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Research paper

Characteristics of NK cells from leukemic microenvironment in MLL-AF9 induced acute myeloid leukemia

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ABSTRACT

NK cells are indispensable components of tissue microenvironment and play vital in both innate and adaptive immunity. The activation and function of NK cells are affected by tumor microenvironments. NK cells are also important players in leukemic microenvironment. However, their characteristics in leukemic microenvironment, including maturation status, phenotype, subpopulations and functional roles especially immunoregulatory potential, have not been well established. Here, we studied these characteristics of NK cells in MLL-AF9 induced mouse acute myeloid leukemia (AML) model. Increase of more mature NK cells were detected in the AML spleen. Splenic AML microenvironment promoted NK cell activation in early and middle stages of leukemia. Cytotoxicity molecules and cytokines were up-regulated in activated NK cells. Furthermore, NK cells from AML microenvironment regulated T cell function, not only by maintaining the activation of $CD4^+$ and promoting the degranulation of cytotoxic $CD8^+$ T cells but also by influencing the differentiation of $CD4^+$ T cells. Moreover, two NK cell subpopulations marked by DNAM-1 (CD226) had distinct cytokine expression patterns but similar regulatory effects on T cells. Collectively, these findings highlight the significance of immunoregulatory role of NK cells, and suggest novel therapeutic potential for leukemia by manipulating NK cell immunoregulatory activity.

1. Introduction

Natural killer (NK) cells are indispensable components of tissue microenvironment, and play vital roles in both physiological and pathological processes (Lanier, 2008). The successfully progressive acquisition of NK cell effector functions is related to their terminal stage functional maturation (Sun, 2016). Some key molecules/pathways important for NK cell terminal stage differentiation have been elucidated (Goh and Huntington, 2017), and the expression of KLRG1 and CD11b on mature NK cells allows the identification of three subpopulations with progressive effector functions (Huntington et al., 2007; Narni-Mancinelli et al., 2011). However, whether the microenvironment of hematopoietic malignancies has an impact on NK cell terminal stage differentiation has not been well established.

Besides innate immune responses, evidence showed that NK cells also participated in adaptive immune responses. On the one hand, NK cells recognized transformed cells by a wide range of activating and inhibitory receptors on their surface, and directly killed transformed cells by releasing perforin and granzyme, or triggering death receptor ligand pathways (Carotta, 2016; Shevtsov and Multhoff, 2016). On the other hand, cytokines and chemokines secreted by activated NK cells were vital for regulating functions of a variety of immune cells to support the adaptive immune response in viral infections (Schuster et al., 2016). Studies showed that DNAM-1 (CD226), an adhesion molecule expressed on approximately half of mouse splenic NK cells (Nabekura et al., 2014a,b), played a fundamental role in anti-tumor immunity by controlling the cytotoxicity of NK cells and IFN-γ production upon interaction by its ligands CD155 and CD112 (Chan et al., 2010; de Andrade et al., 2014). Furthermore, two NK cell subpopulations identified by DNAM-1 had distinct cytokine secretion profiles (Martinet et al., 2015), which implied NK cell subpopulations had different immunoregulatory potential. However, the immunoregulatory effects of NK cells and their subpopulations in malignant microenvironments remain largely unknown.

Both intrinsic and extrinsic factors play crucial roles in the transformation and progression of malignancies (Manjili, 2017; Wargo et al.,

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2016). Mounting evidence demonstrated that the tumor microenvironment (TME) was crucial for tumors (Hanahan and Weinberg, 2011). As cellular component of tumor microenvironments, functional phenotype of NK cells is also affected by microenvironmental signals. Remarkable diversity in activation and function of NK cells is detected among various solid tumors. In most cases, NK cells suppressed tumor progression through anti-tumor immune response (Stojanovic and Cerwenka, 2011). However, their tumoricidal potential were decreased by components in tumor microenvironment including tumor cells, tumor-associated fibroblasts (TAFs), tumor associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), regulatory T cells (Treg cells) as well as cytokines such as IL-10 and TGF-β (Vitale et al., 2014). Compared with solid tumors, leukemia, originating from aberrant expansion of leukemia stem cells (LSCs) (Reinisch et al., 2015), has unique pathological and clinical features. Leukemic microenvironment has great impacts on the progression of leukemia. For example, Notch1-induced T cell leukemia could be potentiated by microenvironmental cues in the spleen (Ma et al., 2014). Furthermore, leukemia-associated macrophages (LAMs), Treg cells and MDSCs were demonstrated to play roles in leukemia progression (Curran et al., 2017; Chen et al., 2015). NK cells were suggested to kill leukemia cells by cytotoxicity mechanism (Boieri et al., 2017; Jin et al., 2016; Satwani et al., 2014). However, terminal stage differentiation, subpopulations and functional characteristics especially immunoregulatory potential of NK cells in leukemic microenvironment have not been elucidated.

