Homeostasis of IL-15 dependent lymphocyte subsets in the liver

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Abstract

IL-15 is a member of the gamma chain family of cytokines (γ_c – CD132). The IL-15 receptor (IL-15R) complex consists of 3 subunits: the ligand-binding IL-15Rα chain (CD215), the β chain (CD122; also used by IL-2), and the common γ chain. The biological activities of IL-15 are mostly mediated by the IL-15:IL-15Rα complex, produced by the same cell and '*trans-presented*' to responder cells expressing the IL-15Rβγ_c. The peculiar and almost unique requirement for IL-15 to be trans-presented by IL-15Rα suggests that the biological effects of IL-15 signaling are tightly regulated even at the level of availability of IL-15. Tissue-specific deletion of IL-15Rα has shown macrophage-and dendritic cell-derived IL-15Rα mediate the homeostasis of different CD8^{+} T cell subsets. Here we show that hepatocyte and macrophage- specific expression of IL-15Rα is required to maintain the homeostasis of NK and NKT cells in the liver. Thus, homeostasis of IL-15-dependent lymphocyte subsets is also regulated by trans-presentation of IL-15 by non-hematopoietic cells in the tissue environment.

Keywords

Interleukin-15; Liver; NAFLD

1. Introduction

Interleukin-15 (IL-15), that was initially characterized as a cytokine required for the maintenance of memory CD8^{+} T cells, has a multi-faceted role in hematopoietic and non-hematopoietic system [1]. IL-15 plays a critical role in the growth and differentiation of T lymphocytes, NK cells, NKT cells and different subsets of innate lymphoid cells [1,2]. Notably, IL-15 promotes the survival of memory CD8^{+} and CD4^{+} T cells [3,4]. The IL-15 receptor (IL-15R) complex consists of 3 subunits: the ligand-binding IL-15Rα chain

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(CD215), the β chain (CD122; also used by IL-2), and the common γ_c chain. The biological activities of IL-15 are mostly mediated by the IL-15:IL-15Rα complex, produced by non-T cells and '*transpresented*' to responder cells expressing the IL-2/15Rβγ_c [5–7]. This peculiar and almost unique requirement for trans-presentation by IL-15Rα suggests that the biological effects of IL-15 are tightly regulated even at the level of IL-15 availability. Most of the effects of IL-15 on lymphocytes can be attributed to the IL-15 that is transpresented by hematopoietic and non-hematopoietic cells [8]. Under steady-state even though transcripts for IL-15 are detected in different organs such as placenta, skeletal muscle, intestine and kidney [9], the expression of the protein is below the level of detection by conventional methods [10]. IL-15Rα is widely expressed in various tissues [11]. As IL-15 exists in the circulation mainly in complex with IL-15Rα, it may be already primed for trans-presentation to cells that express IL-2Rβ and γ_c [12]. The cytoplasmic domain of IL-15Rα, like that of IL-2Rα, appears to be dispensable for signalling. Tissue specific ablation of IL-15Rα has revealed specific, but distinct patterns of requirement for the source of the trans-presented IL-15. For example, NK cells require IL-15 to be trans-presented by both dendritic cells (DCs) and macrophages while central memory CD8^{+} T cells require DC-derived IL-15 [13–15]. However, maintenance of IL-15 dependent T cell subsets in the intestine requires the expression of IL-15Rα by the epithelial cells of the villi [14].

Liver is host to different subsets of lymphocytes such as CD8^{+} T cells, NK cells and NKT cells [16] that are dependent on IL-15 for survival and maintenance [17]. Transcripts for IL-15 have been detected in different cell types of both hematopoietic and non-hematopoietic origin in the liver. Liver resident macrophages called Kupffer cells are one of the major subsets that express IL-15 [10,18]. IL-15 from stellate cells has been shown to support the homeostasis of NKT cells [19], while hepatocytes are a significant source of IL-15:IL-15Rα complex [20]. Thus any of the above mentioned populations could support the survival of IL-15 dependent T cell subsets in the liver.

