The obese liver environment mediates conversion of NK cells to a less cytotoxic ILC1-like phenotype

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Non-alcoholic fatty liver disease (NAFLD) is an obesity-associated disease in which the fatty liver becomes chronically inflamed. Here, we report that in the livers of both humans and mice suffering from NAFLD, NK cells are less able to degranulate. In mice, this is associated with a decreased ability to kill cancerous targets both in vitro and in vivo. On the other hand, perforin-deficient mice suffer from less severe NAFLD, suggesting that perforin-mediated killing is harmful in the obese liver and that the reduction in NK cell cytotoxicity may therefore be protective. The decrease in cytotoxicity is associated with a shift towards a transcriptional profile characteristic of ILC1, increased expression of inhibitory receptors expressed by ILC1, and an altered metabolic profile mimicking that of ILC1. This conversion of NK cells to a less cytotoxic ILC1-like phenotype is at least partially mediated by high levels of TGFβ produced in the obese liver.

NK cells | ILC1 | obesity | NAFLD | immunometabolism | TGFβ
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Introduction

Natural killer (NK) cells are innate lymphoid cells that recognize and kill virally infected and cancerous cells. In mice, NK cells were long defined as Lineage-negative NK1.1$^+$. However, it has recently come to light that, in tissues, cells defined in this way contain at least two distinct populations: circulating, or conventional, NK cells that are CD49a$^-$CD49b$^+$ and tissue-resident NK-like cells that are CD49a$^+$CD49b$^-$ (Peng and Tian, 2017). A population of cells that is in many ways equivalent to mouse tissue-resident CD49a+ NK cells is also present among CD3$^-$CD56$^+$ cells in human livers (Male, 2017). These tissue-resident cells have variously been called tissue-resident NK cells or innate lymphoid cells, type 1 (ILC1): here, we call them ILC1 (Peng and Tian, 2015). The finding that Lin$^-$ NK1.1$^+$ cells in mice and CD3$^-$CD56$^+$ cells in humans are in fact heterogeneous populations means that it is now necessary to re-examine the roles of these cells, distinguishing between NK and ILC1 subpopulations. This is particularly true in the liver, which contains a large number of ILC1.

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of disease, usually associated with obesity, in which excess fat builds up in the liver. Simple steatosis can progress to a chronic inflammatory condition known as non-alcoholic steatohepatitis (NASH), which in turn progresses to fibrosis and ultimately cirrhosis, which increases the risk of developing hepatocellular carcinoma. NASH patients have increased numbers of CD3$^-$CD56$^+$CD57$^+$cells, which are likely to represent circulating NK cells, in their livers (Kahraman *et al*, 2010). The expression of NK cell-activating ligands is also increased in the livers of NASH patients. But the role of NK cells in NASH pathogenesis is not yet clear.

NK cells can limit fibrosis in liver diseases of various etiologies by controlling the activity of hepatic stellate cells (Radaeva *et al*, 2006; Male *et al*, 2017). In a mouse study in which a NASH-like disease was induced using a methionine and choline deficient diet, IFNγ production by NKp46$^+$ NK cells and ILC1 protected against disease (Tosello-Trampont *et al*, 2016). On the other hand, NK cells can be harmful in chronic inflammatory liver disease, where they contribute to pathology by killing hepatocytes (Dunn *et al*, 2007; Beraza *et al*, 2009). In support of this being the case in NASH, mice lacking either TRAIL (an apoptosis-inducing TNF family ligand expressed by NK cells) or its receptor were partially protected against obesity-associated NASH (Idrissova *et al*, 2014; Hirsova *et al*, 2017).

Here, we investigate the activity of NK cells and ILC1 in NAFLD patients, and in a mouse model of NASH. We report that in diseased livers, NK cells are less able to degranulate in both humans and mice. We further show that this is accompanied by a decreased ability of the NK cells to kill cancerous targets, both *in vivo* and *in vitro*. This decrease in cytotoxicity is associated with a shift towards an ILC1-like phenotype, which seems to be at least partially mediated by high levels of TGFβ produced in the obese liver.

Results

NK cells in the livers of NAFLD patients are less able to degranulate than those from healthy controls

A number of research groups have recently identified liver-resident cells in humans, which are in many ways equivalent to mouse liver ILC1 (Male, 2017). We sought to determine how the activity of these cells, as well as conventional NK cells in the liver, is altered in NAFLD and NASH patients. We isolated intrahepatic leukocytes from nine healthy livers, six livers with simple steatosis (livers that showed steatosis

