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Hepatic recruitment of eosinophils and their protective function during acute liver injury

Long Xu[1,2,§], **Yang Yang**[1,§], **Yankai Wen**[1], **Jong-Min Jeong**[1], **Christoph Emontzpohl**[1], **Constance L. Atkins**[1], **Zhaoli Sun**[3], **Kyle L. Poulsen**[1], **David R. Hall**[4], **J. Steve Bynon**[4], **Bin Gao**[5], **William M. Lee**[6], **Jody Rule**[6], **Elizabeth A. Jacobsen**[7], **Hua Wang**[8,†], **Cynthia Ju**[1,†]

[1.] Department of Anesthesiology, McGovern Medical School, University of Texas Health Science Center at Houston, Houston, TX, USA.

[2.] School of Basic Medical Science, Anhui Medical University, Hefei, Anhui, China.

[3.] Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA.

[4.] Department of Surgery, McGovern Medical School, University of Texas Health Science Center at Houston, Houston, TX, USA.

[5.] Laboratory of Liver Disease, National Institute on Alcohol Abuse and Alcoholism, NIH, Bethesda, MD, USA.

[6.] Division of Digestive and Liver Diseases, Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, TX, USA.

[7.] Division of Allergy, Asthma and Clinical Immunology, Mayo Clinic Arizona, Scottsdale, AZ, USA

[8.] Department of Oncology, the First Affiliated Hospital of Anhui Medical University, Hefei, China.

Abstract

Background & Aims—Beyond the classical description of eosinophil functions in parasite infections and allergic diseases, emerging evidence supports a critical role of eosinophils in resolving inflammation and promoting tissue remodeling. However, the role of eosinophils in liver injury and the underlying mechanism of their recruitment into the liver remain unclear.

Methods—Hepatic eosinophils were detected and quantified using flow cytometry and immunohistochemical staining. Eosinophil-deficient (ΔdblGata1) mice were used to investigate the role of eosinophils in three models of acute liver injury. *In vivo* experiments using IL-33$^{-/-}$

†Correspondence: wanghua@ahmu.edu.cn (Hua Wang), changqing.ju@uth.tmc.edu (Cynthia Ju).
§These authors contributed equally to this work.

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mice and macrophage-depleted mice, as well as *in vitro* cultures of eosinophils and macrophages, were performed to interrogate the mechanism of eotaxin-2 (CCL24) production.

Results—Hepatic accumulation of eosinophils was observed in patients with acetaminophen (APAP)-induced liver failure, whereas few eosinophils were detectable in healthy liver tissues. In mice treated with APAP, CCl_4 or concanavalin A, eosinophils were recruited into the liver and played a profound protective role. Mice deficient of macrophages or IL-33 exhibited impaired hepatic eosinophil recruitment during acute liver injury. CCL24, but not CCL11, was increased after treatment of each hepatotoxin in an IL-33 and macrophage-dependent manner. *In vitro* experiments demonstrated that IL-33, through stimulating IL-4 release from eosinophils, promoted macrophages to produce CCL24.

Conclusions—This is the first study to demonstrate that hepatic recruitment of and protection by eosinophils occur commonly in various models of acute liver injury. The data further provide insights into the mechanism of hepatic eosinophil recruitment. The findings support further exploration of eosinophils as a therapeutic target to treat APAP-induced acute liver injury.

Lay summary

The current study unveils that eosinophils are recruited into the liver and play a protective function during acute liver injury caused by APAP overdose. The data demonstrate that IL-33-activated eosinophils trigger macrophages to release high amount of CCL24, which promotes hepatic eosinophil recruitment. The findings implicate the potential of using eosinophils as an effective cell-based therapy for treatment of APAP-induced acute liver injury.

Keywords

Eosinophils; acute liver injury; IL-33; macrophages; CCL24

Introduction

Acute liver injury caused by ischemia/reperfusion (IR), hepatitis virus, autoimmunity, or adverse drug reactions can lead to liver failure (1), for which the only treatment at present is liver transplantation. To expand on therapeutic options, it is important to better understand the underlying mechanisms of acute liver injury, especially endogenous protective pathways that can be harnessed to limit tissue damage.

Eosinophils are traditionally thought to be involved in host response against parasite infection and the pathogenesis of allergic diseases (2, 3). However, emerging evidence has ascribed eosinophils to functions of promoting tissue repair and resolving inflammation. It is reported that eosinophils protect against acute lung injury (4), promote resolution of airway inflammation (5), and airway remodeling (6). The understanding of eosinophils' involvement in liver injury is in its infancy. There are very few published studies and they report contradictory results (7–10). We recently reported that eosinophils were recruited into the liver after IR injury and played a profound hepato-protective function (7). However, it remains unknown how eosinophils are recruited into the liver and what role they play in other acute liver injury conditions.

Although very few eosinophils can be detected in the liver under homeostatic conditions (11), their accumulation in the liver has been observed in several pathological conditions, such as viral hepatitis, hepatic allograft rejection, and drug-induced liver injury (12–14). In addition, the molecular and cellular mechanism accounting for hepatic eosinophil recruitment has not been studied. Eotaxin-1 (CCL11) and eotaxin-2 (CCL24), through acting on CCR3, are the most potent eosinophil chemoattractants. For example, it is reported that the interaction between CCL24 and CCR3 plays a dominant role in eosinophil recruitment to the lung in OVA-induced experimental asthma model (15), whereas CCL11 is more important for basal recruitment of eosinophils to tissues (16). Moreover, both CCL11 and CCL24 contribute to eosinophil infiltration into various types of tumors (17). Interestingly, it has been shown that injection of exogenous IL-33 can significantly increase the number of eosinophils in the blood and various tissues (18, 19). The underlying mechanism, however, is not clear. One study showed that exogenous IL-33 administration could induce CCL24 up-regulation (20). Since IL-33 is an alarmin released during tissue injury, it is a plausible upstream signal in eosinophil recruitment.

In the current study using *in vitro* and mouse models, we uncovered the critical role of an eosinophil/macrophage cross-talk, through the IL-33/IL-4/CCL24 axis, in eosinophil recruitment into injured livers. Moreover, our data demonstrate a hepato-protective function of infiltrated eosinophils in APAP-induced acute liver injury.