Hepatic accumulation of excess fat derived from diet results in a range of asymptomatic pathologies starting from fatty liver (steatosis) to non-alcoholic steatohepatitis (NASH) that eventually lead to cirrhosis and hepatocellular carcinoma [21]. NASH and insulin resistance are extensively associated with the increased presence of pro-inflammatory cytokines in circulation and in adipose tissues and liver. Activated CD8^{+} T and NKT cells have been implicated in the progression of NAFLD to HCC [22]. We have shown recently that absence of IL-15 protected from NAFLD (Cepero-Donates et al., manuscript in this issue). As the mice used in the above-mentioned study did not express IL-15 or IL-15Rα at all, it is difficult to identify the source of IL-15 that contributes to NAFLD. Using hepatocyte- and macrophage- specific deletion of IL-15Rα, we show here that both macrophage and hepatocyte mediated trans-presentation of IL-15 is required to support NK and NKT cell subsets in the liver. We further show that deletion of IL-15Rα in hepatocytes or macrophages is not sufficient to prevent the development of NAFLD.

2. Materials and methods

2.1. Mice

All the mice used were in C57BL/6 background. $Il15^{-/-}$ mice have been already described [23]. $Il15ra^{-/-}$ mice were purchased from Jackson Laboratory and bred into C57BL/6 (Charles River) background for more than ten generations. Conditional $Il15ra$-floxed mice have been described previously [14]. Tissue specific deletion of $Il15ra$ was achieved by crossing these mice *with Alb-Cre or LysM-Cre* transgenic mice (Jackson Laboratory) to ablate the $IL15ra$ gene specifically in hepatocytes ($Il15ra^{fl/fl} Alb-Cre^{+}$) or macrophages ($Il15ra^{fl/fl} LysM-Cre$). Male mice were used in all the experiments. All the experiments were approved by the institutional Animal Ethics committee.

2.2. Isolation of intrahepatic lymphocytes (IHL)

Intrahepatic lymphocytes were isolated using gentleMACS Dissociator (Miltenyi Biotec) according to the instructions of the manufacturer. Briefly, mice were sacrificed and the livers were collected and digested in collagenase digestion buffer (300 CDU-casein digestion unit/ml collagenase IV) using gentleMACS Dissociator. Next, the homogenized liver samples were gently agitated on a rocking shaker for 30 min at room temperature and passed through 40 μm cell strainers. Cells were resuspended in 25 ml of cold 0.5% Bovine serum albumin and 2 mM EDTA in Phosphate buffered saline (PEB) and centrifuged at $50g$ for 5 min at 4 °C to eliminate contaminating hepatocytes. The supernatant was centrifuged at $300g$ for 10 min at 4 °C to collect the lymphocytes. Cells were resuspended in ACK to lyse the red blood cells and washed twice with PEB to prepare the single cell suspension for FACS analysis.

2.3. Flow cytometry

Flow cytometric analyses were performed using standard protocols. Abs against mouse CD3, CD8α, CD8β, CD4, CD44, CD62L, and NK1.1 conjugated to flurochromes were purchased from eBioscience (San Diego, CA), BD Biosciences (San Jose, CA) or Biolegend (San Diego, CA). Mouse CD1d tetramer pre-loaded with PBS57 (an α-GalCer analog; CD1d/PBS57) conjugated to PE was obtained from NIH tetramer Facility. Data was acquired on FACS Canto flow cytometer (BD Biosciences San Diego, CA) and was analyzed using FlowJo software from TreeStar Inc (Ashland, OR).

2.4. Induction of NAFLD in mice

To induce hepatic steatosis, 8-weeks old WT, $Il15^{-/-}$ and $Il15ra^{-/-}$ mice, were maintained on high fat diet (HFD) (Research Diets, New Brunswick, NJ, USA; D12492: 20% kcal protein, 20% carbohydrate and 60% fat). Mice fed with normal control diet (NCD) were used as controls. Mice were maintained on NCD or HFD for 12 weeks before sacrifice. The weight of livers was measured at sacrifice.

2.5. Histology

At sacrifice, livers were kept frozen in OCT for lipid staining. Hematoxylin and eosin or Sudan Black lipid staining was carried out on tissue samples fixed in OCT. The slides were

washed in water and mounted with aqueous mounting media (Vecta-Mount[TM]). Images were taken using automatic tissue slide scanning (Hamamatsu NanoZoomer Digital Pathology (NDP) system).

