Enhanced Spontaneous Anti-Tumor Cytotoxicity of Natural Killer Cells in Cutaneous T-Cell Lymphoma Patients

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Abstract

Cutaneous T-cell lymphoma (CTCL) is characterized by the expansion of malignant CD4+ T cells in the skin. There are two main subtypes of CTCL, mycosis fungoides (MF) and Sézary syndrome (SS). Previous studies have demonstrated defects in cell-mediated immunity, including altered cytokine profiles and decreased neutrophil function, and patients often have recurring bacterial and viral infections. Recent studies have shown increased expression of interleukin (IL)-15 in malignant CD4+ T cells in CTCL patients (Mishra et al., Cancer Discovery, 2016). Since IL-15 can enhance NK cell differentiation and activation, we hypothesized that NK cells from CTCL patients might have phenotypic and functional alterations due to these changes in homeostatic IL-15 levels. To evaluate the absolute number of NK cells in CTCL patients, CD56+Lineage- lymphocytes were quantitated by flow cytometry. SS patients had significantly fewer NK cells as compared to normal donors (mean $\pm$ SEM of absolute cell numbers in normal donors=0.2442 $\pm$ 0.02, n=51; SS=0.1072 $\pm$ 0.02, n=9; MF=0.2161 $\pm$ 0.01, n=112; unpaired t-test normal vs. SS, p=0.007). NK cell counts were significantly associated with overall survival, with the short-term risk of death increasing by 87.5% for every increase in 0.1 of absolute NK cell counts on univariate analysis utilizing the Cox proportional hazards model (Figure 1A; p=0.0413). We then examined the cytotoxic capacity of NK cells (CD56+Lineage-) freshly purified from both MF and SS patients by standard chromium release assay. There was a significantly higher level of specific lysis of tumor cells (K562 target cells) by NK cells from CTCL patients as compared to normal donors (Figure 1B, at 50:1 ratio mean $\pm$ SEM of percent NK cell cytotoxicity in normal vs CTCL = 27.47 $\pm$ 4.76, n=13 vs. 52.54 $\pm$ 4.88, n=12, unpaired t-test, p=0.001). Upon further transcript analysis of NK cell cytolytic mediators from CTCL patients compared to normal donors, we observed significantly increased levels of perforin (mean $\pm$ SEM of relative RNA in normal vs CTCL = 91.39 $\pm$ 9.18, n=3 vs. 485.7 $\pm$ 43.15, n=7, unpaired t-test, p=0.0004) and granzyme B (143 $\pm$ 23.79, n=3 vs. 431 $\pm$ 68.13, n=7 unpaired t-test, p=0.03). In addition, we observed increased levels of interferon-gamma and Fas Ligand (5.149 $\pm$ 0.4775, n=3 vs. 13.93 $\pm$ 1.52, n=7; unpaired t-test p=0.007), indicating NK cells from CTCL patients exhibited consistently elevated activation patterns as compared to normal donor NK cells. We then examined the expression pattern of the IL-15 receptor complex on NK cells in CTCL patients as compared to normal donors by RNA sequencing. There was a significant elevation in IL-15R α (mean $\pm$ SEM of relative RNA in normal vs CTCL = 0.8677 $\pm$ 0.1018, n=3 vs. 1.772 $\pm$ 0.2055, n=7; p=0.03) and IL-15R γ (57.02 $\pm$ 7.525, n=3 vs. 154.7 $\pm$ 12.14, n=7; p=0.001), while IL-15R β was also elevated (128 $\pm$ 23.68, n=3 vs. 223.2 $\pm$ 25.43, n=7; p=0.056). Next we evaluated protein levels of phosphorylated signal transducer and activator of transcription 5 (p-STAT5) in fresh whole blood as the surrogate marker of IL-15 signaling. Preliminary findings demonstrate an increase in p-STAT5 in CTCL patients as compared to normal donors (mean $\pm$ SEM of fluorescent intensity in normal donors vs CTCL patients =1506 $\pm$ 90.82, n=5 vs. 1270 $\pm$ 50.59, n=4; unpaired t-test; p=0.07). These data suggest that NK cell activation is enhanced in patients with CTCL, including MF patients with disease localized to the skin. Further investigations understanding the disconnect between NK cell numbers, activation, and disease control in CTCL patients are warranted.

