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RESEARCH ARTICLE

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Tissue influx of neutrophils and monocytes is delayed during development of trovafloxacin-induced tumor necrosis factor-dependent liver injury in mice

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Abstract

Idiosyncratic drug-induced liver injury (iDILI) has a poorly understood pathogenesis. However, iDILI is often associated with inflammatory stress signals in human patients as well as animal models. Tumor necrosis factor (TNF) and neutrophils play a key role in onset of trovafloxacin (TVX)-induced iDILI, but the exact role of neutrophils and other leukocytes remains to be defined. We therefore set out to study the kinetics of immunological changes during the development of TVX-induced iDILI in the established murine model of acute liver injury induced by administration of TVX and TNF. Initially, TNF stimulated the appearance of leukocytes, in particular neutrophils, into the liver of TVX-treated mice, but even more so in control mice treated with the non-DILI inducing analogue levofloxacin (LVX) or saline as vehicle (Veh). This difference was apparent at 2 hours after TNF administration, but at 4 hours, the relative neutrophil amounts were reduced again in Veh- and LVX-treated mice whereas the amounts in TVX-treated mice remained at the same increased level as at 2 hours. The influx of monocytes/macrophages, which was unaffected in Veh- and LVX-treated mice was markedly reduced or even absent in TVX-treated mice. Unlike controls, mice receiving TVX + TNF display severe hepatotoxicity with clear pathology and apoptosis, coagulated hepatic vessels and increased alanine aminotransferase levels and interleukin 6/10 ratios. Findings indicate that TVX delays the acute influx of neutrophils and monocytes/macrophages. Considering their known anti-inflammatory functions, the disruption of influx of these innate immune cells may hamper the resolution of initial cytotoxic effects of TVX and thus contribute to liver injury development.

KEYWORDS

drug-induced liver injury, inflammation, kinetics, monocytes, neutrophils, TNF, trovafloxacin

1 | INTRODUCTION

Drug-induced liver injury (DILI) is a major concern for healthcare organizations and pharmaceutical companies all over the world, mainly due to the severity and prognosis of these events. Nonetheless, the economic impact associated with the hospitalization of patients and the restricted use of the drug imposed by regulatory agencies cannot be neglected (Chalasani et al., 2015; Fontana et al., 2009).

Some forms of DILI are idiosyncratic in nature and, among them, some involve the immune system during the onset and/or the prolongation of the disease. In particular, pharmaceuticals inducing immune-mediated or idiosyncratic DILI (iDILI) are often not detected by available preclinical and clinical tests alike, revealing their toxic features only during post-marketing pharmacovigilance studies (Lee & Senior, 2005; Watkins & Seeff, 2006). For this reason, many efforts have been spent during the last decade to identify new preclinical tests that can predict, early in drug development, the risk for occurrence of iDILI.

Several animal models underlined that tumor necrosis factor (TNF) administration in combination with known iDILI-associated compounds discloses the hepatotoxicity of these drugs (Buchweitz, 2002; Deng et al., 2006; Dugan & MacDonald, 2010; Lu, Jones,

Harkema, Roth, & Ganey, 2012; Luyendyk, 2003; Zou et al., 2009). Whether these models reproduce the clinical signature of DILI in patients is still matter of debate. In addition, the role of the immune response triggered by TNF in the development of liver injury is not yet well-defined. For this reason, we investigated the immunotoxicological effects of trovafloxacin (TVX) in the established mouse model of TVX-induced iDILI using TNF as a surrogate for inflammation. We emphasized our analyses on the kinetic changes of innate leukocytes in liver, because these leukocytes are known to be involved during the acute phase of inflammation, either as inflammatory or as anti-inflammatory players (Robinson, Harmon, & O'Farrelly, 2016). Characterization of the leukocyte recruitment into the liver will help identifying potential mechanisms involved in the development of iDILI as well as early biomarkers to predict the potential onset of iDILI.

Surprisingly, we found that recruitment of neutrophils and monocytes into the liver was delayed by the combination of TVX + TNF when compared with control mice receiving saline (vehicle, Veh) or the pharmacological analogue levofloxacin (LVX) even in the presence of TNF. Kinetics of the immunotoxicological changes in mice receiving TVX + TNF stresses the importance to investigate further the role of innate leukocytes in the development of iDILI.

2 | MATERIAL AND METHODS

2.1 | Animals

Male, 9–11 week old, C57BL/6 J mice (The Jackson Laboratory, Charles River) were used for all experiments. They were allowed to acclimatize for 1 week in a 12 hour light/dark cycle, and maintained at mean temperature of 23 ± 2°C, 50–55% relative humidity. Acidified drinking water and laboratory food pellets were provided ad libitum.

In vivo studies were approved by the Ethics Committee for Animal Experiments of Utrecht University and complied with governmental and international guidelines on animal experimentation.

2.2 | Chemicals

TVX and LVX were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant murine TNF was purchased from R&D Systems (Minneapolis, MN, USA).

2.3 | Experimental set-up

Mice (n = 6) were fasted 7 hours before treatment. TVX (150 mg kg^{-1}), LVX (375 mg kg^{-1}) or saline as Veh was administered orally 3 hours before recombinant murine TNF injection (50 μg kg^{-1}, intraperitoneally) (Shaw, Beggs, et al., 2009; Shaw, Ganey, & Roth, 2009). Food was available again immediately after TNF administration. Animals analyzed at time point 0 hours did not receive TNF (Figure 1). Dose of TVX was chosen because it was not associated with either hepatic histopathological modifications or serum alanine aminotransferase (ALT) increase. TVX, at selected dose, was capable of interacting with TNF to cause liver injury (Shaw, Beggs, et al., 2009; Shaw, Ganey, & Roth, 2009). As LVX is prescribed at a dose that is 2.5-fold greater than the dose of TVX to achieve a similar therapeutic effect in clinics (Lubasch, Keller, Borner, Koeppe, & Lode, 2000), we decided to keep the same ratio among the administered doses of these fluoroquinolones to conform to the experiments performed by Shaw and colleagues (Shaw, Beggs, et al., 2009; Shaw, Ganey, & Roth, 2009).

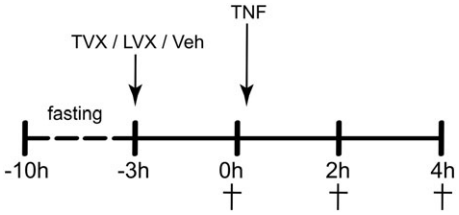

FIGURE 1 Protocol for the treatment of mice in the TVX + TNF model of drug-induced liver injury. Mice were fasted for 7 hours and then administered with an intragastric gavage of the drug solution or saline. Three hours later, mice were either killed (time point 0 hours) or received an intraperitoneal injection of TNF. TNF-injected mice were subsequently killed after 2 or 4 hours. LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

2.4 | Intrahepatic leukocyte isolation

Intrahepatic leukocytes were isolated as previously described by Crispe (Crispe, 2001). Briefly, liver was perfused with 5 ml ice-cold phosphate-buffered saline (PBS) and excised from the animal. Tissue was minced and gently passed through a sieve. The liver slurries were centrifuged, and the pellet resuspended with a collagenase D (0.02%, w/v) and DNAse I (0.002%, w/v) solution. The suspensions were incubated at 37°C for 40 minutes on a reciprocating shaker. Liver leukocytes were isolated by using a 45–67.5% isotonic Percoll density gradient (GE Healthcare, Fisher Scientific, Landsmeer, The Netherlands). Hepatic leukocytes obtained from Veh-only treated mice (without TNF) were used as an internal control. With this, we wanted to rule out small day-by-day differences in the isolation efficiency throughout the experiments.

