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Cancer-associated fibroblasts express CD1d and activate invariant natural killer T cells under cellular stress

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Invariant natural killer T (*i*NKT) cells are part of the family of unconventional T cells, and they recognize glycolipid antigens presented on the MHC class I like molecule CD1 [1]. In humans there are five CD1 molecules named CD1a to CD1e, whereas mice use only CD1d for lipid presentation. Functionally, *i*NKT cells can elicit both cytotoxicity similarly to conventional CD8 T cells and secrete cytokines that influence the adaptive immune response including providing direct T cell help to B cells [2]. In the context of tumors, they can use both release of granzyme B and use Fas-mediated mechanisms for killing of tumor cells. As *i*NKT cells use a restricted TCR repertoire and CD1 molecules are non-heterogenous the risk of graft vs host disease is limited and thus these T cells have been an attractive option for off the shelf immunotherapy to treat cancer [3]. So far clinical trials have mostly focused on using the glycolipid alpha-Galactosyl Ceramide (αGalCer) to activate *i*NKT cells and the outcomes have been varied [3]. Thus, to make immunotherapy using *i*NKT cells more efficient additional information is needed on how these T cells are activated and operate in the complex tumor microenvironment (TME). In mice CD1d is mainly found on antigen presenting cells (APC) including dendritic cells (DC), macrophages and B cells. These cells interact with *i*NKT cells in secondary lymphoid organs to activate them and polarize them to different effector functions. Besides activation by exogenous ligands like αGalCer, *i*NKT cell can also be activated by endogenous glycolipids that are induced by cellular stress and presented by CD1d as shown by using myeloid cells as APCs [4]. In the context of the TME little is known regarding which cell types that express CD1d and to what extent local regulation of *i*NKT cells occur. Also, it is not known if the presentation here is related to cellular stress or if endogenous ligands can be used and presented to pave way for more efficient *i*NKT cell mediated immunotherapy.

To investigate this, we used two mouse cancer models: (i) the B16F10 melanoma model; and (ii) the EO771 breast cancer model. These represent two different cancer types and in addition, EO771 cells express CD1d whereas B16F10 is negative (data not shown). We found that in both these models *i*NKT cells were present in the tumor defined as TCRβ$^+$/NK1.1$^+$/CD1d-tetramer$^+$ cells and could make up to several percent of the total T cell population (Fig. 1A, B and data not shown). Also, the composition *i*NKT cell subtypes were different comparing tumor and draining lymph nodes where the tumor was dominated by *i*NKT1 and *i*NKT17 cells, with few *i*NKT2 cells. Further, we investigated the presence of CD1d expressing cells in the TME that could impose local regulation of *i*NKT cells or be their targets. Surprisingly, we found a subpopulation of CD45 negative cells that was not of tumor origin that expressed CD1d in the B16 model (Fig. 1C). Thus, we set out to identify the CD1d expressing cell type and of the non-tumor and non-immune cells in the TME the different stromal cells populations have and important role to not only provide structure but also modulate the inflammatory response within the tumor. Stromal cells can be divided into endothelial cells, pericytes, and fibroblasts, and subpopulations of these also exists within the TME [5]. To define these and investigate their possible expression of CD1d we used CD31 as a marker for endothelial cells and CD90 and PDPN as markers defining the prototypical subset of matrix-producing cancer associated fibroblasts (CAFs) (Supplementary Fig. S1A) [6]. We found that the majority of the cells expressing CD1d in the TME was CAFs as defined by their co-expression of CD90.2$^+$ and PDPN$^+$ (Fig. 1D). As fibroblasts are very plastic and respond to inflammatory signals we wondered if CD1d could be detected on these cells also in other locations connected to tumor development. However, further investigating this population, we could not find CD90.2+/PDPN+ fibroblast populations in tumor draining or non-draining lymph nodes or in the spleen suggesting that this phenotype is tumor-specific. Also, this population was found in both the B16 and EO771 models (Fig. 1E, F and Supplementary Fig S1B). In this experiment as a control, we also compared CAF from WT mice and mice deficient of CD1d (KO) and found the staining to be specific both in the B16F10 and EO771 model ruling out that CD1d could be transferred from EO771 cells expressing CD1d. We also found the majority of the CAFs in both models to express CD1d (Fig. 1F, G). We subsequently focused on the B16F10 model for the following experiments except when noted. Different subpopulations of CAFs have been described in the TME and these have different roles in shaping the environment being more or less immunomodulatory [7]. Two markers that distinguish subpopulations are PDGFRα and PDGFRβ and expression of these is also of clinical use when predicting outcome for cancer patients. Investigating these

