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Tissue immunology and leukocyte trafficking

Basic

Research Article

Murine iNKT cells are depleted by liver damage via activation of P2RX7

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Invariant natural killer T cells (iNKT) constitute up to 50% of liver lymphocytes and contribute to immunosurveillance as well as pathogenesis of the liver. Systemic activation of iNKT cells induces acute immune-mediated liver injury. However, how tissue damage events regulate iNKT cell function and homeostasis remains unclear. We found that specifically tissue-resident iNKT cells in liver and spleen express the tissue-damage receptor P2RX7 and the P2RX7-activating ectoenzyme ARTC2. P2RX7 expression restricted formation of iNKT cells in the liver suggesting that liver iNKT cells are actively restrained under homeostatic conditions. Deliberate activation of P2RX7 in vivo by exogenous NAD resulted in a nearly complete iNKT cell ablation in liver and spleen in a P2RX7-dependent manner. Tissue damage generated by acetaminophen-induced liver injury reduced the number of iNKT cells in the liver. The tissue-damage-induced iNKT cell depletion was driven by P2RX7 and localized to the site of injury, as iNKT cells in the spleen remained intact. The depleted liver iNKT cells reconstituted only slowly compared to other lymphocytes such as regulatory T cells. These findings suggest that tissue-damage-mediated depletion of iNKT cells acts as a feedback mechanism to limit iNKT cell-induced pathology resulting in the establishment of a tolerogenic environment.

Keywords: liver · NAD · NKT cells · P2RX7 · Tissue-resident

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

The liver is an important immunological organ that functions as a portal between the intestine and the circulation. Nutrient rich blood containing harmless dietary and microbiota derived components as well as pathogens arrives in the liver via the portal vein. In order to tolerate this high immunogenic load and maintain immunosurveillance for pathogens and

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malignant cells, a tight regulation of the hepatic immune system is essential.

Natural Killer T cells (NKT) are innate-like T cells, which are abundant in the liver sinusoids where they represent 20–50% of the murine liver lymphocytes. NKT cells co-express NK cell receptors and rearranged semi-invariant TCRs that recognize glycolipid antigens presented by CD1d, an MHC class I like molecule. Two major subsets of NKT cells can be distinguished based on their TCR repertoire, lipid reactivity, and function. Type I or invariant NKT cells (iNKT) have a restricted repertoire and exert potent proinflammatory effector functions upon activation, while type II NKT cells are more heterogeneous and mainly act immunoregulatory [1].

iNKT cells have been implicated to contribute to the immunosurveillance as well as pathogenesis of the liver. Lack or blockade of iNKT cells impairs clearance of hepatotrophic viruses such as HBV [2] and several bacteria [3]. Furthermore, hepatic iNKT cells mediate inhibition of liver-tumor growth [4]. Bacterial pathogen derived glycolipids from *Sphingomonas*, *Borrelia*, and *Streptococcus pneumoniae* presented by CD1d can activate iNKT cells directly [5–8] and contribute to their protective function. Conversely, aberrant or excessive iNKT cell stimulation can be detrimental. Activation of iNKT cells with ConA or αGalCer induces hepatitis and fatal liver injury [9–12]. In this process, the activated iNKT cells can induce hepatocyte death via Fas-FasL interaction or through release of the cytotoxic proteins perforin and granzyme B. Additionally, production of IFN-γ by stimulated iNKT cells recruits and activates CD4 and CD8 T cells that amplify the inflammatory response.

While activated iNKT cells can induce tissue damage, it is unclear how they respond to the damage and danger signals during inflammation and tissue damage. We have recently shown that tissue-resident CD8 T cells are regulated via the damage receptor P2RX7 [13]. Tissue damage and release of intracellular content into the extracellular space leads to high extracellular concentration of the nucleotides NAD and ATP. Extracellular ATP and NAD activate P2RX7, a cation channel that mediates inflammasome activation and influences the adaptive immune system [14]. Extracellular ATP binds P2RX7 directly, while NAD-mediated P2RX7 activation is catalyzed by the ectoenzyme ARTC2. During prolonged activation, P2RX7 can form a non-selective membrane pore, leading to cell death induction [15,16]. High expression of P2RX7 has also been observed on iNKT cells in the liver as well as in other locations [17]. However, how P2RX7 regulates iNKT cell homeostasis in vivo during steady state and tissue damage is currently unclear.

In this study, we demonstrate that direct activation of P2RX7 by exogenous NAD caused a strong depletion of mature iNKT cells in vivo via iNKT cell intrinsic mechanisms. iNKT cell numbers only recovered slowly after depletion and remained reduced for at least 5 weeks. P2RX7 was also activated under homeostatic conditions and restricted the generation and/or maintenance of hepatic iNKT cells. During toxin induced liver injury iNKT cells were depleted locally in a P2RX7-dependent manner. Our data suggest that tissue damage mediated depletion of iNKT cells may act as a feedback mechanism to limit iNKT cell-induced pathology with long lasting effects on local innate immunity, due to the slow recovery of the iNKT population.