2.5 | Plasma alanine aminotransferase and cytokine levels

Blood from inferior vena cava was collected in pre-coated lithium heparin tubes. Plasma was used to determine ALT activity according to the manufacturer's instructions (article no. MAK052; Sigma-Aldrich). TNF, interleukin (IL)-6 and IL-10 were determined in serum samples by sandwich enzyme-linked immunosorbent assay. Antibodies were from eBioscience (Halle-Zoersel, Belgium) and procedures followed according to the manufacturer's instructions.

2.6 | Histology

Liver and spleen were weighed. Half of the left lateral lobe and half of the spleen was fixed in phosphate-buffered formalin and embedded in paraffin. Five μm sections were cut and stained with hematoxylin–eosin and evaluated in a blind coded fashion by two independent investigators.

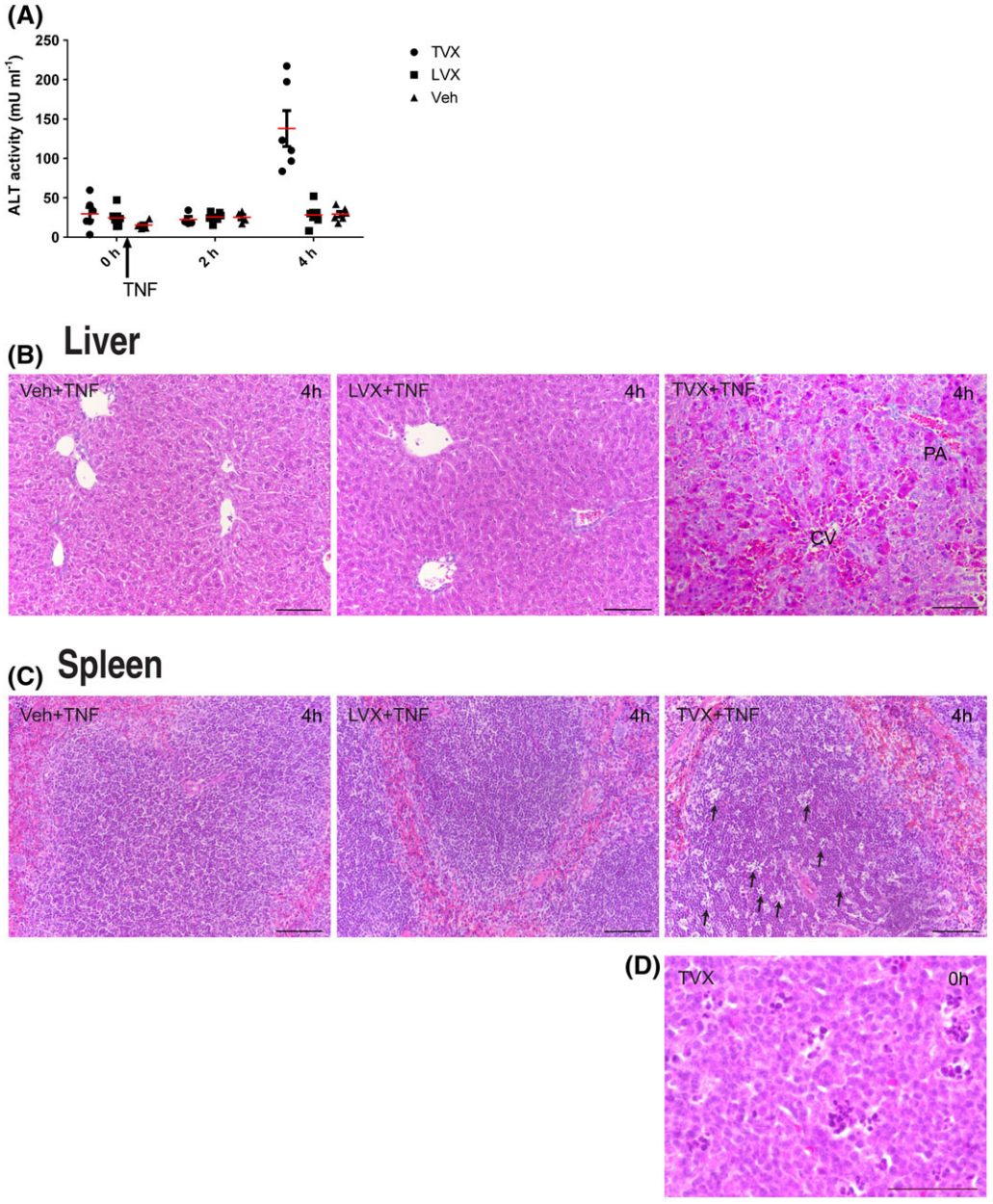

FIGURE 2 Histology and ALT levels. Serum ALT levels of TVX-, LVX- or Veh-treated mice. ALT levels were determined by colorimetric assay for ALT activity (A). Hematoxylin–eosin staining of liver and spleen sections taken 4 hours after TNF injection of mice pretreated with TVX, LVX or Veh (B,C). Arrows indicate focal damage observed in spleen. Apoptotic morphology of splenocytes in TVX-treated mice before injection of TNF (0 hour time point) (D). Representative images selected from six mice per treatment group are shown. Scale bars: (A–C) 100 μm; (D) 50 μm. ALT, alanine aminotransferase; CV, central vein; LVX, levofloxacin; PA, portal area; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

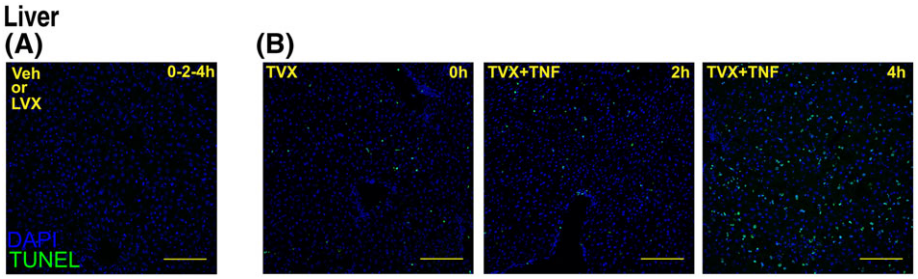

FIGURE 3 DNA fragmentation in liver. TUNEL staining on liver obtained from Veh or LVX (A) or TVX (B) pretreated mice. Liver section depicted in (A) is representative for sections collected at all time points assessed, in presence or absence of TNF for both LVX- or Veh-treated mice. (B) Representative images selected from six TVX-treated mice per treatment group (scale bar = 100 μm). LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

2.7 | Flow cytometry

Leukocytes from spleen were obtained by passing the organ through a 70 µm strainer, after which the collected single cells were washed once. To remove erythrocytes, cell suspensions were incubated with red blood cell lysis buffer (containing NH_4Cl, $KHCO_3$ and Na_2EDTA) for 1 minute, and the remaining cells were washed once with PBS.

