STIMULUS REPORT

blood advances

Highly cytotoxic natural killer cells are associated with poor prognosis in patients with cutaneous T-cell lymphoma

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Key Points

- Paradoxically higher NK-cell activity in CTCL patients is associated with increased expression of phosphorylated STAT5.
- These highly effective NK cells are associated with poor prognosis in patients with leukemic CTCL.

Introduction

Cutaneous T-cell lymphoma (CTCL) is a type of non-Hodgkin lymphoma characterized by the expansion of malignant CD4$^+$ T cells in the skin. There are two main subtypes of CTCL: an indolent form termed mycosis fungoides (MF), which is largely limited to the skin, and Sézary syndrome (SS), an aggressive leukemic variant of the disease which can manifest systemically.[1-3]

Previous studies have demonstrated defects in cell-mediated immunity in CTCL patients, including altered cytokine profiles and impaired neutrophil function, which lead to a high incidence of recurrent bacterial and viral infections as a result of decreased Th1-mediated immunity.[4-9] It has also been reported that natural killer (NK)–cell function is decreased in CTCL patients,[10-14] which could contribute to an overall decrease in the innate immune response to both neoplastic cells and viral or bacterial pathogens. Previous groups have reported that NK cells from SS patients are capable of responding to activation ex vivo, indicating the potential for development of immune-based therapeutics.[15]

Although MF patients often have a prolonged indolent clinical course of disease that requires localized treatment, there are few effective treatments for the successful management of patients with SS. Because of the lack of success with traditional chemotherapeutic approaches, novel immune-based therapeutics are being developed for use in a multitude of hematologic diseases, including CTCL.[4,16-18] Understanding the immune microenvironment in patients with CTCL will be critical to the successful design of targeted therapies for their disease.

Previous studies by our group and by others have shown increased expression of interleukin-15 (IL-15) in malignant CD4$^+$ T cells in CTCL patients.[19] IL-15 acts through a trimeric IL-15R complex to enhance NK-cell maturation and function.[20-22] Indeed, in a first-in-human phase 1 trial in patients with refractory solid cancer tumors, IL-15 treatment induced profound expansion of circulating NK cells (NCT01885897).[23] Considering that IL-15 is produced by malignant cells in CTCL, we sought to study the possible effect of chronically elevated IL-15 on NK-cell function in CTCL patients. In this study, we show that NK-cell activity is significantly enhanced in CTCL, and strikingly, higher NK-cell numbers are associated with increased mortality.

Materials and methods

NK-cell numbers

NK-cell numbers were evaluated by flow cytometric analysis of peripheral blood samples drawn on the same day as the initial diagnostic complete blood cell count with differential and was performed using a 10-color technique with a gating strategy based on CD45 staining and light side scatter characteristics. NK-cell number represents the number of CD56$^+$/CD16$^+$/CD3$^-$ NK cells per microliter. Samples were

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taken from November 2007 through November 2016 from patients at The Ohio State University James Cancer Hospital who were diagnosed with biopsy-proven CTCL (Table 1).

NK-cell isolation and cytotoxicity

NK cells were isolated from fresh peripheral blood samples by negative enrichment (STEMCELL Technologies) followed by sorting on a BD Aria II analyzer. No phenotypic alterations were noted between the presorted and postsorted NK cells. Purified NK cells were co-cultured with chromium-labeled K562 target cells for 4 hours in a standard chromium release cytotoxicity assay.[24] K562 cells were obtained from American Type Culture Collection (ATCC) and were kept in culture less than 1 month. Cells were routinely tested for mycoplasma per routine protocol. Tables 2 and 3 provide clinical information on the patients who participated in NK-cell functional studies.

RNA sequencing analysis

NK cells ($CD56^+$/lineage$^-$) were isolated as described above. Total RNA was isolated from cell samples using standard methods (Active Motif). To generate heat maps of the most differential genes, additional comparative metrics were calculated such as fold-change between averages of fragments per kilobase of transcript per million mapped reads (FPKM) values between groups of interests, and analysis of variance *P* values comparing 2 or more groups. Genes with no values in all samples or a value in only 1 of the 9 samples were removed.

Phosphorylated STAT staining and analysis

Fresh peripheral blood samples were obtained from CTCL patients and age-matched normal donors. Phosphorylated signal of transducer and activator of transcription 3 (pSTAT3) and STAT5 (pSTAT5) were evaluated by direct whole blood antibody labeling (BD Biosciences). Median fluorescence intensity was calculated for each STAT protein.