Here, we studied distribution, terminal stage differentiation, activation, subpopulations and immunoregulatory effects on T cells of NK cells in hematopoietic tissues in MLL-AF9 induced mouse acute myeloid leukemia (AML) model. Increase of more mature NK cells were detected in the AML spleen. Splenic AML microenvironment promoted NK cell activation in early and middle stages of leukemia. Cytotoxicity molecules and cytokines were up-regulated in activated NK cells. Furthermore, NK cells from AML microenvironment regulated T cell function, not only by maintaining the activation of $CD4^+$ and $CD8^+$ T cells but also by influencing the differentiation of $CD4^+$ T cells. Moreover, two NK cell subpopulations marked by DNAM-1 had distinct cytokine expression patterns but similar regulatory effects on T cells. These findings highlight the significance of immunoregulatory role of NK cells, and suggest novel therapeutic potential for leukemia by manipulating NK cell immunoregulatory activity.

2. Materials and methods

2.1. Mice

Six to eight week-old C57BL/6 (CD45.2) mice were obtained from the Animal Centre of the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College. All mice were bred and maintained in the specific pathogen-free certified animal facility of the State Key Institute of Hematology (SKLEH). All experiments were approved by the Institutional Animal Care and Use Committees of SKLEH.

2.2. Mouse AML model

The establishment of the MLL-AF9-induced mouse AML model was described previously (Cheng et al., 2015). Briefly, lineage-negative and Sca-1-positive ($Lin^-Sca\text{-}1^+$) cells were enriched from the BM of C57BL/6J mice and infected with retrovirus carrying MLL-AF9 fusion gene, MSCV-MLL-AF9-IRES-GFP. Then, cells were transplanted into lethally irradiated C57BL/6J recipients. All recipients developed AML. In this study, a non-irradiated mouse AML model was used. Leukemia cells were transplanted *i.v.* into non-irradiated recipient C57BL/6 mice (1×10^6 cells/mouse). All mice suffered from AML, the mice were sacrificed at the indicated time points, and BM and spleen samples were collected for further analysis.

2.3. Flow cytometry and antibodies

The LSR II and LSR Fortessa flow cytometer (both from BD Biosciences) were used for FACS analysis, and FACS Aria III (from BD Biosciences) was used for cell sorting. Monoclonal antibodies (mAbs) against CD3 (17A2, PerCP-Cy5.5-conjugated), CD19 (1D3, PerCP-Cy5.5-conjugated), CD122 (TM-β1, PE-conjugated), NK1.1 (PK136, PE-Cy7-conjugated), NKp46 (29A1.4, APC-conjugated), CD11b (M1/70, APC-Cy7-conjugated), KLRG1 (2F1, APC-conjugated), DNAM-1 (480.1, PE-conjugated) and cell activation cocktail (with Brefeldin A) were from BioLegend. And mAbs against CD4 (GK1.5, PE-Cy7-conjugated), CD8 (SK1, APC-Cy7-conjugated), CD25 (3C7, PE-conjugated), CD62L (MEL-14, APC-conjugated) and IFN-γ (XMG1.2, APC-conjugated) were from BD Bioscience. CCL3 (IC450A, APC-conjugated) was from R&D. Standard protocols were followed for all experiments and Flow Jo software (version 7.6.1) was also used for data analysis.

2.4. In vitro coculture experiment

$DNAM\text{-}1^+$ and $DNAM\text{-}1^-$ NK subpopulations or total NK cells were sorted by flow cytometry from the spleen of normal and leukemic mice on day 15. T cells were sorted from the spleen of normal mice. NK cells were cocultured with T cells in F12 media with 5% FBS at the ratio of 1:2 in 48-well plates, which were pre-coated with 5 μg/ml anti-CD3 (145-2C11, BioLegend) for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C for 72 h. Then, cells were harvested and resuspended in 100 ul PBS. After stained with mAbs at 4 °C, washed with PBS, cells were analyzed by flow cytometry or sorted for further experiments. All culture supplies were screened and selected on the basis of being endotoxin free.

2.5. cDNA synthesis and real time PCR

Cells were lysed and total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was achieved using Super Script First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real time PCR was performed on the StepOne real-time PCR system (Applied Biosystems, Foster City, CA). The ΔΔCt method [$^{\Delta\Delta}Ct = (Ct_{TARGET} - Ct_{GAPDH})_{sample} - (Ct_{TARGET} - Ct_{GAPDH})_{calibrator}$] was used to analyze the expression level of target genes. The sequences for all primers are listed in Table 1.

2.6. RNA sequencing

$DNAM1^+$ and $DNAM1^-$ NK cells were sorted by flow cytometry from spleen samples on day 15 after injection of leukemia cells. Two NK subpopulations were also sorted from normal mice. RNA sequencing (RNA-seq) was carried out by the Beijing Genomics Institute following standard protocols. The library products were sequenced using a BGISEQ-500. Standard bioinformatics analysis was performed by the Beijing Genomics Institute. The RNA-seq data are available in the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE101421.

2.7. Statistical analysis

The results were expressed as mean ± standard error of means (SEM). Analysis was done using GraphPad Prism 6.0 software (San Diego, CA) and SPSS17.0 software package (SPSS, Chicago, IL). Comparisons between two groups were analyzed by unpaired Student's *t*-test, whereas comparisons among more than three groups were analyzed by one-way AVONA with multiple comparison tests. Statistically significant is accepted when the p value is less than 0.05.