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 software. The values are presented as mean ± standard error of the mean. The statistical significance (p value) was calculated by non-parametric comparison between two groups (Mann Whitney test).

3. Results and discussion

3.1. Hepatocyte mediated trans-presentation of IL-15 by IL-15Rα is required to maintain NK cells in the liver

IL-15Rα-mediated trans-presentation of IL-15 is required for the homeostasis of CD8^{+} T cell subsets in the secondary lymphoid organs [14]. Even though the frequency of antigen-specific CD8^{+} T cells is reduced during primary and secondary immune responses in the liver [24], it is not clear whether IL-15 signalling is involved in their maintenance in the liver. We observed that the frequency of CD4^{+} and CD8^{+} T cells are comparable in the livers of WT, $Il15^{-/-}$ and $Il15ra^{-/-}$ mice (Fig. 1A). Similarly, hepatocyte- or macrophage-specific ablation of IL-15Rα did not change the frequency or the total numbers of CD4^{+} and CD8^{+} T cells in the liver (Fig. 1B and C). These observations suggest that basal the homeostasis of T cells in the liver is not influenced by IL-15 signaling.

Studies using IL-15 and IL-15Rα deficient mice and adoptive transfer experiments have shown the requirement for IL-15 in the generation and maintenance of NK and NKT cells [25,26]. Thus in the absence of IL-15 or IL-15Rα these subsets were not detected in the intra hepatic lymphocyte population (IHLs) (Fig. 2A, lower panel). Lineage specific ablation of IL-15Rα have revealed the complexity of IL-15 trans-presentation. In the secondary lymphoid organs, IL-15Rα-mediated trans-presentation by DCs and macrophages contributes to the maintenance of NK cells [14]. In the absence of overt infection, NK cells are located in the sinusoidal regions along with the liver resident macrophages, Kupffer cells (KCs) [16]. Ablation of IL-15Rα expression in macrophages resulted in decreased numbers of NK cells in the liver [14]. Similarly, under conditions where IL-15Rα was not expressed by DCs, the numbers of NK cells was also decreased. However deletion of IL-15Rα expression in both macrophages and DCs did not synergistically bring down the NK cell numbers suggesting that IL-15Rα expressed by other cell types in the liver can trans-present IL-15 and contribute to the maintenance of NK cells.

Hepatocytes constitute 80% of the cells in the liver and express IL-15 and IL-15Rα [20,27]. To determine whether the NK cell homeostasis is maintained by IL-15 from hepatocytes or macrophages, we analysed the intra-hepatic lymphocyte populations following deletion of IL-15Rα in hepatocytes or macrophages by crossing $Il15ra^{fl/fl}$ mice with mice expressing the Cre-recombinase either in liver under the albumin promoter (*Alb-Cre*) or in macrophages under the lysozyme promoter ((*LysM-Cre*). We observed that the frequency and the total numbers of NK cells were significantly reduced in $Il15ra^{fl/fl}Alb-Cre^{+}$ when compared to

Il15ra^{fl/fl}Alb-Cre^− littermate controls (Fig. 2B). As the expression of IL-15 in all other cell types is normal, the spleens of the same mice did not show any difference in the frequency of NK cells (Data not shown). In accordance with the previous report by Mortier et al. [14], the total numbers of NK cells was decreased significantly in *Il15ra^{fl/fl}LysM-Cre^+* when compared to *Il15ra^{fl/fl}LysM-Cre^−* littermate controls (Fig. 2C). Thus, the above results identify hepatocytes as an important cell type that is required for the maintenance of NK cells in the liver.