Results

The absolute number of NK cells in peripheral blood was evaluated in CTCL patients and compared with that in normal donors ($n = 51$). There was no statistical difference in absolute number of NK cells when all patients with CTCL were included (Figure 1A); however, SS patients had on average 57.4% fewer NK cells compared with normal donors (supplemental Figure 1). We then evaluated the association between absolute NK-cell counts and overall survival. NK-cell counts were significantly associated with overall survival ($P = .041$; Figure 1B). To evaluate NK-cell function, NK cells were purified from fresh peripheral blood (Figure 1C) and evaluated for cytotoxic function against K562 target cells.[24] CTCL patients had significantly higher levels of NK-cell cytotoxicity compared with normal donors (Figure 1D). Although these findings differ from those in previous reports, earlier work did not use NK cells isolated from fresh peripheral blood,[10-12] evaluate frozen samples, or use cytokine stimulation.[14]

A comprehensive surface immunophenotypic analysis of NK cells revealed no significant differences (supplemental Figure 2), so we hypothesized that there could be alterations in pathway activation in CTCL patients. Whole transcriptome analysis was completed to evaluate NK-cell activation. A heat map generated by hierarchical clustering of the differentially expressed genes shows the unique patterns of gene expression in NK cells from CTCL patients and healthy donors (Figure 2A). NK cells from CTCL patients had increased expression of cytolytic mediators, including perforin, granzyme A, granzyme B, Fas, and tumor necrosis factor-alpha–related apoptosis-inducing ligand (TRAIL) (Figure 2B). Furthermore, we observed significantly increased cytokine (interferon-γ [IFN-γ]) production in patient NK cells. Using ingenuity pathway analysis, we determined differential expression of key pathways, including IL-15, IL-15/IL-2 receptors, IFN-γ, and several surface activating molecules such as CD2, CD40, FCER1G, CD80, KLR, SELL, and CD244. Furthermore, NF-kB and ICOS were inhibited in our data set (Figure 2C; supplemental Figures 3 and 4).

Our group previously demonstrated that $CD4^+$ T cells from CTCL patients exhibit elevated levels of IL-15, a key cytokine that mediates NK-cell activation and homeostasis.[19] There was significant upregulation of several IL-15 receptor subunits on NK cells (Figure 2D). To further confirm activation of the IL-15 pathway, we evaluated STAT signaling in fresh peripheral blood NK cells by flow cytometry, because IL-15 signals through both STAT3 and STAT5.[25] There was a nonsignificant trend toward increased pSTAT3 expression and a significant upregulation of pSTAT5 in CTCL patients compared with normal donors (Figure 2E). Overall, we propose a mechanism by which malignant $CD4^+$ T cells produce IL-15, which binds to the highly expressed IL-15 receptor complex on NK cells from CTCL patients. NK-cell activation is reflected in enhanced cytotoxicity, STAT5 phosphorylation, and upregulation of downstream effectors (Figure 2F).

Discussion

Although 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. To the best of 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,[25] mediated in part by reduced STAT5 activity. We described increased pSTAT5 and did not identify upregulation of VEGF-A or other immunosuppressive factors. It is possible that there are other yet undiscovered mechanisms of tumor promotion by NK cells, and these may lead to the failure of NK cells to control tumor progression despite elevated cytotoxic activity. It is also possible that there may be additional immunosuppressive mechanisms that are present in the microenvironment of CTCL patients. For example, previous studies by our group and others have demonstrated alterations in NK-cell signaling because of the presence of suppressive myeloid cells such as myeloid-derived suppressor cells and tumor-associated macrophages in cancer patients,[26] and both populations have been reported among CTCL patients.[27-29]

Additional checkpoint inhibitors, such as PD-1, CTLA-4, TIGIT, and TIM-3 may also play a role in decreasing NK-cell function in patients, because previous studies demonstrated that these inhibitors may be increased in the presence of IL-15.[30-32] Transcript analysis of PD-1 did show a moderate increase in

