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Fas/FasL interaction mediates imbalanced cytokine/cytotoxicity responses of iNKT cells against Jurkat cells

Rui Dou[a], Zhenya Hong[b], Xiaosheng Tan[a], Fenfen Hu[a], Yajie Ding[a], Wei Wang[a], Zhihui Liang[a], Rongrong Zhong[c], Xiongwen Wu[a,*], Xiufang Weng[a,*]

[a] Department of Immunology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China
[b] Department of Hematology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China
[c] Queen Mary School, Medical College of Nanchang University, Jiangxi, 330031, China

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ABSTRACT

The rapid antitumor cytokine production and direct cytotoxicity confer invariant NKT (iNKT) cells ideal candidates for cancer therapy. However, the therapeutic potential of iNKT cells in T-cell malignant diseases remains elusive, as antigen presentation by T cells (T-T presentation) has been suggested to induce hyporesponsiveness of iNKT cells. In this study, we found discrepancies in iNKT cell responses against two T cell-origin cell lines (Jurkat and Molt-4). Human iNKT cells exhibited more intensive cytotoxicity and less efficient cytokine production in response to Fas-bearing Jurkat cells than those to the Fas-negative tumor cells (Molt-4 and myeloid-derived K562). The imbalanced cytokine/cytotoxicity responses of iNKT cells against Jurkat cells were CD1d-dependent and relied mostly on Fas/FasL interaction. The impairment in cytokine production could be overcome by Fas/FasL blocking antibodies and exogenous IL-2. Elevated CD1d levels as well as CD1d and Fas co-localization were found in T-cell lymphomas. However, defects in frequency and function of circulating iNKT cells were observed in the patients, which could be partly rescued by exogenous IL-2. Collectively, the Fas/FasL-dependent aberrant iNKT cell responses and the reversibility of the defects suggest the distinct iNKT cell manipulation in CD1d- and Fas-bearing T cell malignancies.

1. Introduction

Invariant natural killer T cells (iNKT) are innate-like T lymphocytes that recognize glycolipid antigens presented by CD1d. They express a semi-invariant TCR-α chain paired with a limited repertoire of TCR-β chains (Vα14Jα18/Vβ2,7,8 in mice and Vα24Jα18/Vβ11 in human) (Godfrey et al., 2010; Bendelac et al., 2007). Despite being relatively low frequency in human, iNKT cells respond quickly to be among the first responders in the scene. On one hand, iNKT cells upregulate co-stimulating receptors and rapidly produce a cytokine 'storm' upon activation, which subsequently activates multiple immune cells including dendritic cells (DC), NK cells, B cells and T cells (Brennan et al., 2013; Nieda et al., 2018). As a result, activation of iNKT cells strongly modulates both innate and adaptive immune responses, and thus augments protective responses against tumors. On the other hand, iNKT cells exert direct cytotoxicity against CD1d-bearing tumor cells in a predominant perforin- and granzyme B-dependent manner, with additional TNF-α and Fas/Fas ligand (FasL)-mediated killing (Kawano et al., 1998; Nicol et al., 2000; Wingender et al., 2010).The indirect improvement of the other immune effectors and direct cytotoxicity against tumor cells endow iNKT cells with antitumor potential for cancer immunotherapy.

Marine sponge-derived agent α-galactosylceramide (α-GalCer or KRN7000) is an iNKT cell specific agonist capable of activating and expanding iNKT cells (Kawano et al., 1997). Intense antitumor immune responses evoked by α-GalCer are observed in various murine tumor models, including lymphoma, melanoma, prostate cancer and colon carcinoma (Bellone et al., 2010; Li et al., 2014; Yoshioka et al., 2012; McEwen-Smith et al., 2015). Therefore, α-GalCer has been employed extensively as an experimental tool to study iNKT cells and used in clinical trials for the treatment of tumors. However, human clinical trials in patients with tumor have failed to achieve strong immune activation post administration of α-GalCer (Ishikawa et al., 2005; Uchida et al., 2008). The autologous DCs loaded ex vivo with α-GalCer show better activation of iNKT cells than free α-GalCer in cancer patients (Ishikawa et al., 2005; Uchida et al., 2008; Chang et al., 2005; Nicol et al., 2011). Although the mechanism behind the differing immune

Abbreviations: iNKT, invariant natural killer T; FasL, Fas ligand; α-GalCer, α-galactosylceramide; APC, antigen presenting cells

* Corresponding author at: Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, 13# Hangkong Rd, Wuhan, Hubei, 430030, China.
E-mail addresses: xiongwenwu@hotmail.com (X. Wu), wengxiufang@hust.edu.cn (X. Weng).