Materials and methods

Human samples

Paraffin-embedded human liver biopsies of explants were obtained from patients diagnosed with APAP-induced liver failure and enrolled in the Acute Liver Failure Study Group (ALFSG). The study was approved by all the study site institutional review boards, including UT Southwestern (STU 062010–126). The bio-repository is maintained by the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK). All patients and their legally authorized representatives had given informed consent for the release of information and use of available tissue samples. Some of the clinical data are shown in supplemental Table 1. Paraffin-embedded human liver biopsies from healthy individuals were obtained through the Liver Tissue Cell Distribution System at the University of Minnesota, which is funded by National Institutes of Health (NIH contract #HHSN276201200017C).

Animals

Breeders of IL-33$^{fl/fl}$-eGFP reporter (cat#030619), Balb/c (cat#000651), and ΔdblGata1 mice (cat#005653) were purchased from the Jackson laboratory. Breeders of IL-33$^{-/-}$ mice (on C57BL/6N genetic background) were obtained from J. Brown (University of Colorado Anschutz Medical Campus). C57BL/6N from Taconic Biosciences were used as WT control mice. All colonies were established and maintained in the UTHealth animal facility. All experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee at UTHealth.

Experimental Method

Eight- to ten-weeks old mice were injected with acetaminophen (APAP, i.p.), carbon tetrachloride (CCl_4, i.p.), or concanavalin A (ConA, i.v.) to induce acute liver injury. Flow cytometric and immunohistochemistry (IHC) analyses were performed to detect hepatic eosinophils. The degrees of liver injuries were determined by measurements of serum ALT levels and evaluation of liver histopathology. Adoptive transfer of bone marrow-derived eosinophils to ΔdblGata1 mice was performed to investigate the hepato-protective function of these cells. The underlying molecular mechanism of hepatic recruitment of eosinophils was explored by multiple *in vivo* and *in vitro* approaches. More detailed methods can be found in the Supplemental Information.

Results

Eosinophils are recruited to the liver after acute injury

Overdose of APAP is a major cause of acute liver failure in the clinic. Although N-acetylcysteine (NAC) can reduce APAP-induced liver injury (AILI), its effectiveness drops sharply around 8h after APAP overdose. Thus, identifying new therapeutic strategies harnessing endogenous protective mechanisms remains an active area of research. To examine if eosinophils are recruited to the liver in patients with APAP-induced liver failure, we obtained liver explant samples and performed IHC staining with an antibody specific for eosinophil peroxidase (EPX). Compared with liver biopsies from healthy individuals, those from patients with AILI displayed a dramatic increase in the number of eosinophils (Fig. 1A, B). Similarly, in a mouse model of AILI, both the percentage and the absolute number of eosinophils in the liver were increased after APAP challenge and peaked at 48h (Fig. 1C). Eosinophils were identified as $CD45^{+}SSC^{hi}SiglecF^{+}CCR3^{+}$ cells by flow cytometric analyses, and as cells expressing major basic protein (MBP) by IHC. (Fig. 1D, E). We observed similar recruitment of eosinophils to the liver after APAP treatment in male and female mice (Fig. 1 and supplemental Fig. 1), suggesting that this phenomenon was not sex-dependent. Moreover, this phenomenon was not limited to AILI, as our data showed that after CCl_4- or ConA-induced acute liver injury, eosinophils were also recruited to the liver (supplemental Fig. 2). These data are the first to reveal that eosinophils are recruited into the liver during AILI and in other animal models of acute liver injury.

Haptic eosinophils protect against acute liver injury

To investigate the role of recruited eosinophils in acute liver injury, we treated WT and eosinophil-deficient mice (ΔdblGata1) with APAP, CCl_4 or ConA. In all three cases, ΔdblGata1 mice developed worsened liver injury at 24h compared with WT mice, as demonstrated by increased serum levels of alanine aminotransferase (ALT) and areas of hepatocyte necrosis (Fig. 2A–C and supplemental Fig. 3). We focused our study on AILI, as it is the most clinically relevant among the three models. To further substantiate the protective function of eosinophils, we adoptively transferred WT bone marrow-derived eosinophils (bmEos) to ΔdblGATA1 mice immediately prior to APAP treatment. The data showed a dramatic reduction of serum ALT levels and the extents of liver necrosis (Fig. 2A–C). Even when the bmEos transfer was delayed to 8h or 12h after APAP treatment, significant attenuation of AILI in ΔdblGATA1 recipient mice was still evident (Fig. 2D–

F). Repeating the adoptive transfer experiments with varying numbers of bmEos revealed that 5 million cells were sufficient to achieve a significant protection against liver injury (supplemental Fig.4). These data demonstrated a profound hepato-protective effect of eosinophils during acute liver injury.

Macrophages play an essential role in eosinophil recruitment after acute liver injury

To understand how eosinophils are recruited into the liver after injury, we set out to determine which cell type(s) are important in this process. It has been reported that platelets are essential for leukocyte recruitment into inflamed tissues (21). However, when we depleted platelets using an anti-CD41 antibody at 24h prior to APAP challenge, we did not observe any effect on the number of eosinophils recruited to the liver (supplemental Fig. 5A, B). Studies of acute peritonitis induced by zymosan or thioglycollate have demonstrated that neutrophils are recruited first at around 4–6h, which is then followed by eosinophil infiltration (22, 23). In many models of tissue inflammation, including AILI, neutrophils and monocytes are among the first leukocytes recruited (24, 25). To investigate whether neutrophils or macrophages are required for eosinophil recruitment, we depleted neutrophils using anti-Ly6G Ab or macrophages using liposome-entrapped clodronate (CLD) at 24h before APAP treatment. Neutrophil depletion did not affect the numbers of eosinophils recruited into the liver (supplemental Fig. 5C, D). However, compared with empty liposome-treated control mice, CLD-treated, macrophage-depleted mice had much fewer eosinophils in the liver after APAP treatment (Supplemental Fig. 6A, B). Likewise, macrophage-depletion also resulted in impaired eosinophil recruitment to the liver in mice treated with CCl_4 or ConA (Supplemental Fig. 6C–F). These data demonstrated that macrophages play a crucial role in eosinophil recruitment during acute liver injury.