Results

Heterogeneous expression of P2RX7 and ARTC2 by iNKT cells

Recent publications demonstrate heterogeneity of iNKT cells in different tissues with respect to function and migration potential [18]. To investigate which iNKT cells receive P2RX7-mediated signaling, we first analyzed the expression of P2RX7 and the P2RX7-activating ectoenzyme ARTC2 on iNKT cells from different tissues. Using CD1d tetramer to identify iNKT cells (Supporting Information Fig. S1A) we found that iNKT cells in the liver and spleen express high levels of P2RX7 and ARTC2, while thymic iNKT cells mostly lack expression of both markers (Fig. 1A and B; Supporting Information Fig. S1B), as previously described [17]. iNKT cells are considered to be largely maintained as tissue-resident cells [19,20]. However, recent parabiosis experiments demonstrate that the expression of CD69 distinguishes $CD69^+$ tissue-resident from $CD69^-$ circulating iNKT cells [21]. In naïve mice, 5–25% of the iNKT cells lacked CD69 expression (Supporting Information Fig. S1C). Similar to CD8 T cells [13], expression of P2RX7 and ARTC2 was selectively increased in the tissue-resident $CD69^+$ fraction of iNKT cells (Fig. 1C and D). Expression of molecules associated with tissue residency, such as CD49a, CXCR6, and LFA-1, was similar on iNKT cells in naïve WT and P2RX7 KO mice (Supporting Information Fig. S1D and E). Other circulating innate cells, such as NK cells also lacked expression of P2RX7 and ARTC2 (Fig. 1E; Supporting Information Fig. S1F and G). Thus, we demonstrate that mature tissue-resident iNKT cells in the periphery specifically express P2RX7 and ARTC2 and accordingly could be responsive to P2RX7-mediated signaling.

P2RX7 expression restricts iNKT cells in the liver

The lack of P2RX7 expression by iNKT cells in the thymus suggests that P2RX7 is acquired after thymic maturation and egress into the periphery. To analyze how P2RX7 deficiency impacts iNKT cell development, we generated mixed bone marrow (BM) chimeras by injection of equal amounts of BM cells from congenically marked WT and P2RX7 KO mice into irradiated recipients. More than 90% of lymphocytes were derived from donor cells in the fully reconstituted mice (Supporting Information Fig. S2A and B). Although equal amounts of WT and P2RX7 KO BM cells were injected, twofold more P2RX7 KO lymphocytes were detected in all analyzed organs of the reconstituted mice (Fig. 2A and B). WT and P2RX7 KO iNKT cells in the spleen and thymus were present at similar ratios as total lymphocytes in the respective organ (Fig. 2A and B). In contrast, we observed a selective dominance of hepatic P2RX7 KO iNKT cells. In the liver, P2RX7 KO cells were increased fourfold compared to WT iNKT cells in contrast

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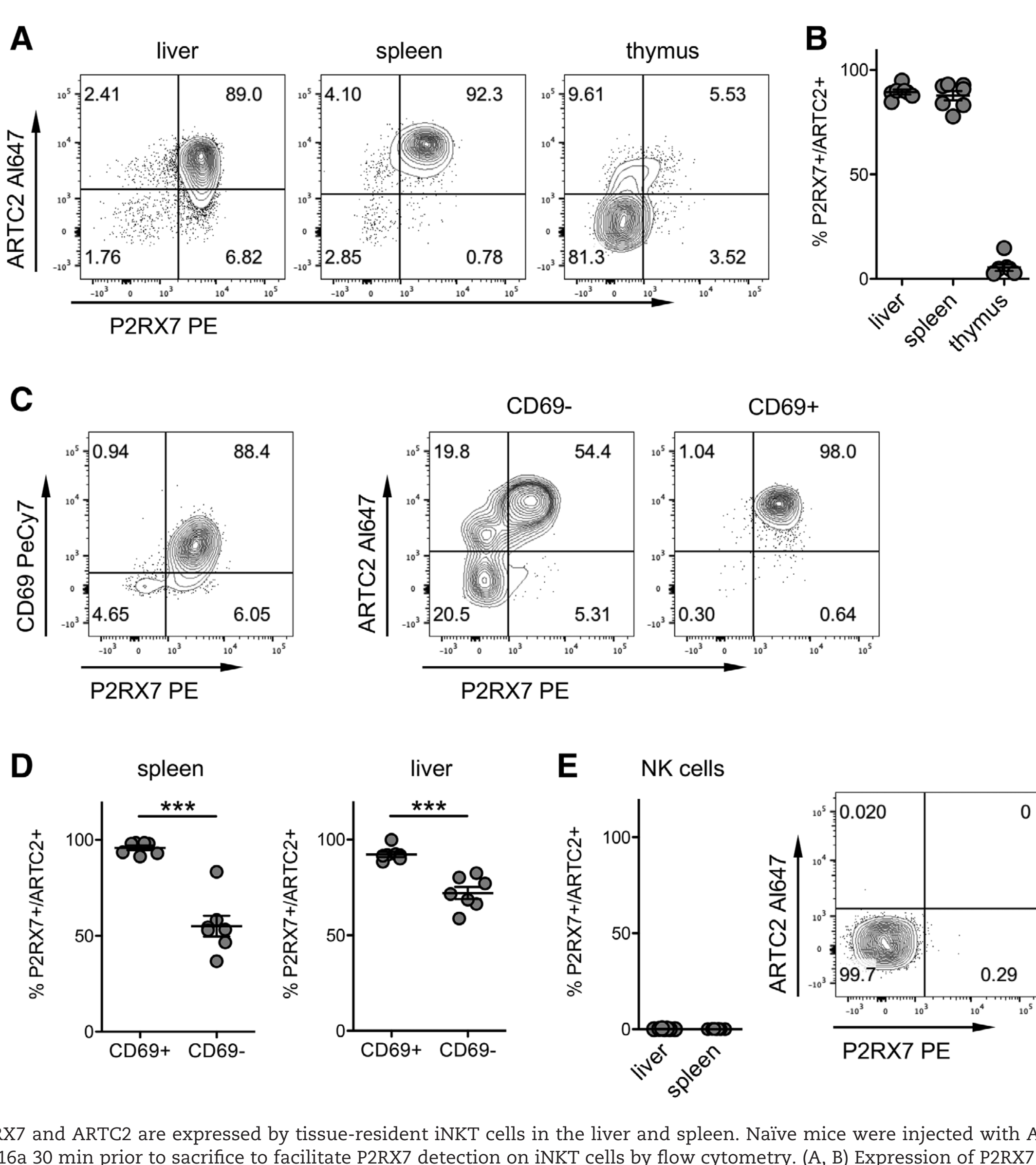

