ORIGINAL ARTICLE

Everolimus enhances TRAIL-mediated anti-tumor activity of liver resident natural killer cells in mice

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SUMMARY

In transplantation, innate immunity plays a pivotal role in immunosurveillance and host defence against microbes and neoplastic cells. Liver-resident NK cells express TNF-related apoptosis-inducing ligand (TRAIL), which distinguishes them from conventional NK cells. In this study, we investigated the impact of mTOR inhibition on liver-resident NK cells in comparison with that on splenic NK cells in a mouse model. In mice that received everolimus (EVR) for 7 days (range: 0.0125–0.25 mg/kg/day), the proportion of splenic NK cells was unchanged, whereas the number of liver NK cells including TRAIL$^+$ NK subpopulation increased for all doses of EVR. Consistently, liver-resident NK cells from the EVR-treated mice displayed enhanced cytotoxicity against TRAIL-sensitive neoplastic cells. EVR treatment inhibited the transition of the immature subset of liver NK cells to a mature state. The negative regulator of NK cells FoxO1 was activated as a consequence of impaired mTORC2-dependent AKT phosphorylation. Activated FoxO1 both reduced T-bet expression and induced TRAIL expression, thereby inhibiting NK cell maturation and promoting the antitumour activity of the immature subset of liver NK cells in response to EVR treatment. These findings indicate that EVR treatment enhances the antitumour activity of immature liver-resident NK cells through TRAIL upregulation.

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Introduction

Natural killer (NK) cells, the third most abundant lymphocyte population, play a vital role in innate immunity and are considered to be a first line of defence for the immune system. In contrast to T and B cells, NK cells do not require priming to exert effector functions on neoplastic cells, modified cells or invading infectious microbes [1,2]. NK cells express a complex array of activating and inhibitory receptors [3], and the predominance of receptor type determines NK cell function. NK cells represent a heterogeneous population, and the NK cell phenotype varies among tissues and organs [4]. For decades, most studies of NK cells have focused on mouse splenic and human peripheral blood NK cells, which are now termed conventional NK (cNK) cells. However, recent studies have focused on tissue-resident NK cells [5]. The liver contains both conventional NK cells and specific liver-resident NK cells. Liver-resident NK cells have a distinct phenotype, characterized by expression of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) [6]. Many transformed cells, including virus-infected and tumour cells, can be attacked by TRAIL [7,8]. We have previously shown that liver-resident NK cells have greater cytotoxic activity against hepatocellular carcinoma (HCC) compare to cNK cells from either peripheral or splenic blood [9].

Mechanistic target of rapamycin (mTOR) is an evolutionary conserved serine/threonine kinase that orchestrates various biological processes as it regulates proliferation, cell survival, differentiation, autophagy, metabolism both in normal and malignant cells [10,11]. The mTOR complex consists of two distinct components that differ in their core components—mTORC1 and mTORC2. mTOR plays an important role in controlling the growth and survival of various types of malignant cells [12]. It has been shown that the mTOR pathway is involved in the regulation of development and function of immune cells, both adaptive and innate [13]. mTOR has been proven to be essential for IL-15-dependent NK cell development and activation, regulation of granzyme and IFN-γ production [14]. The transcriptional factors T-bet and Eomes were also found to be dependent on mTORC1 and mTORC2 molecules [15], and T-bet determines developmental stability in immature NK cells with constitutive expression of TRAIL.

The immunosuppressive regimen currently used after organ transplantation, which includes calcineurin inhibitors (CNIs) combined with antimetabolites and steroids in most transplant centres, efficiently reduces adaptive components [16]. Although, considered a breakthrough, remarkably reducing rejection rate in recipients of organ transplantation, it can put patients at risk of infection, and cancer recurrence or *de novo* formation [17]. During suppressed adaptive immunity, the innate immune cells may play pivotal roles in immune surveillance and defense against microbes and neoplastic cells after transplantation. Hence, the influence of immunosuppressants on innate immunity may need to be elucidated in order to minimize morbidities related to immunosuppressive therapies.

The mTOR pathway is an important target of immunosuppressive and anti-proliferative therapies in fields of transplantation oncology. An mTOR inhibitor (mTORi), everolimus (EVR), was approved as a component of immunosuppression therapy for the prevention of graft rejection in liver transplantation (LT) in Europe, the United States and Japan in 2012, 2013 and 2018, respectively. LT is an oncological preferable approach to HCC, as it can remove all the intrahepatic tumour foci along with the oncogenic cirrhotic liver. The use of mTORi for immunosuppression after LT for HCC has been proposed due to the antitumour activity of these agents [18]. So far, the use of mTORi is encouraging in terms of oncological outcomes for patients who have undergone LT for HCC, for prevention and treatment of HCC recurrence although, definitive data are still awaited [16,19]. In this context, the oncologic effects of mTORi have been proven based in basic and clinical research [16]; however, the impact of mTORi-treatment on innate immunity has been very poorly addressed.

In the present study, we aimed to determine the possible impact of EVR on liver-resident NK cells antitumour activity.

Materials and methods

Mice

C57BL/6J (B6) female mice that were 8–12 weeks old were purchased from CLEA Japan, Inc. (Osaka, Japan). Mice were housed in the animal facility of Hiroshima University, Japan, in a pathogen-free microenvironment. The study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals and the local committee for animal experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University (Permit Number: A15-97). All animal experiments were performed according to the guidelines set out by the US

Transplant International 2020; 33: 229–243

National Institutes of Health (1996). This work was performed in part at the Research Facilities for Laboratory Animal Science, Natural Science Center for Basic Research and Development (N-BARD), Hiroshima University, Japan. Animal welfare was monitored daily by experienced vivarium staff. Procedures were always performed under isoflurane inhalation. All efforts were made to minimize suffering of living animals and animals undergoing euthanasia.

Drug preparation

Everolimus was purchased from Focus Biomolecules (Plymouth Meeting, PA, USA; catalogue # 10-2136, lot # X102100), diluted in PBS at a concentration of 25 mg/ml and stocked at −80 °C. B6 mice were treated with EVR by intraperitoneal injection at one of three different doses (0–0.25 mg/kg) for 1 and 4 weeks.

Isolation of lymphocytes

Liver lymphocytes were prepared according to a previously described method [6]. In brief, after injection of 1 ml phosphate-buffered saline (PBS) supplemented with 10% heparin via the portal vein, the liver was removed by dissection and perfused with 50 ml PBS supplemented with 0.1% ethylene-diamine tetra acetic acid. Blood cells were harvested from the liver perfusate by centrifugation, and erythrocytes were removed using ACK (Ammonium–Chloride–Potassium) lysis buffer. Splenic lymphocytes were prepared as a single-cell suspension by gently crushing the spleens in PBS; erythrocytes were removed by treatment with ACK buffer.

NK cell isolation

Liver lymphocytes were obtained from EVR-treated and untreated mice. NK cells were separated by high-gradient magnetic sorting using a manual Macs separator (Miltenyi Biotec, Auburn, CA, USA). In brief, leucocytes were magnetically labelled using a cocktail of biotin-conjugated monoclonal antibodies against non-NK cells and streptavidin microbeads (Miltenyi Biotec). Isolation of unlabelled NK cells was achieved by depleting the labelled cells. Purity of isolated NK cells was assessed by flow cytometry; the purity of sorted cells was routinely over 90%.