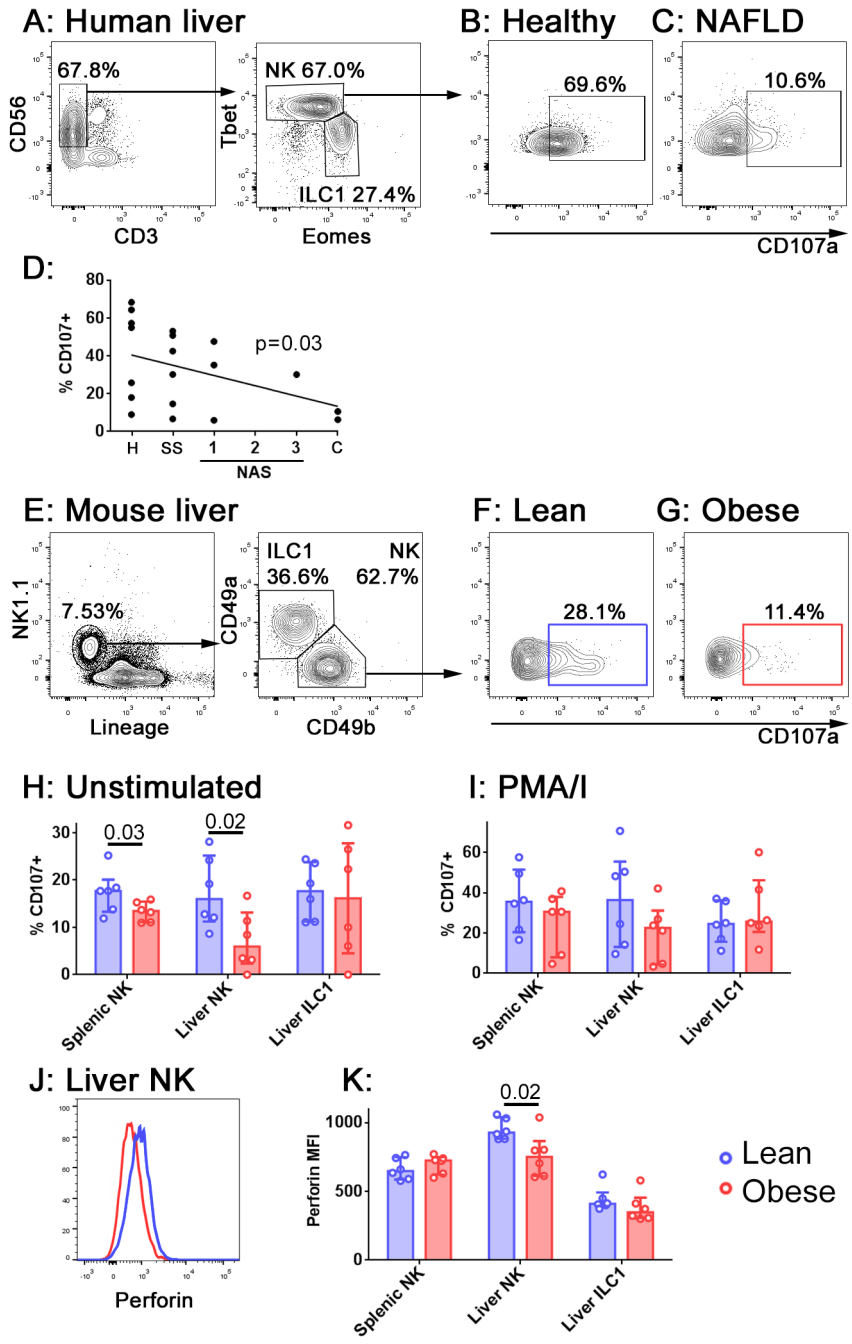

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Fig. 1. NK cells in the livers of obese humans and mice are less able to degranulate. A. Immune cells were isolated from human livers. NK cells were identified by scatter, and as live CD45+ CD56+ CD3- Tbet^{hi} Eomes^{lo} cells. B and C. Intrahepatic leukocytes were cultured for 4h in the presence of anti-CD107a and Brefeldin. Representative CD107a staining of NK cells from a healthy (B) and a NAFLD (C) liver is shown. D. Inverse correlation between CD107a staining and histological score in human livers. H: healthy; SS: simple steatosis; NAS: NASH Activity Score; C: cirrhosis. Significance was determined using Spearman's Rank Correlation. E. Immune cells were isolated from mouse livers. NK cells were identified by scatter, and as live CD45+ Lineage-negative NK1.1+ CD49b+ cells. ILC1 were identified as live CD45+ Lineage-negative NK1.1+ CD49a+ cells. F and G. Intrahepatic leukocytes were cultured for 4h in the presence of anti-CD107a and Brefeldin. Representative CD107a staining of NK cells from a lean (F) and an obese (G) mouse is shown. H and I. CD107a staining in splenic NK, liver NK and liver ILC1 in unstimulated cells (H) and cells stimulated with PMA and ionomycin (I) from lean and obese mice. J. Representative perforin staining in liver NK from a lean (blue trace) and an obese (red trace) mouse. K. MFI of perforin in splenic NK, liver NK and liver ILC1 from lean and obese mice. E - K. n = 6 mice per group; significance was determined using Mann Whitney U Tests; medians and IQRs are shown.

but no inflammation) and six livers displaying various levels of inflammation and fibrosis in addition to steatosis. NK cells release cytotoxic granules containing perforin and granzymes in a process known as degranulation, which can be measured using CD107a staining. By doing this, we found that the ability of NK cells (defined as CD3$^-$CD56$^+$Tbet^{hi} Eomes^{lo}; Cuff, 2016, JI; Figure 1a) to degranulate was inversely correlated with disease severity (Figure 1b-d).

NK cells in the livers of obese mice are less able to degranulate than those in lean mice

To confirm and extend these findings, we next looked at the ability of NK cells (defined as Lineage-negative NK1.1$^+$CD49a$^-$CD49b$^+$) to degranulate in a model of NAFLD in which mice were maintained on an obesogenic diet. Similar to our observations in human livers, we found that NK cells isolated from the livers of obese mice degranulated less than those from the livers of their lean littermates (Figure 1e-h). This was also the case for NK cells isolated from spleens, although the reduction was smaller (a difference in the medians of 4.3% in splenic NK, compared to 10.0% in liver NK; Figure 1h). Following stimulation with PMA and ionomycin, the degranulation of the NK cells from obese mice partially recovered, indicating that the cells retained the ability to degranulate (Figure 1i). However, we also found a significant reduction in the expression of perforin by these cells (Figure 1j,k). This may suggest that even