Table 1. Characteristics of patients diagnosed with biopsy-proven CTCL MF

Patient ID	Diagnosis	Stage at diagnosis	Histology/subtype	Age at diagnosis, y	Sex	Race
9	CTCL, MF	IA	Woringer-Kolopp variant	48	F	White
216	CTCL, MF	IA	$CD30^+$ LPD: primary CTCL γ-δ T-cells	68	M	White
27	CTCL, MF	IA	Hypopigmented	60	F	African American
85	CTCL, MF	IA	Hypopigmented	38	M	African American
65	CTCL, MF	IA	None	40	M	African American
180	CTCL, MF	IA	None	56	M	African American
6	CTCL, MF	IA	None, lyp, and $CD30^+$ LPD	61	M	African American
147	CTCL, MF	IA	Hypopigmented	45	F	Asian
89	CTCL, MF	IA	Folliculotropic	48	F	White
137	CTCL, MF	IA	Granulomatous	67	F	White
28	CTCL, MF	IA	Hypopigmented	38	F	White
38	CTCL, MF	IA	None	41	F	White
42	CTCL, MF	IA	None	49	F	White
52	CTCL, MF	IA	None	57	F	White
55	CTCL, MF	IA	None	41	F	White
61	CTCL, MF	IA	None	82	F	White
73	CTCL, MF	IA	None	64	F	White
82	CTCL, MF	IA	None	50	F	White
107	CTCL, MF	IA	None	58	F	White
111	CTCL, MF	IA	None	56	F	White
144	CTCL, MF	IA	None	51	F	White
145	CTCL, MF	IA	None	42	F	White
150	CTCL, MF	IA	None	28	F	White
152	CTCL, MF	IA	None	47	F	White
183	CTCL, MF	IA	None	53	F	White
200	CTCL, MF	IA	None	37	F	White
215	CTCL, MF	IA	None	50	F	White
226	CTCL, MF	IA	None	19	F	White
166	CTCL, MF	IA	Poikilodermatous	31	F	White
84	CTCL, MF	IA	Poikilodermatous	55	F	White
188	CTCL, MF	IA	Woringer-Kolopp variant	31	F	White
142	CTCL, MF	IA	Follicular	50	F	White
179	CTCL, MF	IA	Follicular T-helper phenotype	60	F	White
50	CTCL, MF	IA	$CD8^+$	58	M	White
32	CTCL, MF	IA	Folliculotropic	37	M	White
36	CTCL, MF	IA	Folliculotropic	61	M	White
54	CTCL, MF	IA	Folliculotropic	81	M	White
64	CTCL, MF	IA	Folliculotropic	25	M	White
100	CTCL, MF	IA	Folliculotropic	62	M	White
130	CTCL, MF	IA	Hypopigmented, $CD8^+$	18	M	White
41	CTCL, MF	IA	None	84	M	White
46	CTCL, MF	IA	None	40	M	White
47	CTCL, MF	IA	None	78	M	White
57	CTCL, MF	IA	None	63	M	White
62	CTCL, MF	IA	None	57	M	White
67	CTCL, MF	IA	None	77	M	White
71	CTCL, MF	IA	None	45	M	White

F, female; LPD, lymphoproliferative disorder; lyp, lymphomatoid papulosis; M, male.

Table 1. (continued)

Patient ID	Diagnosis	Stage at diagnosis	Histology/subtype	Age at diagnosis, y	Sex	Race
80	CTCL, MF	IA	None	26	M	White
95	CTCL, MF	IA	None	60	M	White
99	CTCL, MF	IA	None	74	M	White
103	CTCL, MF	IA	None	82	M	White
108	CTCL, MF	IA	None	42	M	White
120	CTCL, MF	IA	None	62	M	White
124	CTCL, MF	IA	None	57	M	White
128	CTCL, MF	IA	None	54	M	White
131	CTCL, MF	IA	None	37	M	White
139	CTCL, MF	IA	None	26	M	White
162	CTCL, MF	IA	None	51	M	White
174	CTCL, MF	IA	None	76	M	White
181	CTCL, MF	IA	None	53	M	White
189	CTCL, MF	IA	None	44	M	White
191	CTCL, MF	IA	None	34	M	White
197	CTCL, MF	IA	None	70	M	White
223	CTCL, MF	IA	None	72	M	White
146	CTCL, MF	IA	None	48	M	White
199	CTCL, MF	IA	None	63	M	White
4	CTCL, MF	IB	None	52	M	African American
2	CTCL, MF	IB	Poikilodermatous	69	M	African American
203	CTCL, MF	IB	None	46	F	African American
39	CTCL, MF	IB	Hypopigmented	38	M	African American
56	CTCL, MF	IB	None	51	M	African American
77	CTCL, MF	IB	$CD8^+$	58	F	White
26	CTCL, MF	IB	Granulomatous	66	F	White
87	CTCL, MF	IB	None	51	F	White
91	CTCL, MF	IB	None	66	F	White
136	CTCL, MF	IB	None	72	F	White
173	CTCL, MF	IB	None	57	F	White
194	CTCL, MF	IB	None	73	F	White
115	CTCL, MF	IB	$CD8^+$	63	M	White
209	CTCL, MF	IB	$CD8^+$	81	M	White
88	CTCL, MF	IB	Folliculotropic	76	M	White
97	CTCL, MF	IB	Folliculotropic	44	M	White
114	CTCL, MF	IB	Folliculotropic	58	M	White
134	CTCL, MF	IB	Folliculotropic	53	M	White
37	CTCL, MF	IB	None	68	M	White
58	CTCL, MF	IB	None	59	M	White
75	CTCL, MF	IB	None	64	M	White
78	CTCL, MF	IB	None	46	M	White
104	CTCL, MF	IB	None	62	M	White
112	CTCL, MF	IB	None	45	M	White
121	CTCL, MF	IB	None	71	M	White
122	CTCL, MF	IB	None	67	M	White
123	CTCL, MF	IB	None	55	M	White
220	CTCL, MF	IB	None	72	M	White