Flow cytometry

Freshly isolated mononuclear cells were pre-incubated with anti-CD16/32 (2.4G2) mAb to block nonspecific Fcγ II/III receptor binding and then were stained with following diluted fluorescently labelled monoclonal antibodies (mAbs): anti-NK1.1 (PK136), anti-TCRβ chain (H57597), anti-CD11b (M1/70), anti-CD335 (NKp46) and anti-CD69 (H1.2F3) from BD Pharmingen (San Diego, CA, USA); anti-CD27 (LG.3A10) were obtained from Biolegend (San Diego, CA, USA); anti-TRAIL (Clone: N2B2) was obtained from eBioscience (San Diego, CA, USA). Dead cells were excluded by light scatter and propidium iodide or 7-AAD or DAPI staining.

Following the surface staining, anti-Eomes (Dan11-mag) mAb (eBioscience) and anti-T-bet (4B10) mAb (eBioscience) were used for intracellular transcription factor staining using Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, Waltham, MA, USA).

For phospho-flow, fixed liver mononuclear cells were stained with or FoxO1 (C29HA) from Cell Signaling (Danvers, MA, USA), or anti-AKT (pS473) from BD Bioscience (Mountain View, CA, USA). All procedures were performed following the manufacturer's instructions.

The apoptosis-related markers on Hepa 1–6 and YAC-1 cells were also analysed using PE-conjugated anti-mouse CD 262 (DR5; MD 5-1), anti-mouse decoy TRAIL-receptor 3 (DcR1) and 4 (DcR2), or PE-conjugated Rat IgG2, λ1 isotype control antibody, obtained from eBioscience. Dead cells were excluded by light scatter and 7-AAD staining.

BD LSR Fortessa, FACS Canto II Cytometer and a FACSCalibur flow cytometer (BD Bioscience) were used for flow cytometric analysis. Data were analysed using FLOWJO 10.1.7 software (Tree Star, San Carlos, CA, USA).

Cytotoxicity assay

Mouse lymphoma cells (YAC-1) and mouse hepatoma cells (Hepa 1–6), purchased from the RIKEN Cell Bank (Tsukuba, Japan), were used as target cells. The effector cells were freshly sorted liver NK cells obtained from PBS-treated (control) mice and mice that were treated with EVR for 1 and 4 weeks intraperitoneally. YAC-1 and Hepa 1–6 cells were labelled with $Na_2[^{51}Cr]O_4$ and then incubated with effector cells in round-bottom 96-well plates for 4 h. For controls, target cells were incubated either in culture medium to determine spontaneous release or in a mixture of 2% Nonidet P-40 to define maximum ^{51}Cr release. Cell-free supernatants were carefully harvested, and radioactivity from the ^{51}Cr that had been released into the supernatants was

measured using a gamma counter (Aloka ARC-380). The cytotoxicity percentage, as indicated by ^{51}Cr release, was calculated using the following equation: per cent cytotoxicity = [(cpm of experimental release − cpm of spontaneous release)]/[(cpm of maximum release − cpm of spontaneous release)]. In some experiments, the assay was performed in the presence of 10 μg/ml of anti-TRAIL (N2B2) mAb or rat IgG2a λ isotype-matched control mAb, in order to determine the contribution of TRAIL molecule in liver NK cell cytotoxicity against cancer cells.

Statistical analysis

Unpaired Student's *t*-tests and the nonparametric Mann–Whitney *U*-test were performed to compare differences between the two independent groups; $P < 0.05$ was considered statistically significant. Values are expressed as mean ± standard deviation (SD). JMP 11 software (SAS Institute Inc., Cary, NC, USA) was used for all calculations.

Results

Proportion of NK cells in the liver increased after EVR treatment

To examine the impact of mTOR inhibition on lymphopoiesis, EVR was injected i.p. for 1 week at three different doses (0.0125, 0.025 or 0.25 mg/kg), while PBS solution was injected into control group mice. The overall number of mononuclear cells in the liver remained constant in mice that received any dose of EVR, whereas those in the spleen were somewhat decreased compared with control mice that received PBS (data not shown). Flow cytometry analyses revealed that the proportion of $NK1.1^{+}TCR\beta^{-}$ NK cells and $NK1.1^{+}TCR\beta^{+}$ NKT cells increased among mononuclear cells in the liver in mice that received any dose of EVR when compared with control mice that received PBS, whereas those in the spleen were not affected (Fig. 1). The proportions of $NK1.1^{-}TCR\beta^{+}$ T cells in both the liver and spleen were not affected by EVR administration.

Liver $TRAIL^{+}$ NK cells increased in frequency in response to mTOR inhibitor treatment

We further analysed the potential influence of EVR on the phenotypic profile of NK cells in the liver and spleen. As shown in Fig. 2, liver NK cells included cells that expressed CD69—one of the earliest expressed activating markers. The stimulatory receptor NKp46, a natural cytotoxic receptor that can efficiently trigger the release of cytotoxic granules, cytokines, and chemokines upon binding ligands of viral and bacterial origin [20], was expressed in 86.7% liver NK cells (Fig. 2). By contrast, ~5% and ~50% of splenic NK cells expressed CD69 and NKp46, respectively (data not shown). Among TNF family members, TRAIL has recently been shown to be critical for NK cell-mediated antitumour functions [21,22]. Approximately 20% of liver NK cells, but not spleen NK cells, constitutively express TRAIL molecules. After 1 week of EVR treatment, the proportion of CD69-expressing NK cells remained constant both in the liver (Fig. 2) and spleen (data not shown) compared with that in untreated mice. Notably, the proportion of TRAIL-expressing NK cells significantly increased in the liver, whereas TRAIL expression remained undetectable in splenic NK cells after EVR treatment.

TNF-related apoptosis-inducing ligand expression on liver NK cells after a longer period of EVR treatment (4 weeks) remained enhanced, although it was not higher than the 1-week treatment (Fig. 3).

Increased cytotoxicity of liver lymphocytes against TRAIL-sensitive and TRAIL-resistant cancer cells after EVR treatment

We attempted to determine whether EVR treatment promoted NK cell-mediated cytotoxicity against neoplastic cells. By magnetic sorting, NK cells were isolated from liver mononuclear cells of mice treated with EVR (0.25 mg/kg) for 1 week and from control mice. As shown in Fig. 4, the ^{51}Cr release assay revealed that liver NK cells from EVR-treated mice showed significantly higher cytotoxicity against Hepa 1–6 cells, which express surface DR5 but lack DcR-1 and DcR-2 (Fig. 4a), which are ligands for TRAIL, when compared with NK cells from control mice. However, cytotoxicity against YAC-1 cells, which are TRAIL-resistant tumour cells, did not differ between the groups (Fig. 4b). TRAIL neutralizing antibody abrogated the liver NK cells cytotoxicity, while isotype-matched control group kept the liver NK cell function (Fig. 4c). This finding indicates the role of TRAIL molecule in liver NK cell induced cytotoxicity. We also performed additional cytotoxicity assay. Hepa 1-6 TRAIL-sensitive cells were used as target and liver NK cells, obtained from 4 week EVR-treated mice were used as effectors. We found that EVR treatment induced liver NK cell cytotoxicity even after 4 weeks of treatment (Fig. 4d).