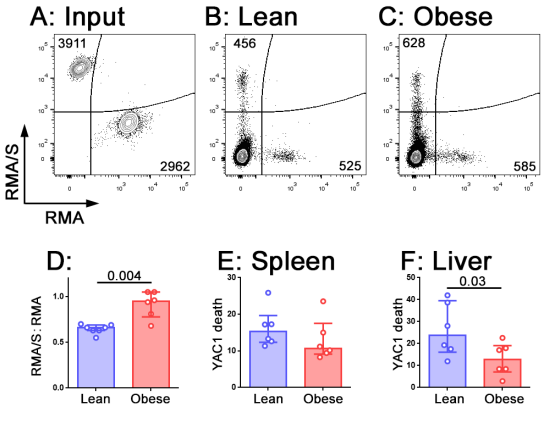

Fig. 2. NK cells from obese mice are less able to kill cancerous targets. A. CTV-labelled RMA/S (NK cell targets) CTB-labelled RMA (recovery control) cells were mixed in a 1:1 ratio and intravenously injected into lean or obese mice. B and C. Cells recovered from the spleens of a representative lean (B) and obese (C) mouse at 4h. D. RMA/S:RMA ratio in recovered splenocytes, normalised to input ratio. E and F. NK cells were sorted from the spleens (E) and livers (F) of lean and obese mice, and cultured with the NK cell target line YAC-1. Cell death at 24h is shown. n = 6 mice per group; significance was determined using Mann Whitney U Tests; medians and IQRs are shown.

if NK from the livers of obese mice can degranulate in response to certain stimuli, they are likely to be less cytotoxic than those from lean animals.

NK cells from obese mice are less able to kill cancer cells

The reduced ability of NK cells in obese mice to degranulate suggests that their ability to kill cancerous target cells will be impaired. To determine whether this was the case *in vivo,* we injected mice intravenously with equal numbers of fluorescently-labelled RMA/s cells (which are MHC class I-negative NK targets) and RMA cells (which are not NK targets and act as a recovery control) (Kärre *et al*, 1986) (Figure 2a). We collected spleens from the recipient mice after 4 hours and determined the number of cells remaining in each population by flow cytometry (Figure 2b,c). The lean mice had eliminated the RMA/s cells with approximately 40% efficiency. In contrast, the obese mice had eliminated them with only 5% efficiency (Figure 2d).

To determine the relative defects in NK cell killing in the spleens and livers of obese compared to lean mice, we sorted NK cells from the two organs and assessed their ability to kill YAC-1 cancerous target cells *in vitro*. NK cells from both the spleens (Figure 2e) and livers (Figure 2f) of obese mice were less able to kill YAC-1 cells than those from their lean littermates, although the defect in killing was only significant (at $p < 0.05$) in NK cells isolated from the liver. Similar to our observations of degranulation, the reduction in target cell killing was greater in NK cells isolated from livers than those isolated from spleens (a difference in the medians of 2.7% in splenic NK cells, compared to 10.1% in liver NK cells).

Perforin-mediated cytotoxicity promotes fibrosis in NAFLD

Clearly, the diminished ability of NK cells to kill target cells in obese animals is likely to limit cancer surveillance. However, since we observed the largest reductions in both degranulation and cytotoxicity in NK cells isolated from the liver,

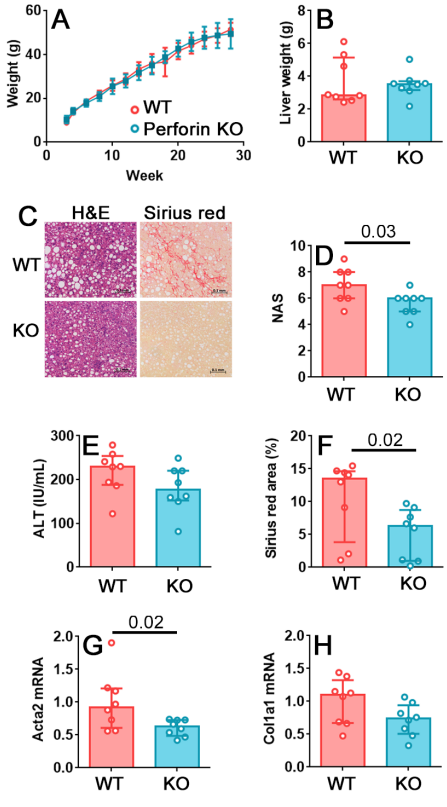

Fig. 3. Perforin-mediated killing promotes fibrosis in NAFLD. A. Growth curves for wild type and Perforin knockout mice on the obesogenic diet. B. Liver weights for wild type and Perforin knockout mice after 24 weeks on the obesogenic diet. C. Representative H&E and Picrosirius red staining in a wild type and a perforin knockout mouse. D. Histological score in wild type and a perforin knockout mouse livers, after 24 weeks on the obesogenic diet. E. Plasma ALT levels. F. Picrosirius red positive area in representative histological fields. G and H. Acta2 and Col1a1 mRNA in livers of wild type and a perforin knockout mouse livers, after 24 weeks on the obesogenic diet. n = 8 mice per group; significance was determined using Mann Whitney U Tests; medians and IQRs are shown.

and since the reduction in perforin expression seemed to particularly affect NK cells in the liver, we sought to define the likely effect of these changes in NAFLD. Given that TRAIL-mediated cytotoxicity exacerbates NAFLD (Idrissova *et al*, 2014; Hirsova *et al*, 2017), we speculated that there might be some advantage to the reduced perforin-mediated cytotoxicity we observed in the obese liver. We hypothesized that perforin-mediated killing is harmful in the liver during obesity, and that the reduction in NK cell degranulation, perforin expression and cytotoxicity could therefore be protective. In this case, we would expect mice lacking perforin to suffer less severe liver disease as a result of obesity. We therefore took a number of measures of liver damage in wild type and perforin-deficient mice that had been kept on the obesogenic diet for 24 weeks. This later timepoint was chosen so that we could compare the development of fibrosis, which is not usually pronounced at 12 weeks, between the two genotypes.