Figure 1. P2RX7 and ARTC2 are expressed by tissue-resident iNKT cells in the liver and spleen. Naïve mice were injected with ARTC2 blocking nanobody s+16a 30 min prior to sacrifice to facilitate P2RX7 detection on iNKT cells by flow cytometry. (A, B) Expression of P2RX7 and ARTC2 on iNKT cells in indicated organs. (C) Identification of tissue-resident ($CD69^+$) and circulating ($CD69^-$) iNKT cells and the co-expression of P2RX7 and ARTC2 on both subsets on iNKT cells in the spleen and (D) quantification. (E) Quantification of co-expression of P2RX7 and ARTC2 on liver NK cells ($NK1.1^+$ $CD335^+$ $CD49a^-$ $CD49b^+$). (A–E) $N = 7$ mice, data pooled from three independent experiments, mean ± SEM, paired t-test, $^{***}p \leq 0.001$.

to a twofold enrichment of P2RX7 KO cells in the total liver lymphocytes (Fig. 2A and B). This suggests that specifically in the hepatic microenvironment P2RX7 on iNKT cells is triggered and limits their generation and/or maintenance. Within the predominating P2RX7 KO iNKT cells in the liver, we observed a lower frequency of circulating CD69- hepatic iNKT cells (Fig. 2C), demonstrating that in a competitive setting P2RX7 selectively restricts the development of tissue-resident iNKT cells in the liver.

Extracellular NAD depletes iNKT cells via P2RX7

The liver-specific P2RX7-dependent phenotype of iNKT cells under homeostatic conditions prompted us to investigate whether also iNKT cells at other locations can respond to P2RX7 triggering. The outcome of P2RX7 activation on iNKT cells has been controversial. Exposure of iNKT cells from the liver or the intestine to NAD in vitro led to the induction of cell death [22]. However, after triggering of P2RX7 by NAD injection in vivo, a strong but transient or no depletion of iNKT cells has been observed in the liver and spleen, while iNKT cells in the intestine were efficiently decreased [21,22]. To analyze the capacity of iNKT cells at different locations to respond to P2RX7 triggering in vivo, we applied NAD in a dose that has previously been proven to induce P2RX7 signaling in other P2RX7 expressing T cells, such as regulatory T cells (Treg) and tissue-resident CD8 T cells [13,23]. We found that injection of NAD induced a 25-fold decrease in iNKT cell frequencies (Fig. 3A) and a 50-fold reduction in iNKT cell number

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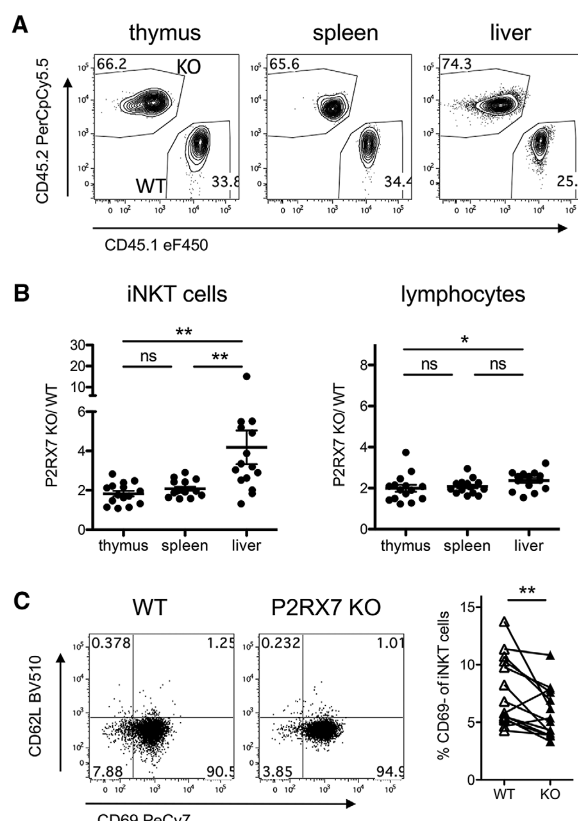

Figure 2. P2RX7 expression restricts iNKT cells in the liver. Mixed BM chimeras of WT and P2RX7 KO cells were analyzed 8–16 weeks post reconstitution. The mice were injected with s+16a nanobodies 30 min prior to sacrifice. iNKT cells were analyzed by flow cytometry. (A) Donor derived iNKT cells in different organs. (B) Fold change of the percentage of P2RX7 KO over WT donor iNKT cells or donor leukocytes. (C) Frequency of CD69 lacking iNKT cells in the liver. (A–C) $N = 15$ mice, data pooled from three independent experiments, (B) one-way ANOVA with Bonferroni's posttest, mean ± SEM, (C) paired *t*-test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

(Fig. 3B) in the liver. iNKT cells were largely maintained in P2RX7 KO mice after NAD administration (Fig. 3A and B), suggesting that the NAD-induced depletion was P2RX7-dependent. Similar to hepatic iNKT cells, iNKT cells in the spleen and BM were also reduced by NAD in a P2RX7-dependent manner (Supporting Information Fig. S3A), suggesting that iNKT cells in most organs can respond to P2RX7-triggering. In contrast, iNKT cell numbers in the thymus were not affected by NAD injection (Fig. 3C), in accordance with the absence of P2RX7 and ARTC2 expression on thymic iNKT cells. Hepatic NK cells, which lack P2RX7 expression (Fig. 1E), were also maintained after NAD treatment (Fig. 3D; Supporting Information Fig. S3B). The few iNKT cells remaining in the liver after NAD treatment in WT mice showed reduced expression of CD69 (Fig. 3E and F). At the same time, $CD69^{-}$ iNKT cells were less efficiently depleted by exogenous NAD (Supporting Information Fig. 3C). The preferential depletion of $CD69^{+}$ iNKT by NAD is in line with the expression pattern of P2RX7 and ARTC2 and demonstrates that tissue-resident iNKT cells are most susceptible to P2RX7-mediated depletion.

