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Lactic acid in tumor microenvironments causes dysfunction of NKT cells by interfering with mTOR signaling

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Cellular metabolism has been shown to regulate differentiation and function of immune cells. Tumor associated immune cells undergo phenotypic and functional alterations due to the change of cellular metabolism in tumor microenvironments. NKT cells are good candidates for immunotherapies against tumors and have been used in several clinical trials. However, the influences of tumor microenvironments on NKT cell functions remain unclear. In our studies, lactic acid in tumor microenvironments inhibited IFNγ and IL4 productions from NKT cells, and more profound influence on IFNγ was observed. By adjusting the pH of culture medium we further showed that, dysfunction of NKT cells could simply be induced by low extracellular pH. Moreover, low extracellular pH inhibited NKT cell functions by inhibiting mammalian target of rapamycin (mTOR) signaling and nuclear translocation of promyelocytic leukemia zinc-finger (PLZF). Together, our results suggest that tumor acidic microenvironments could interfere with NKT cell functions through metabolic controls.

lactic acid, NKT cell, IFNγ, mTOR, PLZF

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INTRODUCTION

NKT cells are CD1d-restricted T lymphocytes, which possess semi-invariant TCR, have effector phenotypes and recognize lipid antigens. Upon activation, NKT cells release abundant Th1 and Th2 cytokines and regulate functions of DCs, macrophages, B cells, NK cells and conventional T cells in a direct or indirect way (Bendelac et al., 2007). As important immune regulators, NKT cells promote the killing effects of NK cells and CTLs against tumor cells and also kill $CD1d^+$ tumor cells directly (Fujii et al., 2013). Thus, NKT cells are ideal targets for anti-tumor immunotherapies. Transferring *in vitro* expanded NKT cells or αGC-pulsed DCs showed significant prolonged mean survival time and stabilization of disease (Motohashi et al., 2006; Motohashi et al., 2009; Motohashi et al., 2011). However, some of those patients with low IFNγ production showed no clinical effects (Motohashi et al., 2011). Additionally, decreased NKT cell numbers and impaired IFNγ productions have been reported previously in cancer patients and are related to poor clinical outcome (Molling et al., 2007; Motohashi et al., 2011; Muhammad Ali Tahir et al., 2001). Thus, optimal IFNγ production is important for the antitumor effects of NKT cells. And dysfunction of NKT cells would dampen their antitumor effects. Antigens and types of antigen-presenting cells are extrinsic factors modulating the cytokine responses of NKT

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cells (Bai et al., 2009; Bai et al., 2012). However, the factors causing dysfunction of NKT cells in tumor microenvironments are still unclear.

Activation of immune cells results in a metabolic reprogramming. Upon activation, naïve T cells undergo a switch from fatty acid oxidation (FAO) and oxidative phosphorylation to glycolysis in supporting their differentiation into effector cells such as Th1, Th2 and Th17 (Barbi et al., 2013). Interestingly, Tregs and memory CD8 T cells utilize oxidative phosphorylation for energy generation. Inhibiting glycolysis or enforcing FAO promotes differentiation of memory CD8 T cells and Tregs (Michalek et al., 2011; Pearce et al., 2009; Sukumar et al., 2013; van der Windt et al., 2012). And increased glycolysis has been shown to promote the functions and development of effector CD8 T cells and Th1 cells (Cham et al., 2008; Chang et al., 2013). Additionally, mammalian target of rapamycin (mTOR) signaling, which senses nutrient availability and controls cellular metabolisms, has been reported to regulate differentiation of distinct T lineages (Pollizzi and Powell, 2015). These results demonstrate a link between cellular metabolism and differentiation and function of immune cells. Thus, immune cells in solid tumors as well as in infection sites and inflammation tissues would show different phenotypes and functions from those in normal tissues due to their distinct metabolic microenvironments. Tumor microenvironments are gradually formed during the progression of tumors as indicated by exhaustion of nutrients and accumulation of lactic acid (Molon et al., 2016; Romero-Garcia et al., 2016), which have been shown to alter the phenotypes and functions of tumor resident immune cells through interfering with their cellular metabolism (Kouidhi et al., 2016; Molon et al., 2016). Nutrient depletion inhibits functions of CTLs and drives DCs and macrophages to immunosuppressive phenotypes by inhibiting glycolysis (Chang et al., 2015; Krawczyk et al., 2010). And accumulation of lactic acid has also been shown to inhibit functions of CTLs and monocytes (Dietl et al., 2010; Fischer et al., 2007; Peter et al., 2015). Although the mechanisms by which cellular metabolism regulates NKT cell functions have not been reported, the dysfunction of NKT cells associated with tumors would hypothetically result from the impaired metabolisms in tumor microenvironments.