There was no difference in weight gain or hepatomegaly between the wild type and perforin knockout mice (Figure 3a,b). However, livers from the perforin-deficient mice displayed a significantly lower histopathological NAS score than wild type controls (Figure 3c,d) and less liver damage

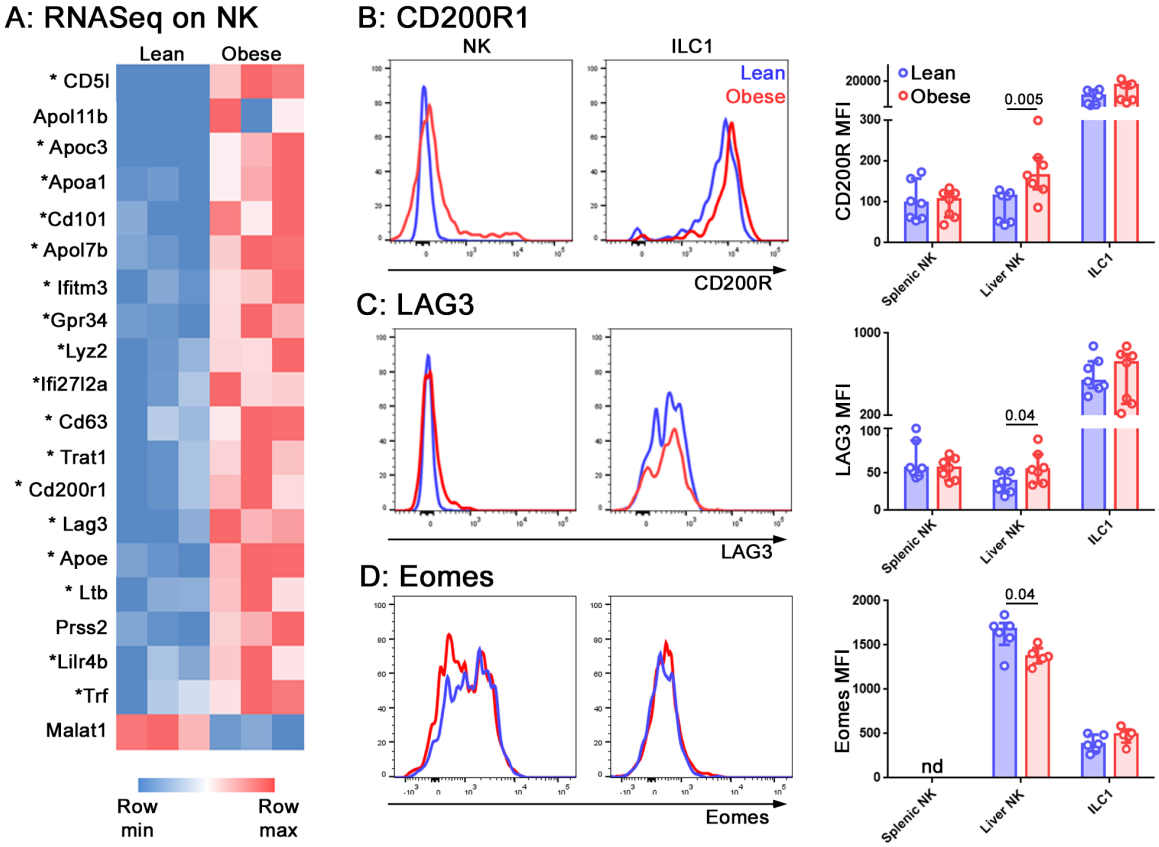

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Fig. 4. NK cells in the livers of obese mice acquire an ILC1-like transcriptional profile. A. NK cells and ILC1 were sorted from the livers of lean and obese mice and examined by RNASeq. Genes that were differentially expressed by > 2-fold, with padj < 0.05, in NK cells of lean versus obese mice are shown. Genes that are also differentially expressed in ILC1 compared to NK (> 2-fold, with padj < 0.05) are marked with an asterisk. B - D. Expression of the inhibitory receptors CD200R1 (B) and LAG3 (C) in NK cells and ILC1 of lean (blue) compared to obese (red) mice. D. Expression of Eomes in NK cells and ILC1 of lean (blue) compared to obese (red) mice. n = 6 mice per group; significance was determined using Mann Whitney U Tests; medians and IQRs are shown.

as determined by circulating alanine transaminase (ALT) although the difference between the two genotypes was not significant (Figure 3e). Fibrosis, determined by Picrosirius red staining, was lower in perforin-deficient mice (Figure 3f) and real time PCR for markers of fibrosis revealed that the livers of the perforin-deficient mice expressed less *Acta2* (alpha smooth muscle actin; Figure 3g) and *Col1a1* (collagen type I alpha 1; Figure 3h) than those of controls, although the difference was only statistically significant for *Acta2*.

NK cells in obese mice display a transcriptional profile characteristic of ILC1

We next sought to define the mechanism by which the reduction in NK cell cytotoxicity occurs. To achieve this, we analyzed the transcriptomes of NK cells and ILC1 in the livers of lean and obese mice by RNASeq. Raw RNASeq data and differentially expressed gene lists are available from the National Center for Biotechnology Information Gene Expression Omnibus under accession no. GSE122828.