For flow-cytometric analysis, cells were first stained with LIVE/DEAD® Fixable Dead Cell Stain (Molecular Probes, Invitrogen, Carlsbad, CA, USA) followed by incubation with anti-CD16/CD32 (clone 2.4G2) to block the fragment crystallizable region receptor (FcR). Next cells were stained with fluorescent-labeled antibodies and stored in 1% paraformaldehyde until analysis. The following antibodies were used: anti-CD45.2 Pacific Blue (clone 104; Biolegend, Uithoorn, The Netherlands), anti-LY6G APC (clone 1A8; Biolegend), anti-F4/80 fluorescein isothiocyanate (FITC; clone BM8; eBioscience), anti-CD11b PE and FITC (clone M1/70; eBioscience), anti-Gr1 APC (clone RBG8C5; eBioscience), CD4 PERCP (clone RM4-5; BD Pharmingen), CD8a PERCP (clone 53-6.7; BD Pharmingen, Erembodegem, Belgium), CD3e FITC (clone 145-2C11; eBioscience), NK1.1 PE (clone PK136; eBioscience), CD49b PE-Cy5 (clone DX5; eBioscience), CD86 PERCP (clone GL-1; Biolegend), MHCII FITC (clone M5/114.15.2; eBioscience), PDCA-1 PE (clone eBio129c; eBioscience), CD11c APC (clone N418; eBioscience), CD62L APC and PE (clone MEL-14; BD Pharmingen), and CD44 PE (clone IM7; eBioscience) in fluorescence-activated cell sorting buffer (PBS containing 0.25% bovine serum albumin, 0.05% NaN_3, 0.5 mM EDTA) for 30 minutes at 4°C.

Data were acquired by means of fluorescence-activated cell sorting Canto II and analyzed using Weasel flow analysis package (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia).

2.8 | Histological TUNEL staining

Immunofluorescence was performed on frozen liver and spleen tissues embedded in optimal cutting temperature compound. Eight µm thick frozen sections were mounted on polylysine-coated glass slides, dried overnight at room temperature and stored at −20°C until use. Briefly, tissue sections were allowed to dry for 2 hours before fixation with 4% formaldehyde in PBS. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay was performed following procedures for cryopreserved tissues reported in manufacturer's instructions (Roche, Woerden, The Netherlands). Neutrophil in liver tissue were stained using rat antimouse Ly6G

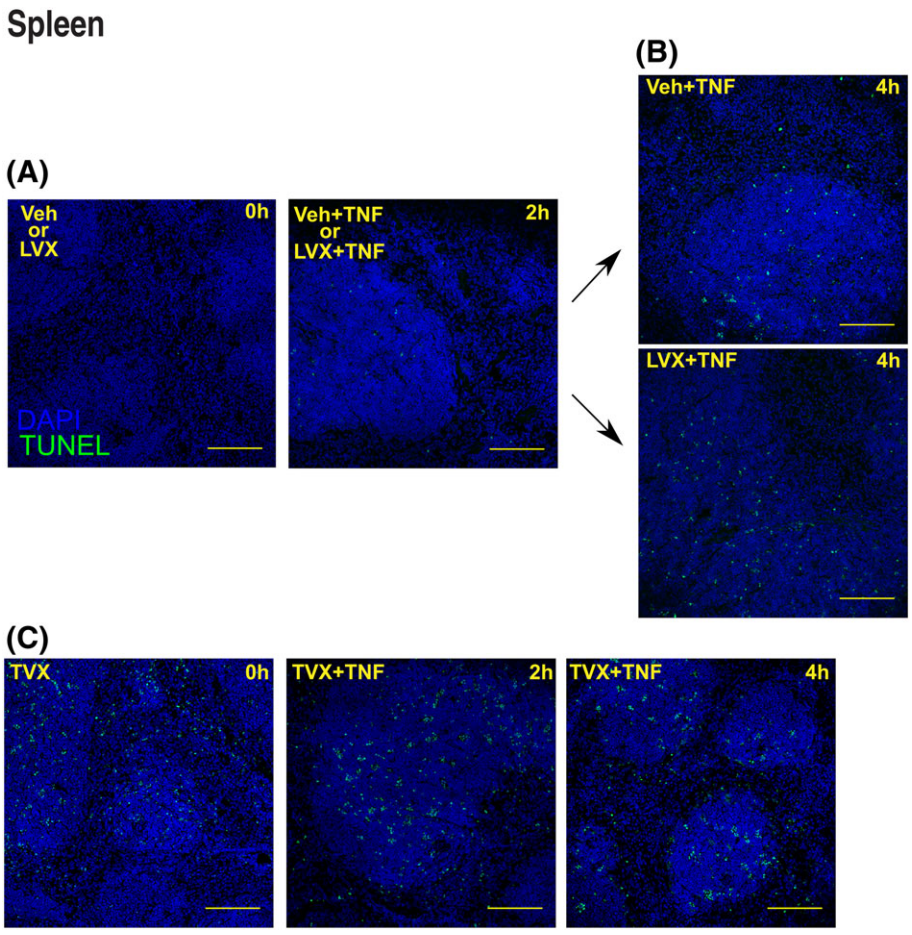

FIGURE 4 DNA fragmentation in spleen. TUNEL staining on spleen (A–C) obtained from Veh-, LVX- or TVX-pretreated mice. Spleen sections in (A) and (B) are representative for both Veh- and LVX-treated mice at the time indicated in figure. Four hours after TNF injection, spleens treated with TNF were positive for TUNEL staining independently of the pretreatment (B,C). (C) Representative images selected from six TVX-treated mice per treatment group (scale bar = 100 µm). LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

(1A8) for 1 hour in PBS/bovine serum albumin 0.1%, after blocking with 5% rat serum for 10 minutes both at room temperature. FITC-conjugated donkey antirat IgG was applied in PBS-Tween20 10% mouse serum for 45 minutes.

Nuclei were identified by DAPI. Images were acquired with Olympus BX-60 microscope equipped with Leica CCD camera (Leica DFC425C) (40×, 0.5 objective).

2.9 | Statistical analyses

Data are presented as means ± standard error of the mean (SEM). Amounts of cells are presented as a percentage of non-TNF and non-drug-treated control animals. Statistical significance for comparisons was determined by one- or two-way analysis of variance with Dunnett's post-hoc test. $P < .05$ was considered statistically significant. All data are analyzed using GraphPad Prism (version 6.07) software (San Diego, CA, USA).

3 | RESULTS

Kinetics of TVX-induced organ changes indicates that liver is affected before TNF exposure and that the spleen is affected as well.

We first set out to characterize the kinetics of damage in liver and spleen tissues induced by TVX. Thus, mice received either saline (Veh) or a single oral dose of TVX (150 mg kg^{-1}) or LVX (375 mg kg^{-1}) followed by a TNF injection after 3 hours. Mice were killed before TNF injection (0 hours), or 2 or 4 hours after TNF injection (Figure 1).

Injection of TNF caused liver damage in TVX-treated mice, but not in Veh- or LVX-treated mice. The treatments did not affect liver weights (not shown) and liver damage was evident at 4 hours after TNF injection and associated with infiltration of inflammatory cells around the central veins and by vast areas of necrotic lesions. The necrotic lesions were mainly in the periportal and midzonal regions of the liver lobules (Figure 2B). Higher magnification revealed that TVX + TNF caused apoptosis of hepatocytes, which was confirmed with TUNEL staining to detect DNA fragmentation (Figure 3B). TVX + TNF also induced hepatic blood congestion and increased levels of serum ALT (Figure 2A). None of these effects was present in the livers of Veh- and LVX-treated mice.

Liver tissues of TVX-treated mice collected at 2 hours after TNF injection were free from major lesions or leukocytes (data not shown) and serum ALT levels were not enhanced (Figure 2A). Remarkably, TVX without TNF (i.e., 3 hours after TVX administration, $t = 0$ in Figure 1) also caused a modest but clear increase of TUNEL positivity in the hepatocyte nuclei (Figure 3B). However, this was not accompanied by histopathologically apparent hepatic changes (data not shown).