IL-33 facilitates hepatic eosinophil recruitment in a macrophage-dependent manner

To understand the mechanism by which macrophages promote eosinophil recruitment, we hypothesized that damage associated molecular patterns (DAMPs) might be important in activating macrophages to recruit eosinophils. High mobility group box 1(HMGB1) and IL-1α are widely reported as DAMPs in models of sterile tissue injury, including AILI. More recently, IL-33 has been identified as another DAMP or alarmin released during tissue injury (26–28). To examine if any of these DAMPs triggers eosinophil recruitment to the liver, we injected i.v. HMGB1, IL-1α, and IL-33 to naive WT mice and measured the number of eosinophils in the liver after 24h. As shown in Fig. 3A, B, exogenous IL-33, but not IL-1α or HMGB1 induced eosinophil recruitment to the liver. We next hypothesized that if IL-33 played a role in eosinophil recruitment during AILI, it should be released after APAP challenge. Indeed, our data showed a significant increase in serum levels of IL-33 after APAP treatment (Fig. 3C). To further confirm that IL-33 was necessary for eosinophil recruitment, we subjected IL-$33^{-/-}$ mice to APAP-, CCl_4- and ConA-induced acute liver injury. Compared with WT mice, IL-$33^{-/-}$ mice displayed dramatically reduced eosinophil recruitment into the liver after injury (Fig. 3D–F and supplemental Fig. 7). These data provide strong evidence that IL-33 is essential in inducing eosinophil recruitment after acute liver injury.

To determine if IL-33-mediated eosinophil recruitment is dependent on macrophages, we depleted macrophages in naive mice at 24h before IL-33 treatment. As shown in Fig. 4B, IL-33-induced hepatic recruitment of eosinophils was abrogated when macrophages were depleted. Furthermore, we found that injecting exogenous IL-33 could restore hepatic eosinophil recruitment in IL-33$^{-/-}$ mice, but not when macrophages were depleted (supplemental Fig. 8).

Although the release of IL-33 has been reported in animal models of liver injury and in patients with liver diseases (29, 30), the cellular source of IL-33 release has not been identified. To determine which cells release IL-33 during AILI, we treated IL-33-GFP reporter mice with APAP and isolated hepatocytes and liver non-parenchymal cells (NPCs) after 24h. Flow cytometric analyses identified two IL-33-positive cell types that could potentially release IL-33. Liver sinusoidal endothelial cells (LSECs, CD45^{-}CD146^{+}) accounted for about 65% of IL-33^{+} cells, and the remaining were CD45^{+}F4/80^{+} macrophages (Fig. 4A). To further determine which of the two cell types contributed to IL-33 release, we depleted macrophages and found no effect on the serum levels of IL-33 (Fig. 4B). The data implied that LSECs are more likely the cellular source of IL-33 release.

IL-33 plays a role in macrophage production of CCL24

To address the question of what macrophages produce to recruit eosinophils, we investigated CCL11 and CCL24, which are widely reported eosinophil-specific chemoattractants (31). We observed an increase in mRNA but not protein levels of CCL11 after CCl_4 treatment; however, neither mRNA nor protein levels of CCL11 were altered after APAP- or ConA-treatment (Fig. 5A, B and supplemental Fig. 9A–D). In contrast, both hepatic mRNA and serum protein levels of CCL24 were markedly increased after treatment of each hepatotoxin (Fig. 5C, D and supplemental Fig. 9E–H). To determine if CCL24 was produced by macrophages, we measured the serum level of CCL24 in control and macrophage-depleted mice. The data showed that macrophage-depletion dramatically reduced level of CCL24 in all three models of acute liver injury (Fig. 5D, and supplemental Fig. 9F, H), indicating an important role of macrophages in CCL24 production.

Since our data suggested that IL-33-induced macrophage activation plays a critical role in eosinophil recruitment, we tested if IL-33 was important in triggering CCL24 expression by macrophages. We found that the serum levels of CCL24 were significantly lower in APAP-treated IL-33$^{-/-}$ mice than WT mice (Fig. 5E). Moreover, exogenous IL-33 induced CCL24 production in naive mice, but not in macrophage-depleted mice (Fig. 5F). These data suggested that IL-33, released during acute liver injury, plays an important role in promoting CCL24 production by macrophages, thereby mediating eosinophil recruitment into the liver.

IL-33, through activating eosinophils, indirectly promotes CCL24 production by macrophages.

To investigate whether IL-33 directly activates macrophages to produce CCL24, we treated bone marrow-derived macrophages (BMDM) *in vitro* with IL-33. As shown in Fig. 6A, IL-33 did not directly induce CCL24 release, suggesting that another cell type may be involved. Because IL-33 functions through its receptor, suppression of tumorigenicity 2

(ST2), we set out to identify ST2-expressing cells in the liver. The data revealed that almost all $ST2^+$ cells were eosinophils (Fig. 6B). To examine if eosinophils are involved in inducing CCL24 production by macrophages, we isolated eosinophils from the livers of APAP-treated mice by using magnetic-activated cell sorting (MACS). We then cultured purified eosinophils or the remaining cell mixture (not containing eosinophils) with BMDM in the presence of IL-33. The data showed that eosinophils were required for IL-33-induced CCL24 expression by macrophages (Fig. 6C).

To further elucidate the mechanism, we cultured BMDM with conditional media (CM) collected from IL-33-stimulated eosinophils and found induction of CCL24 (Fig. 6D). The data suggested that soluble factor(s) released from IL-33-activated eosinophils were important. We and others have shown that IL-33 stimulates eosinophils to release IL-4 and IL-13 (7, 32). To determine whether IL-4 and/or IL-13 played a critical role in CCL24 production by macrophages, we added anti-IL-4 or anti-IL-13 neutralizing antibody to the CM. As shown in Fig. 6E, neutralizing IL-4, but not IL-13, abrogated CCL24 production by macrophages. Interestingly, we found that IL-4, but not IL-13, was depleted from the CM after culturing BMDM for 24h (Fig. 6F), suggesting that IL-4 was consumed by macrophages. Furthermore, treatment of BMDM with the same concentration IL-4 or IL-13 showed that IL-4 induced a much stronger response than IL-13 in terms of Stat6 activation (Fig. 6G). Taken together, these data demonstrated that IL-4, but not IL-13, released by eosinophils after IL-33 stimulation played a key role in CCL24 production by macrophages.