18 of the 20 genes identified as being significantly overexpressed (FC > 2, padj < 0.05) in NK cells from obese mice, compared to those from their lean littermates, were also overexpressed in ILC1, compared to NK cells (Figure 4a, genes characteristic of ILC1 are marked with an asterisk). We particularly noted that transcripts encoding the inhibitory receptors CD200R1, LAG3 and CD101, all of which are involved in limiting immune pathology in inflammatory diseases (Hoek *et al*, 2000; Fernandez *et al*, 2007; Snelgrove *et al*, 2008; Anderson *et al*, 2016) were increased in NK cells from obese mice compared to lean, as well as in ILC1 compared to NK cells. We confirmed the increase in expression at the protein level for CD200R1 (Figure 4b) and LAG3 (Figure 4c). CD101 was increased in ILC1 compared to NK cells, but we did not detect a difference in its expression in NK cells from obese compared to lean animals (not shown).

NK cells and ILC1 express the transcription factor Tbet at roughly similar levels, although they rely on it to different extents (Sojka *et al*, 2014). On the other hand, the transcription factor Eomes is expressed only by NK cells and specifies the NK cell lineage (Daussy *et al*, 2014). In the light of the ILC1-like transcriptional profile expressed by NK cells in obese mice, we examined the expression of these two transcription factors and found that Eomes was underexpressed in NK cells in obese mice, again reminiscent of ILC1 (Figure 4c).

NK cells in obese mice are metabolically reprogrammed

Gene ontogeny analysis on the differentially expressed transcripts (Figure 4a) further revealed that the most overrepresented pathway in the NK cells of obese mice, compared

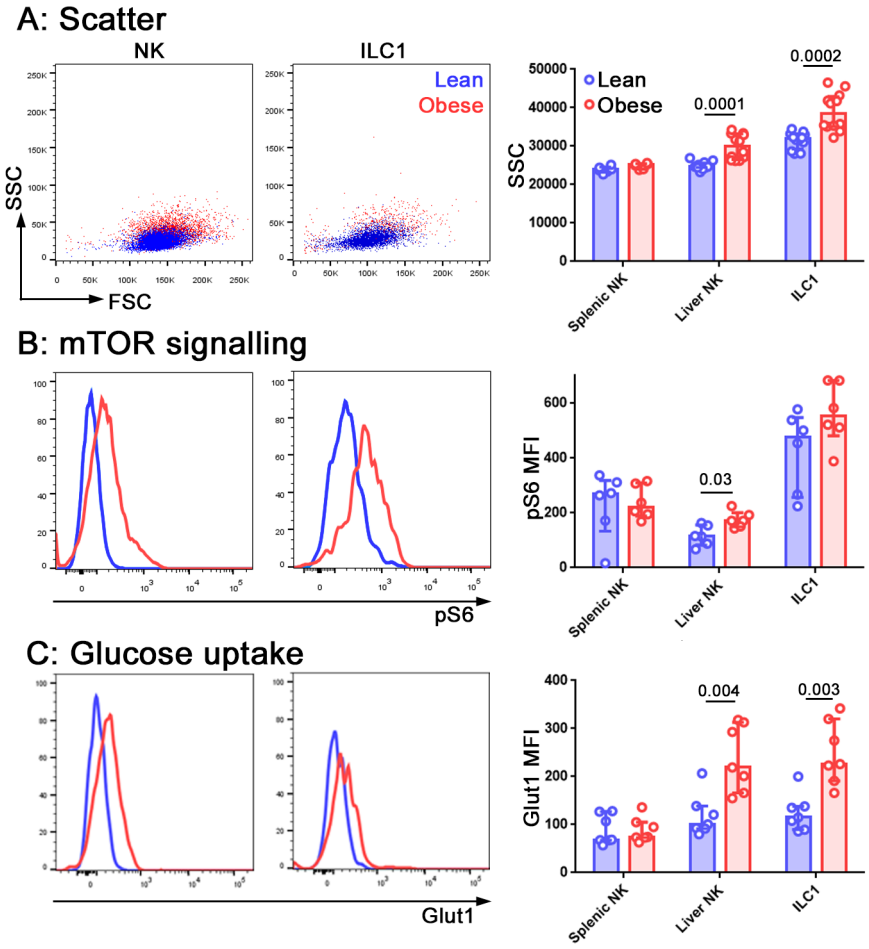

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Fig. 5. NK cells in the livers of obese mice are metabolically reprogrammed. Forward and side scatter (A), pS6 staining (B) and Glut1 staining (C) of freshly isolated NK cells and ILC1 from lean (blue) and obese (red) mice. n = 12 mice per group (A), 6 mice per group (B) and 7 mice per group (C); significance was determined using Mann Whitney U Tests; medians and IQRs are shown.

to those of lean littermates, was Triglyceride catabolic processes (p = 3.65 x 10^{-3}). We therefore examined the cells for signs of metabolic reprogramming.

Scatter has been used as an indicator of metabolic alteration in NK cells (Castro *et al*, 2018) and we found that side scatter increased in NK cells from the livers of obese mice (Figure 5a).

NK cells use mTOR for nutrient sensing and it also has an important role in immune regulation (O'Brien and Finlay, 2019). The phosphorylation of ribosomal S6 protein (pS6) is used as an indicator of mTORC1 signaling and we found that pS6 was increased in NK cells freshly isolated from the livers of obese compared to lean mice (Figure 5b). Notably, in both these cases, the metabolic alterations that we observed in the NK cells served to make them more ILC1-like, similar to our findings at the transcriptional level. We also observed an increase in the expression of the glucose transporter Glut1 (Figure 5c).