Here, we showed that accumulation of lactic acid in tumor environments caused dysfunction of NKT cells as indicated by dampened IFNγ production and IL4 production to a less extent. Moreover, we demonstrated that low pH in culture medium was responsible for the dysfunction of NKT cells caused by lactic acid accumulation. Moreover, we observed impaired mTOR signaling and nuclear localization of promyelocytic leukemia zinc-finger (PLZF) in NKT cells in low pH culture medium. Our results suggest that acidification of tumor environments causes dysfunction of NKT cells by interfering with mTOR signaling.

RESULTS

Lactic acid in tumor microenvironment inhibits NKT cell functions

Lactate accumulation and acidification of extracellular environment are hallmarks of solid tumors. Tumor cell lines BT474, HCC1954, MDAMB231 and B16F10 were cultured for two days, and the different colours of culture medium indicated different amount of lactic acid productions (Figure 1A). To study the influences of tumor microenvironments on

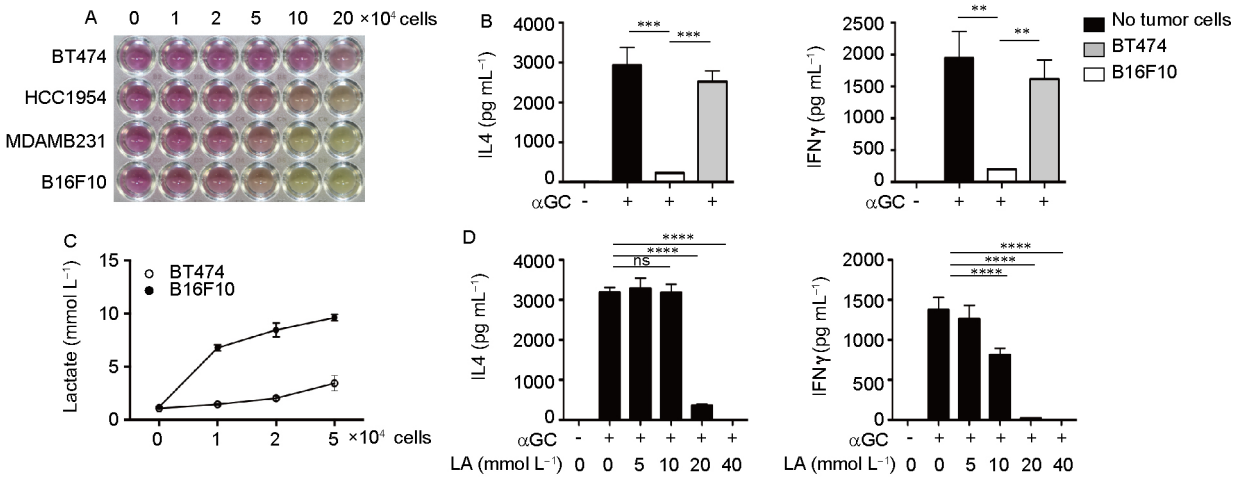

Figure 1 Lactic acid in tumor microenvironments inhibits functions of NKT cells. A, Productions of lactic acid from indicated tumor cell lines, as shown by colour of culture medium after two days culture. Data are representative of three independent experiments. B, IL4 and IFNγ productions from NKT cells activated by RBL.CD1d cells and αGC in the absence or presence of indicated tumor cells (2×10^5 cells). Data are pooled from two independent experiments. C, Lactic acid productions from B16F10 cells and BT474 cells at indicated numbers after 24 h culture. The data are pooled from three independent experiments. D, IL4 and IFNγ productions from NKT cells activated by RBL.CD1d cells and αGC in the absence or presence of lactic acid at indicated concentrations. The data are pooled from two independent experiments. Error bars are mean±SE. **, P<0.01; ***, P<0.001; ****, P<0.0001.