Intrinsic P2RX7 signaling renders iNKT cells susceptible to NAD induced depletion

To investigate whether the observed NAD-induced loss of iNKT cells was mediated by iNKT cell intrinsic P2RX7 expression, we treated mixed BM chimeras containing WT and P2RX7 KO cells with NAD. WT iNKT cells in mixed BM chimeras were strongly diminished in the liver after NAD application. In contrast, P2RX7 KO iNKT cells and iNKT cells in the thymus were resistant to NAD treatment (Fig. 4A). Accordingly, the WT compartment of iNKT cells only minimally contributed to iNKT cells in the liver after NAD application compared to control mice or P2RX7 negative thymic iNKT cells (Fig 4B and C). These results confirm that cell intrinsic signals induce the depletion of P2RX7-expressing iNKT cells by extracellular NAD in vivo.

Tissue damage depletes iNKT cells locally in the injured organ

Extracellular nucleotides such as NAD and ATP, which can activate P2RX7, are locally increased at sites of tissue damage. High doses of the analgesic acetaminophen, also termed paracetamol (*N*-acetyl-*p*-aminophenol, APAP), cause significant hepatotoxicity and induce severe acute liver damage with necrosis of hepatocytes in humans and mice.[24] To investigate the impact of local tissue damage on iNKT cells, we analyzed livers of APAP-treated mice. Liver injury in APAP-treated mice induced a twofold reduction in the frequency and absolute number of iNKT cells in the liver (Fig. 5A and B; Supporting Information Fig. S4A). Splenic iNKT cells were unaffected by induction of liver injury (Fig. 5C; Supporting Information Fig. S4B), which suggests that the activity of extracellular NAD and ATP is retained locally in the liver after tissue damage. In line with this, we also observed liver-specific depletion of other P2RX7-sensitive cells, such as Treg, after liver injury induction (Supporting Information Fig. 4C and D). In a second set of experiments, we compared the susceptibility of WT and P2RX7 KO iNKT cells to APAP-induced liver damage. Both strains showed a similar level of APAP-induced liver injury, as measured by serum ALAT levels (Supporting Information Fig. S4E). We observed a substantial reduction of liver iNKT cells in WT but not in P2RX7 KO mice after APAP treatment (Fig. 5D; Supporting Information Fig. S4F). Confirming our earlier findings, splenic iNKT cells as well as liver NK cells were not impaired by liver injury induction in both WT and P2RX7 KO mice (Supporting Information Fig. S4G and H). Thus, liver damage depletes local iNKT cells by triggering of P2RX7.

iNKT cells are slowly reconstituted after depletion

The substantial depletion of iNKT cells after NAD injection allowed us to investigate the repopulation of the liver by iNKT cells. Remarkably, iNKT cell numbers only recovered slowly over the next 5 weeks after the initial depletion (Fig. 6A and B). The impact

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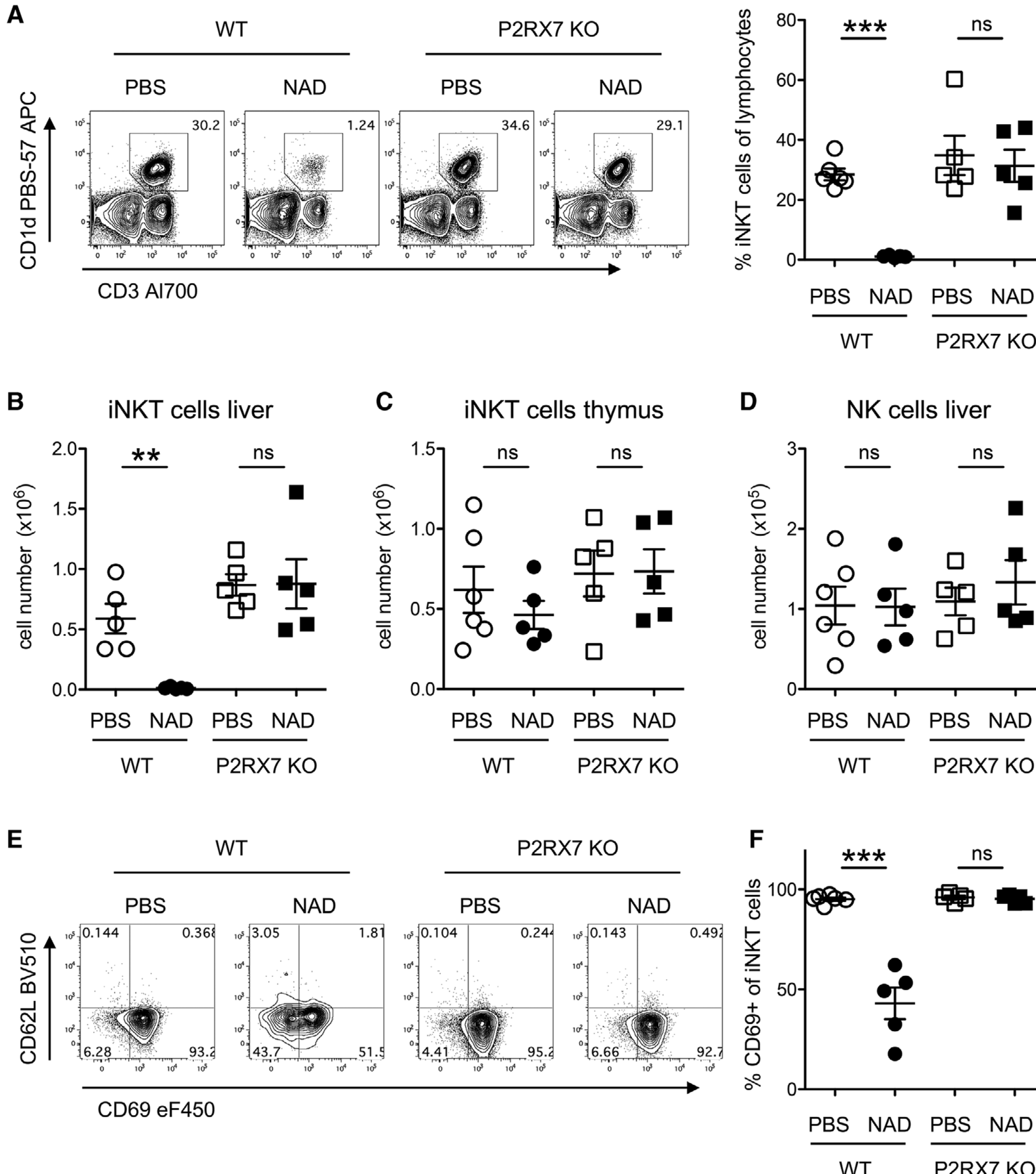

