiming Ouyang, and Joao Pedras Vasconcelos for critically reading drafts of the report.

Abbreviations used

ABC	Abacavir
aCD4Ab	Anti-CD4 antibody
ALT	Alanine transaminase
DC	Dendritic cell
DILI	Drug-induced liver injury
DMSO	Dimethyl sulfoxide
FDA	US Food and Drug Administration
FLX	Flucloxacillin
GZMB	Granzyme B
H&E	Hematoxylin and eosin
HLA	Human leukocyte antigen
KO	Knockout
KC	Kupffer cell
LN	Lymph node
LSEC	Liver sinusoidal endothelial cell
MFI	Mean fluorescence intensity

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MHC	Major histocompatibility complex
mMHC	Murine MHC
NPC	Nonparenchymal cell
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
RA	Retinoic acid
T_{EM}	Effector memory T
Tg	HLA-B*57:01 transgenic mice
Tg/DKO	HLA-B*57:01 transgenic/H2-K^b D^b and PD-1 double-KO mice
Tg/KO	HLA-B*57:01 transgenic/H2-K^b D^b KO mice
Treg	Regulatory T
WT	Wild type

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Key messages

- FLX presentation by HLA-B*57:01 in mice leads to $CD8^+$ T-cell activation with hepatotoxic potential, which is restricted by the presence of liver tolerogenic cells *in vivo*.
- $CD4^+$ cells, presumably Treg cells, and PD-1 expression limit HLA-B*57:01–dependent immune responses to FLX.
- HLA-B*57:01 transgenic mice are promising systems to understand DILI and tolerance.

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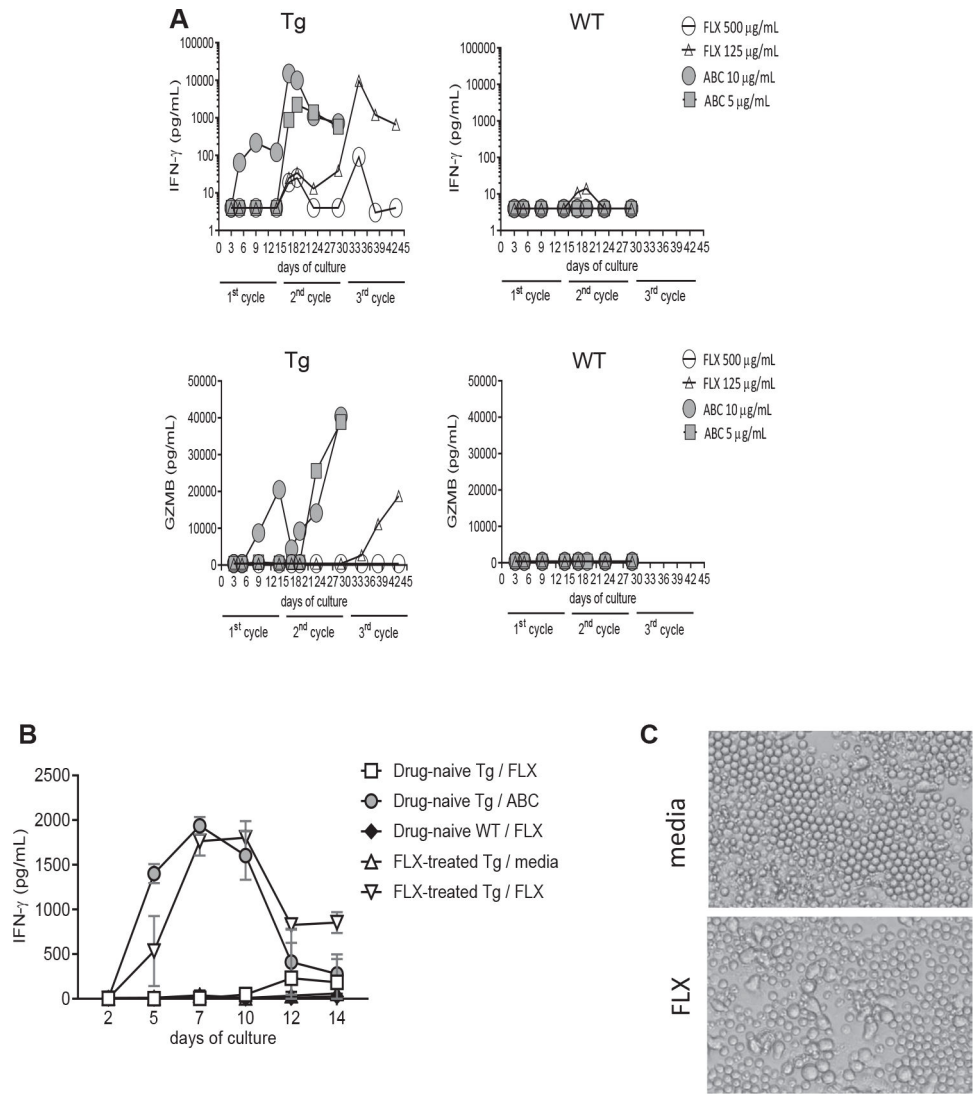