NKT cell functions, NKT cells plus RBL.CD1d cells in upper chamber were co-cultured with tumor cells in lower plate in the presence of αGC in transwell systems. Two tumor cell lines with different lactic acid productions were chosen based on the colour of culture medium. B16F10 cells releasing high level of lactic acid significantly inhibited IFNγ and IL4 productions from NKT cells in transwell co-culture system. Whereas BT474, which produced less amount of lactic acid, showed no influence on cytokine productions from NKT cells (Figure 1B and C). To further investigate whether accumulation of lactic acid alone would result in the dysfunction of NKT cells, NKT cells and RBL.CD1d cells were co-cultured in the presence of αGC with or without lactic acid treatment. Consistently, lactic acid inhibited IFNγ and IL4 productions in a dose dependent way, and more profound influence was observed on IFNγ than on IL4 (Figure 1D).

Extracellular acidification inhibits NKT cell functions independent of TCR signaling

Lactic acid accumulation causes acidification of extracellular environment. Thus, the dysfunction of NKT cells induced by lactic acid could be a result of dropped extracellular pH. To test this possibility, we adjusted medium pH back to 7.3 in lactic acid treated groups by adding NaOH. Interestingly, NaOH was capable to restore the IFNγ and IL4 productions (Figure 2A). Next, we changed the pH of culture medium to