Figure 3. Extracellular NAD depletes iNKT cells in liver but not thymus. Naïve WT or P2RX7 KO mice were injected with NAD or PBS as control and iNKT cell analyzed by flow cytometry 24 h post injections. (A) Frequency of iNKT cells within live lymphocytes in the liver. (B and C) Absolute cell number of iNKT cells in (B) liver and (C) thymus as well as (D) NK cells in the liver. (E) Phenotype of iNKT cells in the liver. (F) Quantification of tissue-resident CD69^{+} iNKT cells in the liver. (A–F) $N = 4$–6 mice per group, data pooled from three independent experiments, mean ± SEM, unpaired *t*-test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

of the NAD application remained evident up to at least 36 days after NAD injection. At this time-point, NAD treated mice still contained fivefold less iNKT cells in the liver compared to non-treated controls. Other P2RX7 expressing cells, such as Treg that are depleted with similar efficiency by NAD treatment, were fully recovered after 14 days (Fig. 6C). The slow reconstitution of iNKT cells after depletion via P2RX7 suggests that tissue damage events induce long-lasting gaps in the local immune defense by depleting iNKT cells in tissues.

Discussion

iNKT cells are important for the homeostasis of the liver and their aberrant activation causes liver injury via destruction of hepatocytes. During tissue injury, different damage signals are released that regulate repair activities of parenchyma and immune cells. Here, we provide evidence for a feedback mechanism that acts on iNKT cells after induction of tissue damage. Triggering of the nucleotide receptor P2RX7 by extracellular NAD or ATP, which

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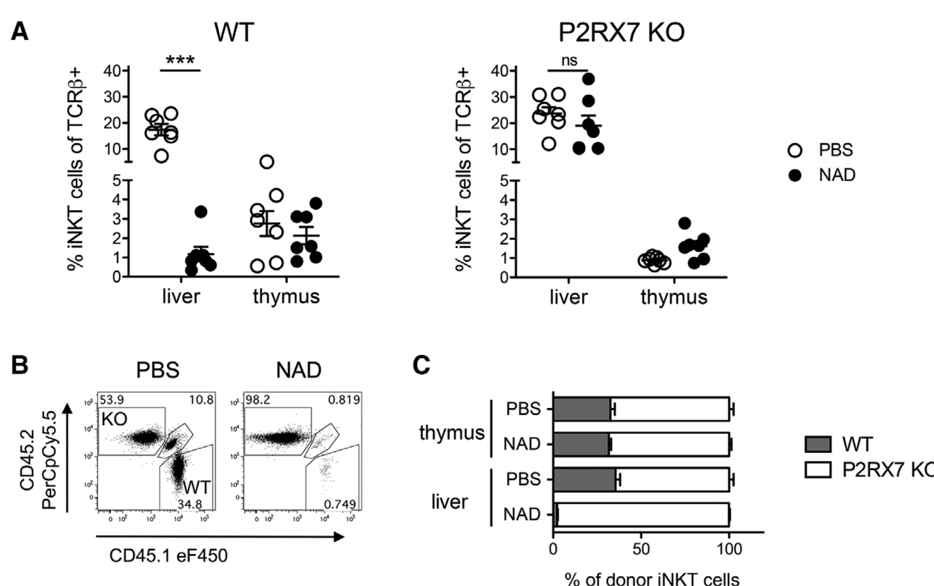

Figure 4. Intrinsic P2RX7 signaling renders iNKT cells susceptible to NAD induced depletion. Mixed BM chimeras of WT and P2RX7 KO cells were injected with NAD or PBS as control 12–16 weeks post reconstitution and 30+ days post infection with LCMV. Twenty-four hours post application of NAD or PBS, mice were injected with s+16a nanobodies and sacrificed 30 min later. iNKT cells were analyzed by flow cytometry. (A) Frequency of iNKT cells in WT and P2RX7 KO T cells. (B) Liver iNKT cells in the mixed BM chimeras with WT CD45.1^{+} cells and P2RX7 KO CD45.2^{+} (KO) cells. (C) Quantification of the contribution of WT and P2RX7 KO donor iNKT cells. (A–C) N = 7 mice per group, data pooled from two independent experiments, mean ± SEM, unpaired *t*-test, $^{***}p \leq 0.001$.

increase during tissue damage, lead to the induction of P2RX7-dependent cell death and consequently elimination of pro inflammatory iNKT cells.