FIG 1.

Drug-reactive CD8$^+$ T-cell responses are enriched in FLX-primed Tg mice. CD8$^+$ T cells were isolated from spleens of drug-naive HLA-B*57:01$^+$ (Tg) and WT control animals (A and B) or FLX-treated Tg mice (B) and cultured with irradiated autologous feeders at a 1:2 ratio in the presence or absence of FLX or ABC (125 μg/mL of FLX and 10 μg/mL of ABC in *(B)*). IFN-γ and GZMB levels were measured at different time points by ELISA. *(A)* Data shown are from 1 representative of 2 independent experiments. *(B)* IFN-γ values are presented as means ± SEMs of results from 3 animals per group. **(C)** Cell morphology changes observed by optical microscopy (20×) at day 4 of culture of CD8$^+$ splenocytes from a representative FLX-primed animal, in the absence or presence of 125 μg/mL of FLX.

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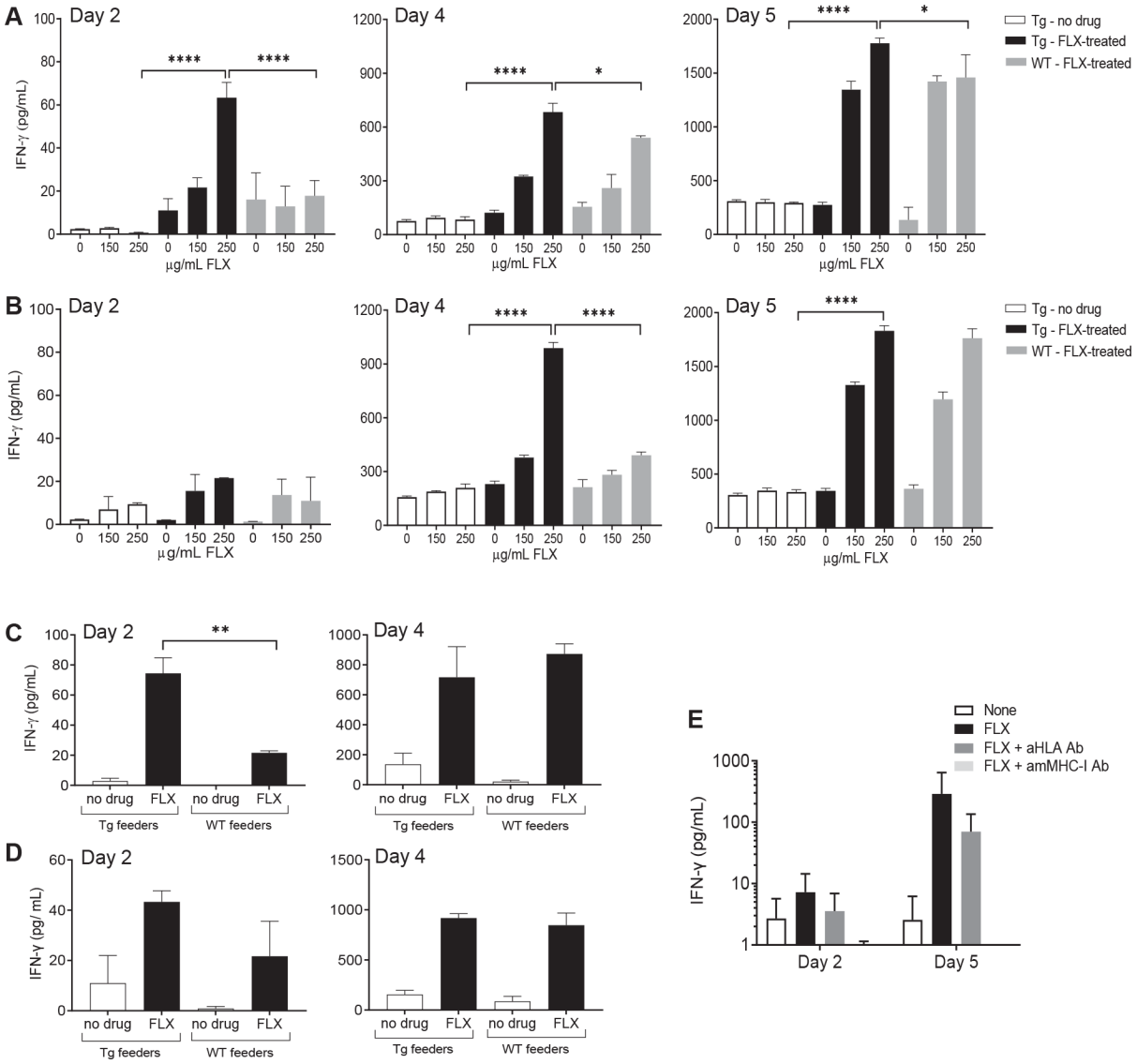