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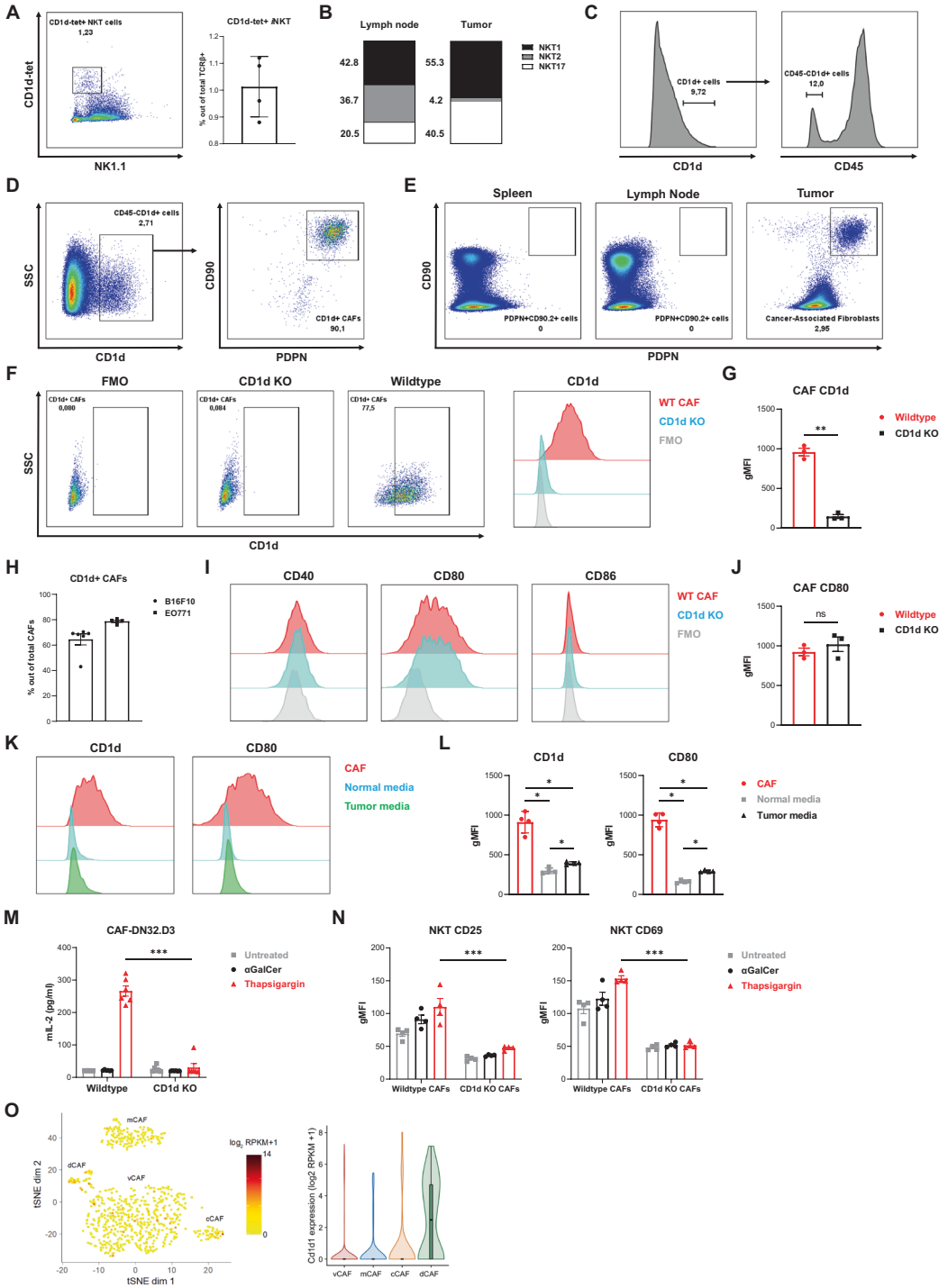