F, female; LPD, lymphoproliferative disorder; lyp, lymphomatoid papulosis; M, male.

Table 1. (continued)

Patient ID	Diagnosis	Stage at diagnosis	Histology/subtype	Age at diagnosis, y	Sex	Race
227	CTCL, MF	IB	None	62	M	White
217	CTCL, MF	IB	Follicular T-helper phenotype	36	M	White
167	CTCL, MF	IB	None	78	M	White
204	CTCL, MF	IB	Poikilodermatous	61	F	Hispanic/Latino
132	CTCL, MF	IB	None	79	M	Hispanic/Latino
206	CTCL, MF	IIA	Poikilodermatous	41	M	White
33	CTCL, MF	IIB	Large cell transformation	50	F	African American
30	CTCL, MF	IIB	None	66	F	African American
157	CTCL, MF	IIB	Folliculotropic	69	M	African American
168	CTCL, MF	IIB	Folliculotropic	53	M	African American
156	CTCL, MF	IIB	None	80	F	White
90	CTCL, MF	IIB	Folliculotropic	62	M	White
63	CTCL, MF	IIB	None	88	M	White
185	CTCL, MF	IIB	None	75	M	White
153	CTCL, MF	IIB	Tumor	64	M	White
141	CTCL, MF	IIB	Large cell transformation	59	M	White
109	CTCL, MF	IIIB	Erythrodermic	55	F	White
213	CTCL, MF	IIIB	Erythrodermic	65	M	African American

F, female; LPD, lymphoproliferative disorder; lyp, lymphomatoid papulosis; M, male.

the NK cells isolated from CTCL patients (supplemental Figure 5), and because K562 cells are known to express low levels of the PD-1 ligand (PD-L1), this inhibitory effect might not be observed in vitro.[33] Analysis of inhibitory ligand expression on CTCL cells in circulation in SS patients or in skin biopsies from MF patients suggests that novel potential therapeutic targets could be focused on removing this barrier to immune cell activation.

It is known that γ cytokines such as IL-7 and IL-15 are important for CTCL progression.[19,34,35] Indeed, as disease progresses, neoplastic $CD4^+$ T cells express higher levels of IL-15.[19] Furthermore, IL-15 overexpression alone can induce CTCL in a murine model of the disease.[19] IL-15 is also known to stimulate NK-cell proliferation and cytotoxicity via phosphorylation of STAT5.[13,36-38] Thus, we proposed that IL-15 derived from the neoplastic $CD4^+$ T cells in CTCL may contribute to increased NK-cell cytotoxicity observed in patient samples. Indeed, transcriptome analysis reveals upregulation of IL-15–induced signaling pathways, and protein levels of pSTAT5 were significantly elevated in patients with CTCL, further confirming this relationship between increased NK-cell activation and cytotoxicity in CTCL patients. However, recent reports by multiple groups have demonstrated that continuous exposure to IL-15 can lead to a decrease in NK-cell proliferation, cell cycle arrest, and NK-cell exhaustion, suggesting that chronic exposure to IL-15 could also lead to immune-cell exhaustion in CTCL patients.[39,40] It is also possible that although NK cells from peripheral blood exhibit higher levels of cytotoxicity, those localized in skin lesions where