FIG 2.

CD8$^+$ T-cell reactivity to FLX is driven by both HLA-B*57:01 and mouse MHC-I molecules following different kinetics. (**A** and **B**) CD8$^+$ T cells from LNs *(A)* and spleens *(B)* of untreated Tg mice or FLX-treated Tg and WT mice and cultured with irradiated autologous splenocytes at 1:2 ratio for up to 5 days. Feeders were incubated overnight with the indicated drug concentration before irradiation. FLX was also added to cocultures at the same concentration. IFN-γ release was measured by ELISA. Data represent means ± SEMs of values from 3 animals per group. (**C** and **D**) CD8$^+$ T cells isolated from LN *(C)* or spleen *(D)* of FLX-treated animals were cultured with autologous (Tg) or WT feeders under conditions indicated in the graph. FLX was provided at a concentration of 150 µg/mL. IFN-γ levels in culture supernatants were measured by ELISA at days 2 and 4 of culture. (E) Splenic CD8$^+$ T cells from drug-treated Tg mice were cultured with 150 µg/mL of FLX *in vitro* with autologous feeders in the presence or absence of 10 µg/mL anti-HLA or anti-mouse MHC-I (H2-K^b and H2-D^b) antibodies. IFN-γ concentration in culture supernatants

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was measured by ELISA. Values represent means + SEMs of 3 animals per group. *$P \leq .05$, **$P \leq .01$, ***$P \leq .001$, ****$P \leq .0001$,1-way ANOVA.

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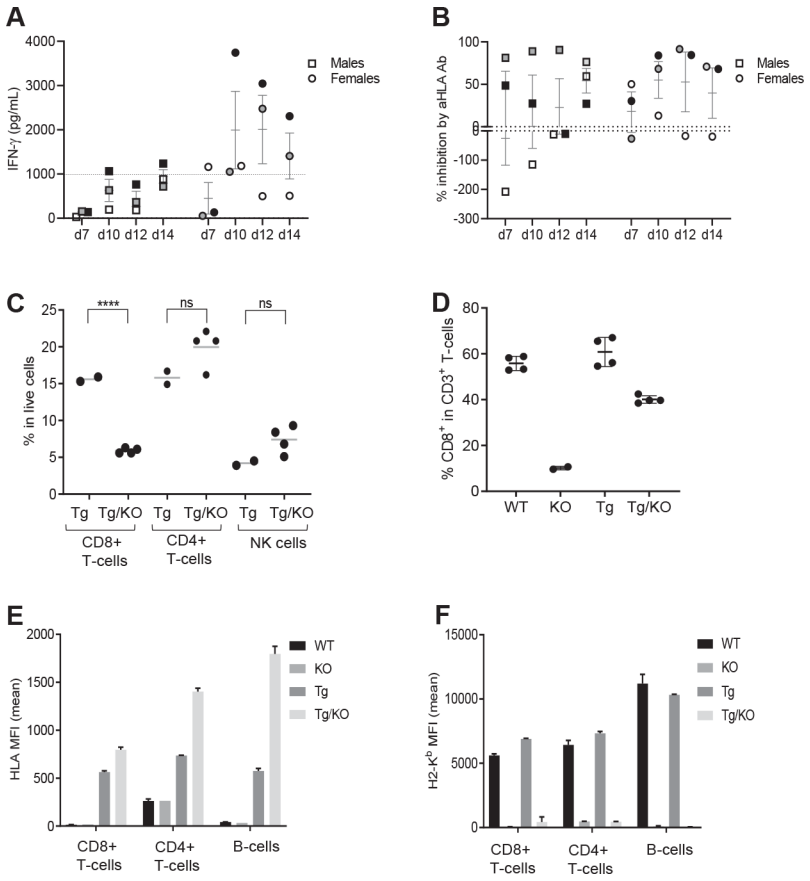

FIG 3.