Notably, TVX + TNF-induced tissue damage was not restricted to the liver, as evident from reduction of splenic cell numbers and from focal signs of cell death observed in splenic white pulp by hematoxylin–eosin staining and confirmed by nuclear TUNEL positivity (Figures 2C and 4C). Nuclear TUNEL positivity and apoptotic morphology was also observed in splenocytes of mice treated with TVX at 0 hours (before TNF injection, Figures 4C and 2D respectively). TNF also caused modest DNA fragmentation in the spleens of Veh-treated mice at 4 hours (Figure 4B), but not in the livers.

3.1 | Tumor necrosis factor elicits aberrant cytokine production in trovafloxacin-exposed mice

As the ratio of serum concentrations of IL-6 and IL-10 has been identified as a potential prognostic marker of the severity of different inflammatory conditions, we further investigated the kinetics of these cytokines (de Brito et al., 2016; Nagaki et al., 2000; Stensballe et al., 2009; Taniguchi et al., 1999). We also monitored the serum concentration of TNF, which will be of mixed endogenous and exogenous origin, but in turn can also stimulate secretion of the aforementioned cytokines (Libert, Brouckaert, Shaw, & Fiers, 1990; van Der Poll, Marchant, & van Deventer, 1997).

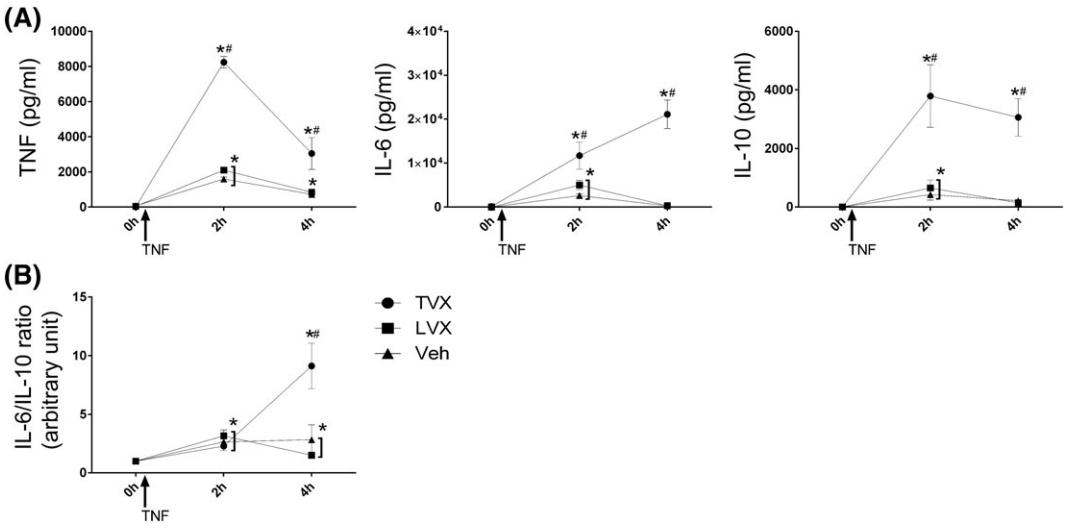

FIGURE 5 Serum cytokine levels of TNF, IL-6 and IL-10. Cytokines were determined in serum at indicated time points. (A) Levels of TNF, IL-10 and IL-6, and (B) ratio of IL-6/IL-10. *When compared to Veh only (no TNF, Veh-treated at 0 hours); #when compared to LVX + TNF and Veh + TNF, * or #$P < .05$. ●, TVX; ■, LVX; ▲, Veh; LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

TNF administration by itself, but also in combination with LVX, elicited a mild increase in serum levels of TNF, IL-6 and IL-10 when compared with animals not receiving TNF. The combined TVX + TNF treatment caused a profound increase of the serum concentrations of the above-mentioned cytokines when compared with LVX or Veh + TNF (Figure 5). Remarkably, although the serum levels of TNF and IL-10 were already decreasing again in all groups at 4 hours, this was less apparent in the TVX-treated group, which in addition showed further increased levels of IL-6. Consequently, the IL-6/IL-10 ratio was highly increased at 4 hours in TVX-treated mice, when mortality (which occurred in one of six animals between 2 and 4 hours) and severe liver damage were observed.

The mild liver damage observed at time point 0 hours, before TNF injection, was not associated with increased serum levels of TNF, IL-6 or IL-10 (Figure 5, time point 0 hours).

3.2 | Leukocyte influx in liver and spleen of trovafloxacin-treated mice appeared delayed

Several scientific studies describe the importance of a well-controlled leukocyte recruitment and activity in the regulation of inflammation (Gabrilovich, 2017; Pillay, Tak, Kamp, & Koenderman, 2013; Soehnlein & Lindbom, 2010). We first assessed the amount of sequestered CD45.2 leukocytes in the liver and spleen induced by the treatments.

As expected (Vollmar & Menger, 2009), all the groups receiving TNF showed an increased amount of sequestered leukocytes in the liver with a marked increase at 2 hours after TNF injection. This increase was reduced again at 4 hours, except in TVX-pretreated mice, where we found a fourfold increase in the amount of leukocytes ($CD45.2^+$ cells) relative to controls (Figure 6B).

Remarkably, the presence of leukocytes at 2 hours was significantly lower in TVX-treated groups compared to Veh- or LVX-treated groups (Figure 6B). TVX disturbs leukocyte recruitment in the liver indicating that it causes a delay in the TNF-mediated hepatic sequestration of leukocytes at 2 hours.

In addition, LVX and Veh induced an increase in the percentage of viable cells isolated from livers, whereas TVX reduced the viability of the isolated cells to 80% of control at 4 hours (Figure 6B).

The amounts and viability of leukocytes in the spleen did not change significantly in control groups but were significantly reduced in TVX-treated mice, again indicating that TVX affects leukocytes also systemically (Figure 6C).

