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Targeting CD147 for T to NK Lineage Reprogramming and Tumor Therapy

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ABSTRACT

CD147 is highly expressed on the surface of numerous tumor cells to promote invasion and metastasis. Targeting these cells with CD147-specific antibodies has been validated as an effective approach for lung and liver cancer therapy. In the immune system, CD147 is recognized as a co-stimulatory receptor and impacts the outcome of thymic selection. Using T cell-specific deletion, we showed here that in thymus CD147 is indispensable for the stable αβ T cell lineage commitment: loss of CD147 biases both multipotent DN (double negative) and fully committed DP (double positive) cells into innate NK-like lineages. Mechanistically, CD147 deficiency results in impaired Wnt signaling and expression of BCL11b, a master transcription factor in determining T cell identity. In addition, functional blocking of CD147 by antibody phenocopies genetic deletion to enrich NK-like cells in the periphery. Furthermore, using a melanoma model and orthotopic liver cancer transplants, we showed that the augmentation of NK-like cells strongly associates with resistance against tumor growth upon CD147 suppression. Therefore, besides its original function in tumorigenesis, CD147 is also an effective surface target for immune modulation in tumor therapy.

1. Introduction

T cell development is a process that restricts lineage choices of common lymphoid precursors (CLPs) and lymphoid-primed multipotent precursors (LMPPs) in a step-by-step fashion. The thymus is a permissive microenvironment for T-cell lineages by providing spatiotemporally structured signals to instruct CLPs and LMPPs to differentiate in a fixed direction (Ciofani and Zuniga-Pflucker, 2007). These signals drive lymphoid precursors to become early thymic progenitors (ETP) cells, to become CD4/CD8 double negative (DN, DN2-DN3-DN4), to become CD4/CD8 double positive (DP), then finally to become either CD4 or CD8 single positive thymocytes (SP). Recent progress has unveiled that thymic T-lineage commitment and maintenance requires activation of several key transcription factors (Yui and Rothenberg, 2014). Among them, Bcl11b plays a dominant role in each step of thymocyte development. During phase I of the lineage choice decision (ETP to DN2a) (Yui and Rothenberg, 2014), the loss of Bcl11b results in the continuous expression of genes that maintains stemness and multipotency and differentiation of early thymocytes into the myeloid and NK lineages (Ikawa et al., 2010; Li et al., 2010a; Li et al., 2010b). In addition, Bcl11b-deficient cells that have passed through the first stage of lineage choice and have committed to the T cell lineage are still susceptible to reprogram into an NK-like cell lineage(Kastner et al., 2010; Li et al., 2010b). While the lineage-specifying transcription factors for T cells have been extensively studied, upstream mechanisms have remained to be investigated. Specifically, it is intriguing to know how these transcription factors are regulated by external signals, and wha surface receptors are responsible for lineage stability in committed T cells.

CD147, also known as Basigin or EMMPRIN, is a highly glycosylated immunoglobulin superfamily protein. Under physiological conditions, this transmembrane protein is widely expressed and plays fundamental roles in various hematopoietic and non-hematopoietic cell lineages (Fok et al., 2014; Hahn et al., 2015; Kaushik et al., 2015; Pennings and Kritharides, 2014). In various cancers, levels of CD147 expression are highly elevated and are associated with poor prognosis (Als et al., 2007; Caudroy et al., 1999; Sienel et al., 2008; Xiong et al., 2014). Beyond its role as a tumor biomarker, CD147 is considered as a bona-fide tumor associated antigen (TAA) because of its intrinsic regulation in tumorigenesis (Biswas et al., 1995; Sweeny et al., 2013; Tang et al., 2005; Wang et al., 2015). Besides its critical role in tumorigenesis, CD147 is also identified as a signal receiver for immune modulation, especially in T cell development and activation. Throughout the entire T cell lineage, from ETPs to naïve T cells and to fully differentiated memory cells, CD147 is highly expressed (Kirsch et al., 1997; Renno et al., 2002). Blocking CD147 with a specific monoclonal antibody partially arrested thymocyte development at both the DN3 to DN4 transition and DP to $CD4^+$ SP selection (Renno et al., 2002), and the latter phenotype was validated by Lck-Cre driven CD147 genetic deletion (Yao et al.,

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2013). These developmental abnormalities could indicate that loss of CD147 impairs pre-TCR/TCR signaling; but it may alternatively suggest that CD147 functions in other critical pathways that facilitate thymocyte development besides TCR signaling.

In this study, we analyzed thymocyte development in a mouse strain that carries the conditional CD147 allele and transgenic Cre recombinase under the control of the proximal Lck promoter ($CD147^{T\text{-}KO}$ mice (Yao et al., 2013)). Using this model, we made a surprising discovery that the loss of thymic cellularity and diminished cells of the T-cell lineage was accompanied by an increase of various lymphocyte populations with innate immunological functions, such as γδ T cells, NKT-like cells, and NK-like cells. We further assessed the lineage conversion capacity of these cells, dissected their associated molecular mechanism, and determined that CD147 could serve as an immune modulatory target for antibody-mediated cancer therapy.

2. Materials and Methods

2.1. Mice

$CD147^{T\text{-}KO}$ conditionally knocked out mice were developed according to a standard gene targeting approach in ES cells, and were used in previous experiments (Kuno et al., 1998). $CD147^{T\text{-}KO}$ mice were of a mixed C57BL/6 J and 129S5 genetic background. Normal female C57BL/6 mice were purchased from the Fourth Military Medical University, Laboratory Animal Center, and white C57BL/6 mice were obtained from the Biomedical Analysis Center of the Third Military Medical University. The detail information can be found in *Supplemental materials and methods*.