CD8$^+$ T cells from FLX primed Tg/KO cells have a delayed HLA-dependent response to FLX *in vitro* due to decreased levels of CD8$^+$ T cells influenced by lack of H2-K^{b}D^b. (**A** and **B**) Splenic CD8$^+$ T cells from FLX-primed Tg/KO animals (males and females, n = 3 each) were cocultured with drug-pulsed irradiated autologous feeders (matching sex) and 250 μg/mL of FLX for 14 days. *(A)* IFN-γ levels in culture supernatants were measured by ELISA at indicated time points. *(B)* Inhibition of IFN-γ release was represented as percentage of cytokine level between cultures pretreated with or without anti-HLA antibody. **(C)** Percentage of CD8$^+$ and CD4$^+$ T lymphocytes and NK cells in spleen of Tg and Tg/KO mice. **(D)** Percentage of splenic CD8$^+$ T lymphocytes in total CD3$^+$ T cells in mice of different backgrounds. (**E** and **F**) MFI for HLA B/C *(E)* and H2-K^b *(F)* molecules expressed in blood lymphocyte populations of different mouse strains. **** $P \leq .0001$, unpaired *t* test. *NK*, Natural killer.

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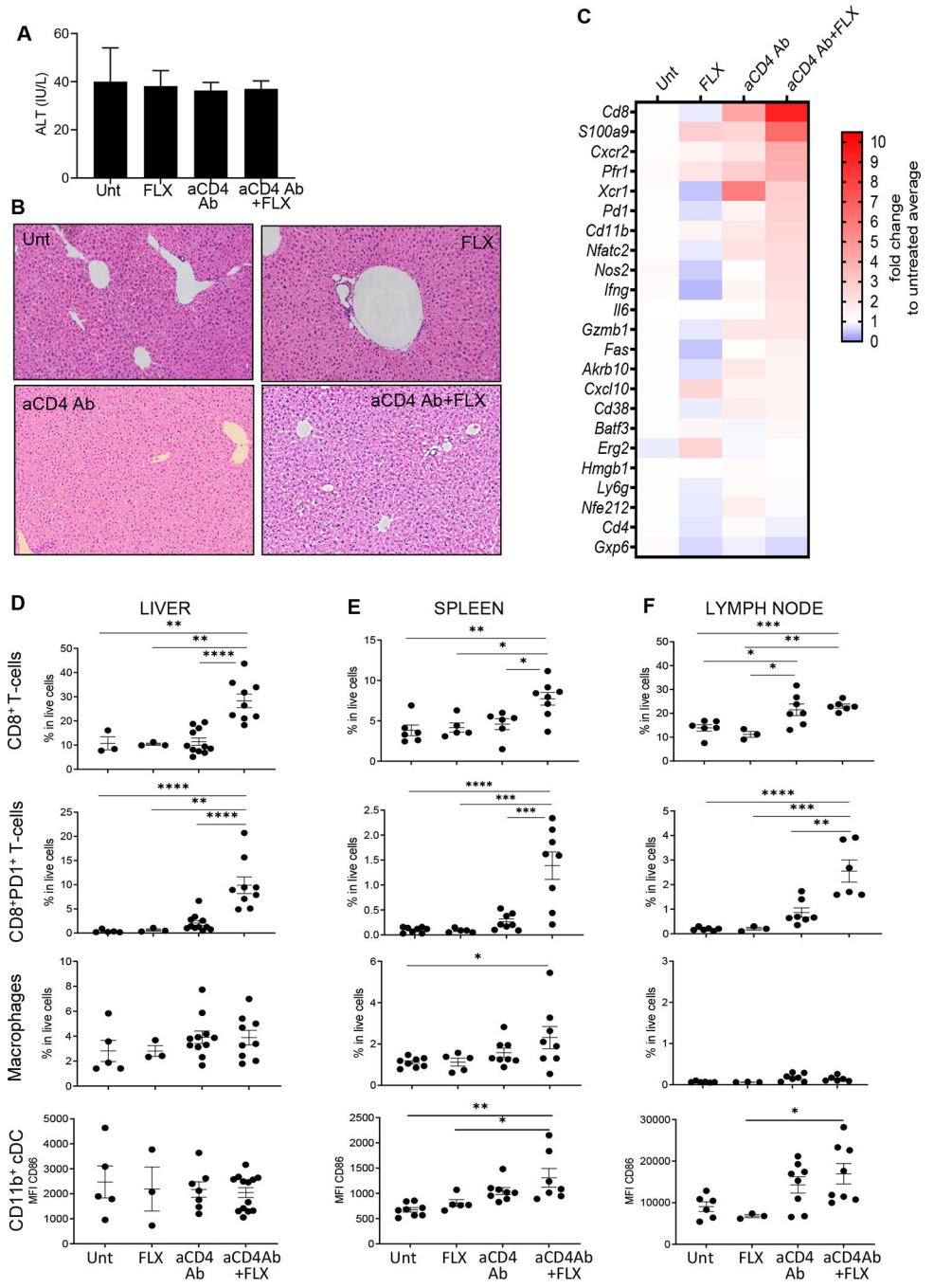