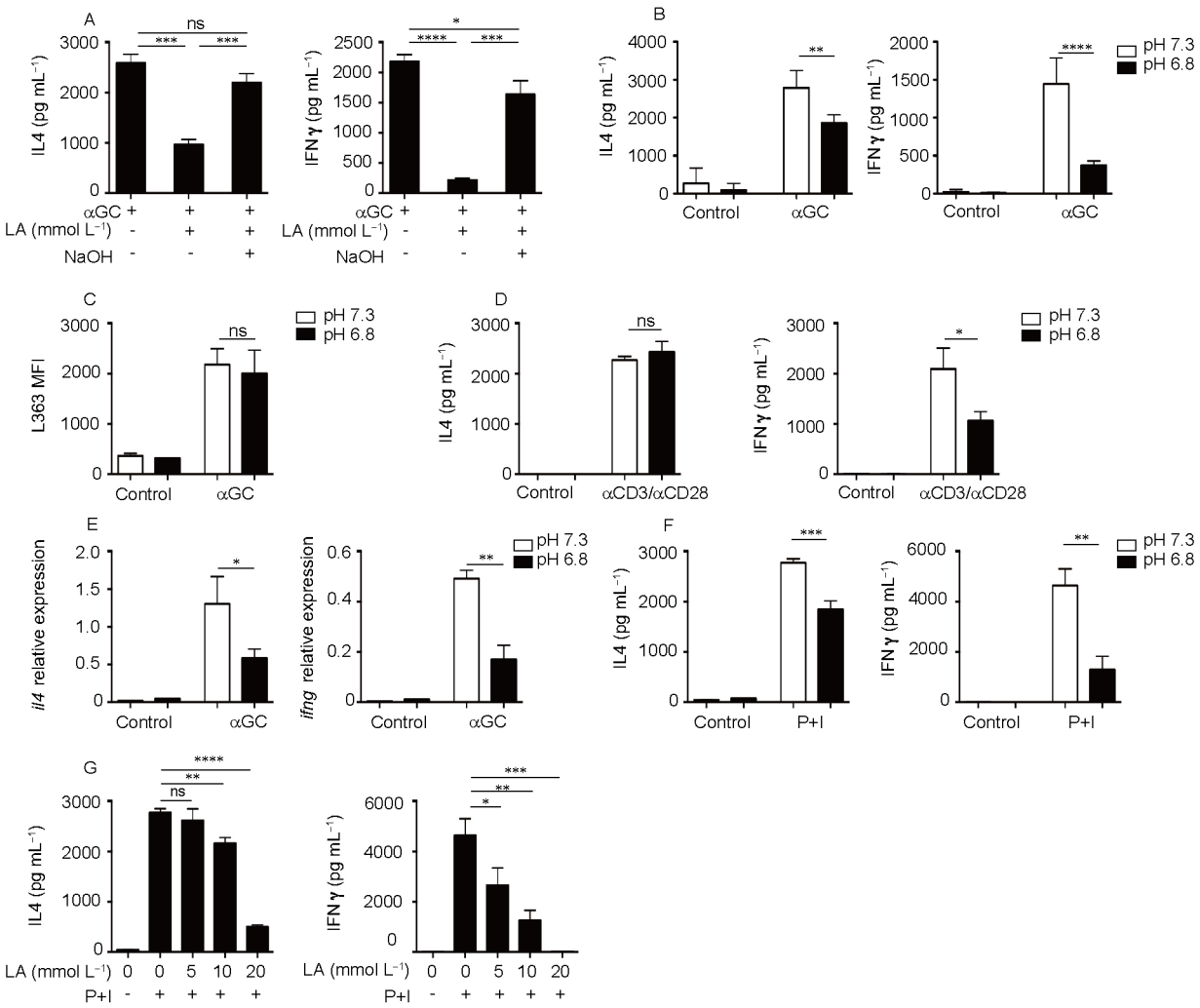

Figure 2 Low pH inhibits functions of NKT cells independent of TCR signaling. A, Cytokine productions from NKT cells activated by RBL.CD1d cells and αGC in the absence or presence of lactic acid (20 mmol L^{-1}) and with or without pH adjusted back to 7.3 by adding NaOH. The data are representative of two independent experiments. B, Cytokine productions from NKT cells activated by RBL.CD1d cells and αGC at pH 7.3 or pH 6.8. The data are pooled from two independent experiments. C, CD1d-αGC on cell surface was detected by L363. Mean fluorescence intensity of L363 was shown. The data are representative of two independent experiments. D, Cytokine productions from NKT cells activated by plate-coated anti-CD3 and anti-CD28 at pH 7.3 or pH 6.8. The data are representative of three independent experiments. E, mRNA levels of cytokines in NKT cells activated at pH 7.3 or pH 6.8 by RBL.CD1d cells pulsed with αGC. The data are representative of three independent experiments. F–G, Cytokine productions from NKT cells activated by PMA (50 ng mL^{-1}) and ionomycin (1 μmol L^{-1}) in pH 7.3 or pH 6.8 culture medium (F), or in the absence or presence of lactic acid (G). The data are representative of two independent experiments. Error bars are mean±SE. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

6.8 by adding HCl without lactic acid, mimicking the pH in tumors *in vivo*. NKT cells and RBL.CD1d cells were co-cultured in presence of αGC in normal medium or in pH 6.8 medium. Consistently, extracellular acidification inhibited IFNγ and IL4 productions from NKT cells (Figure 2B). And more profound influence was observed on IFNγ.These results suggested that change of pH but not accumulation of lactate caused dysfunction of NKT cells. To exclude the possibility that dysfunction of NKT cells was due to the impaired antigen presentation, we measured the level of CD1d-αGC complex on RBL.CD1d cell surface with antibody L363. Similar amount of surface CD1d-αGC complex was detected in cells culturing at pH 7.3 and pH 6.8 (Figure 2C). And in antigen presenting cell-free system, NKT cells were stimulated by plate-coated anti-CD3 and anti-CD28 in pH 7.3 or pH 6.8 medium, and down-regulation of IFNγ production from NKT cells was also observed at pH 6.8 (Figure 2D).Whereas no effect on IL4 production was observed. Thus, extracellular acidification inhibited NKT cell functions independent of influence on antigen presentation. Additionally, we showed that low pH inhibited cytokines at the transcriptional level (Figure 2E). To further investigate whether low pH influenced TCR signaling, we stimulated NKT cells with PMA plus ionomycin bypassing the TCR signaling in pH 6.8 medium or in the presence of lactic acid. And under both conditions, we observed dysfunction of NKT cells (Figure 2F and G). Thus, extracellular acidification inhibited NKT cell cytokine productions by modulating pathways downstream TCR signaling.

Extracellular acidification does not change cell viability

To investigate whether the dysfunction of NKT cells was due to increased cell death in acidic environment, we stained NKT cells with propidium iodide (PI) after 24 h stimulation in normal medium or in pH 6.8 medium. Decreased dead cells were observed at pH 6.8, excluding the possibility that increased mortality caused dysfunction of NKT cells (Figure 3).