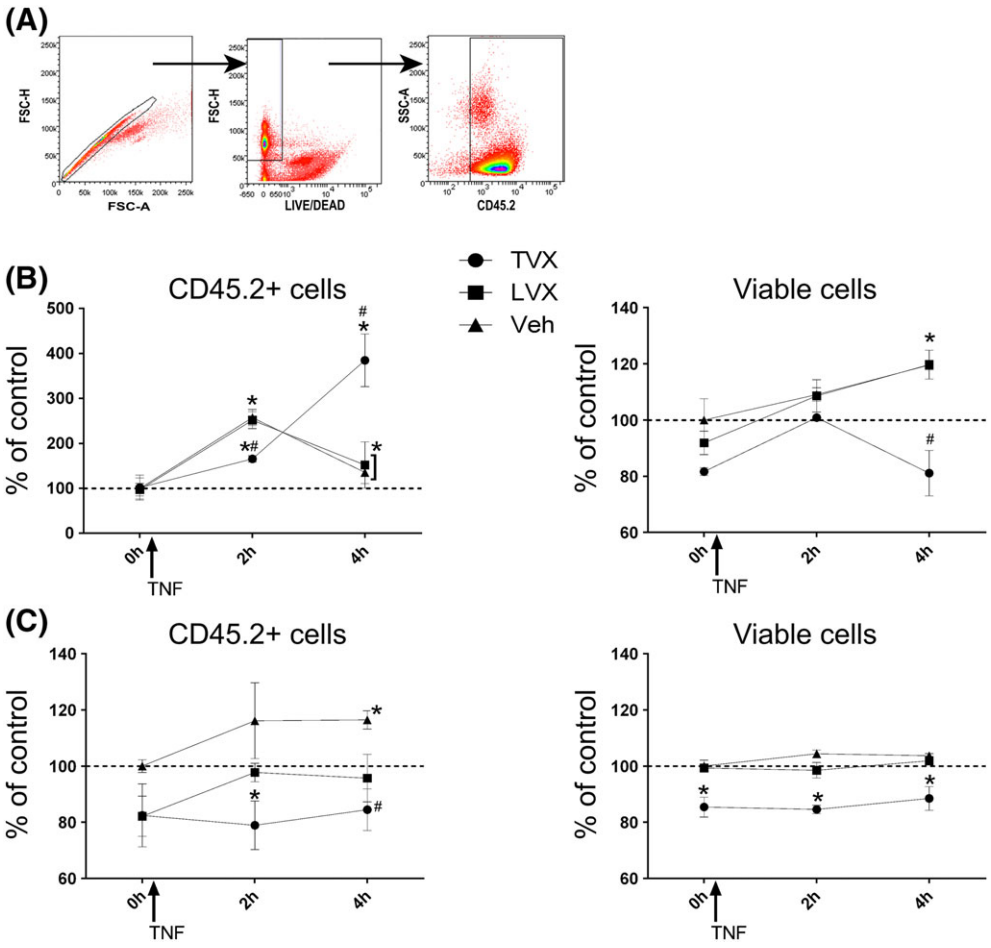

FIGURE 6 Leukocyte cellularity of liver (B) and spleen (C). Amounts and viability of leukocytes isolated from liver (Figure 6B) or spleen (Figure 6C). Leukocytes were characterized as displayed in Figure 5(A); *when compared to Veh only (no TNF, dashed line); #when compared to LVX + TNF and Veh + TNF, * or #$P < .05$). ●, TVX; ■, LVX; ▲, Veh; LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

3.3 | Trovafloxacin specifically delays liver influx of neutrophils and monocytes

Neutrophils and monocytes are among the first cellular players in the response to an inflammatory trigger (Ribeiro et al., 2015), but their recruitment to the site of inflammation also represents the first anti-inflammatory event (Soehnlein & Lindbom, 2010). For this reason, we focused on further evaluating the amount of these leukocytes in comparison with natural killer/T-cell fractions and other cells (e.g., dendritic cells, B cells). In Table 1, we show the data of the evaluated cell types in various exposure groups, whereas Figure 7 displays the distribution of the main leukocytes subtypes and other cells.

As mentioned in Section 3.2, TNF administration caused a clear change in the percentages of the total leukocyte population present in the liver, and it appeared that these differences between TVX- and LVX- or Veh-treated groups were most remarkable in the neutrophil and monocyte fractions (Figure 7). Notably, all groups receiving TNF displayed a marked increase in the amount of neutrophils (i.e., $Ly6G^+$ cells; Figure 8 and Table 2) at 2 hours. This increase was highest in LVX-treated mice, followed consecutively by Veh- and TVX-treated mice. Remarkably, immunofluorescent staining with the use of anti-Ly6G antibody showed poor positivity on liver sections obtained from TVX + TNF-treated mice 2 hours after TNF injection (Figure 8D), whereas $Ly6G^+$ cells well populated the hepatic tissue derived from LVX + TNF- and Veh + TNF-treated mice.

Four hours after TNF administration, the amount of neutrophils in the liver had returned towards background levels in LVX- and Veh-treated mice. In contrast, however, upon TVX administration, the neutrophil amount was not yet reduced to control levels at 4 hours and even significantly higher when compared to other treatments (Figure 8).

The amount of hepatic inflammatory macrophages, identified as $Gr1^+CD11b^{hi}F4/80^{lo}$ leukocytes, was clearly increased in LVX- and Veh-treated mice 2 hours after TNF injection (Figure 9B, Table 2) and had returned to background level again at 4 hours. No differences were observed between these two groups of treatment, but again TVX appeared to abrogate the recruitment of these monocytes/macrophages (Figures 7 and 9B, Table 1) resulting in decreased amounts at both 2 and 4 hours. Remarkably, compared to LVX or Veh, TVX also caused a significant reduction in the number of monocytes/macrophages already before injection of TNF (Figure 9B, right).

3.4 | Trovafloxacin also affects tumor necrosis factor-induced neutrophil and monocyte recruitment in the spleen

The amount of neutrophils isolated from in the spleen was reduced in the TVX-treated mice both at 2 and 4 hours after TNF administration (Figure 8C). In contrast, amounts of splenic neutrophils in Veh- or LVX-treated groups were increased at 2 hours and, different from what we observed in the liver, also at 4 hours.

Similarly to what we observed in the liver, TVX also affected amounts of monocytes/macrophages in the spleen. Indeed, the amount of splenic monocytes/macrophages observed at 4 hours after TNF injection was completely suppressed by the administration of TVX.

TABLE 1 Composition of leukocytes isolated from liver: at each time point hepatic lymphocytes were isolated by 45–67.5% isotonic Percoll density gradient and prepared for flow cytometry analysis

			% $CD45.2^+$ cells							
			0 hours			2 hours			4 hours	
Cell type		Phenotype	Veh	LVX	TVX	Veh	LVX	TVX	Veh	LVX	TVX
Lymphocytes	T lymphocytes	$CD45.2^+/CD3^+/NK1.1^-$	17.50 ± 1.57	17.00 ± 1.63	18.41 ± 0.85	11.78 ± 1.90	5.10 ± 0.79*	8.04 ± 0.24	24.03 ± 1.29	16.14 ± 1.48*	17.12 ± 1.53*
	NK	$CD45.2^+/CD3^-/NK1.1^+$	12.00 ± 0.48	12.87 ± 1.72	18.89 ± 1.49*	6.28 ± 0.44	3.69 ± 0.59	5.53 ± 0.38	19.78 ± 0.88***	7.23 ± 2.76	14.08 ± 1.93 ***
	NK(T)	$CD45.2^+/CD3^+/NK1.1^+$	9.28 ± 3.27	13.49 ± 0.53	8.24 ± 1.51	4.52 ± 0.60	2.26 ± 0.20	5.87 ± 0.54	5.74 ± 0.51***	18.67 ± 1.28	7.11 ± 1.78***
Neutrophils		$CD45.2^+/Ly6G^+$	4.93 ± 0.69	12.85 ± 0.72	7.76 ± 0.98	50.83 ± 0.92***	76.97 ± 1.56	63.68 ± 1.20***	13.66 ± 2.03	22.46 ± 6.14	33.47 ± 5.76*
Monocytes		$CD45.2^+/F4\text{-}80^{+\,low}/CD11b^{+\,int}$	2.90 ± 0.11	2.69 ± 0.05	1.66 ± 0.01*	2.67 ± 0.14	3.78 ± 0.28	0.48 ± 0.02**	2.38 ± 0.49	2.66 ± 0.49	0.48 ± 0.02**
Others		Non-specific $CD45.2^+$	53.39 ± 4.99	41.10 ± 4.12	45.04 ± 1.94	23.92 ± 0.26	8.20 ± 2.67	16.40 ± 1.45	34.41 ± 1.91	32.84 ± 2.03	27.74 ± 1.82

LVX, levofloxacin; NK, natural killer cells; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle.

Different cell phenotypes are presented in table as percentage of the total $CD45.2^+$ cells (mean ± SEM, *when compared to Veh with or without TNF depending on the time point assessed; **when compared to LVX + TNF and Veh + TNF, ***when compared to LVX + TNF, * or ** or ***$p < .05$).