Fig. 1 Cancer-associated fibroblasts express CD1d and can activate invariant natural killer T cell under cellular stress condition. **A** Flow cytometric dot plot and bar graph show the existence (right panel) and proportion (left panel) of TCRβ+CD1d-tet+ invariant natural killer T (iNKT) cells in mouse melanoma ($n = 4$). **B** The comparison of composition of the subtypes of iNKT cells in lymph node and tumor. **C** Flow cytometric histograms show part of the CD1d+ cells in tumor are non-immune cells. **D** Flow cytometric dot plots show the CD45-CD1d+ cells in mouse melanoma are mostly cancer-associated fibroblasts (CAFs). **E** The existence of the CD90.2+PDPN+ fibroblasts are specific in mouse tumors but not found in lymphoid organs like spleen or lymph node. **F**, **G** CAFs express high level of CD1d on surface. **F** Flow cytometric dot plots and histogram show the positive staining of CD1d on CAFs. **G** Representative bar graph shows the absolute expression level (quantified and shown as gMFI) of CD1d on CAFs compared with CD1d KO. Data are shown from WT ($n = 3$), CD1d KO ($n = 3$). **H** The proportion of CD1d+ CAFs in mouse melanomas ($n = 6$) and in breast tumors ($n = 5$). **I**, **J** CAFs also express CD80 but not CD40 or CD86 as a costimulatory molecule. **I** Representative flow cytometric histograms of the expression of CD40, CD80 and CD86 on CAFs. **J** The CAFs expression of CD80 is not affected by the CD1d KO. Data are shown from WT ($n = 3$), CD1d KO ($n = 3$). **K**, **L** Magnetically isolated CAFs lost CD1d and CD80 expression when in vitro cultured in normal media while tumor-supplemented media restore the surface expression ($n = 4$). **M**, **N** Magnetically isolated CAFs were co-cultured with iNKT hybridoma cell line DN32.D3 or with isolated primary splenic iNKT cells after given α-GalCer or cellular stressed by Thapsigargin. Level of secreted IL-2 in coculture supernatant was measured by ELISA (n = 6) and the expression of activation markers on primary iNKT cells were analyzed by flow cytometry ($n = 4$). **O** A single-cell RNA sequencing dataset of mouse breast cancer also detected CD1d+ CAFs at transcriptional level. All data are shown as mean ± SEM, *P* values were calculated using Mann-Whitney test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ns no significance)