We found that tissue-resident and circulating iNKT cells differ in the capacity to respond to local tissue damage signals. In contrast to circulating CD69- iNKT cells, nearly all tissue-resident iNKT cells in the spleen and liver co-expressed P2RX7 and the P2RX7-activating ectoenzyme ARTC2. In line with our findings, elevated P2RX7 expression has been previously reported on tissue-resident iNKT cells in the intestine [21]. Similar expression patterns of P2RX7 and ARTC2 in circulating versus resident cells are also observed for other lineages of adaptive and innate immune cells. Tissue-resident pathogen-specific CD8 T cells express higher levels of both markers compared to circulating memory T cells. Similarly, liver-resident innate lymphoid cells contain elevated levels of P2RX7 and ARTC2 mRNA, while expression is limited in the closely related circulating NK cells [13]. This expression pattern suggests that P2RX7 and ARTC2 are an integrated part of the tissue-residency program. Therefore, P2RX7 and ARTC2-driven elimination upon tissue injury may represent a general feature of resident lymphocytes during destructive insults.

Our results show that thymic iNKT cells largely lack P2RX7 and ARTC2 expression. This implies that this trait is acquired later in differentiation or specific environmental cues that drive the induction of P2RX7 and ARTC2 are missing in the thymus. It was previously demonstrated, that the functionally and transcriptionally distinct iNKT1, iNKT2, and iNKT17 cells also express P2RX7 and ARTC2 differentially [17], suggesting that there might be additional heterogeneity in the P2RX7-dependent regulation of iNKT cells.

Triggering of P2RX7 with NAD or high concentrations of ATP in vitro induces cell death of iNKT cells [22]. Consequently, cell death has also been observed in P2RX7 and ARTC2 expressing T cell populations such as Treg, tissue-resident memory CD8 T cells, and iNKT cells ex vivo after extraction of the cells from the tissues [13,25,26]. Accordingly, blockade of P2RX7 or the P2RX7-activating ectoenzyme ARTC2 increases the viability of isolated iNKT cells for in vitro and in vivo applications [17,26]. Our results demonstrate that activation of P2RX7 by exogenous NAD initiates similar downstream effects in vivo and causes an efficient and long-lasting depletion of iNKT cells in a wide range of tissues. P2RX7 was intrinsically required for the induction of iNKT cell death, demonstrating that P2RX7 expression on iNKT cells drives NAD-induced iNKT cell depletion. Furthermore, we observed a more pronounced depletion of CD69^{+} tissue-resident iNKT cells, correlating with high expression of P2RX7 and ARTC2 in this population. Injection of lower doses of NAD has been shown to lead to the depletion of intestinal iNKT cells, but no or less efficient iNKT cell depletion in the liver and spleen [21,22]. This suggests that the local NAD concentrations as well as expression levels of P2RX7 and ARTC2 determine the sensitivity of iNKT cells to nucleotide-mediated depletion at different locations. Additionally, the outcome of P2RX7 activation might depend on the cellular state of the iNKT cell. While NAD treatment in naïve mice limits the consecutive induction of iNKT cell mediated liver injury, NAD exacerbates liver damage when applied after iNKT cell activation [22]. Accordingly, additional signals might modulate the susceptibility of P2RX7-mediated regulation of iNKT cells.

Whether P2RX7 expressed by iNKT cells is engaged during steady state and impacts on iNKT cell generation or maintenance was previously unclear. Using mixed BM chimeras, we demonstrated that iNKT cell intrinsic P2RX7-signaling restricts iNKT cells in the liver under homeostatic conditions. A detrimental role of P2RX7 for the development of iNKT cells in the intestine has previously been reported [21]. The impact of P2RX7-mediated signaling on iNKT cell generation has also been shown indirectly. CD38 is an ectoenzyme that mediates the degradation of extracellular NAD and accordingly is a negative regulator of P2RX7 signaling. In line with this, mice lacking CD38 contain decreased numbers of iNKT cells [27]. Reducing P2RX7 activation by P2RX7 or ARTC2

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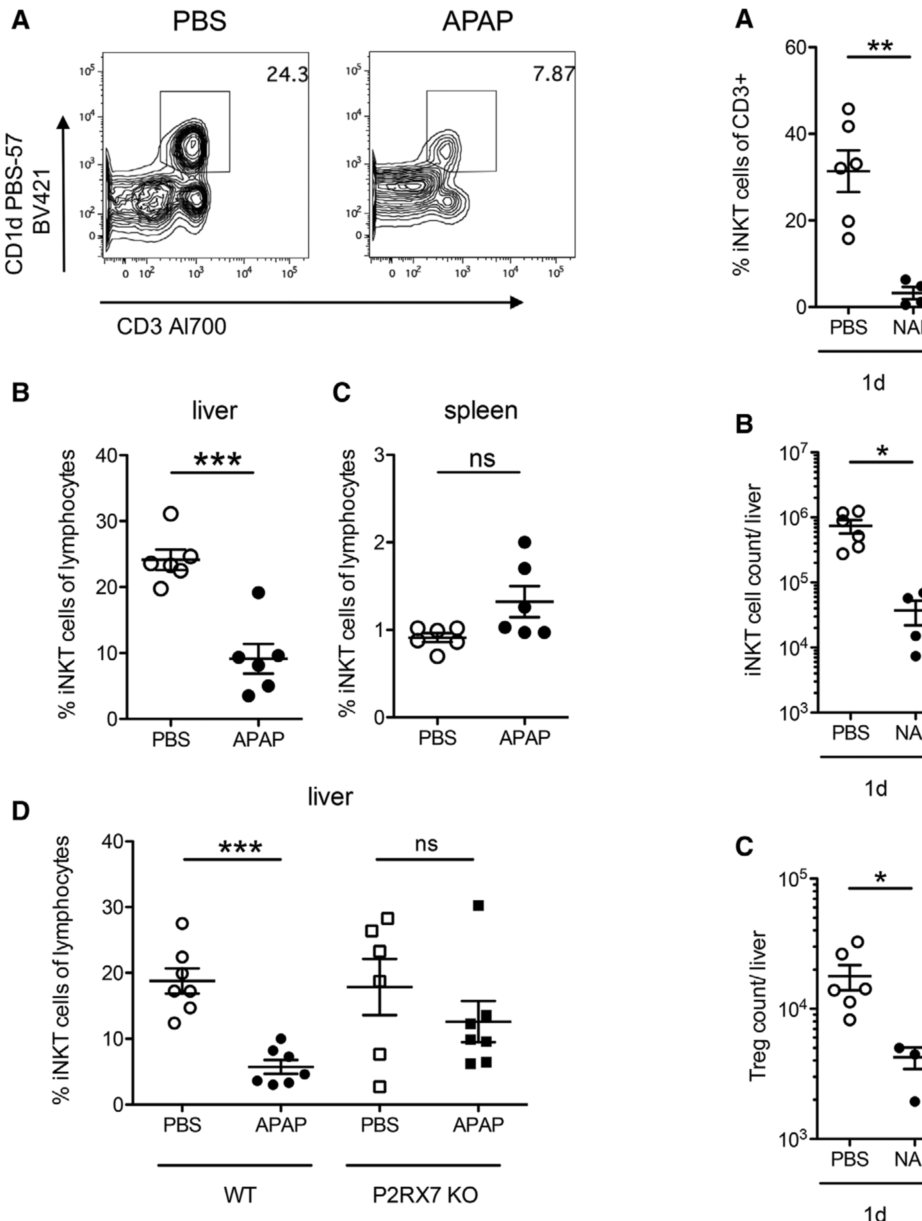