Discussion

The current study demonstrates that eosinophils are recruited to the liver during acute injury and play a protective role in multiple modes of liver injury. IL-33, which is up-regulated and released by LSEC during acute liver injury, acts on eosinophils and initiates the positive feedback loop involving a cross-talk between eosinophils and macrophages. IL-33 activates eosinophils to release IL-4. IL-4 plays a critical role in inducing macrophages to produce CCL24, which in turn recruits more eosinophils. Furthermore, our data also show that the recruited eosinophils confer hepato-protection against liver injury caused by APAP overdose.

IL-33 expression in injured livers has been investigated by IHC staining. It was found to be mainly expressed in endothelial cells in CCl_4-induced liver injury, but predominantly in hepatocytes in ConA-induced hepatitis (33). In an LPS-induced inflammation model, IL-33-positive staining demarcates the liver sinusoids, implying endothelial cell expression of IL-33 (34). It is known that IL-33 is released as an alarmin during tissue injury (28). We observed a significant elevation of IL-33 in the serum of mice after APAP treatment, similar to our previous finding of IL-33 release during hepatic IR injury (7). However, the cellular source of IL-33 release was unknown. The current study using IL-33-GFP reporter mouse and macrophage depletion approach identified LSEC as a main source of IL-33 release during APAP-induced liver injury.

Two recent genome-wide association studies have highlighted a correlation between IL-33 and the number of circulating eosinophils (35, 36). One study discovered certain sequence

variants in IL-33 strongly correlating with blood eosinophil numbers and the risk of developing asthma (36). Another study found that a loss of function mutation of IL-33 was associated with reduced blood eosinophil numbers (35). Studies in mice reported that multiple injections of IL-33 over a 7-day period could significantly induce eosinophil accumulation in various tissues, such as the spleen and bone marrow (18, 19). IL-33-induced eosinophilia was also found in the lung when IL-33 was administered intranasally for 3 days (37). These studies provide direct evidence that IL-33 induces eosinophilia. However, because the IL-33 treatments were extended for several days in these studies, it was unclear whether IL-33-induced eosinophilia was due to eosinophil recruitment into the tissues or enhanced eosinophil differentiation from progenitor cells in the bone marrow. Our finding that eosinophils accumulate within 24h after IL-33 injections strongly suggests that IL-33 drives eosinophil recruitment to the liver.

The notion that IL-33 might act directly as an eosinophil chemoattractant has been negated by an *in vitro* study of eosinophil migration in response to IL-33 (38). Our data are consistent with an indirect action of IL-33 on eosinophil recruitment, such that IL-33-activated eosinophils, through releasing IL-4, promote CCL24 production by macrophages to recruit more eosinophils in a feed-forward loop. Although the role of IL-4 in promoting macrophages to produce CCL24 has been reported (39–41), our study is the first to link IL-33-activated eosinophils with IL-4 release and CCL24 production by macrophages. Interestingly, a previous study showed that although IL-33 could not induce eosinophil recruitment into the lung of $ST2^{-/-}$ mice, adoptive transfer of WT eosinophils to $ST2^{-/-}$ mice actually triggered the infiltration of $ST2^{-/-}$ eosinophils into the lung. Although the study did not define the mechanism, our findings would suggest that the transferred WT eosinophils responded to IL-33, thereby promoting macrophages to release CCL24 and recruiting eosinophils regardless of ST2 expression (37).

Our study highlights a cross-talk interaction between eosinophils and macrophages that plays a key role in eosinophil recruitment to sterile inflamed liver. This interaction is important and applies to several distinct biological conditions. For example, eosinophil-derived IL-4 or IL-13 promotes alternative activation of macrophages (M2) in adipose tissues, thereby maintaining glucose homeostasis (42). IL-4-activated macrophages promote eosinophilia, important in anti-helminth infection in mice (43). A more recent study documented a cooperative interaction between eosinophils and tissue-resident macrophages in cutaneous leishmaniasis, in which eosinophils were required to maintain a M2-like phenotype of macrophages and that the macrophages promoted eosinophil recruitment to the dermis (39).

There have been very few studies of the role of eosinophils in acute liver injury and the results are contradictory (7–10, 44). For example, one study of ConA-induced hepatitis showed that eosinophil-depletion by anti-CCR3 antibody reduced ConA-induced injury, implicating a pathological role of eosinophils (10). This study used repeated high doses of anti-CCR3 antibody, raising the question whether in addition to eosinophils, other $CCR3^{+}$ cells that may be affected. Another study showed that $ST2^{-/-}$ mice developed more severe ConA-induced hepatitis and pretreatment with IL-33 prevented liver injury in WT mice (44). Our finding that IL-33 induces hepatic eosinophil recruitment suggests that the

protective effect of IL-33 may be attributed, in part, to eosinophils. Moreover, we found that eosinophil-deficient mice developed exacerbated liver injury caused by APAP, CCl_4, or ConA treatment. These data together with our previous observation in hepatic IR injury strongly support a protective role of eosinophils in acute liver injury (7).

In summary, the present study for the first time demonstrates hepatic recruitment of eosinophils after APAP overdose and a profound hepato-protective function of these cells against AILI. The data also provide novel insights into the molecular and cellular mechanisms involved in hepatic eosinophil accumulation. The findings suggest that strategies to promote eosinophil recruitment and/or adoptive transfer of eosinophils may represent a novel cell-based therapeutic approach to treat patients with APAP overdose-induced liver failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement:

The data that support the findings of this study are available on request from the corresponding author.

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Highlights

- Eosinophils are recruited to the liver in multiple models of acute injury and play a protective role.
- A cross-talk interaction between eosinophils and macrophages plays an important role in eosinophil recruitment to the injured liver in a feed-forward loop.
- IL-33 is selectively released from liver sinusoidal endothelial cells during APAP-induced liver injury.
- IL-33, through stimulating IL-4 release from eosinophils, promotes macrophages to produce CCL24.