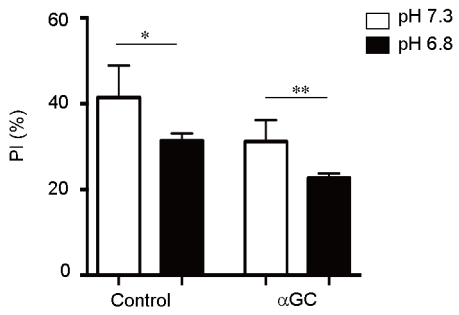

Figure 3 Low pH does not influence the viability of NKT cells. Percentages of PI^+ NKT cells after activation with RBL.CD1d cells and αGC in pH 7.3 or pH 6.8 medium for 24 h. The data are pooled from two independent experiments. Error bars are mean±SE. *, $P<0.05$; **, $P<0.01$.

Extracellular acidification interfered with mTOR signaling in NKT cells upon activation

mTOR signaling has been critically linked to cellular metabolisms and immunity by previous studies (Powell et al., 2012; Vitiello et al., 2015; Weichhart et al., 2015). Activation of mTOR signaling was observed in NKT cells upon activation. However, impaired mTOR signaling was detected in NKT cells activated in pH 6.8 medium or in the presence of lactic acid, as indicated by less amounts of p-S6 and p-4E-BP1 (Figure 4A and B). And pH 6.8 medium dampened mTOR signaling to a lesser extent than 20 mmol L^{-1} lactic acid did, which was in consistence with their influences on cytokine productions. It has been reported that mTOR signaling regulates development and cytokine productions of NKT cells via controlling nuclear translocation of transcription factor PLZF (Shin et al., 2014). PLZF is a signature transcription factor for NKT cells, which controls development and effector programs of NKT cells (Savage et al., 2008). Nuclear localization is important for the functions of PLZF. In mTORC1 signaling deficient mice, diminished nuclear localization of PLZF and diminished cytokine productions were observed in NKT cells (Shin et al., 2014). In consistence with previous studies, we also detected less nuclear translocation of PLZF in NKT cells activated in the presence of lactic acid (Figure 4C). Thus, our results suggested that acidic tumor microenvironments caused dysfunction of NKT cells by interfering with mTOR signaling and PLZF nuclear translocation.

DISCUSSION

Immune evasion of tumor is the major reason for tumor progression. The underlying mechanisms include defective antigen presentations, impaired expression of costimulatory molecules, increased inhibitory mediators and accumulation of immunosuppressive cells (Liu and Cao, 2015; Mapara and Sykes, 2004). Moreover, warburg effect of tumor cells results in special microenvironments especially in solid tumors, which are characterized by nutrient depletion and low pH (Vander Heiden et al., 2009). Metabolic regulations on differentiation and function of immune cells have been reported recently. Similar as tumor cells, M1 macrophages, DCs, Th1 cells and CTLs utilize glycolysis for energy supply. Whereas the pro-tumor immune cells, such as M2 macrophages, regulatory DCs and Tregs, undergo oxidative phosphorylation (Biswas, 2015; Wang et al., 2014). And accumulation of lactic acid would influence cell functions by interfering with glycolysis. Together, competition for nutrients and accumulation of lactic acid would inhibit the development and functions of anti-tumor cells but favor the pro-tumor cells (Chang et al., 2015; Dietl et al., 2010; Fischer et al., 2007). Thus, tumor microenvironments could be another explanation for the failed immune surveillance