Figure 5. Tissue damage depletes iNKT cells locally in the injured organ. Mice were injected with acetaminophen (APAP) to induce liver injury or were treated with PBS as control. (A and B) Quantification of iNKT cells in the liver or (C) spleen of naïve WT mice 24 h after APAP/ PBS treatment. (D) Frequency of iNKT cells in WT or P2RX7 KO LCMV immune mice 24 h after treatment with PBS or APAP. (A–C) $N = 6$ per group, one experiment, (D) $N = 6$–7 mice per group, data pooled from two independent experiments, (B–D) unpaired *t*-test, mean ± SEM, $^{***}p \leq 0.001$.

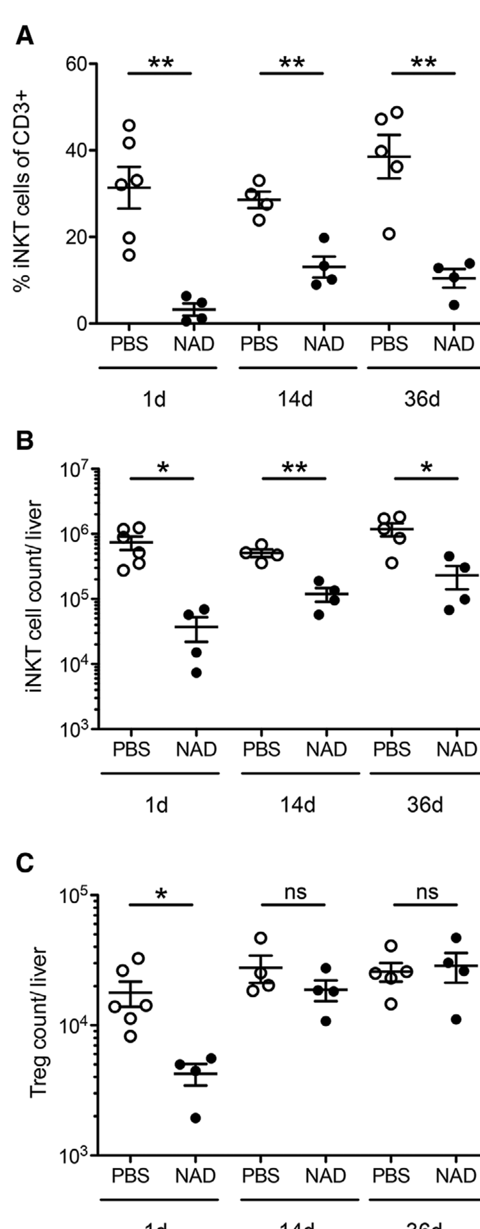

Figure 6. iNKT cells in the liver are slowly reconstituted after depletion. WT mice were injected with NAD or PBS as control. At different time points after injection iNKT cells and Treg in the liver were quantified by flow cytometry. (A) Frequency and (B) absolute number of iNKT cells, (C) absolute counts of Treg (FoxP3$^+$/Helios$^+$) in the liver. (A–C) $N = 4$–6 mice per group and time point, data pooled from two independent experiments, mean ± SEM, unpaired *t*-test, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

blockade normalizes iNKT cell numbers in CD38 KO mice, showing that the effect of CD38 is driven by P2RX7 [27,28]. On the other hand, the vitamin A metabolite retinoic acid is a positive regulator of P2RX7 signaling and has been shown to induce P2RX7 expression on T cells [29]. Dietary restrictions of vitamin A reduced P2RX7 expression on iNKT cells and increased iNKT cell numbers [21]. These data support our conclusion that also under steady state conditions local increases of extracellular NAD occur and restrict iNKT cell populations in a P2RX7-dependent manner. However, we observed that restriction of WT iNKT cells occurred selectively in the liver, but not in the spleen, while the level of P2RX7 and ARTC2 expression on iNKT was similar in both organs. This might suggest that P2RX7 triggering occurs more often in the liver than in the spleen. The liver is closely connected to the intestine via the portal vein and is therefore exposed to high concentrations of dietary and gut commensal products. These agents could trigger low level inflammatory events with local increases

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of extracellular NAD and ATP in the liver that could lead to iNKT cell depletion.