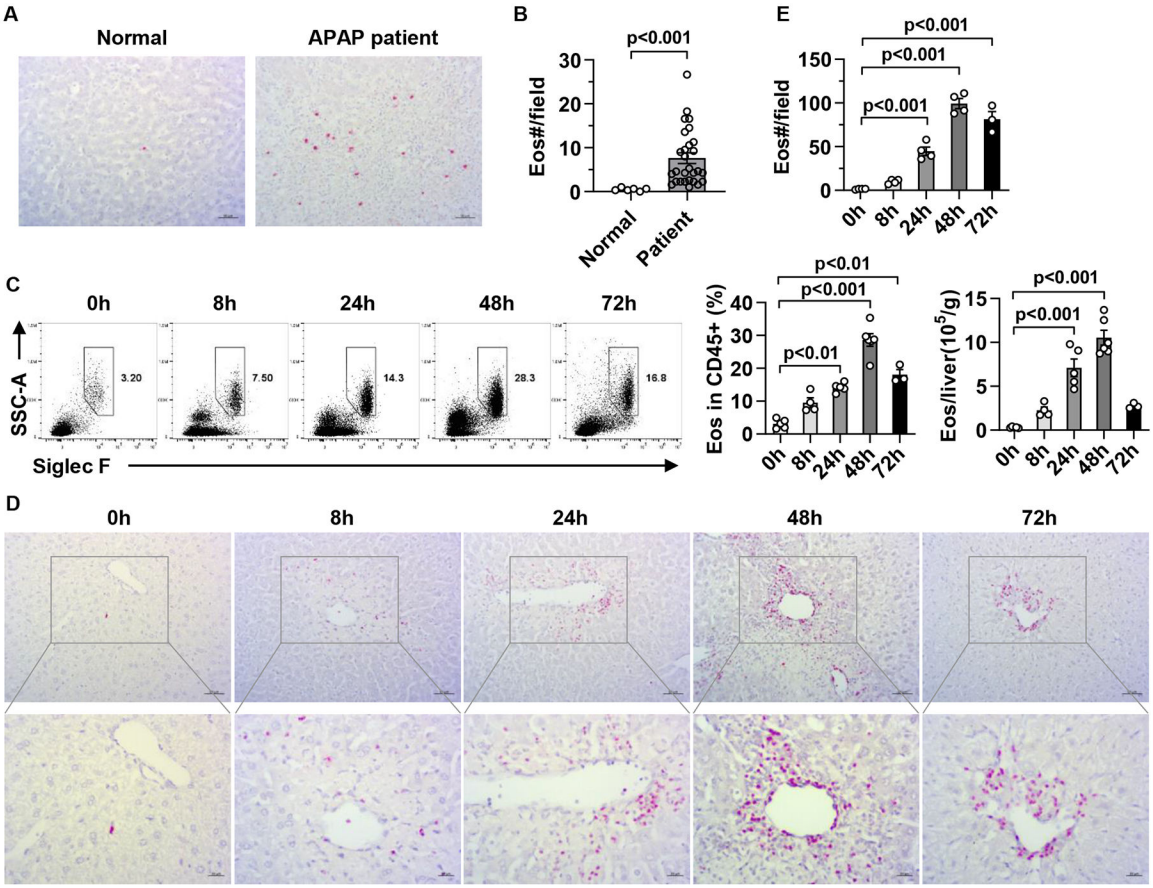

Fig.1. Eosinophils accumulate in the liver after APAP treatment.

(**A, B**) Eosinophils in liver samples from individuals with normal livers (Normal, n=6) and patients with APAP-induced liver failure (APAP patient, n=28) were detected by IHC staining using anti-human EPX antibody (scale bar, 50μm). The numbers of eosinophils per field (776×582 pixels) were quantified. (**C-E**) Male C57BL/6 mice were injected i.p. with APAP. (**C**) The percentage and absolute number of eosinophils in the liver were measured after APAP treatment by flow cytometry (n=3–6/group). Eosinophils were identified as $CD45^+CD11b^+SSC^{hi}$Siglec F^+CCR3^+ cell. (**D, E**) Eosinophils were detected by IHC staining using anti-mouse MBP antibody and quantified (n= 3–4/group, scale bar, 50μm). Two-tailed unpaired Student's t test was performed in B. One-way ANOVA with Tukey post hoc test was performed in C and E.