markers, we found that the CD1d expressing CAFs expressed higher level of PDGFRβ than PDGFRα and that it was a uniform population (Supplementary Fig. S2A). Another marker that has been reported to subdivide CAF subsets is CD26 which is a serine protease that modulate cell growth, adhesion, and metastasis of tumor cells [8]. We found that a subpopulation of CD1d positive CAFs expressed CD26 and that there was a positive correlation with higher CD1d and higher CD26 expression (Supplementary Fig. S2B). Activated *i*NKT cells do not need potent co-stimulation to perform effector functions when engaging CD1d but we contemplated the possibility that initial activation and regulation could occur locally. Thus, we investigated if CD1d-expressing CAFs would also express costimulatory molecules. Using flow cytometry, we investigated presence of CD40, CD80 and CD86 that have all been shown to be important for *i*NKT cell activation [9]. We found that CD1d positive CAFs within both tumor types express CD80 but not CD40 or CD86 (Fig. 1H, I and Supplementary Fig S1C). It has been shown that *i*NKT cells can activate APCs to be more efficient through interactions with CD1d and thus modulate their interactions with other T cells. However, we found that the expression of CD80 was independent of expression of CD1d (Fig. 1J). To investigate if tonic signals from the TME were needed for CD1d and CD80 expression we isolated CAFs and put them in in vitro cultures for 48 h (Supplementary Fig. S2C). We found that when left in culture the CAFs lost both CD1d and CD80 expression, but the expression could be partly rescued when the media was tumor-conditioned, supporting that TME-derived factors drive the interaction of CAFs with *i*NKT cells (Fig. 1K, L). Next, we setup an in vitro co-culture system to investigate if freshly isolated CAFs from the tumor expressing CD1d could activate *i*NKT cells. We used the *i*NKT cell hybridoma cell line DN32.D3 as these cells release IL-2 to the supernatant as a measure of activation. When we added the exogenous glycolipid αGalCer to the co-cultures we could not find any evidence for *i*NKT cell activation suggesting that CAFs could not efficiently present this lipid on CD1d (Fig. 1M). Loading of CD1d can be mediated through uptake of glycolipids via several mechanisms. This includes phagocytosis and uptake via the LDL-receptor which is expressed by CAFs. However, we could not find evidence for this type of presentation using this system. The other possibility is that CAFs would present endogenous glycolipids that are induced by cellular stress. To test this, we used drug-induced cellular ER-stress by treating CAFs with Thapsigargin (TG). We found that TG-treated CAFs could indeed present via CD1d and activate *i*NKT cells as we saw IL-2 production in the cultures after over night incubation (Fig. 1M). This show that CAFs has the capacity to present and activate *i*NKT cells locally in the TME during cellular stress responses. We further confirmed this using sorted *i*NKT cells from spleen of naïve mice and found that these could be activated with upregulation of CD25 and CD69 in a CD1d dependent manner (Fig. 1N). We next investigated wither CD1d are expressed on a subpopulation of CAFs using a dataset from single cell RNA sequencing of a breast cancer model (MMTV-PyMT) [5]. We did find evidence for expression in a proportion of cells primarily in the developmental (dCAF) subpopulation which has a genetic signature suggesting epithelial origin (Fig. 1O). Finally, investigating human single cell RNA sequencing data sets we found CD1d to be expressed by human PDPN and Thy1 positive fibroblasts in Gastric cancer (Supplementary Fig. S3A). This upregulation was tumor specific and further evaluation of inflammatory skin disease (Atopic Dermatitis and Psoriasis) in connection to pathological skin development programs [10] (Supplementary Fig. S3B, C).

Taken together our findings demonstrate that CAFs express CD1d as well as the costimulatory molecule CD80 and that this expression is driven by the TME. Also, the in vitro experiments show that CD1d expressed by CAFs can present self-lipids under conditions of cellular stress to NKT cells. This could occur during different stages of tumor growth related to hypoxia or when patients go through treatments that induce local cell death including chemotherapy or immunotherapy. Further studies are needed to investigate if this would contribute to treatment efficacy or if local regulation imposed by CAF-activated *i*NKT cells would work against the treatment. Also, it will be of interest to examine other cancer types and the value of using presence of CD1-expressing CAFs to determine treatment outcomes. Recently many studies have used high throughput technologies to dissect CAF heterogeneity and found several distinct subpopulations. Of note is that one of these populations have been found to also express MHC class II molecules and these have been found in both pancreatic and lung cancer [11]. In the study investigating CAFs from pancreatic tumors it was shown that the interaction with CD4 positive T cells was driving expansion of regulatory T cells. This could be potentially further enhanced by *i*NKT cells as it has been shown that they can enhance and influence regulatory T cell function at least in response to foreign ligands [12]. On the other hand, regulatory T cells can also induce *i*NKT cell anergy and stop their capacity to kill tumor cells. Finally, it will be of importance to evaluate how cellular stress responses modulate clinical outcomes of treatment and here we now show a direct link between stromal cells and unconventional T cells that could be employed.

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AUTHOR CONTRIBUTIONS

MCIK and SP conceived the original idea, designed the project and wrote the manuscript with input and revisions by YP, JS and KP. YP and SP performed animal and in vitro experiments. JS performed data analysis and bioinformatics.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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