TGFβ in the obese liver inhibits NK cell degranulation

The conversion of NK cells to a less cytotoxic ILC1-like phenotype has been reported in a number of situations (Cuff *et al*, 2016; Gao *et al*, 2017; Cortez *et al*, 2017). In all these cases, TGFβ was shown to be a key cytokine driving the conversion. We therefore hypothesized that TGFβ in the obese liver might be mediating the reduction in cytotoxic ability and the acquisition of ILC1-like features that we observed. *Tgfb1* transcript was increased in the livers of obese compared to lean mice (Figure 6a) and we also observed increased TGFβ 1 protein in conditioned medium from obese compared to lean livers (Figure 6b) and in the plasma of obese mice (Figure 6c).

When NK cells from the spleens of lean animals were cultured for 24 hours in conditioned medium from either lean or obese mouse livers, we observed that their ability to degranulate was impaired following culture in obese, compared to lean, liver conditioned medium and we were able to recapitulate this phenotype by culturing the NK cells in recombinant TGFβ 1 (Figure 6d). Furthermore, the addition of an anti-panTGFβ antibody to the cultures partially rescued the ability of the NK cells to degranulate following culture with obese liver conditioned medium (Figure 6e), suggesting that TGFβ in the obese liver is at least partially responsible for limiting the ability of NK cells to degranulate.

Discussion

It is becoming increasingly apparent that NK cells are dysfunctional in obesity (O'Shea *et al*, 2010; Tobin *et al*, 2017; Michelet *et al*, 2018), and that this could be one mechanism that accounts for the link between obesity and cancer (Michelet *et al*, 2018). These studies have largely focused on

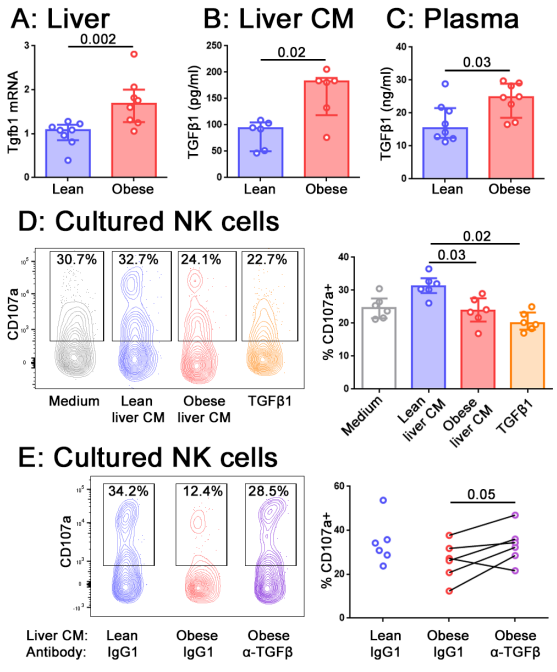

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Fig. 6. TGFβ in the livers of obese mice limits the ability of NK cells to degranulate. A. Tgfb1 mRNA in the livers of lean and obese mice, normalised so that mean Tgfb1 transcript expression in the livers of lean mice = 1. B,C. TGFβ1 in lean and obese liver conditioned medium (B) and lean and obese mouse plasma (C) measured by ELISA. n = 8 mice per group (A, C) and 6 conditioned media per group (B); significance was determined using Mann Whitney U Tests; medians and IQRs are shown. D. Splenic NK cells were cultured for 24h in medium alone, lean or obese liver conditioned medium, or 10 ng/ml TGFβ1. For the last 4h of culture, CD107a antibody and Brefeldin A were added to media. n = 6 culture conditions per group; significance was determined using Mann Whitney U Tests with Holm's correction for multiple comparisons; medians and IQRs are shown. E. Splenic NK cells were cultured as described in D, with the addition of 10 µg/ml anti-TGFβ antibody or isotype control. n = 6 culture conditions per group; significance was determined using a Wilcoxon signed ranks test, where each pair is obese liver conditioned medium from the same mouse, with anti-TGFβ antibody or isotype control. Degranulation in NK cells cultured in lean liver conditioned medium with isotype control are shown for comparison, but were not statistically tested.

NK cells in the blood, but in this study we looked at how NK cells in the liver change during obesity, and the impact that this may have not only on cancer immunosurveillance, but also on NAFLD pathogenesis.

We found that, in obesity, NK cells in the livers of both humans and mice are less able to degranulate and kill target cells. This is consistent with a number of reports that NK cells taken from the blood of obese humans (O'Shea *et al*, 2010; Tobin *et al*, 2017; Michelet *et al*, 2018) or the spleens of obese mice (Michelet *et al*, 2018) are less cytotoxic than those taken from their lean counterparts. We further show that the NK cells of obese mice are less able to kill cancerous target cells *in vivo*. In a recent high profile study, NK cells that had been cultured in fatty acids before being adoptively transferred into a B16 melanoma-bearing host were less able to control the cancer than control NK cells (Michelet *et al*, 2018). Obese mice were also less able to control B16 metastases than their lean counterparts, a phenotype that was associated with reduced NK cell infiltration into metastatic foci. Our finding that obese mice are less able to control RMA/S cells confirms these findings using another type of cancerous target.

In humans, NK cell dysfunction in obesity is associated with metabolic reprogramming (Tobin *et al*, 2017; Michelet *et al*, 2018). Similarly, we observed metabolic changes in NK cells taken from the livers of obese mice. In a pediatric cohort, obesity was associated with increased Glut1 and pS6 expression (Tobin *et al*, 2017), whereas in adults, obesity was associated with decreased 2-NBDG uptake (a proxy for glucose uptake) and pS6 expression (Michelet *et al*, 2018). This suggests that the way in which NK cells are metabolically dysregulated in obesity may change over time. Interestingly, the NK cells from our obese mice more closely resembled those from the pediatric cohort and this may reflect the fact that both these children and our mice had both been obese for a relatively short time, compared to those in the adult human cohort.