Table 2. Characteristics of patients with biopsy-proven CTCL SS

Patient ID	Diagnosis	Stage at diagnosis	Age at diagnosis, y	Sex	Race	Clonality on initial flow cytometric analysis
69	CTCL, SS	IVA1	51	M	White	84% $CD3^+/CD26^-$ T-cells; 82% $CD4^+/CD26^-$; 75% $CD7^+/CD26^-$
76	CTCL, SS	III	82	F	White	22% $CD3^+/CD4^+/CD7^-$ T-cells
86	CTCL, SS	IVA1	77	F	White	92% $CD3^+/CD4^+/CD26^-$; 22% $CD3^+/CD4^+/CD7^-$
96	CTCL, SS	IVA1	67	M	White	82% $CD3^+/CD26^-/CD2^-$
135	CTCL, SS	IVA1	59	F	White	92% $CD3^+/CD26^-$
164	CTCL, SS	IVA1	60	F	White	63% $CD3^+/CD4^+/CD7^+/CD26^-$; 34% $CD3^+/CD4^-/CD8^-/CD7^-/CD26^-$
187	CTCL, SS	IVA1	64	M	African American	60% $CD3^+/CD26^-$; 50% CD3 biphasic/$CD7^-$
224	CTCL, SS	IVA1	82	M	White	98% $CD2^+/CD4^+/CD5^+/CD3^-/CD7^-/CD8^-/CD26^-$
227	CTCL, SS	IVA1	56	M	White	93% $CD4^+/CD26^-$

Table 3. Characteristics of patients with CTCL, treatment, and experimental assay performed

Patient ID	Assay	Sex	Race	CTCL type	Stage at sample procurement	Treatment at time of collection
MF1	Flow cytometry	M	White	MF	IA	Imiquimod
MF2	Flow cytometry	F	White	MF	IA	Desoximetasone
MF3	Flow cytometry	M	White	MF	IA	Clobetasol
U16-2023	Cytotoxicity assay	M	White	MF	IB	PUVA, triamcinolone, imiquimod
U16-2101	Cytotoxicity assay	M	White	MF	IB	Bexarotene
U16-2102	Cytotoxicity assay	M	White	MF	IB	Bexarotene
U16-2225	Cytotoxicity assay	M	White	MF	IIB	Gemcitabine/doxorubicin
U16-2228	Cytotoxicity assay	M	White	MF	IIB	Bexarotene
U16-2237	Cytotoxicity assay	F	African American	MF	IA	Topical steroids
U17-0092	Cytotoxicity assay	M	White	MF	IB	Bexarotene
U17-0093	Cytotoxicity assay	F	White	MF	IB	Topical steroids
U17-0094	Cytotoxicity assay	M	White	MF	IA	nbUVB
U17-0095	Cytotoxicity assay/RNA sequencing	M	White	MF	IA	Bexarotene
U17-0253	Cytotoxicity assay/RNA sequencing	F	White	MF	IA	Topical bexarotene
U17-0254	Cytotoxicity assay/RNA sequencing	F	White	MF	IB	nbUVB
U17-0314	Flow cytometry	F	African American	SS	IVB	Romidepsin
U17-0315	Flow cytometry	F	White	MF	1A	Topical steroids
U17-0316	Flow cytometry	F	White	MF	IB	Valchlor gel
U17-0341	Flow cytometry	M	White	MF	1A	Oral or topical bexarotene
U17-0342	Flow cytometry	M	White	MF	1A	Mechlorethamine gel, clobetasol
U17-0343	Flow cytometry	M	White	MF	1A	Mechlorethamine gel, TAC
U17-0350	Flow cytometry	F	African American	MF	IA	Topical steroids
U17-0352	Flow cytometry	F	White	MF	IA	Oral bexarotene
U17-0353	Flow cytometry	M	White	MF	1B	Interferon, nbUVB, TAC, clobetasol
U17-0543	RNA sequencing	M	White	SS	1VA	Romidepsin, IPH 4102
U17-0593	RNA sequencing	M	White	MF	1B	Gemcitabine/ doxorubicin
U17-0594	RNA sequencing	M	White	SS	IVA1	Topical steroids
U17-0595	RNA sequencing	M	White	MF	IA	TAC
U17-1613	Phospho-specific flow cytometry	F	White	MF	IIB	Brentuximab
U17-1614	Phospho-specific flow cytometry	M	White	SS	1VA2	Bendamustine + brentuximab vedotin
U17-1615	Phospho-specific flow cytometry	M	White	MF	IA	nbUVB
U17-1616	Phospho-specific flow cytometry	F	White	MF	1B	TAC, nbUVB
U17-1654	Phospho-specific flow cytometry	M	White	MF	1A	Clobetasol
U17-1657	Phospho-specific flow cytometry	M	White	MF	IA	TAC
U17-1658	Phospho-specific flow cytometry	M	White	MF	1A	TAC, topical imiquimod
U17-1649	Phospho-specific flow cytometry	F	White	MF	1B	Romidepsin
U17-1651	Phospho-specific flow cytometry	F	White	MF	1B	nbUVB, TAC, interferon, oral bexarotene
U17-1652	Phospho-specific flow cytometry	F	White	MF	IA	Topical bexarotene
U17-1653	Phospho-specific flow cytometry	M	White	MF	IA	TAC
U17-1819	Phospho-specific flow cytometry	M	White	MF	IA	Romidepsin
U17-1936	Phospho-specific flow cytometry	M	White	SS	IVA	Bexarotene and interferon
U17-1937	Phospho-specific flow cytometry	M	White	SS	IIIA	None
U17-1938	Phospho-specific flow cytometry	F	White	MF	1A	None

nbUVB narrow-band ultraviolet B light therapy; PUVA, psoralen and ultraviolet A (light therapy); Tac, triamcinolone cream.

localized levels of IL-15 may be higher have additional defects that would render them ineffective at lysing the $CD4^+$ malignant T cells.