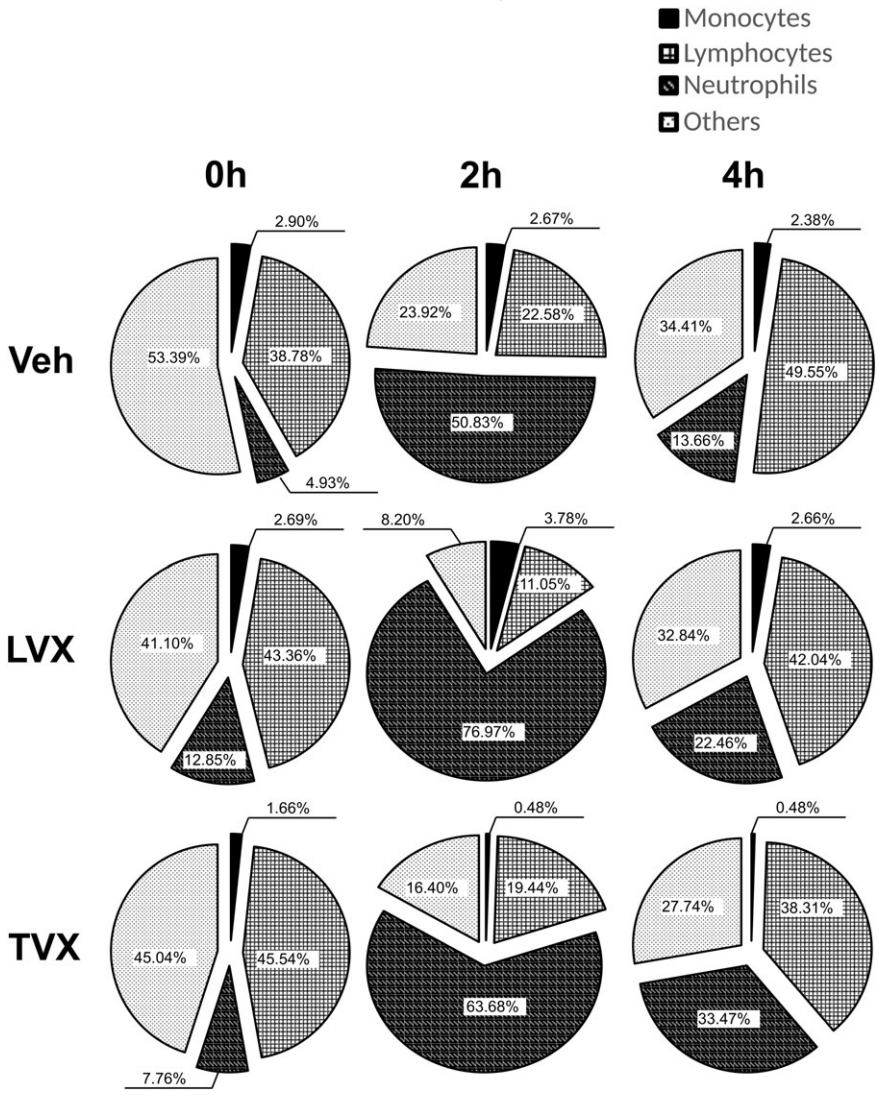

FIGURE 7 Distribution of various leukocyte subsets. Distributions in percentage of total leukocytes isolated from livers of TVX-, LVX- or Veh-treated mice at indicated time points. (Others = non-specific CD45.2$^+$ cells.) LVX, levofloxacin; TVX, trovafloxacin; Veh, vehicle

4 | DISCUSSION

To determine the role of the immune system in iDILI we focused on the evaluation of kinetics of leukocyte appearance in the liver in the well-established TVX model. Our kinetics data of leukocyte changes in the liver demonstrates that, at 2 hours, TNF induces the strongest neutrophil influx into livers of LVX- and Veh-treated mice whereas in comparison the neutrophil influx was only slightly increased in TVX-treated mice. At 4 hours, however, neutrophil amounts in Veh- of LVX-treated mice were reduced again, whereas those in TVX-exposed mice were still slightly increased, resulting in higher neutrophil amounts than in the other groups at the same time point. This led to us to conclude that TVX may cause a delay in the TNF-induced leukocyte recruitment into the liver. We excluded the possibility that the decreased number of neutrophils at 2 hours in mice receiving TVX + TNF was due to decreased viability observed in the spleen. Indeed, the number of splenic neutrophils (which may represent the first neutrophils recruited in the inflamed liver due to contiguity of these organs) in TVX-treated animals was even slightly increased when compared with the other treatment groups. In addition, this delaying effect of TVX may also hinder influx of monocytes/macrophages, as these leukocytes were not at all increased in livers of mice treated with TVX, but clearly present in livers of Veh- and LVX-treated mice.

Usually, leukocytes are recruited to the site of inflammation aiming at resolving the damage and the inflammation (Ortega-Gómez, Perretti, & Soehnlein, 2013; Soehnlein & Lindbom, 2010). In particular, after the induction of tissue damage, residential hepatocytes and macrophages (Kupffer cells) start producing cytokines to recruit mainly neutrophils and monocytes/macrophages, which in turn release cytokines, reactive oxygen species, proteases but also control cytokine levels via different mechanisms. At the site of inflammation, neutrophils release either soluble TNF receptors, capable of capturing the free fraction of TNF (Lantz, Björnberg, Olsson, & Richter, 1994; Steinshamn & Bemelmans, 1995), and alpha defensins that inhibit mRNA translation in macrophages (Brook et al., 2016). Importantly, phagocytosis of apoptotic neutrophils (also called efferocytosis; Michlewska, Dransfield, Megson, & Rossi, 2009) by monocyte/macrophages represents one of the initial anti-inflammatory events capable to regulate inflammation. In particular, it has been demonstrated that impairment of neutrophil sequestration into the liver and lack in their efferocytosis increases the early proinflammatory response (Holub et al., 2009). In addition, depletion of neutrophils in mice with anti-Ly6G antibody