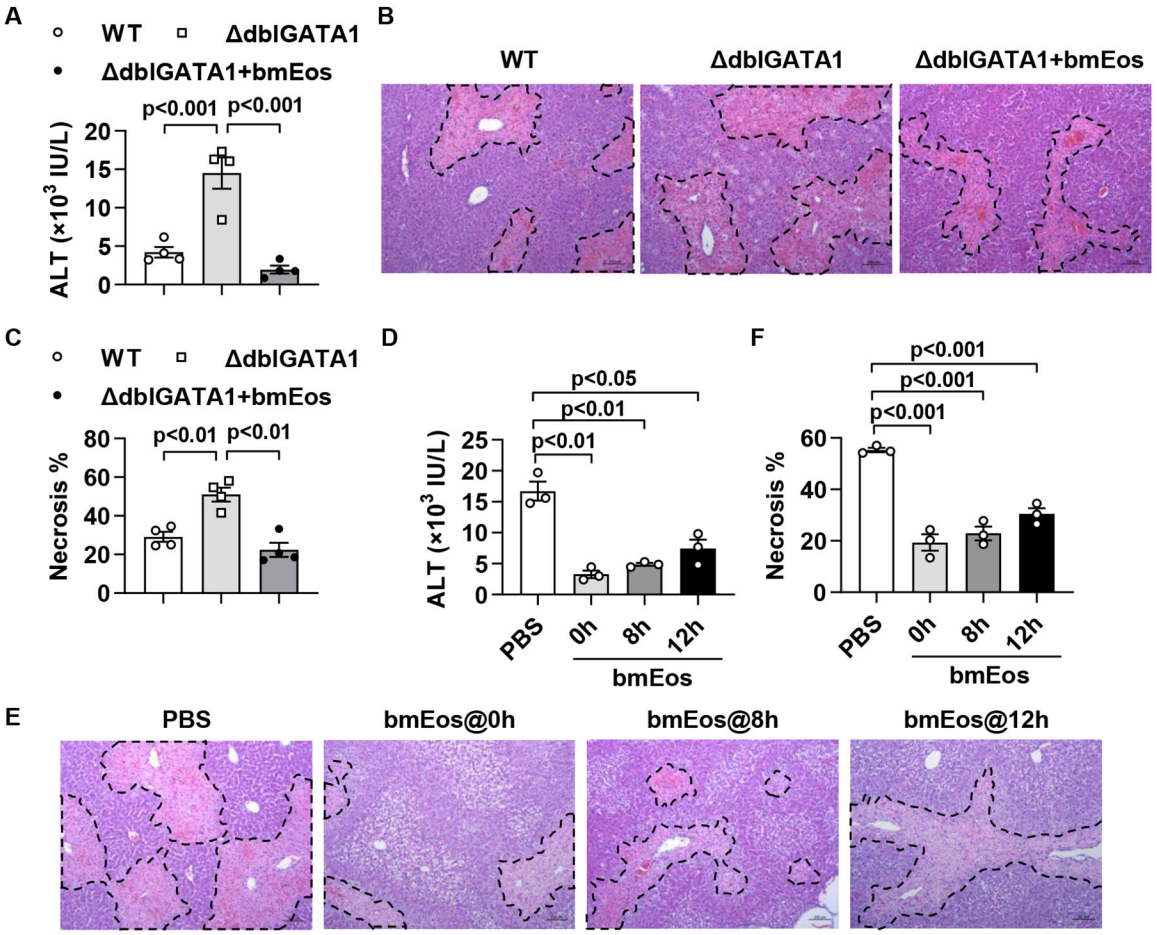

Fig. 2. Eosinophils protect against APAP-induced liver injury.

(**A** to **C**) WT Balb/c and ΔdblGATA1 mice were i.v. injected with PBS or WT bmEos (7.5×10^6) immediately before treatment with APAP (n=4/group). Serum ALT levels were measured, and liver necrosis (scale bars, 100μm) was evaluated and quantified at 24h after APAP treatment. (**D** to **F**) ΔdblGATA1 mice were i.v. injected with PBS or WT bmEos (7.5×10^6) at 0h, 8h, or 12h after APAP treatment (n=3/group). Serum levels of ALT were measured and liver necrosis (scale bars, 100μm) was examined at 24h after APAP treatment. One-way ANOVA with Tukey post hoc test was performed in A, C, D, and F.

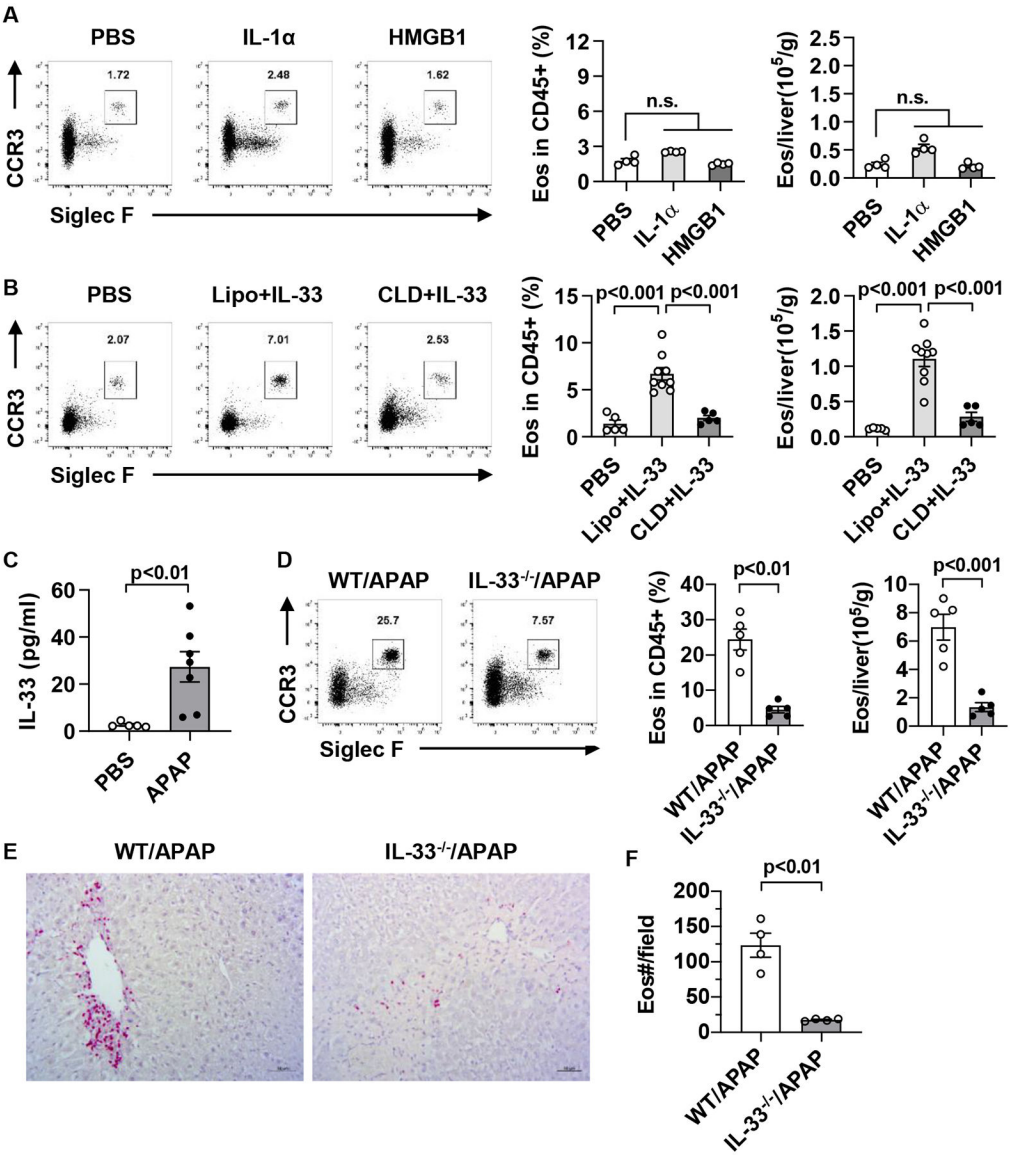

Fig. 3. IL-33 promotes eosinophil recruitment to the liver.