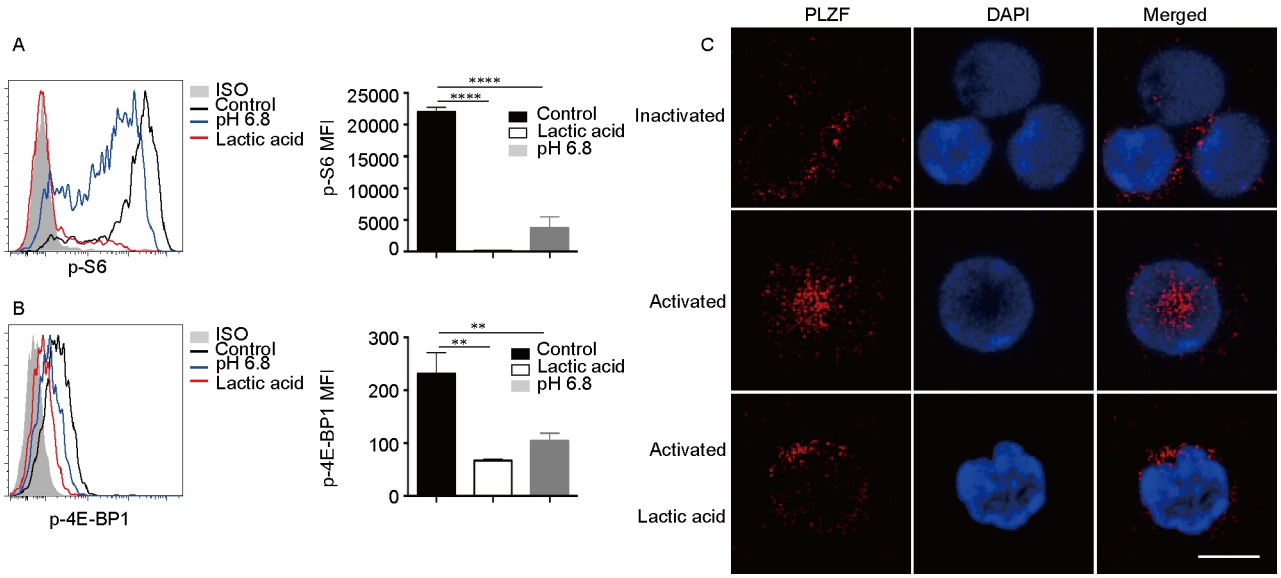

Figure 4 Low pH impairs mTOR signaling and translocation of PLZF in activated NKT cells. A and B, Phosphorylation of S6 (A) and 4E-BP1 (B) in NKT cells activated by plate-coated anti-CD3 and anti-CD28 for 24 h in pH 7.3 or pH 6.8 medium or in the presence of 20 mmol L^{-1} lactic acid. Data are representative of two independent experiments. C, Localization of PLZF in NKT cells inactivated or activated by plate-coated anti-CD3 and anti-CD28 in the presence or absence of lactic acid (20 mmol L^{-1}). Red, PLZF; blue, 4′,6-diamidino-2-phenylindole, DAPI. Scale bars, 5 μm. Error bars are mean±SE. **, $P<0.01$; ****, $P<0.0001$.

against tumors.

NKT cells, as the bridge of innate and adaptive immunity, serve as the first line of body defense. In addition to killing the CD1d expressing tumor cells directly, activation of NKT cells has been shown to promote the functions of NK cells and CTLs (Fujii et al., 2013). Due to these advantages, NKT cells are good candidates for immunotherapies against tumors. However, our results indicate a significant influence of tumor microenvironments on NKT cell functions. Dysfunction of NKT cells, especially diminished IFNγ production, was induced by low pH in tumors (Figure 2). These results could explain the abnormal functions of NKT cells associated with tumors and the failed NKT cell-based immunotherapies. Additionally, we demonstrated that low pH impaired activation of mTOR signaling and downstream PLZF translocation (Figure 3). mTOR signaling has been previously shown to regulate nuclear localization of PLZF, which is important for NKT cell effector functions (Shin et al., 2014). Moreover, mTOR signaling and lactic acid have been reported to regulate glycolysis (Dietl et al., 2010; Powell et al., 2012). Thus, our results do not exclude the possibility that other factors influencing cellular metabolism, such as glycolysis, would also regulate NKT cell functions in tumor microenvironments. The mechanisms by which mTOR signaling regulates PLZF localization remain unclear. It has been reported that mTORC1 but not mTORC2 regulates nuclear localization of PLZF (Prevot et al., 2015).

It is not clear whether activating NKT cells and interfering with the production of lactic acid from tumor cells would result in a synergic effect. However, recent studies on anti-PD1 treatment demonstrate a very promising future for therapies targeting on both tumor immunity and tumor microenvironments. In addition to the blockade of inhibitory receptors, anti-PD1 treatment inhibits tumor growth by directly regulating tumor cell metabolism and reducing the glucose exhaustion in tumor microenvironments, which promotes CTL functions on the other side (Chang et al., 2015). Thus, except for the immune cells and checkpoint molecules, the tumor microenvironments would also be targets of immunotherapies. Normal tissue microenvironments are required to maintain the function of anti-tumor cells and also may be important for the migration of anti-tumor cells. Combination therapies are required in the future.