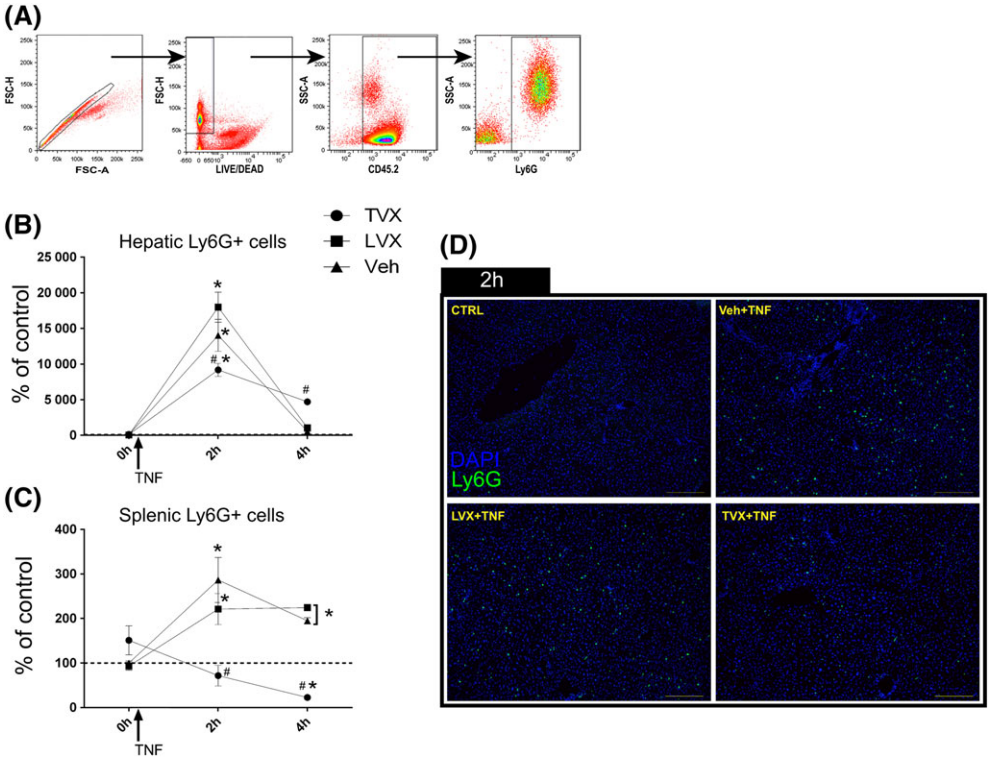

FIGURE 8 Neutrophil amounts in liver and spleen. Livers and spleens obtained from mice at each time point indicated were processed to isolate leukocytes. Cells were counted and prepared for flow cytometry. Neutrophils were identified as depicted in Figure 8(A) as $CD45.2^+/Ly6G^+$ live cells. Amount of cells are presented as percentage of cells counted in samples derived from mice receiving only the Veh (no TNF, dashed line). Flow cytometry data were obtained from experiments performed with four animals per group. Liver sections (8 μm) were obtained from liver of mice (four animals per group) pretreated with TVX, LVX or only the Veh 2 hours after TNF injection. TVX + TNF 2 hours after the cytokine injection showed less $Ly6G^+$ cells when compared to the other treatments receiving TNF (Veh + TNF and LVX + TNF); *when compared to Veh only (no TNF, dashed line); #when compared to LVX + TNF and Veh + TNF; * or #$P < .05$) (scale bar = 100 μm). ●, TVX; ■, LVX; ▲, Veh; CTRL, control; LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

led to increased serum concentrations of TNF in response to lipopolysaccharide (LPS), confirming the role for neutrophils in the regulation of inflammation among others by capturing TNF (Daley, Thomay, Connolly, Reichner, & Albina, 2008). An impairment in the interplay of professional phagocytes may expose an endangered tissue to a higher risk of leakage of intracellular contents due to secondary necrosis (Brauner, Schett, Herrmann, & Muñoz, 2013; Savill & Fadok, 2000). Secondary necrosis is a particularly dangerous phenomenon in short living cells such as neutrophils, as harmful mediators released from these cells can establish a vicious circuit of cell death, inflammation and tissue damage (Brauner et al., 2013). Interestingly, depletion of monocytes/macrophages in a model of endotoxemia was associated with a 20-fold increase in secreted TNF by leukocytes (Daley et al., 2008), which is in support of the regulatory role of monocytes/macrophages on neutrophil activity.

Our data may be explained in view of this anti-inflammatory cascade of events. In both control groups, Veh- and LVX-treated groups, no damage (e.g., low ALT, absence of necrosis or apoptosis) was observed and levels of TNF remained very low, whereas a clear, but temporary, influx of neutrophils and of monocyte/macrophages occurred. Therefore, the mere influx of neutrophils into the liver is not the cause of liver injury but may rather contribute to the prevention of damage. On the other hand, sustained presence of neutrophils combined with hampered influx of monocytes/macrophages may be responsible, at least to a great extent, for the liver injury in case of TVX. We therefore propose that the delay in neutrophil recruitment combined with complete absence of monocyte/macrophage recruitment represents a key phenomenon in the induction of TVX-mediated liver damage. The lack in monocyte/macrophage recruitment combined with the neutrophil sequestration in the liver may represent an obstacle to the resolution of inflammation, because TNF-induced apoptotic cells are not effectively cleared (Salamone et al., 2001; Soehnlein & Lindbom, 2010; Tsuchida et al., 1995). Although the mechanisms behind the TVX-mediated reduction of sequestered neutrophils and monocytes/macrophages into the liver needs further elucidation, current findings could explain the lack in clearance of TNF upon TVX exposure as described in a previous study in this mouse model (Shaw, Beggs, et al., 2009). In addition, our findings may be linked to the known procoagulatory effects of TVX as coagulation may hamper the influx of leukocytes from the liver blood vessels into the liver tissue. Indeed, previous studies on the model of TVX + LPS-induced liver damage showed that the combination of the fluoroquinolone with the bacterial component increases sinusoidal fibrin deposition in mice. Moreover, mice lacking plasminogen activator-inhibitor 1 and mice pretreated with heparin were less sensitive to TVX + LPS-mediated toxicity, showing a significant decrease in serum ALT and histopathological modification of the liver (Shaw, Fullerton, Scott, Ganey, & Roth, 2009). By contrast, inhibition of thrombin

TABLE 2 Composition of living cells isolated from liver: at each time point hepatic lymphocytes were isolated by 45–67.5% isotonic Percoll density gradient and prepared for flow cytometry analysis

Cell type		Phenotype	Number of cells								
			0 hours			2 hours			4 hours		
			Veh	LVX	TVX	Veh	LVX	TVX	Veh	LVX	TVX
Lymphocytes	T lymphocytes	$CD45.2^+/CD3^+/NK1.1^-$ ($\times10^6$)	0.25 ± 0.03	0.17 ± 0.03	0.35 ± 0.10	0.60 ± 0.10	0.48 ± 0.10	0.42 ± 0.07	0.26 ± 0.05	0.25 ± 0.03	0.39 ± 0.06
	NK	$CD45.2^+/CD3^-/NK1.1^+$ ($\times10^6$)	0.26 ± 0.07	0.23 ± 0.05	0.32 ± 0.08	1.05 ± 0.12	0.65 ± 0.13*	0.60 ± 0.05*	0.32 ± 0.04	0.25 ± 0.07	0.48 ± 0.05
	NK(T)	$CD45.2^+/CD3^+/NK1.1^+$ ($\times10^6$)	0.19 ± 0.09	0.18 ± 0.07	0.14 ± 0.05	0.42 ± 0.07	0.29 ± 0.04	0.42 ± 0.02	0.07 ± 0.02	0.10 ± 0.02	0.19 ± 0.04
Neutrophils		$CD45.2^+/Ly6G^+$ ($\times10^6$)	0.07 ± 0.02	0.04 ± 0.01	0.10 ± 0.02	7.39 ± 1.18	9.47 ± 1.11*	4.39 ± 0.48	0.15 ± 0.06	0.43 ± 0.26	3.14 ± 0.39
Monocytes ($Gr\text{-}1^+$)		$CD45.2^+/F4\text{-}80^{+\ low}/CD11b^{+\ int}/Gr\text{-}1^+$ ($\times10^5$)	0.17 ± 0.03	0.11 ± 0.04	0.08 ± 0.04	2.03 ± 1.07	2.91 ± 1.50	0.32 ± 0.16	0.27 ± 0.23	0.33 ± 0.24	0.27 ± 0.19

LVX, levofloxacin; NK, natural killer cells; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle.

Different cell phenotypes were quantified and presented in number as indicated for each cell phenotype (mean ± SEM, *when compared to vehicle with or without TNF depending on the time point assessed; **when compared to LVX + TNF and Veh + TNF, * or **$P < .05$).

was not associated with a decreased number of hepatic neutrophils in TVX + LPS-treated mice, excluding the possibility that the observed neutrophil accumulation in the liver must rely exclusively on fibrin deposition (Shaw, Fullerton, et al., 2009).