Transplant International 2020; 33: 229–243

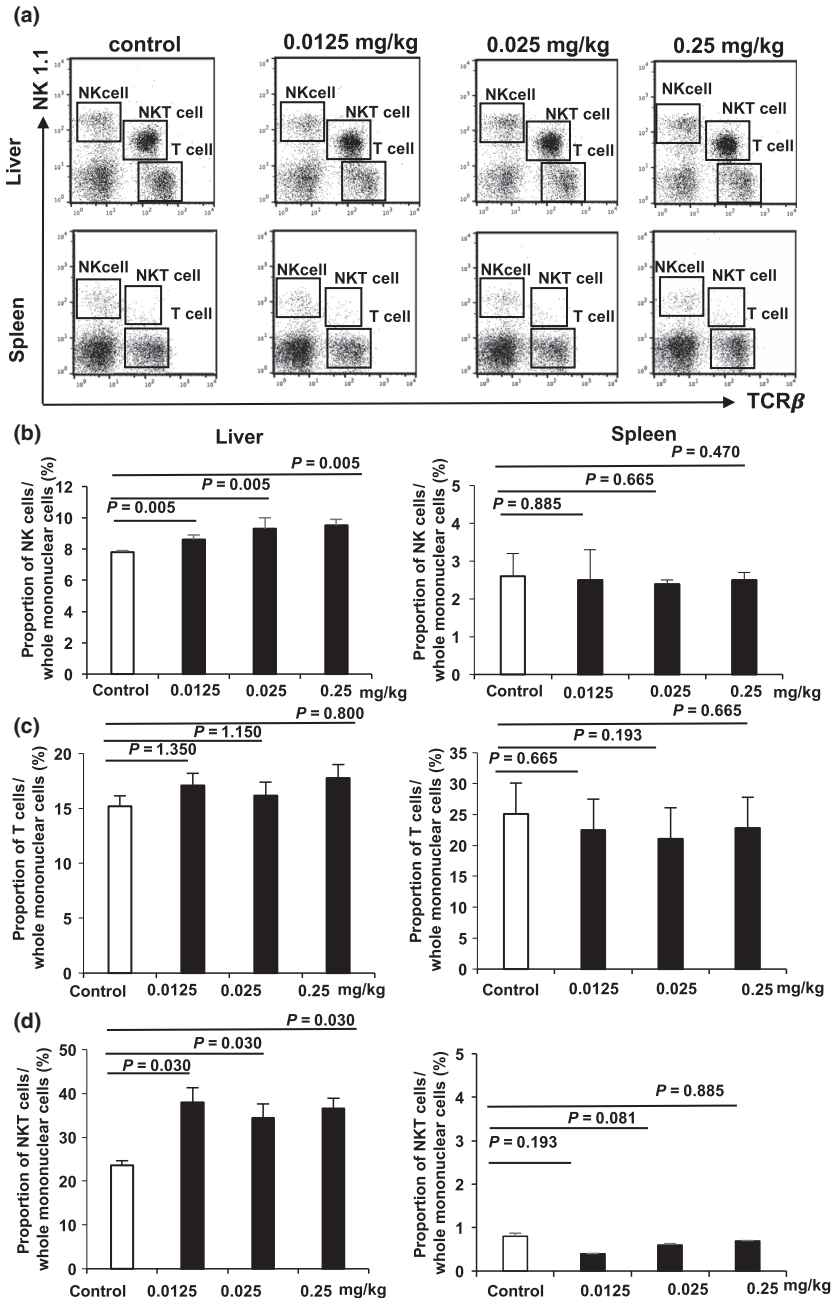

Figure 1 Liver and splenic lymphocytes were isolated from untreated control or EVR-treated mice. (a) Lymphocytes were stained with anti-NK 1.1, anti-TCRβ and propidium iodide. Representative flow panels show the proportion of TCRβ$^-$ NK 1.1$^+$ NK, TCRβ$^+$ NK 1.1$^+$ NKT cells and TCRβ$^+$ NK 1.1$^-$ T cells in the liver and spleen. The bar graph shows the average of the proportion of NK cells in control or EVR-treated mice. Proportions of NK cells (b), T cells (c) and NKT cells (d) in the liver and spleen are shown. Data are expressed as mean ± SD (n = 4 per group). Significant difference between means were assessed using the nonparametric Mann–Whitney U-test. $P < 0.05$ was considered statistically significant. EVR, everolimus; NK, natural killer.

EVR inhibited the transition of CD27$^+$ CD11b$^-$ to CD27$^+$ CD11b$^+$ NK cells in the liver and enhanced TRAIL expression on immature/intermediate stage NK cells

Natural killer cells have been considered to represent heterogeneous population, among which maturational stages can be divided based on CD27 and CD11b surface expression. It has been proposed that CD11b^{low} CD27^{low}, CD11b^{low} CD27^{high}, CD11b^{high} CD27^{high} and CD11b^{high} CD27^{low} (hereafter referred to as double negative: DN, CD27 single positive: CD 27 SP, double positive: DP, and CD11b single positive: CD11b SP NK cells, respectively) are discrete stages of *in vivo*