2.2. Reprogramming of T cells to NK-like Cells In Vitro

Myeloid cells were obtained from mouse bone marrow after injection of 5-FU for 4 days, and DP cells were sorted by FACS. 10^5 cells/well of myeloid cells or DP cells were cultured on OP9-DL1 stromal cells in media (alpha-MDM with 20% FCS, 1% penicillin/streptomycin, and 2 mM L-glutamine, 5 × 10–5 M β-mercaptoethanol, 10 mM Hepes buffer, sodium pyruvate, 5 ng/ml IL-7 (R&D), and 27.5 ng/ml Flk2/Flt3 (R&D)). Every 3 days, half of the media was replaced with new media for 7 to 21 days. DN3 or DP thymocytes were sorted by FACS, and likewise co-cultured with OP9-DL1 in T cell culture media (RPMI-1640, 10% FCS, 1% penicillin/streptomycin, 2 mM L-glutamine, 5 ng/ml IL-7, 27.5 ng/ml Flk2/Flt3) at 3000 cells per well in 24-well plates. 100 ng/ml huIL-2 was supplemented in T cell medium to promote NK-like cell proliferation. Every 3 days, half of the media was replaced with fresh T cell media with IL-2.Every seven days, cells were collected by vigorous pipetting, filtered through cell strainers and transferred to new tissue culture plates pre-seeded with fresh OP9-DL1 stromal cells. Cells were collected and analyzed by FACS after 14 days.

2.3. Reprogramming of Single Thymocyte to NK-like Cells

Single DN3 thymocyte was sorted directly into individual wells in a 96-well plate pre-seeded with OP9-DL1 stromal cells in T cell medium supplemented with 100 ng/ml huIL-2.Medium was changed every 3 days. After 12 days cells were analyzed by flow cytometry.

2.4. Cytotoxicity Assays for Converted NK-like Cells

Target cells, B16-F10 melanoma cells, were maintained in RPMI-1640,10% FCS, 1% penicillin/streptomycin, 2 mM L-glutamine. NK-like cells were generated *in vitro* with OP9-DL1 feeder cells as described above. To perform the cytotoxicity assay, target cells were washed and incubated with 0.1 μCi $Na_2^{51}CrO_4$ for 45 mins at 37 °C. ^{51}Cr-loaded cells were then washed and mixed with to-be-tested effector cells at various ratios, and then incubated for 4 h at 37 °C before the supernatant was tested for chromium release in a scintillation counter. Percent specific lysis was calculated as (experimental release − spontaneous release) / (maximum release − spontaneous release) × 100.

2.5. Retroviral Transduction of Mouse Bone Marrow Cells

The day before transduction, PLAT-E packaging cells were plated at 1×10^6cells/well of a 6-well plate in DMEM with 10% FCS. After 24 h, the cells were transfected with MSCV-Puro-2Xins-mG-Mock vectors carrying TCF1 and Neo cDNAs using Fugene 6 transfection reagent (Roche) according to the manufacturer's instructions. 24 h after transfection, medium was replaced and the plate was transferred to 32 °C for retrovirus production. The viruses were collected at 48 h and 72 h, and filtered with a 0.45 μm filter before transduction. Twenty-four hours after transduction, the medium was replaced. Mouse bone marrow cells were seeded at 8×10^5 cells per 100 mm dish. After 24 h, virus-containing supernatants derived from these Plat-E cultures were filtered through a 0.45 μm cellulose acetate filter (Schleicher & Schuell) and supplemented with 4 μg/ml polybrene (Nacalai Tesque). Target cells were incubated in the viral/polybrene-containing supernatant for a minimum of 4 h. After infection, the cells were replated in 10 ml fresh medium. 3 days after infection, G418 was added at a final concentration of 0.3 mg/ml, and the GFP^+ cells were sorted by FACS Aria.

2.6. In Vivo Tumor Transplantation

6 week old female C57BL/6 mice were used in all experiments. A murine *in situ* hepatoma model was generated by intraperitoneally anesthetizing mice with 50 mg/kg of pentobarbital. The mice were fixed, and their abdomens dissected to expose their liver. 1×10^6 viable Hepa16 cells or Hepa16-IRES cells in 0.05 ml DMEM were intrahepatically injected into murine liver. 10 mg/kg of CD147 antibody (R&D, Clone # 116318) was used for treatment starting on day 3, and treatment was given every three days for two weeks. Small animal imaging was performed on hepatoma-bearing mice on day 3, 7, 14, 28 and 42, and the livers were removed at day 3, 7 and 14, and were weighed to determine the tumor growth. The number of NK cells were quantified using flow cytometry and immunofluorescence. Black C57BL/6 mice were used in melanoma model. 5×10^4 viable B16-F10 cells resuspended in 0.02 mL DMEM were subcutaneously injected, and tumor size was detected starting on day 6. 10 mg/kg of CD147 antibody treatment was carried out from day 1, and treatment was given every three days for four times. The number of NK cells were again quantitated using flow cytometry and immunofluorescence.

2.7. Statistical Analysis

Graphpad Prism software was used to analyze the data. Means, S.D. and the probability (p) were presented in some figures. ANOVA were used to compare when divided into more than two groups, and student's t-test was used to assess the comparisons between two groups. Comparisons of tumor-growth curves were assessed by analysis of variance. $^*p < 0.05$ was considered significant.

The other methods can be found in *Supplemental materials and methods*.

3. Results

3.1. Loss of CD147 Biases Developing T cells Into NKT-like Lineages

In fetal thymic organ culture, suppression of CD147 resulted in a transitional block of developing thymocytes from the DN to DP stage. In another report, this blockade was established as early as the DN1 stage (Renno et al., 2002). Through the proximal Lck promoter-driven Cre expression, we previously reported that the loss of CD147 dampened thymic cellularity in various populations (Yao et al., 2013). We

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