The increased IL-6/IL-10 ratio, reflecting the inflammatory condition in TVX-induced liver damage, also supports the hypothesis that a disruption in the neutrophil/monocyte partnership occurs in this model. Previously, Shaw et al. (2009; Shaw, Ganey, & Roth, 2009a) have already demonstrated increases of serum levels of the proinflammatory IL-6 and anti-inflammatory IL-10 cytokines in TVX-treated mice, but here we show that kinetics of these cytokines in the serum differs, resulting in the increased IL-6/IL-10 ratio. Several studies (Filardy et al., 2010; Michlewska et al., 2009; Ribeiro-Gomes et al., 2004) show that monocytes/macrophages start producing IL-10 in response to neutrophil efferocytosis. As hepatocytes and possibly Kupffer cells will continue producing IL-6 under TVX(+TNF)-induced conditions, the alteration in the IL-6/IL-10 ratio may represent an additional effect resulting from the disrupted phagocyte partnership.

The TVX-induced suppression in neutrophil and monocyte/macrophage recruitment in spleen at 2 and 4 hours underlines that the effect of TVX is not limited to the liver. It is tentative to speculate that splenic neutrophils migrate to the liver when the damage caused by the TVX + TNF combination takes place. Regardless of this, the effects on the spleen highlight that, despite the peculiar effects of TVX on the liver, leukocyte populations in other organs are also affected. What this means in relation to immunotoxicological or clinical effects of TVX in other organs such as the skin, is not known.

We also demonstrate that TVX has clear effects before TNF injection, i.e., low level of DNA fragmentation in liver and reduced amounts of macrophages in the spleen. It is known that TVX by itself induces compound-specific effects in hepatocytes (such as DNA damage caused by topoisomerase II inhibition) (Beggs et al., 2015; Beggs, Fullerton, Miyakawa, Ganey, & Roth, 2014; Liguori, Blomme, & Waring, 2008), although the cytotoxic (i.e., apoptosis) effects of TVX in vitro also depend on the presence of TNF (Beggs et al., 2014; Cosgrove et al., 2009). However, we cannot exclude that low levels of TNF, e.g., induced by subtle leakage of LPS from the gut, are involved in these effects. Nevertheless, although positivity for the TUNEL reaction in this model does not discriminate between apoptosis (potentially induced by TVX and TNF combination) and DNA damage, here we demonstrated to the best of our knowledge for the first time that TVX alone in vivo induced early toxic effects on the liver.

Together, our findings indicate that TVX delays the acute influx of neutrophils and monocytes/macrophages. Considering their known anti-inflammatory functions, the disruption of influx of these innate immune cells may hamper the resolution of initial cytotoxic effects of TVX and thus contribute to the development of liver injury. As already stated before by Shaw and colleagues (Shaw, Fullerton, Scott, Ganey, & Roth, 2009), the combinations of effects by TVX may be the basis of the idiosyncrasy of this particular form of iDILI, and the hampered influx of anti-inflammatory leukocytes may add to the complexity of this disease. In addition to the contribution of neutrophil elastase in the onset of TVX + LPS-induced liver injury as previously demonstrated by Shaw et al. (2009a), our findings

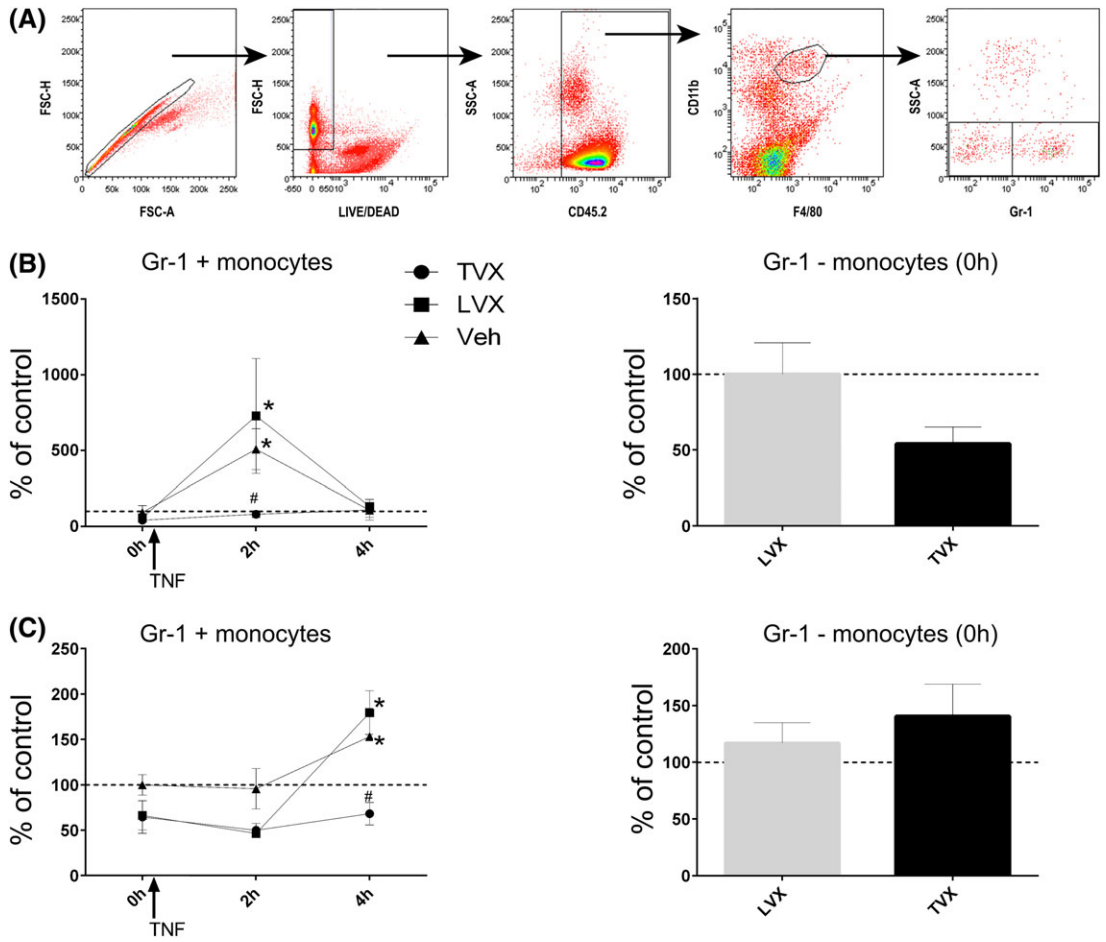

FIGURE 9 TVX reduced monocyte recruitment into the liver (B) and spleen (C). Livers obtained from mice at each time point indicated were processed to isolate hepatic leukocytes. Cells were counted and prepared for flow cytometry. Monocytes were identified as depicted in Figure 9(A) (CD45.2$^+$/F4-80$^+$ low/CD11b$^+$ and Gr1$^+$ "classical," Gr1$^-$ "non-classical"). Amount of cells are presented as percentage of cells counted in samples derived from mice receiving only the Veh (no TNF). Flow cytometry data were obtained from experiments performed with four animals per group (A). (* when compared to Veh only (no TNF); #when compared to LVX+TNF and Veh+TNF; * or #$P < .05$). ●, TVX; ■, LVX; ▲, Veh; LVX, levofloxacin; TNF, tumor necrosis factor; TVX, trovafloxacin; Veh, vehicle

delineate a second role for neutrophils in the model of TVX + TNF. In this respect, we believe that early recruitment of neutrophils and monocytes in the inflamed organ aims to resolve inflammation, whereas their prolonged permanence may trigger protease release with consequent tissue damage. For this reason, further investigations to identify mechanisms explaining the observed disruption of leukocyte sequestration may ultimately add to predict idiosyncratic DILI.

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CONFLICT OF INTEREST

The authors did not report any conflict of interest.

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