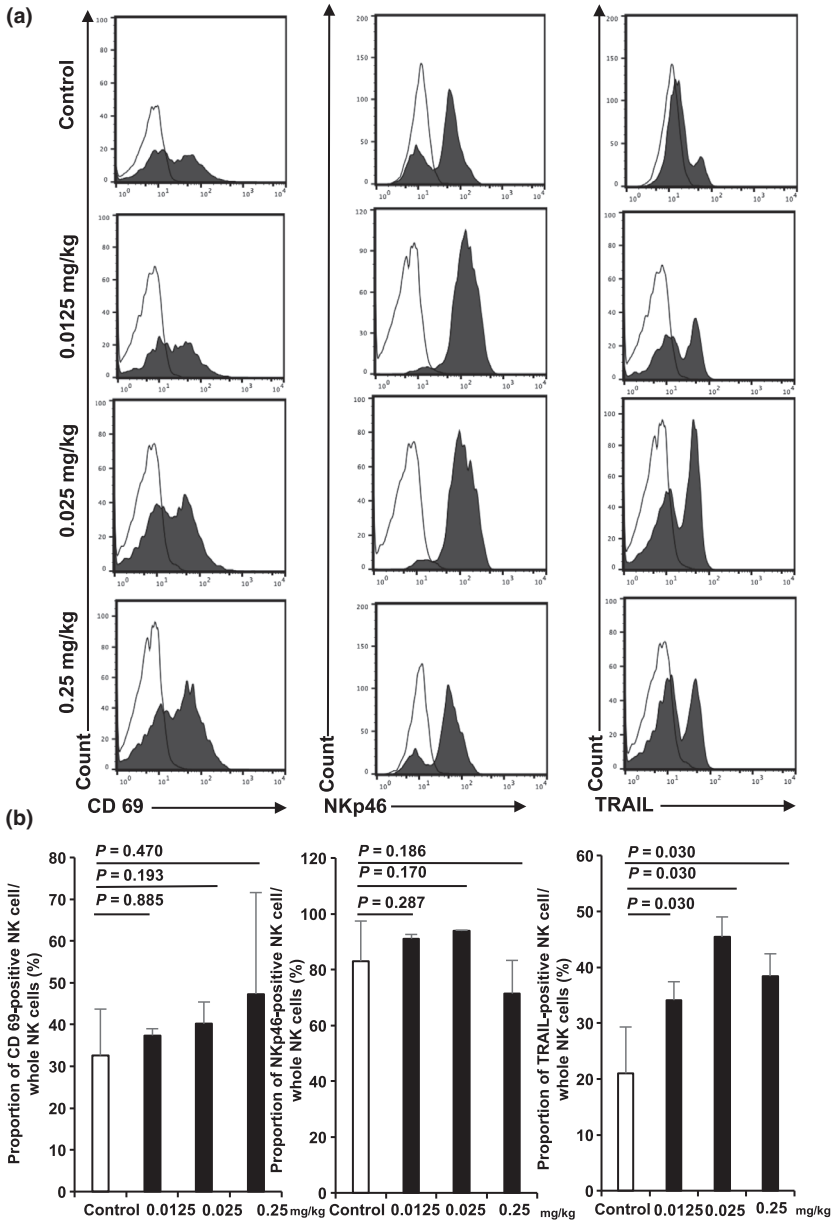

Figure 2 The liver TCRβ$^-$ NK1.1$^+$ NK cells obtained from control and EVR-treated mice were selected by gating on flow cytometry analysis. Expression levels of CD69, NKp46 and TRAIL on those cells were analysed. (a) Representative histograms for the expression of CD69 (left), NKp46 (middle) and TRAIL (right) are shown for the indicated EVR concentration (0–0.25 mg/kg). (b) The bar graph shows the average of each marker expressed by NK cells in control or EVR-treated mice. Data are expressed as mean ± SD (n = 4 per group). Significant difference between means was assessed using the nonparametric Mann–Whitney U-test. $P < 0.05$ was considered statistically significant. EVR, everolimus; NK, natural killer; TRAIL, TNF-related apoptosis-inducing ligand.

maturation that follow a pathway from a DN (immature) to a CD11b SP (mature) stage [23]. By using those maturational markers, we analysed the frequency of NK cell subsets in the liver and spleen in mice with or without EVR treatment to elucidate the role of mTOR inhibition in NK cell development and maturation (Fig. 5). We found that EVR treatment significantly increased the proportion of the immature CD27 SP subset in the liver, while reducing that of the mature DP cell subset when compared with those in control mice. By contrast, EVR treatment significantly increased the proportion of the mature CD11b SP subset in the spleen, while the DP intermediate stage subset was decreased after EVR treatment (Fig. 5). A previous study demonstrated that acquisition of TRAIL occurs at the earliest stage of definitive NK cell development [24].

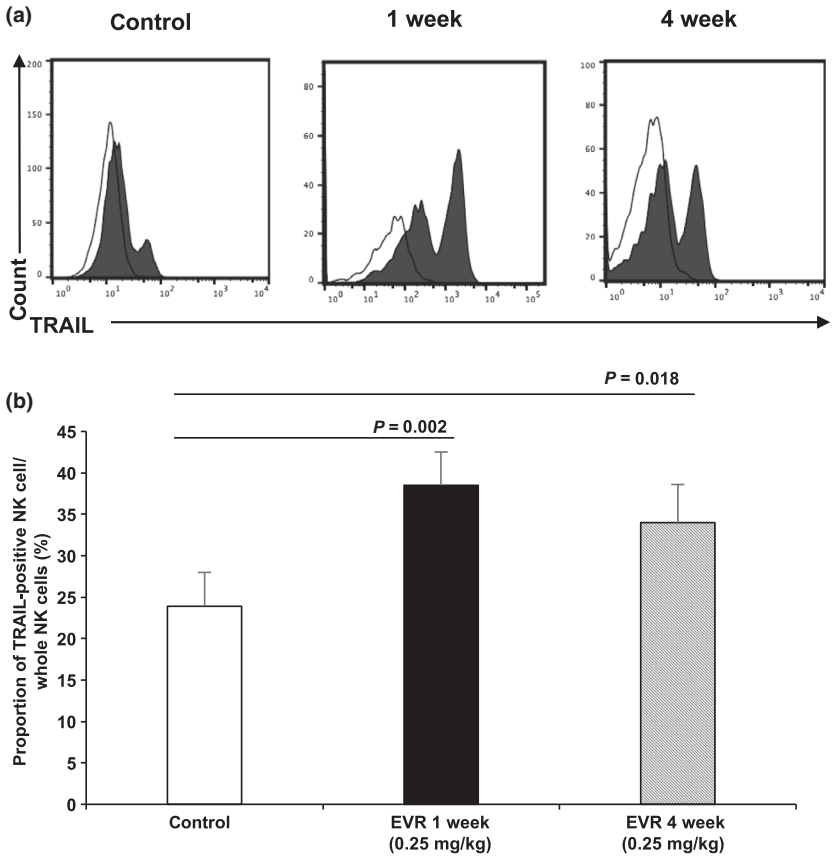

Figure 3 The liver TCRβ$^-$ NK1.1$^+$ NK cells obtained from control, 1- and 4-week EVR-treated mice were selected by gating on flow cytometry analysis. Expression levels of TRAIL on those cells were analysed. (a) Representative histograms for TRAIL expression are shown for the indicated EVR treatment period. (b) The bar graph shows the average of TRAIL expression by NK cells in control or EVR-treated mice. Data are expressed as mean ± SD (n = 5 per group). The significant difference between means was assessed using the nonparametric Mann–Whitney U-test. $P < 0.05$ was considered statistically significant. EVR, everolimus; NK, natural killer; TRAIL, TNF-related apoptosis-inducing ligand.

To further investigate the influence of EVR treatment on NK cell maturation, we analysed TRAIL expression, as another marker of immaturity, on each NK cell maturational subset in the spleen and liver of untreated control and EVR-treated mice (Fig. 6). We found that the proportion of TRAIL-expressing NK cells among DN and CD27 SP liver NK cell subsets increased in the EVR-treated group, while that among mature DP and CD11b SP liver NK cell subsets was unaffected (Fig. 6). Thus, EVR treatment promotes TRAIL expression on NK cells at the immature and intermediate stages, but not at later stages in the liver.

Everolimus enhanced TRAIL expression on liver-resident NK cells through impaired mTORC2-AKT^{Ser473}–FoxO1 signalling

To identify the molecular pathway by which mTOR inhibition blocked the maturation of immature CD27 SP to mature DP and CD11b SP NK cells in the liver, we searched for mTOR-dependent negative regulators of NK cell development. FoxO1 was previously established to be a transcription factor critical for NK cell development. FoxO1 is a member of the FoxO family transcription factors and is abundantly expressed by immature NK cells. Mice that lack FoxO1 exhibit an increased frequency of the most mature CD11b SP NK cells and a decreased CD27 SP NK cell population [25]. FoxO1 activation is dependent upon AKT^{Ser473} phosphorylation. Prolonged EVR treatment can suppress the assembly of mTORC2, which results in inhibition of AKT phosphorylation on its Ser 473 site [26]. AKT has been shown to inactivate members of the FoxO family of transcription factors by inducing their exit from the nucleus [27,28]. Among FoxO family proteins, FoxO1 and FoxO3 are considered as negative regulators of NK cell development [25]. Experiments with cancer cell lines have established that the level of FoxO proteins is positively correlated with TRAIL expression, that is FoxO1 overexpression results in increased levels of TRAIL [29]. Taking in account the mechanisms described