NK cells develop in the bone marrow, mature in the periphery and reside predominantly in blood, splenic red pulp and in the sinusoidal regions of the liver [28]. Hepatocytes have been shown to be the major producers of IL-15/IL-15Rα complexes in the liver following viral infections through the production of the transcription factor, IRF-1 [20]. Following certain viral infections, NK cells undergo 1000-fold expansions in the liver [28]. Given the requirement of cell-to-cell contact for IL-15 signaling in NK cells, it is plausible that hepatocytes activate NK cells by upregulating IL-15Rα to support the activity of NK cells. NK cells express high levels of CD122, indicating that IL-15 signaling is required throughout their development [29]. IL-15 signals upregulate Bcl-2 family of anti-apoptotic proteins to maintain their survival as overexpression of Bcl-2 in NK cells can compensate for the absence of IL-15 [30,31]. IL-15/IL-15Rα complexes also support the expansion of NK cells [28]. Bone marrow chimera experiments have shown that IL-15 and IL-15Rα need to be expressed on the same cell to support NK cells [32]. Studies using tissue specific ablation of IL-15Rα expression has revealed further intricacies in the requirement for IL-15 in NK cells. Mortier et al. [14] showed that DC and macrophage specific trans-presentation of IL-15 is required to support NK cells in the spleen, liver and lungs. However, despite the absence of IL-15Rα expression on macrophages and DCs, the frequency of NK cells was diminished only by 50% in the liver [14]. Our results show that in addition to DCs and macrophages, survival of NK cells in the liver requires trans-presentation of IL-15 by IL-15Rα expressed on hepatocytes (Fig. 2B). It is probable that expression of IL-15Rα by the parenchymal non-hematopoietic cells of any given tissue contribute to the maintenance of NK cells.

3.2. Hepatocyte- and macrophage-mediated trans-presentation of IL-15 by IL-15Rα is required to maintain NKT cells in the liver

A subset of NK1.1^+ cells also co-express CD3/TCR complex (NKT cells) and are selected in the thymus on CD1, a non-classical MHC class I like molecule [33]. Type 1 NKT cells (iNKT) express a restricted invariant T-cell receptor α-chain (TCRα; Vα14-Jα18 in mice) in combination with certain Vβ chains (Vβ8.2, Vβ7 or Vβ2 in mice) that recognize the CD1d restricted glycosphingolipid antigen, α-galectosylceramide. Type 1 NKT cells can be identified by staining with CD1d tetramer loaded with α-galectosylceramide or its analog, PBS57 [34]. Type II NKT cells are also restricted by CD1d but they express restricted, but diverse TCR repertoire. In mice, NKT cells are dependent on IL-15 for development and maintenance and are present in abundance in the liver where they have been observed to be associated with hepatic stellate cells [19,35]. Here we examined whether IL-15Rα expression by hepatocyte or macrophages is required to support NKT cells. To our surprise, NKT cell numbers were significantly reduced in *Il15ra^{fl/fl}Alb-Cre^+* when compared to

Il15ra^{fl/fl}Alb-Cre^− mice (Fig. 2B, middle panel). NKT cells also required the expression of IL-15Rα on macrophages for their maintenance in the liver (Fig. 2C, middle panel). On the other hand, survival of iNKT cell subset was dependent on hepatocyte-, but not macrophage mediated trans-presentation of IL-15Rα in the liver (Fig. 2B and C, right panels). These observations show that trans-presentation of IL-15 by parenchymal cells in the liver plays an important role in the homeostasis of NKT cells.

The requirement for IL-15 in NKT cell homeostasis appears to be different from that of NK cells (Fig. 2). In contrast to NK cells, NKT cell development can be sustained by IL-15Rα expressed on non-hematopoietic cells [36,37]. Similarly, IFN-γ production by NKT cells following α-galactosylceramide stimulation is deficient in *Il15ra^{−/−}* mice suggesting that IL-15 signalling also regulates their activation [33,37]. In contrast to NK cells, IL-15 mediated survival of NKT cells is not mediated primarily through the upregulation of anti-apoptotic factors [30]. In the periphery, studies using bone marrow chimeras and transgenic models suggest that IL-15Rα expression is equally important in both hematopoietic and non-hematopoietic cells [36]. Our results show that hepatocyte- and macrophage- mediated presentation of IL-15Rα is essential for the maintenance of NKT cells in the liver (Fig. 2). However iNKT cells appear to be less dependent on IL-15Rα present on macrophages for their homeostasis.