Results

Figure 1. NK cell numbers are associated with overall survival in CTCL

Figure 1. (A) The absolute number of NK cells in CTCL patients and healthy normal donors was determined by flow cytometry (Total CD56/16+/Lineage-cells). **(B)** We next evaluated the association between absolute NK cell counts and overall survival of early and late stage CTCL patients. Interestingly, NK cell counts were significantly associated with overall survival, with the short-term risk of death increasing by 87.5% for every increase in 0.1 of absolute NK cell counts on univariate analysis utilizing the Cox proportional-hazards model (p=0.041).

Results (cont.)

Figure 2. NK cells from CTCL patients exhibit increased cytotoxicity against tumor targets ex vivo

Figure 2. NK cells from normal healthy donors or CTCL patients were isolated from peripheral blood and sorted to purity. **(B)** NK cell function was assessed by standard chromium release assay, using purified NK cells as effectors cells at the ratio indicated and K562 cells as the tumor targets. NK cell cytotoxicity was significantly enhanced in CTCL patients as compared to normal donors.

Figure 3. NK cells from CTCL patients display activated transcriptional profile compared to healthy donors

Figure 3. NK cells were isolated from CTCL patients and normal donors and evaluated by RNA sequencing. **(A)** Heatmap of differentially regulated transcripts between normal and CTCL patients (n=5 MF, n=2 SS*, and n=3 normal donors). **(B)** Mediators of cytolytic activity and NK cell activation were evaluated in all CTCL patients (n=7) as compared to normal controls (n=3).

Figure 4. Ingenuity pathway analysis of NK cells in CTCL

Figure 4. An IPA upstream functional analysis was performed in enriched data set from the NK cells. Red indicates upregulation in CTCL patients as compared to normal donors, while green indicates reduced expression.

Results (cont.)

Figure 5. IL-2 and IL-15 signaling pathways are altered in CTCL patients.

Figure 5. Purified NK cells were evaluated by RNA sequencing analysis (n=7 CTCL vs. n=3 normal donors). **(A)** RNA expression of receptor components required for both IL-2 and IL-15 signaling were evaluated in CTCL patients and compared with normal donors. **(B)** Protein expression of phosphorylated STAT3 (pSTAT3) and STAT5 (pSTAT5) on NK cells was determined in freshly obtained whole blood samples from CTCL patients and matched normal donors by flow cytometry. Graph indicates median fluorescent intensity (MFI) of normal (gray bars, n=7) as compared to CTCL patients (green bars, n=15).

Figure 6. CD4+ malignant cells produce IL-15 which may lead to enhanced NK cell activation and function.

Figure 6. Schematic of interaction between malignant CD4+ T cells in CTCL patients producing IL-15, which binds to the upregulated IL-15 receptor complex on NK cells and enhances downstream activating pathways in NK cells.

Conclusions

Conclusions. While our initial goal with this work was to define the functional capacity of freshly isolated NK cells in CTCL patients, our comprehensive analysis of both NK cell function and expression profiles uncovered intriguing results. One of the most interesting findings of this study was the significant association between NK cell number and CTCL patient survival. Similar to previous reports, we find significantly decreased NK cell numbers in leukemic CTCL patients when compared to healthy donors, but to our knowledge this is the first description of higher NK cell numbers in a malignancy being associated with decreased short-term survivability. The cause of this significant clinical relationship is unknown. Few recent studies have described the tumor-promoting potential of NK cells through their ability to upregulate certain oncogenic pathways such as VEGF-A, an activity mediated in part by reduced STAT5 activity. Notably, we describe increased pSTAT5 and did not identify upregulation of VEGF-A or other common tumor-promoting factors in our RNA sequencing analysis of NK cells from CTCL patients. In addition, we did not see increased expression of immunosuppressive factors such as IL-10 or TGF β in NK cells from CTCL patients. It is possible that there are other yet-undiscovered mechanisms of tumor promotion by NK cells, which may respond to the tumor microenvironment or specific signals from tumor cells themselves. These mechanisms may lead to failure of NK cells to control tumor progression despite elevated cytotoxic activity. In addition, further investigation of multiple facets of NK cell recognition is warranted, however it is clear the overall immunosuppressive microenvironment in CTCL patients contributes to the insufficiency of patient NK cells to effectively control CTCL progression.

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