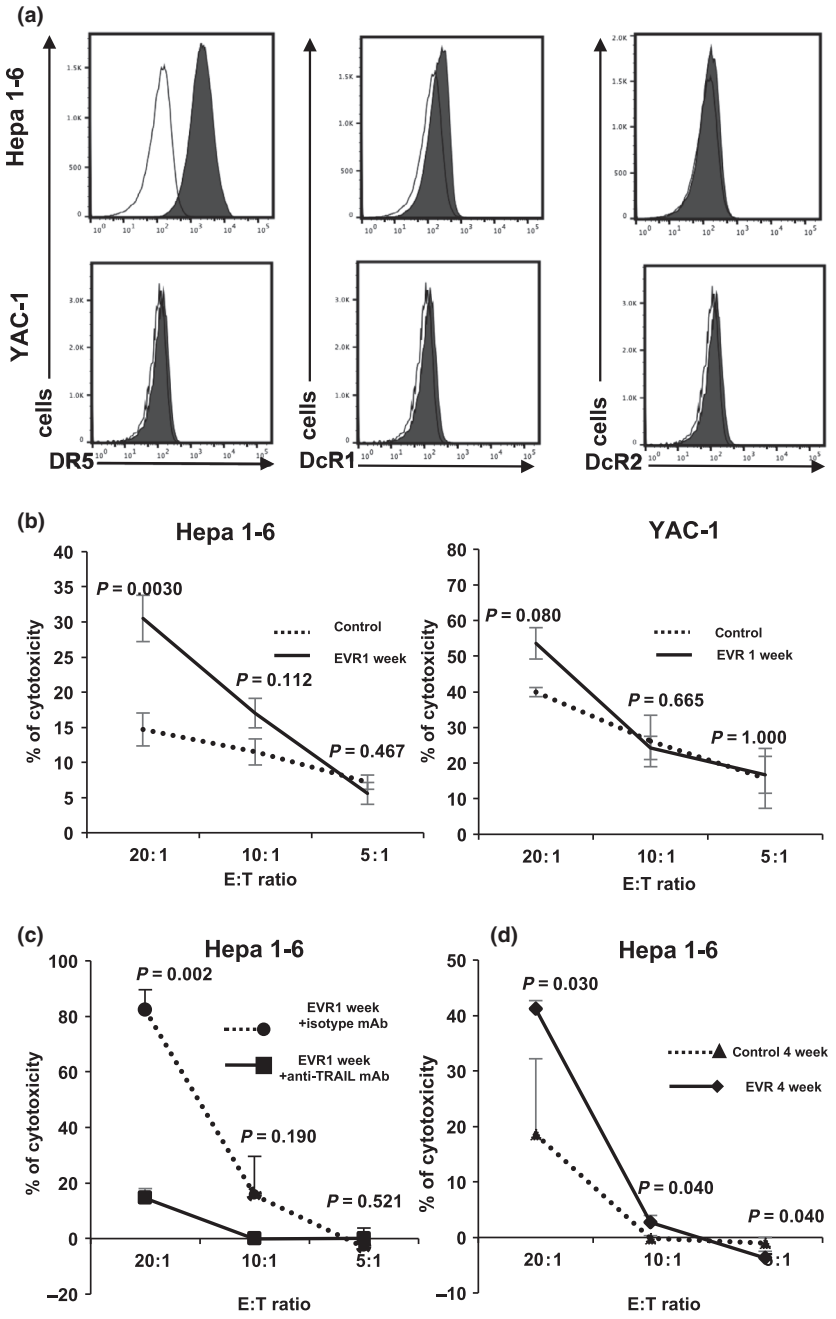

Figure 4 The cytotoxicity of liver NK cells against TRAIL-sensitive and TRAIL-resistant cancer cells after EVR treatment. (a) Decoy receptors (DcR1, DcR2) and death receptor (DR) 5 expression levels on Hepa 1–6 cells (upper) and YAC-1 cells (bottom) are showed. The filled histograms indicate those expressions, whereas the open histograms indicate negative staining with isotype-matched control mAb. (b) Cytotoxic activity of freshly isolated liver NK cells from control (dotted line) and mice treated with EVR for 1 week (solid line) against TRAIL-sensitive Hepa 1–6 cells (left) and against TRAIL-resistant YAC-1 cells (right) was measured using the ^{51}Cr release assay as described in material and methods. (c) Cytotoxicity of liver NK cells, from mice treated with EVR for 1 week, against TRAIL-sensitive Hepa 1–6 cells in the presence (solid line with square cap) or absence (dotted line with round cap) of TRAIL neutralizing mAb. (d) Cytotoxic activity of freshly isolated liver NK cells from mice treated with EVR for 4 weeks (solid line) and PBS-treated control mice (dotted line) against TRAIL-sensitive Hepa 1–6 cells was measured using the ^{51}Cr release assay. The effector to target (E:T) ratios were 20:1, 10:1 and 5:1. The percentage cytotoxicity was calculated as the percentage of specific ^{51}Cr release, as described in the material and methods section. Data are presented as mean ± SD from triplicate samples of assays repeated four times. Significant difference between means was assessed using the nonparametric Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant. EVR, everolimus; NK, natural killer; TRAIL, TNF-related apoptosis-inducing ligand.

Transplant International 2020; 33: 229–243

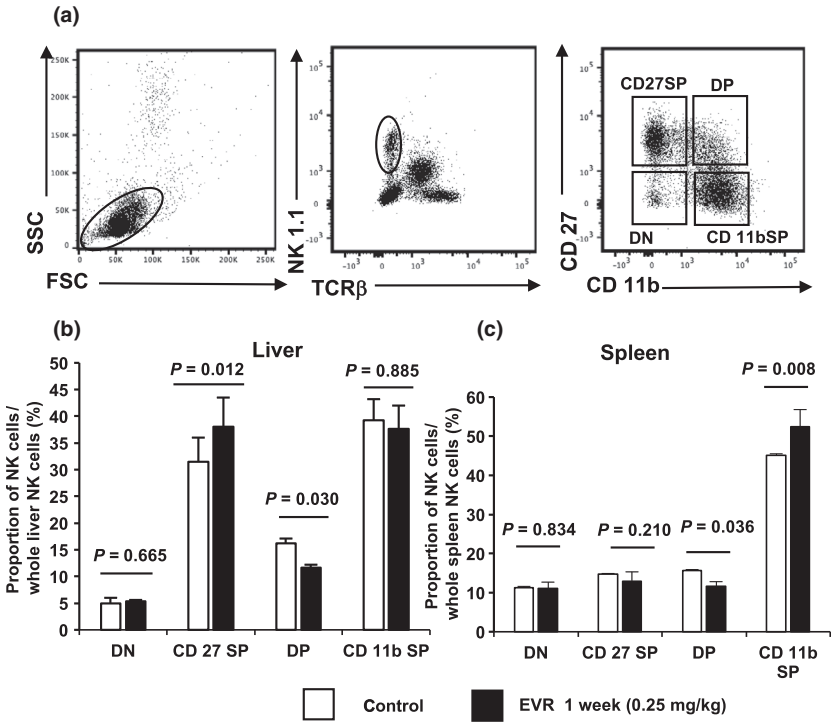

Figure 5 Liver and spleen lymphocytes were isolated from control or EVR-treated mice. TCRβ^{-} NK1.1^{+} NK cells were stained with anti-CD27 and anti-CD11b to divide NK cells into different stages of maturation. (a) Representative flow panel shows the proportion of CD11b^{low} CD27^{low} (DN), CD11b^{low} CD27^{high} (CD27 SP), CD11b^{high} CD27^{high} (DP) and CD11b^{high} CD27^{low} (CD11b SP). (b) The frequency of NK cell subsets in the liver (left) and spleen (right) in control or EVR-treated mice. The bar graphs show mean ± SD for five independent experiments. Significant difference between means was assessed using the nonparametric Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant. EVR, everolimus; NK, natural killer.

above, we investigated the impact of EVR treatment on FoxO1 expression on NK cells by analysing the level of FoxO1 and phosphorylated AKT in all liver NK cell subsets. After 7 days of EVR treatment, the level of phosphorylated AKT was significantly decreased on DN, CD27 SP and DP NK cells, whereas it was unaffected on the mature CD11b SP subset (Fig. 7). Predictably, the level of active FoxO1 protein was elevated within the immature liver NK cell subset, while levels of FoxO1 on mature DP and CD11b SP remained intact (Fig. 7). These data indicate that EVR treatment enhances TRAIL expression on liver NK cells through impaired AKT phosphorylation and consequent hyperactivation of FoxO1.