FIG 4.

FLX treatment of Tg/KO mice leads to mild liver inflammation if $CD4^+$ T cells, including Treg cells, are depleted. FLX was administered to Tg/KO mice with or without aCD4Ab. All animals were treated with RA. At day 10 after initiation of drug treatment, liver inflammation was evaluated by serum levels of ALTs **(A)**, H&E staining of fixed liver sections (representative mouse per group) **(B)**, and gene expression analysis of perfused liver by real-time PCR (geomean of n = 3–6 mice per group) **(C)**. **(D)** Infiltrating liver leukocyte populations were isolated from perfused livers and characterized by FACS. Spleen

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(E) and LN **(F)** cell suspensions were obtained and analyzed with the same FACS antibody panel (n = 3–9 mice per group). Dead cells were excluded from analysis. Macrophages are $CD11b^{hi}F4/80^{+}$; cDC $CD11b^{+}$ are $CD19^{-}CD11c^{+}CD8^{-}$ cells (see gating strategy in Fig E5). $*P \leq .05$, $**P \leq .01$, $***P \leq .001$, $****P \leq .0001$, 1-way ANOVA. *FACS*, Fluorescence-activated cell sorting.

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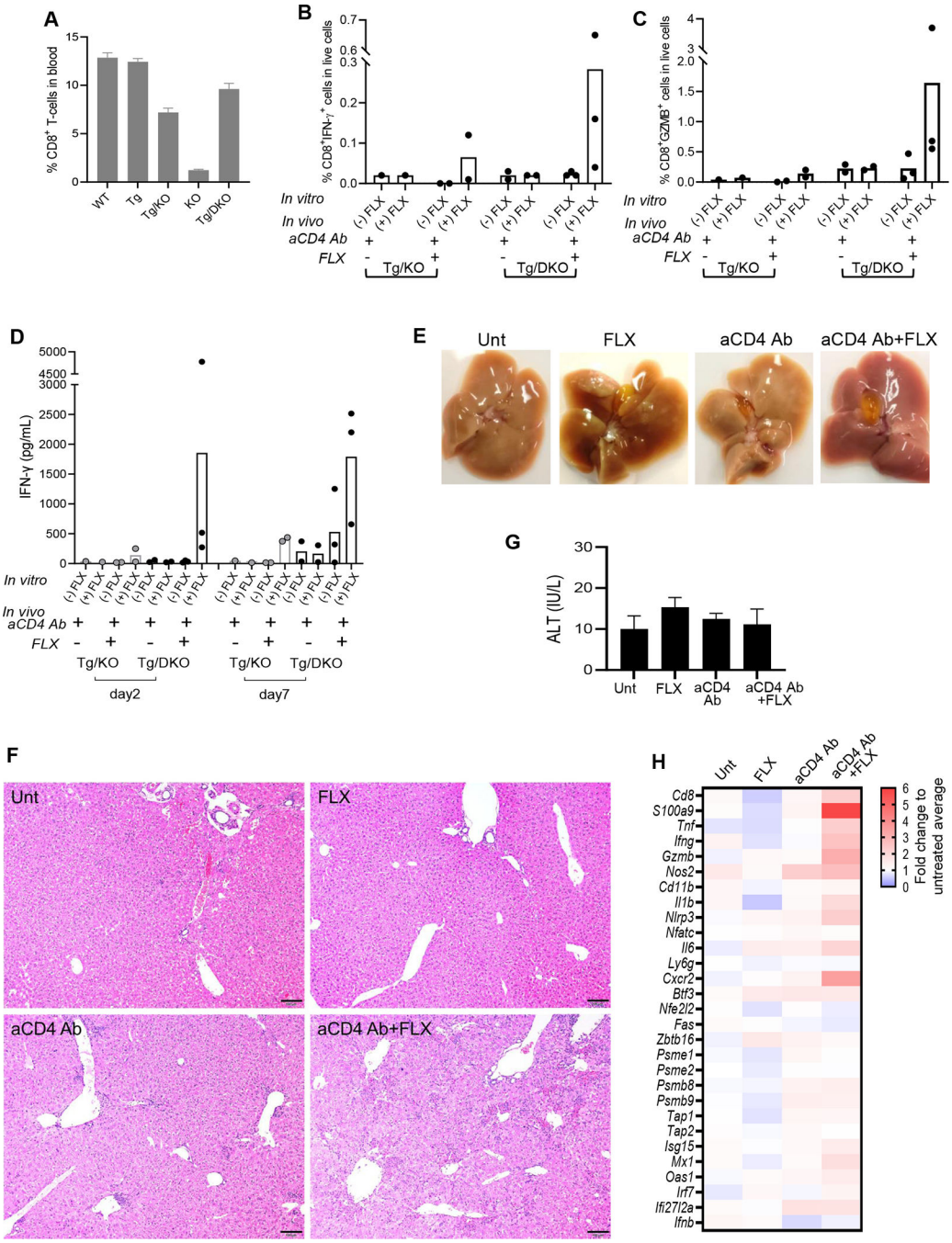