3.3. Deletion of IL-15Rα in hepatocytes or macrophages does not prevent the development of non-alcoholic fatty liver disease (NAFLD)

Given the important role of IL-15 in the activation of different immune subsets in the liver, we showed that the absence of IL-15 or IL-15Rα prevented the development of NAFLD in mice maintained on high fat diet (HFD) (Cepero-Donates et al., this issue). Macrophages play an important role in promoting the inflammatory cascade in metabolic syndrome. Deletion of *Pparg* in macrophages prevents the development of type 2 diabetes but not hepatic steatosis [38]. On the other hand, deletion of *Tnfa* in macrophages reduced steatosis and non-alcoholic steatohepatitis in mice maintained on methionine/choline-deficient diet [39]. As IL-15 induces *Tnfa* expression in rheumatoid arthritis [40], we assessed whether deficiency of IL-15Rα in macrophages or hepatocytes is sufficient to prevent the development of NAFLD in mice maintained on HFD. Body mass and livers of *Il15ra^{fl/fl}LysM-Cre^+* and *Il15ra^{fl/fl}Alb-Cre^+* mice maintained on HFD showed significant increase in weight compared to littermate controls (Fig. 3A). Furthermore, lipid accumulation was observed in the livers of these mice as seen from the H&E and Sudan Black staining (Fig. 3B, data not shown). These observations suggest that absence of IL15-Rα expression on hepatocyte or macrophages is not sufficient to prevent NAFLD.

We have observed that the accumulation of CD8^+ T, NK and NKT cells is increased in the livers of mice maintained on HFD. However, whole body deficiency in IL-15 or IL-15Rα protected mice from NAFLD (Cepero-Donates et al., this issue). These mice were characterized by reduction in inflammation as well as alterations in fatty acid oxidation in the liver. Even though NK and NKT cells are significantly reduced in both groups of mice (total body ablation of IL-15Rα and hepatocyte or macrophage restricted deletion) the outcome of the disease phenotype is completely different. These observations rather indicate

that the presence or absence of NK or NKT cells have minimal impact on NAFLD. The role of NKT cells in NAFLD is controversial [41,42]. Our observations suggest that inflammation, rather than any given cell type, combined with modulation of metabolic characteristics of the liver by IL-15 determines the progression of NAFLD. Detailed analysis of the role of IL-15 in metabolism and diabetes will help designing rational immunotherapeutic approaches to treat NAFLD.