Accumulation of immature subsets of NK cells in the liver in EVR-treated mice was associated with decreased T-bet activity

A previous study demonstrated that hepatic hematopoiesis restricts Eomes expression and limits NK development to the T-bet-dependent, immature stage, whereas medullary hematopoiesis permits Eomes-dependent NK maturation in mice [24]. We investigated the impact of EVR treatment on the expression of T-bet and Eomes, which are both T-box transcriptional factors and are considered to be critical for NK cell maturation, on liver NK cells at various stages of maturation. We found that EVR treatment resulted in a significant reduction of T-bet in immature subsets (DN and CD27 SP) and of Eomes levels in all populations of liver NK cells (Fig. 8). Taken together with the finding that the terminal stages of maturation are incomplete in the absence of T-bet [24], our findings suggest that the accumulation of immature subsets of NK cells in the liver of EVR-treated mice is caused by reduced T-bet activity.

Discussion

The immunoregulatory effects of mTOR inhibition vary among immune cells [30]. Many previous studies have explored the role of mTOR and downstream effectors in T-cell differentiation [31]. By contrast, limited data are available on the role of mTOR in NK cell differentiation. Previous studies have revealed that external

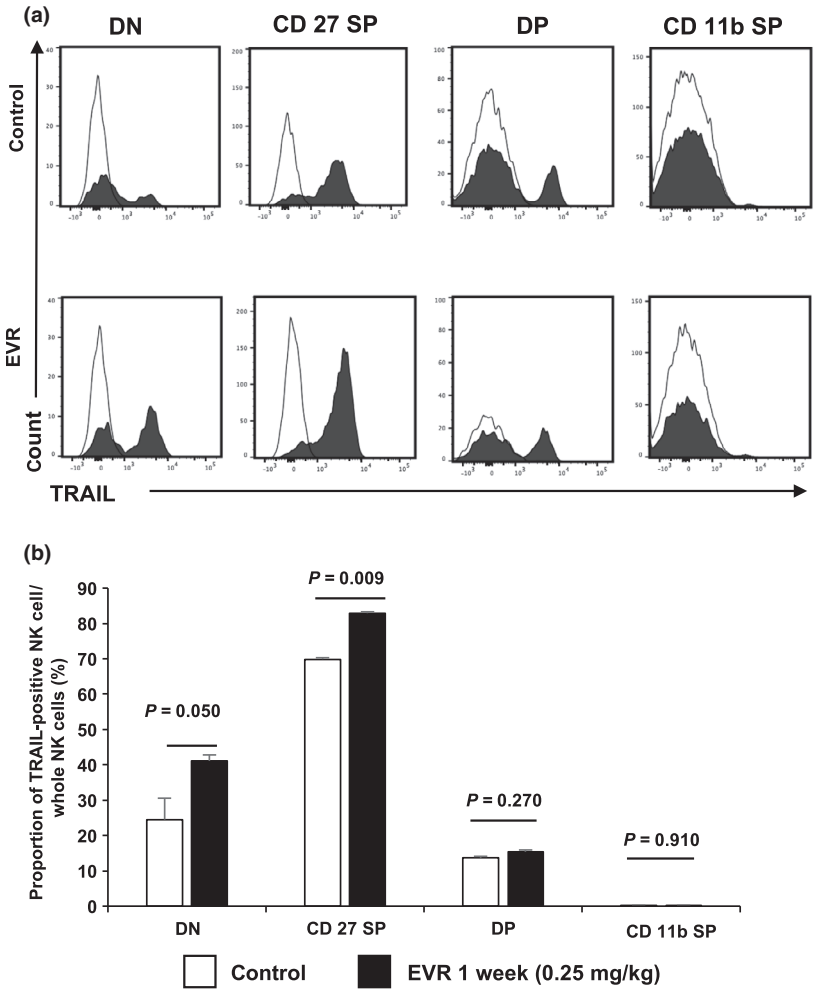

Figure 6 Liver TCRβ$^-$ NK1.1$^+$ NK cells were stained with anti-CD27 and anti-CD11b mAbs. TRAIL expression on each liver NK cell subset before and after EVR treatment was analysed. (a) Representative histograms for TRAIL expression of each subset are shown in control (upper) and EVR-treated (lower) mice. (b) The bar graph shows the average expression for TRAIL-positive NK cells of each subset in control or EVR-treated mice. Data in the bar graph show mean ± SD of five independent experiments. Significant difference between means was assessed using the nonparametric Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant. EVR, everolimus; NK, natural killer; TRAIL, TNF-related apoptosis-inducing ligand.

factors, such as growth factors and various cytokines (e.g. IL-15, IL-2 and IL-12), as well as intrinsic transcription factors (e.g. Tbx21 and Eomes) are required to control NK cell differentiation, maturation and effector functions [24]. Furthermore, mTOR acts as a central integrator that regulates anabolic growth and proliferation in response to both extracellular and intracellular signals [10,11]. mTOR forms the catalytic subunit of two structurally distinct complexes, mTORC1 and mTORC2, that mediate separate but overlapping cellular functions [32]. Recent studies involving rapamycin treatment or mTOR deletion indicate that the kinase mTOR controls a key checkpoint in NK cell differentiation, and its activation occurs downstream of IL-15 and requires a negative signal from Tsc1 [14]. However, how inhibition of mTOR signalling mediates the cellular functions of NK cells remains unclear.

Another group previously reported that mTOR deficiency can lead to a severe NK cell developmental block, which is considered to be a negative effect of mTOR inhibitors [32]. They found that mTOR inhibitor treatment reduced the expression of granzyme A and B in conventional NK cells and impaired NK cell reactivity towards missing-self targets. In contrast, our present study revealed that EVR treatment increased TRAIL expression exclusively on liver-resident NK cells and significantly increased the cytotoxicity of these cells against targets with TRAIL/Apo2L death-inducing receptors, but not missing-self targets. Susceptibility to TRAIL-induced apoptosis is thought to be related to the