The reason for this counterintuitive finding is not known; however, we speculate that although NK cells are maintained in a hyperactive state in CTCL patients, malignant cell recognition is impaired. It is

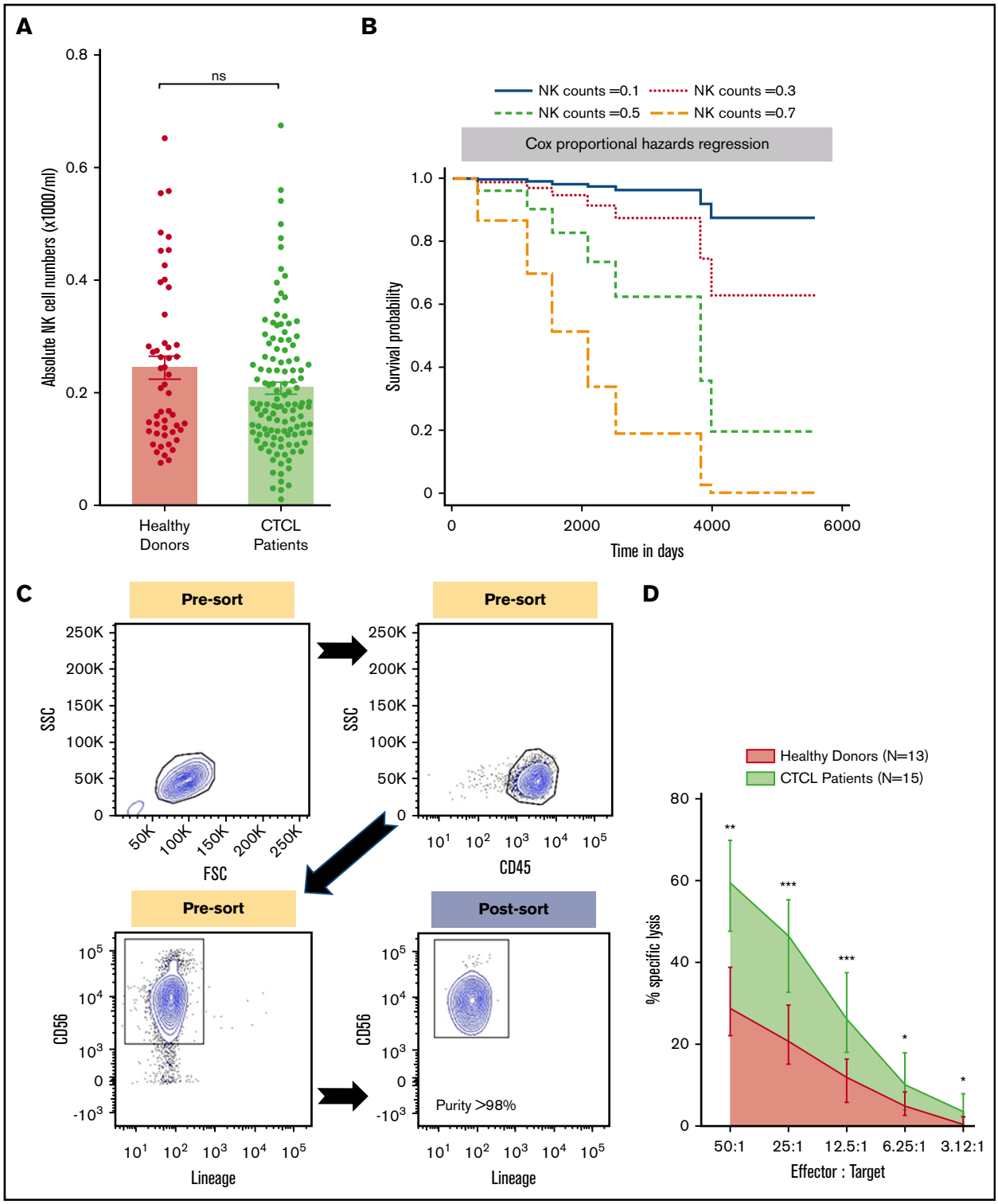

Figure 1. NK-cell number and correlation with CTCL patient survival. (A) Absolute NK-cell numbers were calculated in normal donors (n = 51; mean ± standard error of the mean [SEM], 0.2442 ± 0.02) and CTCL patients (n = 121; 0.208 ± 0.01; $P = .08$). (B) Kaplan-Meier curves for overall survival at possible absolute NK-cell counts in CTCL patients (n = 121). (C) NK cells, CD56$^+$/lineage (CD3/CD14/CD20), were isolated from freshly obtained peripheral blood samples from CTCL patients and normal control donors. (D) Purified NK cells were co-cultured with K562 leukemic targets in a standard chromium release assay at indicated ratios. Data are presented as mean ± SEM. *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$; unpaired 2-tailed Student *t* test. FSC, forward scatter; ns, not significant; SSC, side scatter.