FIG 5.

Enhanced drug reaction and liver histopathology in Tg/DKO mice after aCD4Ab + FLX treatment. **(A)** Flow cytometry analysis of $CD8^+$ T cells in blood of drug-naive animals of different strains. **(B-D)** Tg/KO or Tg/DKO animals (n = 2–3 per group) were treated with aCD4Ab or aCD4Ab + FLX. Frequency of splenic IFN-γ^+CD8^+ *(B)* and $GZMB^+CD8^+$ *(C)* T cells was measured by flow cytometry analysis after day 10 of drug *in vivo* treatment and subsequent culture in the presence or absence of 250 μg/mL of FLX for 16 hours before adding brefeldin A for 4 hours. **(D)** IFN-γ levels in culture supernatant of total splenocytes

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restimulated with or without 250 μg/mL of FLX for 2 or 5 days. **(E-H)** Tg/DKO mice were treated as described in Methods. *(E)* Liver and gallbladder at euthanasia (1 representative animal). *(F)* H&E staining of fixed liver sections (scale bar 5 100 μm) (1 representative mouse per group). Additional sections showed more foci of inflammation in aCD4Ab–treated mice than untreated or FLX control groups, but inflammation and hepatocellular injury were most prominent in the aCD4Ab + FLX group (Fig E4). **(G)** Serum ALT at day 10 of treatment (n = 3 per group). (H) Gene expression analysis of perfused liver by real-time PCR (geomean of n = 3–12 mice per group).