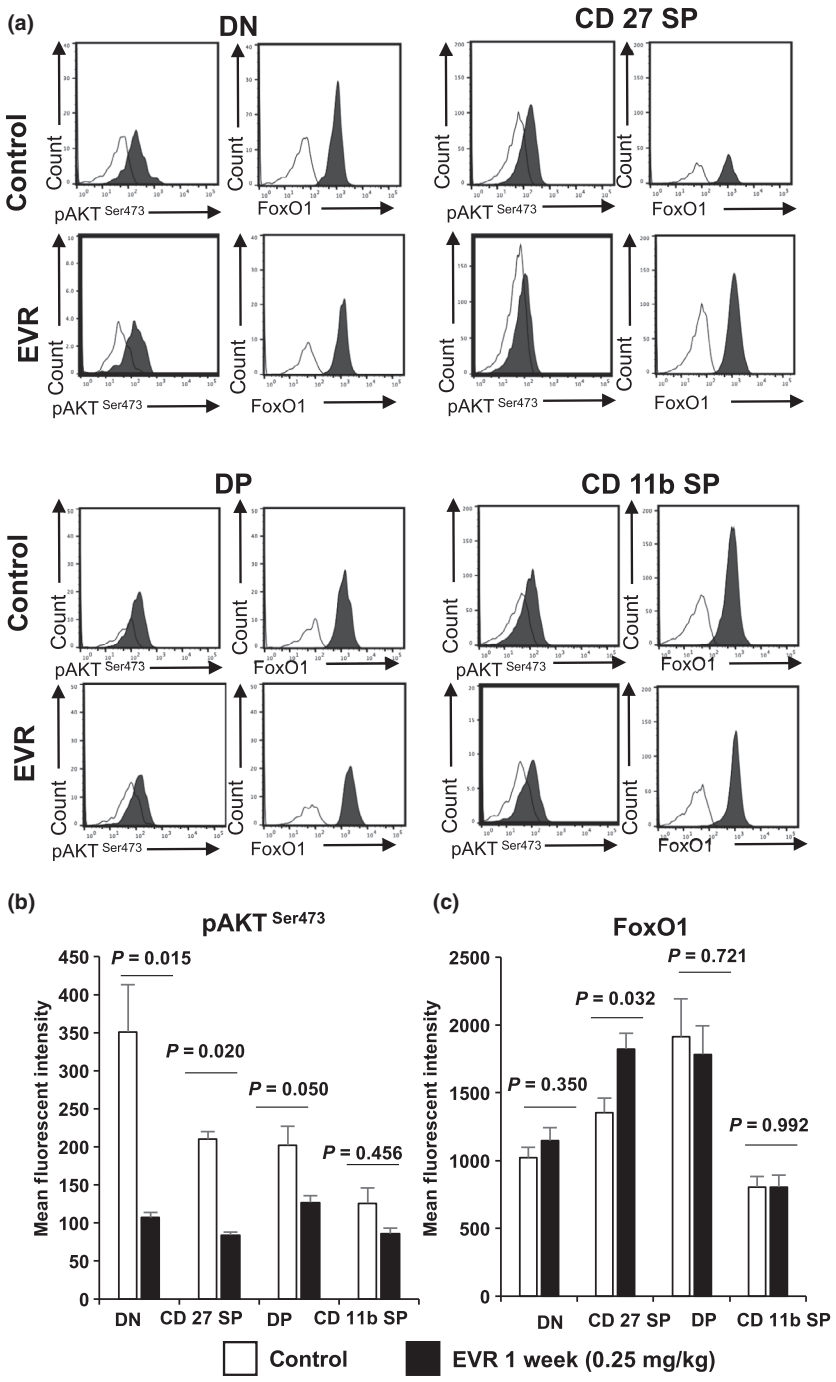

Figure 7 Liver TCRβ$^-$ NK1.1$^+$ NK cells were stained with anti-CD27 and anti-CD11b antibodies to detect cell surface expression. Anti-phosphorylated AKT^{Ser473} and anti-FoxO1 mAbs were used for intracellular staining. (a) Representative histograms show the expression of pAKT-Ser473 and FoxO1 expression for each subset. (b) The bar graph shows the MFI of pAKTSer473 on each subset in control or EVR-treated mice. (c) The bar graph shows the MFI of FoxO1 on each subset in control or EVR-treated mice. Bar graph data are shown as mean ± SD of four independent experiments. Significant difference between means was assessed using the nonparametric Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant. EVR, everolimus; NK, natural killer.

expression levels of multiple receptors on target cells. TRAIL binds to at least four receptors, two of these are death-inducing receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) that contain cytoplasmic death domains and signal apoptosis, whereas two other death-inhibitory receptors (TRAIL-R3/DcR1 andTRAIL-R4/DcR2) lack a functional death domain and do not mediate apoptosis; all of these receptors have similar ligand affinities, and

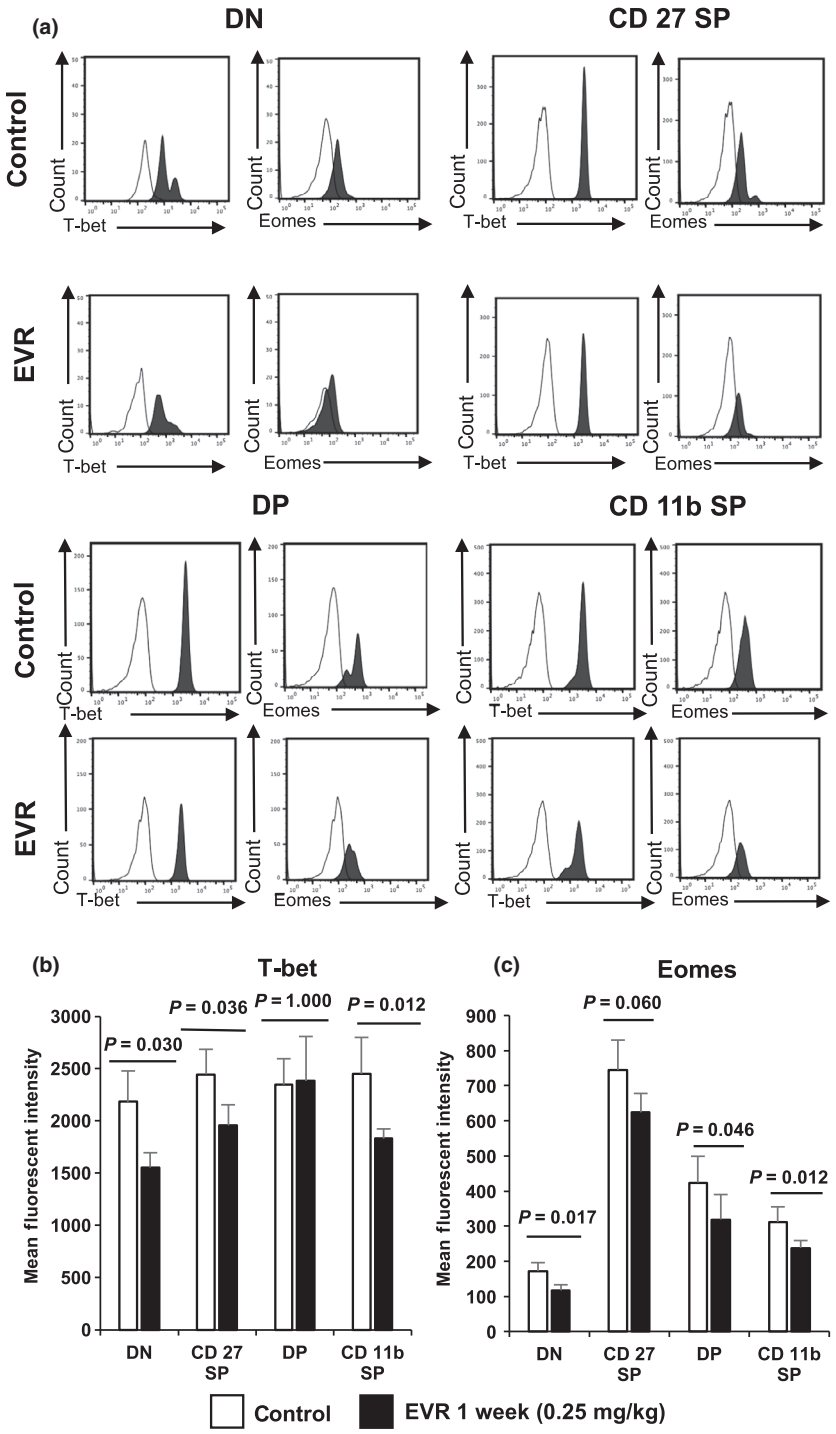

Figure 8 Liver TCRβ$^-$ NK1.1$^+$ NK cells were stained with anti-CD27 and anti-CD11b mAbs to detect cell surface expression. Anti-T-bet and anti-Eomes mAbs were used for intracellular staining. (a) Representative histograms show the expression levels of T-bet and Eomes for each subset. (b) The bar graph shows the MFI of T-bet expression on each subset in control or EVR-treated mice. (c) The bar graph shows the MFI of Eomes on each subset in control or EVR-treated mice. Data in the bar graphs are shown as mean ± SD of four independent experiments. Significant difference between means was assessed using the nonparametric Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant. EVR, everolimus; NK, natural killer.