The altered metabolic profile of the NK cells in the obese mice was somewhat reflective of the metabolic profile of ILC1. We also observed an ILC1-like transcriptional profile and expression of certain inhibitory receptors that are characteristic of ILC1, including CD200R1, which has recently been proposed to be a better marker of ILC1 than CD49a (Weizman *et al*, 2017). NK cells are converted into less cytotoxic ILC1-like cells in the tumor microenvironment, a tumor immunevasion strategy mediated by TGFβ (Gao *et al*, 2017). Similarly, TGFβ promotes the conversion of NK cells to ILC1 in the mouse salivary gland (Cortez *et al*, 2017) and in recently-transplanted human livers (Cuff *et al*, 2016). We confirmed previous reports that TGFβ is highly expressed in the obese liver (Yadav *et al*, 2011; Mouralidarane *et al*, 2013; Hart *et al*, 2017) and further showed that conditioned medium from obese livers could limit the ability of NK cells to degranulate, mirroring the phenotype that we observe in NK cells taken from obese livers, in a TGFβ-dependent manner. This is consistent with previous reports that TGFβ limits NK cell cytotoxicity (Viel *et al*, 2016; Zaiatz-Bittencourt *et al*,

2018), although in contrast to one of these reports (Viel *et al*, 2016) we observed an increase, rather than a decrease, in pS6 expression. This may reflect differences in the kinetics of altered mTORC1 signaling in short-term *versus* longer term TGFβ exposure (Zaiatz-Bittencourt *et al*, 2018) but is also likely to be attributable to differences in the liver environment other than TGFβ production. For example high levels of fatty acids may interact with TGFβ to produce a different metabolic phenotype (Michelet *et al*, 2018).

The reduction in NK cell cytotoxicity in obesity is likely to be detrimental to tumor immunosurveillance, but one key question is whether it is helpful or harmful in the context of NAFLD. We observed greater reductions in degranulation and cytotoxicity in the liver than in the spleen. Further, TRAIL-mediated cytotoxicity is known to promote pathogenesis in NAFLD (Idrissova *et al*, 2014; Hirsova *et al*, 2017). Therefore, we postulated that the decrease in degranulation, and thus perforin-mediated cytotoxicity, that we observed in the obese liver might protect against liver disease. In support of this idea, perforin-deficient mice suffered from less severe NAFLD. Since these animals lack perforin globally, we cannot say with certainty that the protection is not mediated at least in part by defective T cell cytotoxicity. Indeed if, as we suggest, the liver is acting to protect itself against immunopathology by producing TGFβ, it is likely that T cell cytotoxicity will also be reduced. Nevertheless, the protection from liver disease that we observe in the absence of perforin does suggest that defect in NK cell degranulation we observe in the obese liver may act to protect it. NK cell-derived ILC1-like cells in tumors produce angiogenic and growth factors (Gao *et al*, 2017), so it is tempting to speculate that the ILC1-like cells we see emerging from the NK cell population in the obese liver may have a similar reparative effect.

It has been suggested that NK cell metabolism could be targeted to prevent cancer (O'Brien and Finlay, 2019). In the light of recent findings that NK cells are less cytotoxic in obesity, and that this is at least partially mediated by metabolic changes, it might be tempting to consider using such treatments as a way to break the well-established link between cancer and obesity (Park *et al*, 2014). However, our finding that perforin-mediated killing is harmful in NAFLD sounds a note of caution about such approaches, which may result in adverse effects in the liver.

Materials and Methods

Human liver biopsies

Liver biopsies were taken from livers that were destined for transplantation, but that were discarded because of long warm ischemic time, vascular abnormalities, or tumors elsewhere in the patient (n = 9), or because they displayed signs of NAFLD or NASH on histopathologic examination (n = 10). Biopsies were also taken from livers explanted during transplantation for NASH (n = 2). Cells were isolated from human liver samples and degranulation assessed as previously described (Cuff *et al*, 2016). Ethical approval for use of human liver samples was obtained through the Royal Free Hospital Biobank (National Health Service Research Ethics Committee approval no. 11/WA/0077, study no. 9455).

Mice

Female C57BL/6J (bred in house) or Prf1$^{-/-}$ mice (Charles River; RRID MGI:5576721) were randomized at weaning onto standard chow (RM1) or a highly palatable obesogenic diet consisting of 22.6% fat, 23.0% protein and 40.2% carbohydrate (w/w); diet code 824018 - '45% AFE fat' Special Dietary Services, Essex, UK), supplemented with sweetened condensed milk (Nestle) *ad libitum* (Oben *et al*, 2010).

After 12 or 24 weeks on the diet, mice were sacrificed by direct cervical dislocation. To determine the severity of liver disease, blood was collected by cardiac puncture and centrifuged at 10,000 *x*g for 10 minutes at room temperature to isolate serum for ALT measurement and pieces of liver were fixed in 10% neutral buffered formalin, fixed, sectioned and stained with H& E or Picrosirius red. Sections were blinded and scored by a hepatopathologist, using the Brunt-Kleiner NASH activity score (NAS; Brunt *et al*, 2011) (Supplementary figure 1). Spleens were collected and splenocytes isolated as previously described (Cuff and Male, 2017). Intrahepatic lymphocytes were isolated using an adaptation of the method from Cuff and Male, 2017. Briefly, finely minced liver tissue was collected in RPMI 1640 medium (Life Technologies brand; Thermo Fisher Scientific, Hudson, NH) and passed through a 70 μm cell strainer. The suspension was spun down (500 × g, 4°C, 10 minutes) and the pellet resuspended in RPMI 1640 medium. The cell suspension was layered over 24% Optiprep (Sigma-Aldrich) and centrifuged without braking (700 × g, RT, 20 minutes). The interface layer was taken and washed in HBSS without Ca2+ Mg2+ (Lonza, distributed by VWR, Lutterworth, UK) supplemented with 0.25% bovine serum albumin (Sigma-Aldrich, Hammerhill, U.K) and 0.001% DNase I (Roche, distributed by Simga-Aldrich).