(**A**) C57BL/6 mice were injected i.v. with PBS, IL-1α, or HMGB1 twice on one day spaced out by 8h (0.5 μg/mouse each dose). The percentage and number of hepatic eosinophils were measured by flow cytometry at 24h after first dose (n=4/group). (**B**) C57BL/6 mice were injected i.v. with PBS (n=5) or IL-33 (n=9) twice on one day spaced out by 8h (0.5 μg/mouse each dose). Some IL-33-treated mice were injected with CLD (n=5) to deplete macrophages or empty liposomes (Lipo) as control (n=9) at 24h prior to the initial IL-33 treatment. All mice were sacrificed at 24h after the first dose of IL-33 for the measurement of the percentage and number of hepatic eosinophils. (**C**) C57BL/6 mice were injected i.p. with PBS (n=5) or APAP (n=7). After 24h, serum levels of IL-33 were measured by ELISA. (**D**, **E**) WT and IL-33$^{-/-}$ mice were injected i.p. with APAP. After 48h, eosinophils in the liver were measured using flow cytometry (**D**, n=5/group) and IHC staining (**E-F**,

n=4/group, scale bar, 50μm). Two-tailed unpaired Student's t test was performed in A to D, and F. n.s. indicates no statistical significance.

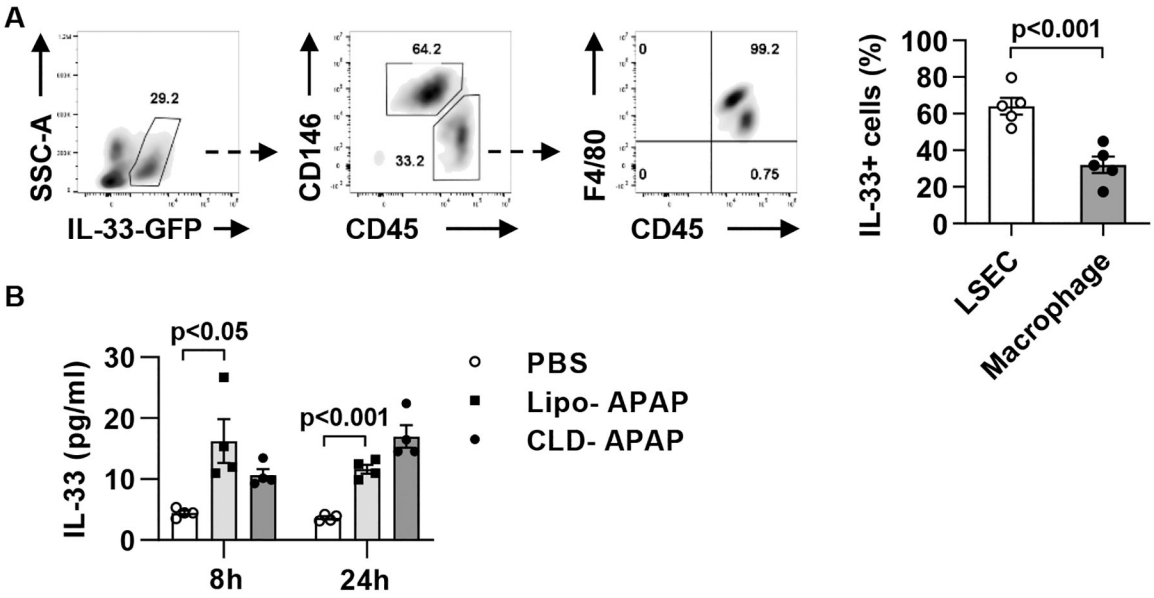

Fig. 4. Liver sinusoidal endothelial cells are the main source of IL-33 release during AILI.

(**A**) IL-33-GFP reporter mice were injected i.p. with APAP (n=5). After 24h, liver non-parenchymal cell were isolated and analyzed for GFP^+ cells by flow cytometry. (**B**) WT C57BL/6 mice were injected i.p. with PBS (n=3) or APAP (n=8). The APAP-treated mice were divided into two groups (n=4/group) treated with empty liposomes (Lipo) or CLD to deplete macrophages 24h prior to APAP challenge. All mice were sacrificed at 24h after APAP treatment and serum levels of IL-33 were measured by ELISA. Two-tailed unpaired Student's t test was performed in A and B.

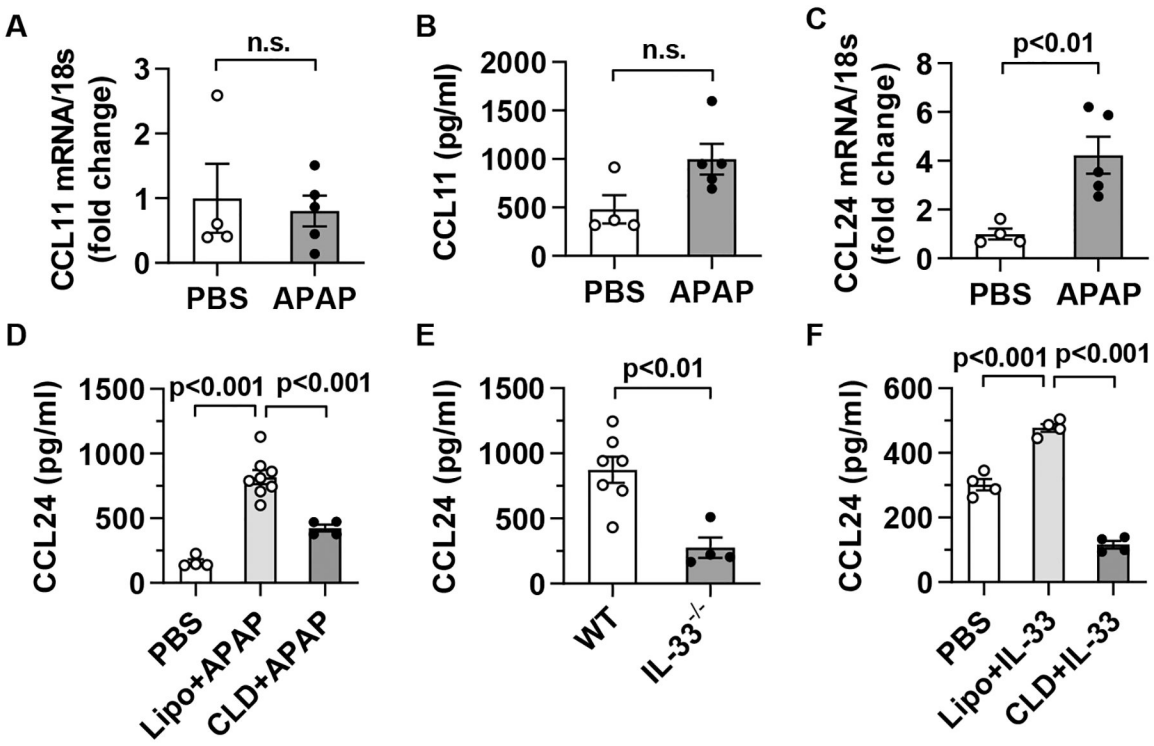

Fig. 5. CCL24, but not CCL11, is up-regulated in a macrophage-dependent manner during AILI.