the latter receptors may act as decoys. The preferential expression of these decoy receptors in normal tissues suggests that TRAIL may be useful as an antitumour agent that induces apoptosis in cancer cells, while sparing normal cells. We have previously demonstrated that moderately/poorly differentiated HCCs express

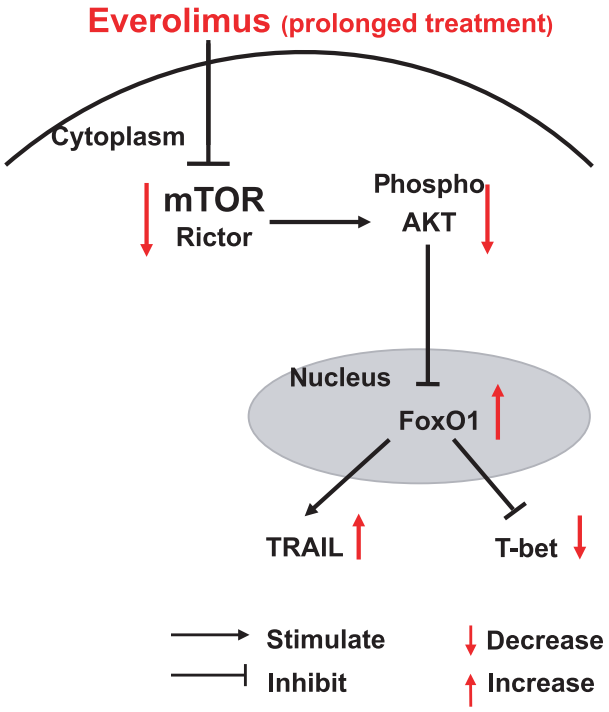

Figure 9 The EVR-sensitivity of liver NK cells. FoxO1 is dependent on AKT phosphorylation, which is well known to be downstream of the mTORC2 complex. mTORC2 can phosphorylate AKT on its Ser 473 site, then phosphorylated AKT can inactivate FoxO1 transcription factors by inducing their exit from the nucleus. FoxO1 regulates NK cell development through T-bet. TRAIL has been found to be a direct target of FKHR (FoxO1), as it has been demonstrated that FoxO1 overexpression results in increased TRAIL expression. Therefore, EVR treatment decreased levels of active AKT, which resulted in a significant elevation of intranuclear FoxO1 protein levels in NK cells. Liver NK cells are enhanced by EVR treatment through the mTORC2-AKT^{Ser47}–FoxO1 pathway, in particular because of hyperactive FoxO1. EVR, everolimus; NK, natural killer; TRAIL, TNF-related apoptosis-inducing ligand.

remarkable levels of DR4 and DR5, but do not express DcR1 or DcR2, indicating a susceptibility to TRAIL-expressing NK cell-mediated cell killing, while normal liver tissues do not express all those receptors [9]. Hence, our demonstration that EVR treatment increased TRAIL expression on liver-resident NK cells likely reflects clinical studies where patients who received mTOR inhibitor-based immunosuppression therapy showed a better survival rate and lower recurrence rate in the HCC group [33]. To address the clinical relevance of our data presented as described above, further *in vivo* studies may be required.

The sequential development of NK cell subsets can be classified based on the surface expression of CD11b and CD27: CD11b^{low} CD27^{high} (CD27 SP), CD11b^{high} CD27^{high} (DP) and CD11b^{high} CD27^{low} (CD11b SP). The liver mononuclear cells contained a remarkable proportion of immature CD27 SP NK cells. We found that EVR treatment impaired the transition of CD27 SP to DP NK cells and enhanced TRAIL expression on the immature CD27 NK cells (Figs 5 and 6). To address the difference of EVR-sensitivity in the sequential development of NK cell subsets, we analysed FoxO1 levels on each NK cell subset. FoxO1, a transcription factor of the forkhead family, is dependent on AKT phosphorylation, which is a well-known downstream molecule of the mTORC2 complex, and thus, it is sensitive to mTOR inhibition. EVR and other rapalogues were thought to inhibit the mTORC1 complex. However, it has been proven that long-term EVR and rapalogue treatment can disrupt the assembly of mTORC2 [34]. mTORC2 phosphorylate AKT on its Ser 473 site, and phosphorylated AKT can inactivate FoxO1 transcription factors by inducing their exit from the nucleus [35]. Therefore, EVR treatment decreased levels of active AKT, resulting in significant elevation of intranuclear FoxO1 protein levels in NK cells. Deng *et al.* reported that FoxO1 regulates NK cell development through T-bet. In a murine model, FoxO1 binds to the T-bet promoter region. FoxO1 and T-bet inversely correlate with each other. T-bet suppresses CD27 expression, thereby promoting transition from a CD27 positive immature state to a CD27 negative mature state of NK cells [24]. We found that EVR significantly decreased T-bet expression (Fig. 8). Hence, hyperactive FoxO1 proteins likely suppress T-bet function, which is thought to block the transition of CD27 SP NK cells to a more mature DP phenotype. It has been shown that in certain cell types, FoxO1 can induce proapototic ligands, including TRAIL. TRAIL has been found to be a direct target of FKHR (FoxO1), as it has been demonstrated that FoxO1 overexpression results in increased levels of TRAIL [36]. Those are all consistent with results obtained in a model where TRAIL expression on liver NK cells is enhanced by EVR treatment through the mTORC2-AKT^{Ser473}–FoxO1 pathway, due to hyperactive FoxO1 (Fig. 9).

Interestingly, the proportion of TCRβ^{+} NK 1.1^{+} NKT cells was also significantly increased in the liver after EVR treatment (Fig. 1d). Within the NKT cell population there is maturational, phenotypic and functional diversity [37]. NKT cell development divided into four stages, based on CD 24, CD 44 and NK 1.1 surface marker expression. It has been reported that mTOR is selectively required for early development of iNKT cells.

mTORC1 deficient mice showed increased frequency of stage 0 iNKT cells compared to wild-type mice [38]. mTOR inhibition in the liver may block NKT cell maturation and increase the frequency of immature NKT cells. Further study may determine the impact of everolimus treatment on liver NKT cell maturation and function.

In conclusion, we have demonstrated that EVR treatment enhances the antitumour activity of immature liver-resident NK cells through TRAIL upregulation. Immature and mature subsets of liver NK cells have different levels of sensitivity to EVR treatment, mainly because of differential AKT activity and FoxO1 expression.

Authorship

JS: participated in research design, experimental procedures, data analysis and writing the paper. YT: participated in research design, writing the paper. NT: edited the paper. MO: edited the paper. HO: participated in research design, writing the paper.

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Conflict of interest

The authors declare no conflicts of interest that might bias this work.

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