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responses is unclear, it has been hypothesized that the type of APCs plays an important role related to their CD1d levels and stimulatory capacity. The CD1d level in tumor cells is an important determinant for iNKT cell responses. Tumors with low CD1d expression are insufficiently recognized by iNKT cells, while those with high CD1d level provide favorable targets (Haraguchi et al., 2018). The selective presentation of glycolipid antigens by different CD1d-expressing cell types has also been suggested to be critical for iNKT cell function (Schmieg et al., 2005). Moreover, a requirement for CD28 costimulatory signaling is proposed in optimal responses of iNKT cells (Hayakawa et al., 2001). Nonetheless, sufficient iNKT cell-dependent cytotoxicity and cytokine production are induced by CD1d-bearing solid tumor cells lacking costimulatory molecules (Shimizu et al., 2007). These observations raised the question as to what degree of iNKT cells rely on costimulatory signals and other molecules for their antitumor effects.

Although CD1d expression on mature T cells is barely detectable, cutaneous T-cell lymphoma and the widely used T cell-origin cell lines, including mouse EL4 cells and human Jurkat cells, express high levels of CD1d. Consistent with the critical role of CD1d level in iNKT cell antitumor effect, a previous study shows competent anti-EL4 T-cell lymphoma effects of iNKT cells in mouse (Bassiri et al., 2014). However, inhibition of antitumor immunity by iNKT cells in a T-cell lymphoma model is reported (Renukaradhya et al., 2006). Also, aberrant upregulation of CD1d on T cells has been shown to induce hyporesponsiveness of iNKT cells (Zimmer et al., 2006). Different from efficient T-cell responses initiated by professional antigen presenting cells (APCs), antigen presenting by T cells (T-T antigen presentation) induces an initial T cell activation followed by anergy status, which is attributed to defects in costimulatory signals (Taams et al., 1999). Whether T-T antigen presentation affects the antitumor potential of iNKT cells in T-cell malignant diseases remains vague, especially in human cases.

In this study, discrepancy in iNKT cell cytokine/cytotoxicity responses was found against two malignant T cell lines (Jurkat and Molt-4). Human iNKT cells exhibited intensive cytotoxicity but impaired cytokine responses against CD1d- and Fas-expressing Jurkat cells. Exogenous IL-2 helped to overcome the impaired antitumor cytokine production against Jurkat cells and rescue the defects of iNKT cells from T lymphoma patients, indicating a novel aspect of iNKT-cell based treatment for CD1d- and Fas-bearing T-cell malignant diseases.

2. Materials and methods

2.1. Cell lines

The Jurkat (ATCC, TIB-152™) and Molt-4(ATCC, CRL-1582™) are human T leukemia cell lines. K562 (ATCC, CCL-243™) cells are myeloid leukemia cells. The CD1d transfectants were generated through transfecting encoding gene of human CD1d into Jurkat, Molt-4, and K562 cells. Transfectants expressing highest CD1d level in each cell lines were cloned.

2.2. Study population

Blood samples from 13 patients with T-cell lymphomas and 20 healthy donors were collected from Tongji Hospital and Wuhan Blood Center, respectively. Paraffin-embeded lymphoma sections from 18 patients with T-cell lymphoma and 3 patients with B-cell lymphoma were obtained from tissue bank of Tongji Hospital, Wuhan, China. Patient characteristics are listed in Table 1.

2.3. Isolation and in vitro stimulation of PBMCs

PBMCs from patients and healthy donors were isolated by Ficoll density gradient centrifugation as described before (Weng et al., 2007). 3×10^7 PBMCs were simulated with 200 ng/ml α-GalCer (KRN7000, Avanti) and IL-2 (Peprotech) (Watarai et al., 2008). Recombinant IL-2 was used at 50U/ml.