Acknowledgments

Abbreviations

NAFLD   non-alcoholic fatty liver disease

NASH   non-alcoholic steatohepatitis

IHL   intrahepatic lymphocytes

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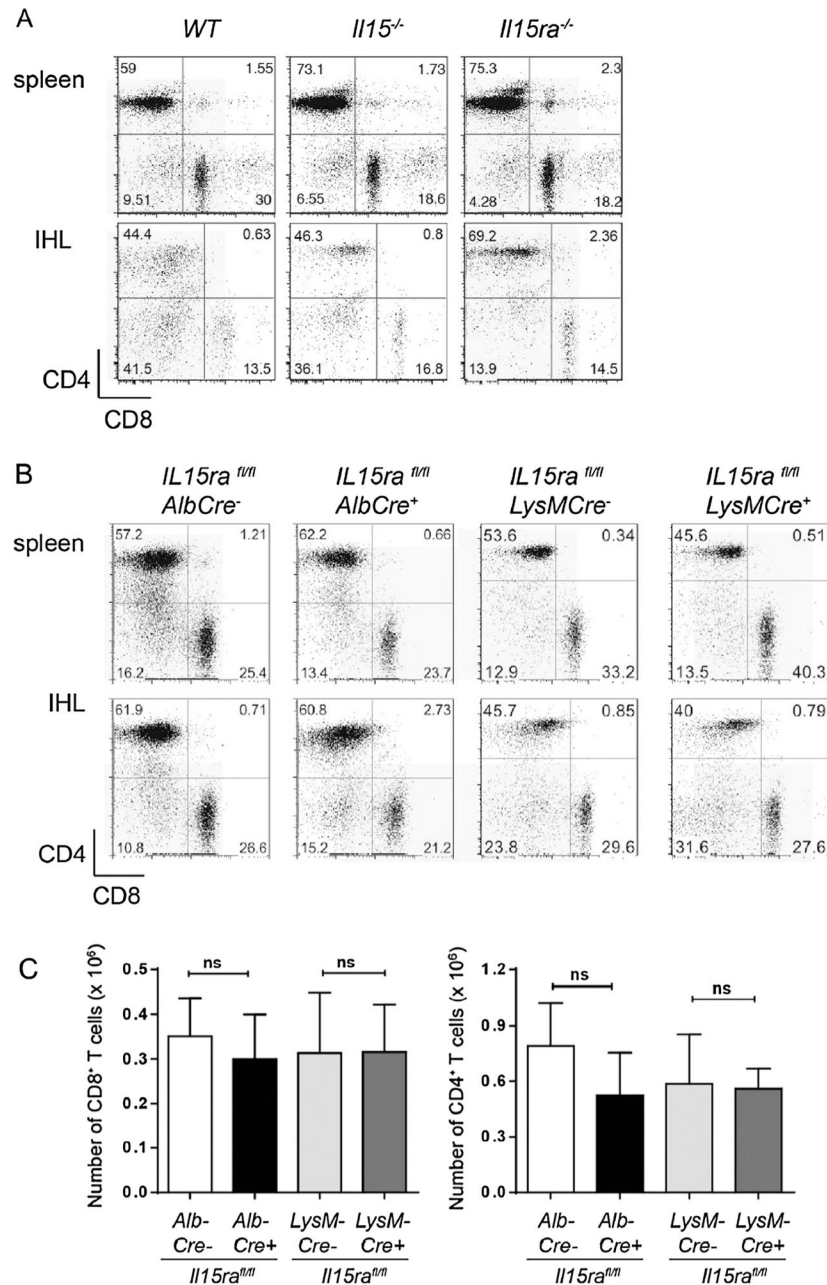
Fig. 1.
Absence of IL-15 or IL-15Rα does not alter the frequency of CD8⁺ T cells in the liver. Lymphocytes were isolated from spleen and liver of age matched (A) WT, *Il15*⁻/⁻ and *Il15ra*⁻/⁻ mice or (B) hepatocyte- and macrophage-specific deletion of IL-15Rα and phenotyped for CD4⁺ and CD8⁺ T cells. Representative data from 4 individual mice per group is shown. (C) Absolute numbers of CD4⁺ and CD8⁺ T cells were calculated from 4 mice per group. Values are expressed as the means ± SEM. Mann Whitney test; ns – not significant.
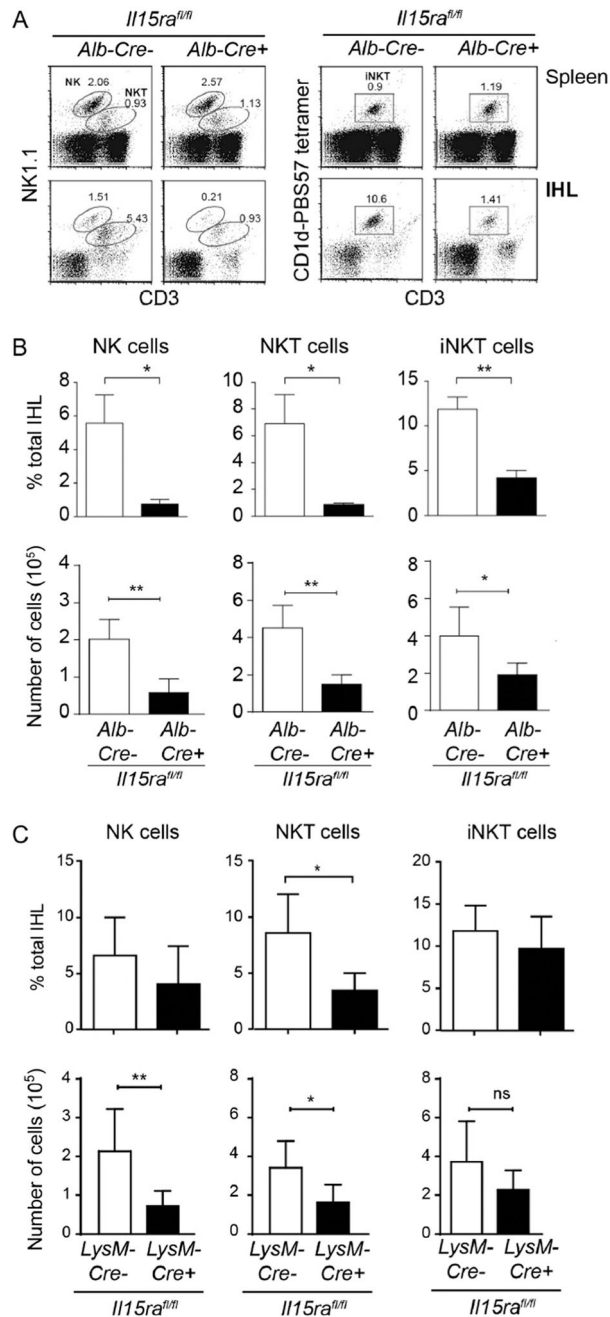
Fig. 2. IL-15Rα expression in hepatocytes and macrophages is required for the maintenance of NK and NKT in the liver