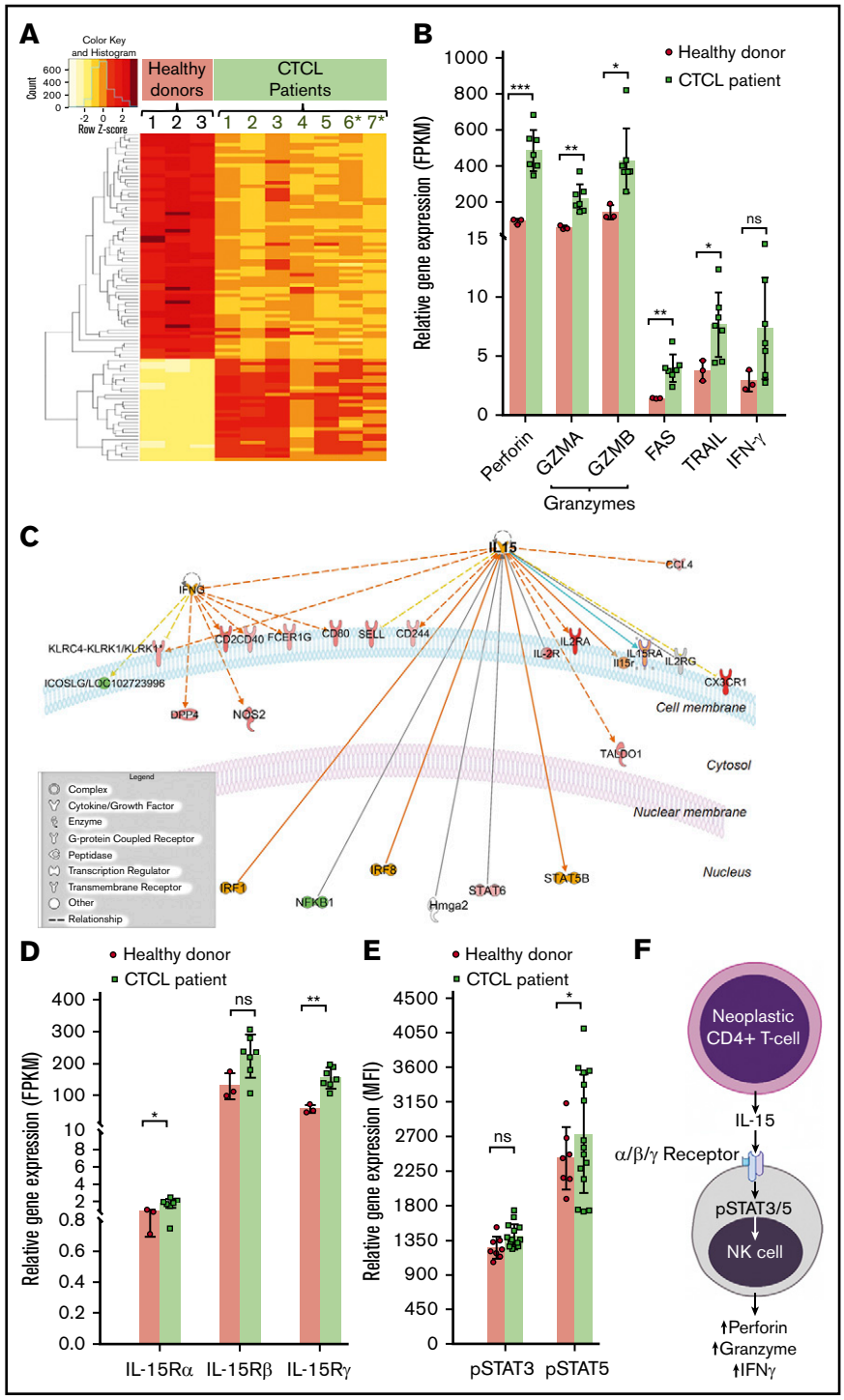

Figure 2. Differential RNA expression analysis in CTCL patients. NK cells were isolated from CTCL patients and normal donors and evaluated by RNA sequencing. (A) Heat map of differentially regulated transcripts among normal donors (n = 3) and CTCL MF (n = 5) and CTCL SS (n = 2) patients. (B) Mediators of cytolytic activity and NK-cell activation were evaluated in all CTCL patients compared with normal controls. Perforin mean ± SEM of transcript in healthy donor vs CTCL patients for NK cells (91.39 ± 9.18 [n = 3] vs 485.7 ± 43.15 [n = 7]; $P = .0004$), granzyme A (GZMA) (58.15 ± 6.605 [n = 3] vs 218.7 ± 29.78 [n = 7]; $P = .0094$), granzyme B (GZMB) (143 ± 23.79 [n = 3] vs 431 ± 68.13 [n = 7]; $P = .03$), Fas (1.413 ± 0.01849 [n = 3] vs 3.967 ± 0.4395 [n = 7]; $P = .0063$), tumor necrosis factor-alpha–related apoptosis-inducing ligand (TRAIL) (3.756 ± 0.4925 [n = 3] vs 7.634 ± 1.033 [n = 7]; $P = .0477$), and IFN-G (2.861 ± 0.5007 [n = 3] vs 7.325 ± 1.621 [n = 7]; $P = .1218$). (C) An ingenuity pathway analysis upstream functional analysis was performed in an enriched data set from the NK cells. Red indicates upregulation in CTCL patients compared with normal donors; green indicates reduced expression. (D) RNA expression of receptor components required for both IL-2 and IL-15 signaling were evaluated in CTCL patients and compared with that in normal donors (IL-15Rα: mean ± SEM of relative RNA in normal vs CTCL, 0.8677 ± 0.1018 [n = 3] vs 1.772 ± 0.2055 [n = 7]; $P = .03$; IL-15Rγ, 57.02 ± 7.525 [n = 3] vs 154.7 ± 12.14 [n = 7]; $P = .001$. IL-15Rβ was also elevated (128 ± 23.68 [n = 3] vs 223.2 ± 25.43 [n = 7]; $P = .056$). (E) Protein expression of pSTAT3 and pSTAT5 on NK cells was determined in freshly obtained whole blood samples from CTCL patients and matched with that of normal donors by flow cytometry. Graph indicates mean fluorescence intensity (MFI) of normal (gray bars [n = 7]) compared with CTCL patients (green bars [n = 14]); pSTAT3: normal, 2417 ± 154.1 (n = 7) vs CTCL, 2734 ± 199; $P = .32$; pSTAT5: normal, 1253 ± 50.23 (n = 8) vs CTCL, 1405 ± 64.35; $P = .028$. (F) 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. Data are presented as mean ± SEM. *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$; unpaired 2-tailed Student *t* test. FPKM, fragments per kilobase million.