2.4. Reagents and flow cytometry

PBS57/CD1d tetramer was kindly gifted by NIH tetramer core facility. Immunophenotyping was performed using the following fluorescence-conjugated mAbs: CD3 (UCHT1, Biolegend), CD14 (M5E2, Biolegend), Fas (DX2, BD Biosciences), FasL (NOK-1, Biolegend), CD1d (51.1, BD Biosciences), CD80 (2D10.4, eBioscience), CD83 (HB15e, eBioscience), CD86 (BU63, Biolegend), perforin (B-D48, Biolegend), and granzyme B (GB11, BD Biosciences). Cells were stained with PBS57/CD1d tetramer and corresponding antibodies. Cytokine levels in co-culture supernatants were measured using the cytometric bead array (CBA) system (BD Biosciences) according to the product instruction. Data were collected using FACSVerse (BD Biosciences) and analyzed by FlowJo software (Tree Star Inc).

2.5. Transient Fas siRNA transfection

Jurkat-CD1d cells were transfected with 100 nM of Fas-specific siRNA (Santa Cruz Biotechnology) by using Amaxa Nucleofector (Lonza). After 48 h, total RNA was extracted by TRIzol reagent (Thermo Fisher Scientific) and reverse transcription were performed by using a transcription kit (TaKaRa). Primers for real-time PCR were as follows: Fas, forward 5′-AGGGAAGCGGTTTACGAGTG-3′, and reverse 5′-GATG CCCAGCATGGTTGTTG-3′; GAPDH, forward 5′-CAGTCCATGCCATCAC TGCCACCCAG-3′, and reverse 5′- CAGTGTAGCCCAGGATGCCCTT GAG-3′. Real-time PCR was performed to monitor Fas gene expression knockdown. Cell lysates prepared from untransfected or siRNA transfected cells were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted with anti-Fas antibody (ZB4, GeneTex) to assess knockdown of Fas expression.

2.6. Cells sorting and in vitro functional assay

T cells and monocytes were positively sorted by FACS sorting (BD FACS Aria III) using antibodies against CD3 and CD14 respectively. α-GalCer/IL-2 expanded iNKT cells were positively enriched by APC labeled PBS57/hCD1d tetramer, followed by MACS sorting with anti-APC microbeads (Miltenyi Biotec) according to the product instruction. Primary T/monocytes, tumor cell lines and Fas-specific siRNA transfected Jurkat-CD1d cells were pulsed with vehicle or α-GalCer (200 ng/ml) for 8 h and used as stimulators. The tumor cell lines and their transfectants were further stained with CellTrace™ Violet (Thermo Fisher Scientific) according to the product instruction and used as targets for cytotoxicity assay.

1×10^5 sorted iNKT cells were co-cultured with 5×10^4 stimulators or targets for 16 h in the presence of agonistic anti-human CD28 (CD28.2, Biolegend), or blocking antibodies against anti-human CD1d (51.1, Biolegend), anti-human Fas (ZB4, GeneTex), and anti-human FasL(10 F2, GeneTex). Supernatants were collected for IFN-γ, TNF-a level detection. PI (BD Biosciences) staining was performed to determine the cell death of CellTrace™ Violet stained cells. Cytotoxicity (%) was calculated by subtracting % PI positive cells in control groups from that in experimental groups.

2.7. Immunofluorescence analysis

The sections were stained with primary antibodies against CD1d (NOR3.2, Bio-Rad Laboratories), Fas(DX2, Biolegend)and CD3 (HIT3a, Servicebio technology CO.) for 1 h at room temperature, followed by secondary staining with CY3 goat anti-mouse IgG (Servicebio technology CO.) and Alexa Flour 488 goat anti-rabbit IgG (Servicebio technology CO.). Whole sections were scanned by Pannoramic MIDI (3D HISTECH). More than 3 images without overlap were taken from $CD3^+$ T-cell enriched area in each section, followed by integrated

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