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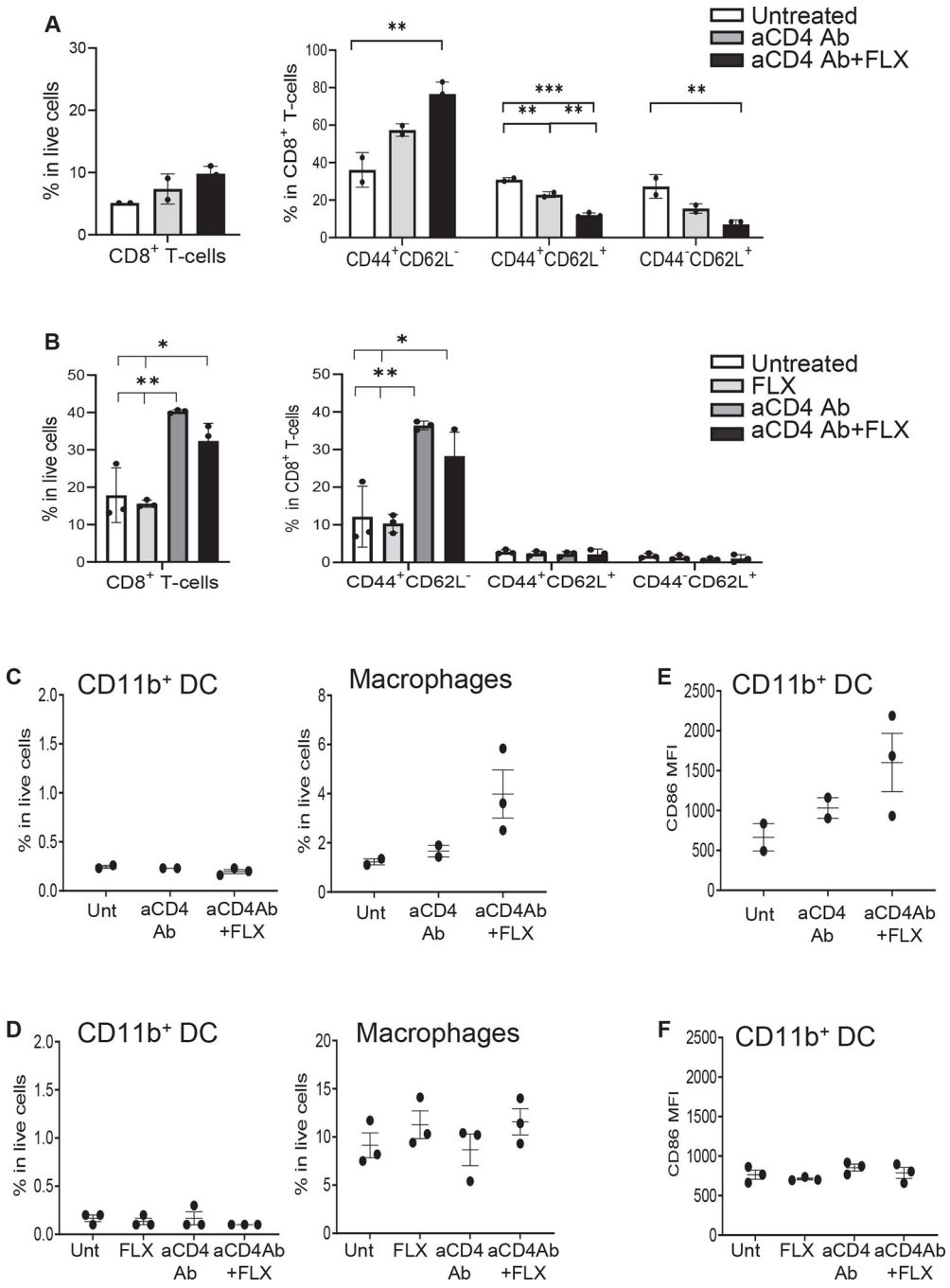

FIG 6.

Leukocyte profiling in spleen and liver of Tg/DKO mice. Animals received FLX and/or aCD4Ab. Livers were perfused before cell isolation. (**A** and **B**) Flow cytometry analysis of $CD8^+$ T-cell subsets obtained from spleen (*A*) or liver (*B*) of Tg/DKO mice. (**C** and **D**) Frequency of DC ($CD11b^+CD11c^+CD8^-$) and macrophage ($F4/80^+CD11b^+$) in spleen (*C*) and liver (*D*). (**E** and **F**) MFI of $CD86^+CD11b^+$ DCs isolated from spleen (*E*) and liver (*F*) of the same animals in (*C*) and (*D*), respectively.