Lymphocytes were isolated from spleen and liver of age matched (A and B) hepatocyte- and (C) macrophage-specific deletion of IL-15Rα and phenotyped for NK and NKT cells. (A) Representative data from 4 individual mice per group is shown. (B and C) The frequency (upper panels) and the absolute numbers (lower panels) of NK, NKT and iNKT cells were calculated from 4 mice per group. Values are expressed as the means ± SEM. Mann Whitney test; $p < 0.05$(*); ns – not significant.
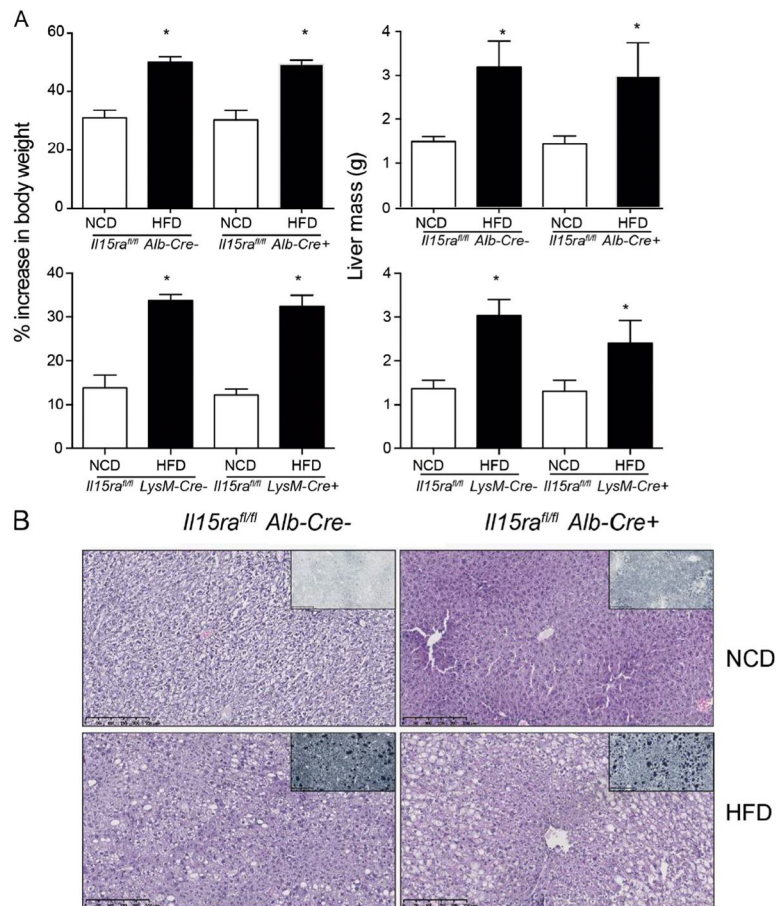
Fig. 3. Hepatocyte- and macrophage-specific deletion of IL-15Rα does not prevent lipid accumulation in the liver

(A) Body (left panels) and liver (right panels) weight was measured in mice of indicated genotype that were maintained on either normal control diet (NCD) or high fat diet (HFD) for 12 weeks. Values are expressed as mean ± SD. Mann Whitney test $N = 4$–8 per group; $p < 0.05$(*). (B) Sections of livers collected from the indicate mice maintained on NCD or HFD for 12 weeks were stained with hematoxylin and eosin or Sudan Black. Representative images from at least 4 mice for each group are shown. Magnification 10×.