Toxin-, pathogen-, or trauma-induced tissue damage can lead to elevated levels of extracellular NAD and ATP and can enhance the regulatory impact of P2RX7. Indeed, we found that iNKT cells were strongly reduced in the liver after toxin-induced liver injury in a P2RX7-dependent manner. Increased apoptosis and decreased numbers of hepatic iNKT cells have also been observed in other models of liver injury, such as after chronic CCl_4 treatment and nonalcoholic fatty liver disease [30,31]. This suggests that events of trauma and tissue injury impact iNKT cell mediated protection and shape iNKT cell localization. In vivo imaging demonstrated that iNKT cells change their migratory behavior when encountering tissue injury. iNKT actively avoid regions of tissue damage and necrotic sites in the first 4 h after injury infliction, while other immune cells such as neutrophils readily enter [32]. iNKT cells migrated into necrotic areas only 48–72 h after injury infliction. Sensing of locally elevated concentrations of ATP or NAD might contribute to the repelling of iNKT cells from the site of injury.

After depletion, we observed a very slow recovery of iNKT cells, while other T-cell populations, such as Tregs, quickly replenished. This slow return of iNKT cells after depletion in vivo was also observed by others [33]. We have previously demonstrated that P2RX7 triggering also leads to depletion of tissue-resident CD8 T cells [13]. These results demonstrate that tissue injury induces a local depletion of different tissue-resident T cell populations and therefore might impair local immunity. Additionally, the faster recovery of Treg populations suggests that tissue damage locally shifts the immunologic balance from an inflammatory to a tolerogenic milieu to support non-inflammatory responses upon future insults. Activation of iNKT cells can cause liver damage and iNKT cells are implicated in the development of immunopathology for several types of liver injury [34]. We provide data that recognition of tissue damage via P2RX7 provides a feedback signal that regulates iNKT cells by inducing cell death after recognition of extracellular NAD. Exclusion of iNKT cells from locations of tissue damage could be a regulatory mechanism to prevent aberrant immunopathology and promote tissue regeneration.

Materials and methods

Mice

C57BL/6JRj mice were purchased from Janvier or bred in the animal facility of the NKI (Netherlands Cancer Institute). P2RX7 KO (B6.129P2-*P2rx7*^{tm1Gab}/J) and CD45.1 (Ptprc^{a}Pepc^{b}/BoyJ) were purchased from Jax mice and maintained in the animal facility of the Netherlands Cancer Institute (NKI). Mice were used when they were 8–16 weeks old. For the generation of mixed BM chimeras B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ × C57BL6/JRj (Ly5.1 × Ly5.2) recipient mice were irradiated (2 × 5 Gy) and reconstituted with intravenous (i.v.) transfer of 2×10^7 BM cells containing P2RX7 KO and CD45.1 cells in a 1:1 ratio. The injected BM samples were analyzed before transfer and contained 50.8, 44.4, and 45.5% of P2RX7 KO cells in the respective experiment. Mixed BM chimeras were used 8–16 weeks after reconstitution. Chimerism of lymphocytes was confirmed before usage. All mice were maintained under SPF conditions. Animal experiments were approved by the national and local authorities and conducted according to institutional (NKI) and national guidelines.

In vivo treatments

For triggering of P2RX7 in vivo, mice were injected i.v. with 60 mg NAD (Sigma) in PBS or NaCl adjusted to pH 7. Injection with PBS of NaCl was used as control. To induce liver injury male mice were fasted overnight, weighed, and 400 mg acetaminophen (Sigma) per kg body weight in PBS at a concentration of 15 mg/mL was injected intraperitoneally (i.p.). Equivalent volumes of PBS were injected as a control. For some experiments, mice were infected i.p. with 1×10^5 pfu LCMV Arm or 1×10^5 pfu rLCMV OVA [35] (kindly provided by Doron Merkler, University of Geneva). Detection of P2RX7 was facilitated by injecting mice i.p. or i.v. with 50 μg of ARTC2.2 blocking nanobody s+16a (Biolegend) 30 min before sacrifice [26].

Tissue preparation

After extraction organs were stored in cold PBS with 0.5% FCS. Single cell suspensions of spleen, thymus, and liver were generated by passing organs over 70 μm cell strainer with cold PBS with 2% FCS. The isolated lymphocytes were purified by density centrifugation on an isotone 60%/40% Percoll gradient (GE Healthcare). Contaminating erythrocytes were removed using RBC lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA).

Flow cytometry

Cells were incubated with antibodies at 4°C for 30 min in the dark. Antibodies were purchased from Biolegend, eBioscience, or Novus Biologicals. iNKT cells were identified by binding of CD1d tetramer loaded with the αGalCer analog PBS-57 (provided by the NIH Tetramer Facility). Exclusion of dead cells was performed with live/dead fixable near-IR dead cell stain kit (molecular probes). After staining, cells were analyzed directly or fixed with the eBioscience Transcription Factor Staining Buffer set according to manufacturer's specifications. Samples were acquired on an LSR Fortessa flow cytometer (BD) and data were analyzed using FlowJo (Tree star). Flow cytometric analysis were conducted in accordance to the guidelines used for immunologic studies [36].

ALAT measurement

Serum was prepared by centrifuging coagulated blood two times for 10 min at 2000 × *g*. ALAT was measured on the COBAS 8000

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by the clinical chemistry laboratory (Amsterdam UMC, location VUmc).

Statistical analysis

Statistical analysis was done using Graphpad Prism. The used test is indicated in the figure legends. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Data are displayed at mean ± SEM if not indicated otherwise. The N in the figure legends represents biological replicates.

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Abbreviations: **APAP**: N-acetyl-para-aminophenol/acetaminphen · **iNKT cell**: invariant natural killer T cell · **Treg**: regulatory T cells

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