(**A**-**D**) C57BL/6 mice were injected i.p. with PBS or APAP (n=4–5/group). After 24h, CCL11 and CCL24 mRNA expression in liver tissues (**A, C**) and protein levels in sera (**B, D**) were measured by qPCR and ELISA, respectively. Some mice were treated with CLD to deplete macrophages at 24h prior to APAP challenge. (**E**) WT (n=7) and IL-33$^{-/-}$ (n=4) mice were treated with APAP, and after 24h serum levels of CCL24 were measured. (**F**) C57BL/6 mice were treated with PBS or IL-33 as described in Fig. 3B. Some IL-33-treated mice were injected with CLD to deplete macrophages following the same protocol described in Fig. 3B. All mice were sacrificed at 24h after the first dose of IL-33 and serum levels of CCL24 were measured (n=4/group). Two-tailed unpaired Student's t test was performed in A to F. n.s. indicates no statistical significance.

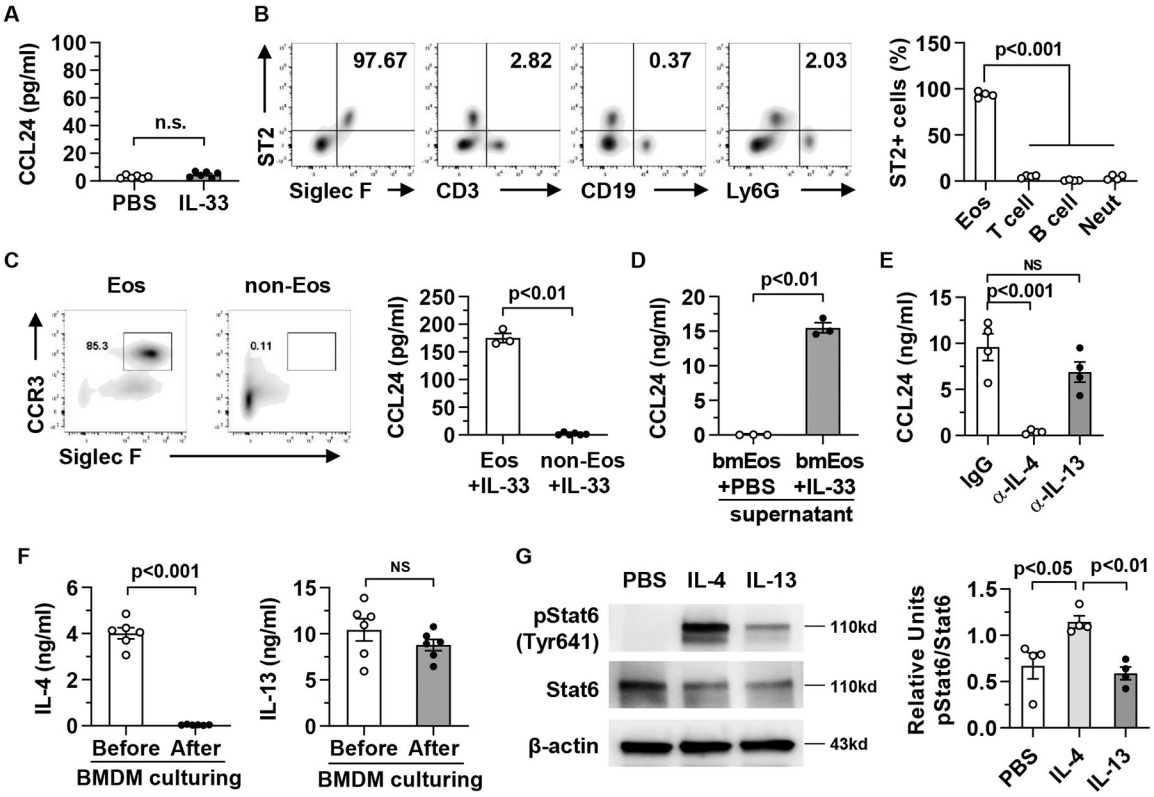

Fig. 6. IL-33-stimulated eosinophils, through release of IL-4, induce CCL24 production by macrophages

(**A**) BMDMs (4×10^6 cells/ml per well), generated from C57BL/6 mice, were stimulated with mouse recombinant IL-33 (20 ng/ml). After 24h, CCL24 protein levels in the supernatants were measured by ELISA. (**B**) Liver mononuclear cells (MNCs) were isolated from WT mice (n=4) at 24h after APAP treatment and analyzed for ST2 expression by flow cytometry. (**C**) From liver MNCs, isolated at 24h after APAP treatment, eosinophils (Eos) were purified by Siglec F-positive selection using MACS and the purity is around 85%. The Siglec F-negative portion of cell mixture (non-Eos) was also collected. Eos and non-Eos (1×10^6 cells/ml per well) were cultured together with BMDMs (4×10^6 cells/well) in presence of IL-33. After 24h co-culturing, CCL24 protein levels in the supernatants were measured by ELISA. (**D**) Bone marrow-derived eosinophils (bmEos, 4×10^6 cells/ml per well) were stimulated with PBS or IL-33 (20 ng/ml). After 24h, the conditioned media (CM) were collected and used to culture BMDMs (4×10^6 cells/ml per well) for another 24h. CCL24 protein levels in the BMDM culture supernatants were measured by ELISA. (**E**) The culturing system was set up as described in D with the addition of anti-IL-4 antibody or anti-IL-13 antibody in the CM collected from IL-33-stimulated bmEos. (**F**) The protein levels of IL-4 and IL-13 in the CM from IL-33-stimulated bmEos were measured before and after culturing of BMDMs. (**G**). BMDMs was treated with PBS, mouse recombinant IL-4 or IL-13 for 1h. Expression levels of phosphorylated-Stat6, Stat6, and β-actin were measured and quantified (n=4/group). Two-tailed unpaired Student's t test was performed in A to D,

and F. One-way ANOVA with Tukey post hoc test was performed in E and G. n.s. indicates no statistical significance.