Animal husbandry and experimental procedures were performed according to UK Home Office regulations and institute guidelines, under project license 70/8530.

In vivo NK cytotoxicity assays

RMA and RMA/s thymoma cells (cultured in Iscove's modified Dulbecco's medium supplemented with 10% FCS; Life Technologies) were labeled with Cell Trace Blue or Cell Trace Violet, respectively, according to the manufacturer's instructions (Thermo Fisher Scientific). 10^7 cells of each cell type were injected intravenously into control or obese mice. After 4h, recipients were sacrificed by direct cervical dislocation and the spleens were examined for fluorescent target cells.

Ex vivo NK cell activity assays

For degranulation and cytokine production assays, total intrahepatic leukocytes or splenocytes were cultured for 4 hours at 10^7cells/mL in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-ME, MEM nonessential amino acids, penicillin, and streptomycin (all Life Technologies brand; Thermo Fisher Scientific). Brefeldin A (1 μg/mL; Sigma-Aldrich) and anti-

CD107a (1:100, eBioscience, San Diego, CA) were added to all conditions. Experiments were carried out in either the presence or absence of PMA and ionomycin (25 ng/mL and 1 µg/mL, respectively; Sigma-Adrich).
For cytotoxicity assays, 15,000 sorted NK cells were cultured with 75,000 YAC-1 target cells in 100 µL RPMI supplemented as before for 24h.
Cell death was measured using an LDH cytotoxicity assay (Abcam, Cambridge, U.K.), according to the manufacturer's instructions. Readings were normalized between the medium only (0% cell death) and lysis buffer (100% cell death) controls.

Cell culture experiments
Liver conditioned medium was produced by culturing 500 mg of finely minced liver tissue in serum-free M199 medium (Gibco brand, Thermo Fisher Scientific) for 48h. The medium was cleared of debris by being passed through a 70 µm strainer and by centrifugation at 500 x*g* for 5 minutes at 4. Splenic NK cells were cultured for 24h in RPMI supplemented as before mixed 3:1 with liver conditioned medium or M199 medium. TGFβ1 (10 ng/mL, PeproTech, Rocky Hill, NJ), anti-mouse TGFβ (10 µg/mL, clone 1D11 R&D Systems, Minneapolis, MN) or isotype control antibody (10 µg/mL, clone 11711 R&D Systems) were added as indicated.

Flow cytometry
Details of antibodies used in the study are given in Supplementary table 1. The lineage cocktail for mouse cells consisted of CD3, CD8α, CD19 and Gr1. Dead cells were excluded using fixable viability dye eFluor 450 (eBioscience) (4°C, 15 minutes). Surface staining was carried out in PBS supplemented with 1% FCS (4°C, 15 minutes). Intracellular staining was carried out using Human FoxP3 Buffer (BD Biosciences, Oxford, UK), except for pS6 staining, which was carried out using Cytofix/cytoperm (BD Biosciences). Data were acquired on an LSRFortessa II (BD Biosciences) and analyzed using FlowJo v.X.0.7 (Tree Star, Ashland, OR, USA). Sorting was carried out on an Aria (BD Biosciences).

Real time PCR
Liver sections were dissected directly into RNAlater and RNA was extracted using an RNeasy Lipid Tissue Mini kit (both from Qiagen, Manchester, U.K.). cDNA was made using a Transcriptor First Strand cDNA Synthesis kit (Roche, Welwyn Garden City, U.K.) and real-time PCR was performed using TaqMan (Applied Biosystems, Warrington, U.K.) primer/probe sets recognizing Hprt1 (Mm00446968_m1), Acta2 (Mm01546133_m1), Col1a1 (Mm00801666_g1) and Tgfb1 (Mm01178820_m1).

RNASeq
Total RNA was extracted from sorted cells using an RNeasy Micro kit (Qiagen) and libraries were prepared from 2 ng of total RNA using the NEBNext low input kit (New England Biolabs, Hitchen, U.K.). Libraries were assessed for correct size distribution on the Agilent 2200 TapeStation and quantified by Qubit DNA High Sensitivity assay (Thermo Fisher Scientific) before being pooled at an equimolar concentration. Samples were sequenced on a NextSeq 500 (Illumina, Essex, U.K). Differential expression analysis was carried out using SARTools (Varet *et al*, 2016), filtering at padj < 0.05 and FC > 2.

TGFβ1 ELISA
Total TGFβ1 protein concentrations in plasma and liver conditioned medium were determined using a mouse TGF-beta1 DuoSet ELISA (R&D Systems), according to the manufacturer's instructions.

Statistics
The data was analyzed and found not to be normally distributed. Significance was therefore determined using non-parametric tests: Mann Whitney U Tests (for unpaired data), Wilcoxon signed rank tests (for paired data) or Spearman's tests (for correlations). P values < 0.05 are reported. Bars represent medians and interquartile ranges; individual data points are also plotted.

Supplementary material
Supplementary Table 1 gives full details of all the antibodies used for flow cytometry in the study. Supplementary Figure 1 demonstrates that mice kept on the obesogenic diet become obese and develop NAFLD.

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