also possible that the ability of NK cells to form an effective immune synapse and polarize actin and cytolytic granules is altered in the microenvironment of CTCL patients. In support of this theory, a key mediator of NK-cell polarization, phosphatase and tensin homolog (PTEN), is significantly overexpressed in NK cells from CTCL patients in the RNA sequencing analysis (data not shown). We have previously demonstrated a role for PTEN in the organization of the components of the immunologic synapse and the appropriate convergence of cytolytic granules.[41] It is also possible that the process of NK-cell recognition of malignant CD4$^+$ T cells is altered in CTCL patients. Previous studies by Bouaziz et al[15] suggest that NK cells are potentially able to be activated to kill autologous CTCL cells, which suggests that malignant CD4$^+$ T cells are susceptible to NK-cell killing, but additional mechanisms of inhibition such as the ones discussed above prevent this in CTCL patients. Although further investigation of multiple facets of NK-cell recognition is warranted, it is clear that the overall immunosuppressive microenvironment in CTCL

patients contributes to insufficiency of patient NK cells to effectively control CTCL progression.

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Authorship

Contribution: B.M.-B., L.C., H.C.M., Y.Y., G.L., N.D., and A.M. assisted with experimental design and implementation; E.M. and D.K. assisted with statistical modeling and bioinformatics analysis; P.P., B.W., and S.H. assisted with patient identification and sample acquisition; N.C., D.A.L., and A.M. assisted with ingenuity pathway analysis; B.M.-B., R.K., and A.M. assisted with writing and analyzing experiments; and A.G.F., P.P., and M.A.C. provided critical design and manuscript assistance.

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