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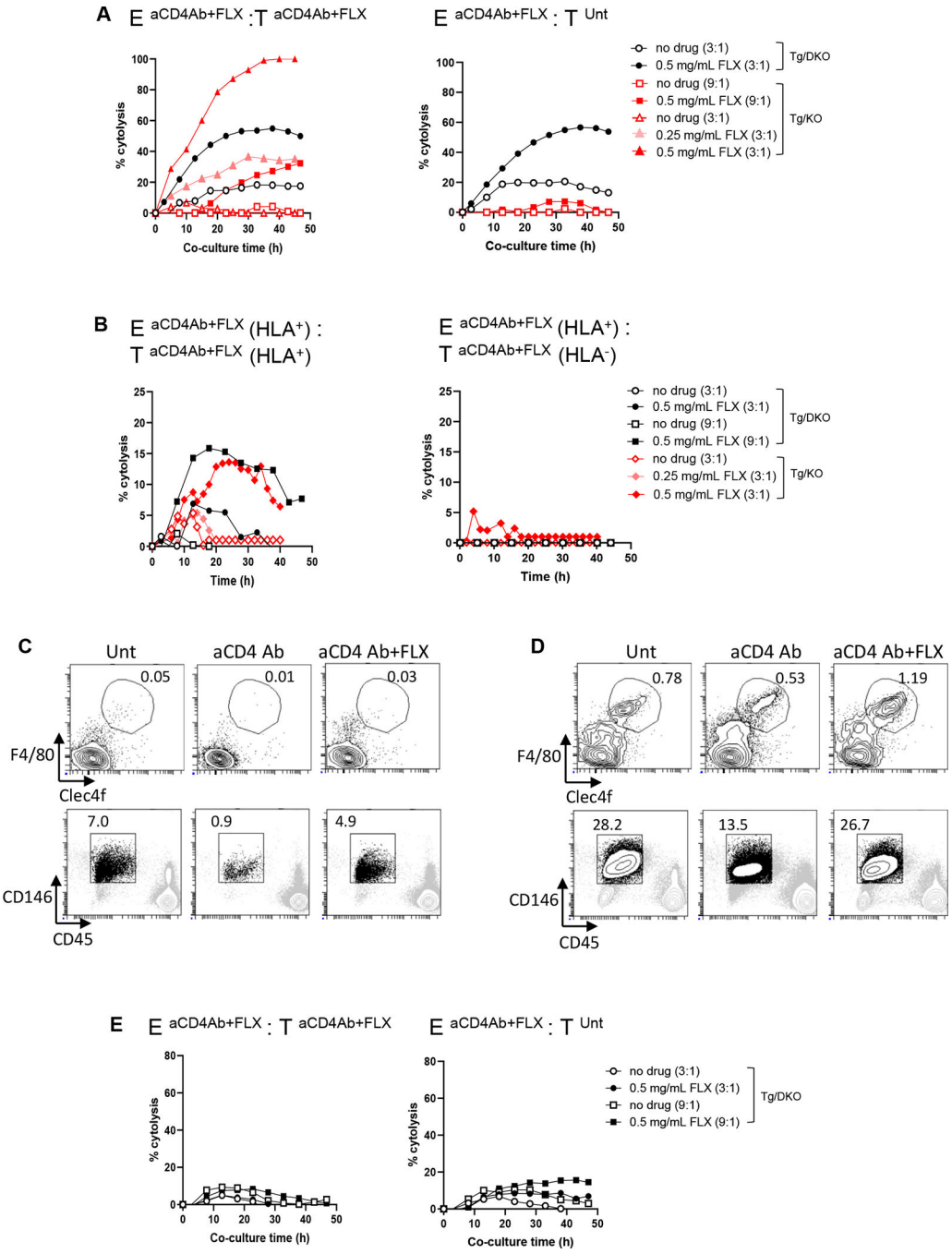

FIG 7.

In vitro hepatocyte cytolysis by FLX-reactive leukocytes of mice treated with aCD4Ab + FLX is influenced by drug reexposure, HLA expression, and presence of liver tolerogenic cells. Tg/DKO and Tg/KO animals were treated with aCD4Ab + FLX or left untreated as per our 10-day standard protocol. (**A** and **B**) Primary hepatocytes were isolated from perfused livers and seeded in E-plates (target cells [*T*] at 20,000 cells per well) for 18 hours. Subsequently, liver leukocytes from aCD4Ab + FLX–treated mice (effector cells [*E*]) were added to cultures at an *E:T* ratio of 3:1 or 9:1 (*E* and *T* from same mouse strain).

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Superscript annotations correspond to *in vivo* treatment of animals from which *E* and *T* cells were isolated. Selected cocultures were exposed to FLX. Percentage cytolysis was measured as a function of impedance, as indicated in the Methods section in the Online Repository. In (*B*), *T* were isolated from HLA^+ or HLA^- mice from Tg/KO or Tg/DKO strains. E were from HLA^+ animals. Different *symbols* correspond to different animals. (**C** and **D**) Frequency of KCs ($F4/80^+Clec4f^+$) and LSECs ($CD146^+CD45^-$) in cell suspensions of enriched liver leukocytes (*C*) or bulk NPC (*D*) from perfused livers of untreated or treated Tg/DKO animals. (**E**) Cytolysis was evaluated as indicated in (*A*) but with cocultures of hepatocytes and NPC from perfused livers of Tg/DKO mice.