MATERIALS AND METHODS

Mice

Vα14 Tg.*cxcr6*$^{gfp/+}$ mice were previously described and provided by Dr. Albert Bendelac (Bai et al., 2012; Scanlon et al., 2011). Mice were housed under specific pathogen-free conditions. All animal procedures were approved by the University of Science and Technology of China (USTC) Institutional Animal Care and Use Committee. And all experiments were performed in accordance with the approved guidelines.

Cell culturing

The B16F10 cells, MDAMB231 cells, BT474 cells and HCC1954 cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% FBS. RBL.CD1d cells and NKT cells were cultured in RPMI-1640 medium (Gibco)

supplemented with 10% FBS and 50 μmol L^{-1} βME.

Cell enrichment

Vα14 Tg.*cxcr6*$^{gfp/+}$ mice were sacrificed and spleens were minced and passed through 70 μm meshes. Cells were blocked with anti-CD16/32 for 15 min, and stained with anti-CD4-APC for 30 min on ice. NKT cells were enriched with anti-APC microbeads (Miltenyi Biotec, Germany). In some experiments, purified NKT cells were obtained by sorting as $CD4^+$ and GFP^{hi} cells from livers of *Vα14* Tg.*cxcr6*$^{gfp/+}$ mice.

Activation of NKT cells

For NKT cell activation, 3×10^4 RBL.CD1d cells and 4×10^4 enriched NKT cells were co-cultured in the presence of αGC (1 μg mL^{-1}) or DMSO as control for 24 h. Cytokines in supernatant were detected by CBA kit (BD, USA). To study the NKT cell functions in tumor microenvironments *in vitro*, indicated tumor cells (2×10^5 cells per well) were seeded in lower plate and enriched NKT cells were co-cultured with RBL.CD1d cells in upper chamber (Millipore, USA) in the presence of αGC (1 μg mL^{-1}). In cell-free stimulation assays, sorted NKT cells (2×10^5 per well) were stimulated with plate-coated anti-CD3 (10 μg mL^{-1}) and anti-CD28 (10 μg mL^{-1}) for 24 h. Alternatively, purified NKT cells were stimulated with 1 μmol L^{-1} ionomycin and 50 ng mL^{-1} PMA for 24 h.

Flow cytometry

Cells were suspended in PBS buffer containing 1% BSA, and blocked with anti-CD16/32 for 15 min on ice, then stained with monoclonal antibody for 30 min on ice. Monoclonal antibodies were listed here: purified anti-CD16/32 (93), anti-CD4 (GK1.5), anti-TCRβ (H57-597). All antibodies were purchased from BioLegend (USA). CD1d-PBS57 tetramer is provided by National Institute of Health (NIH) Tetramer Core Facility. To detect intracellular phospho-S6 (pS6), or phospho-4E-BP1 (p4E-BP1), sorted NKT cells were fixed with 2% PFA and permeabilized with 90% pre-cooling methanol. Cells were stained with rabbit anti-pS6 (Ser235/236, Cell Signaling Technology, USA) or rabbit anti-p4E-BP1 (T37/46, Cell Signaling Technology) antibodies. For antigen presentation assays, RBL.CD1d cells were cultured in the presence of αGC (1 μg mL^{-1}) at different pH for 24 h. Cells were digested by trypsin and washed with PBS buffer. After blocking, RBL.CD1d cells were stained with antibody against CD1d-αGC complex (L363). Cells were acquired by FACSVerse (BD). And data was analyzed with FlowJo 7.6 software.

Confocal

Sorted NKT cells were stimulated by plate-coated anti-CD3 (10 μg mL^{-1}) and anti-CD28 (10 μg mL^{-1}) for 12–14 h in culture dishes. Cells were fixed with 4% PFA for 15 min, and then permeabilized with 0.1% Triton X-100 for 30 min. Cells were washed and stained with PE-conjugated anti-PLZF (4 μg mL^{-1}) and DAPI (1.5 μg mL^{-1}) for 4 h on ice. Images were collected with an LSM 710 (Zeiss, Germany) inverted microscope. Data was analyzed with ImageJ.

Statistical analysis

Error bars represent SE. The statistical significance of differences between two groups was determined by the unpaired two-tailed student's *t* test.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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