Full-length Article

A population of atypical CD56[−]CD16⁺ natural killer cells is expanded in PTSD and is associated with symptom severity

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ABSTRACT

Introduction: Post-traumatic stress disorder (PTSD) has been associated with immune disturbances, including a higher incidence of infections and autoimmune diseases as well as a net pro-inflammatory state. Natural killer (NK) cells, a key component of the innate immune system, have been less well-studied in PTSD despite their importance in immunity.

Methods: We studied two independent samples of combat-exposed male war veterans with or without PTSD, the first ("Discovery Sample") to generate hypotheses, and the second ("Validation Sample") to replicate the findings. The Discovery Sample was comprised of 42 PTSD subjects and 42 controls. The Validation Sample was comprised of 25 PTSD subjects and 30 controls. Participants had fasting, morning blood samples collected for examination of the frequency of NK cell subsets, determined by flow cytometry. The current and lifetime Clinician Administered PTSD Scale (CAPS) was used to assess symptom severity. Statistical analyses were adjusted for age and BMI.

Results: PTSD subjects compared to controls had (i) a significantly higher relative frequency of atypical CD56[−]CD16⁺ NK cells in the Discovery Sample ($p = 0.027$), which was replicated in the Validation Sample ($p = 0.004$) and the combined sample ($p < 0.001$), and (ii) a non-significantly lower relative frequency of CD56^{bright}CD16[−] NK cells in the two samples ($p = 0.082$; $p = 0.118$), which became statistically significant in the combined sample ($p = 0.020$). Further, within subjects with PTSD of both samples, the relative frequency of atypical CD56[−]CD16⁺ NK cells was near significantly positively correlated with lifetime PTSD severity ($p = 0.074$).

Discussion: This study is the first to characterize NK cell subsets in individuals with PTSD. The results suggest that combat-exposed men with PTSD exhibit an aberrant profile of NK cells with significantly higher frequencies of an atypical population of CD56[−]CD16⁺ cells and possibly lower frequencies of the functional CD56^{bright}CD16[−] NK cell subsets. Higher proportions of dysfunctional CD56[−]CD16⁺ cells have been reported in certain chronic viral infections and in senescent individuals. It is possible that this could contribute to immune dysfunctions and prematurely senescent phenotypes seen in PTSD.

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1. Introduction

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Post-traumatic stress disorder (PTSD) is a debilitating mental illness characterized by re-experiencing distressing memories of an initial traumatic event, avoidance, negative cognitions and

mood, and hyperarousal (American Psychiatric Association, 2013). In addition to the traditional psychiatric symptoms, individuals with PTSD have a substantially higher incidence of various disorders including those reflecting immune senescence or dysfunction, such as autoimmune diseases and infections (Levine et al., 2014). Several studies, in fact, show that PTSD individuals have increased innate immune responses and a low-grade systemic pro-inflammatory state with increased circulating pro-inflammatory markers that are positively related to psychopathological severity (Levine et al., 2014; Bauer et al., 2010; Lindqvist et al., 2014).

Natural killer (NK) cells, a key component of the first-line antiviral and anti-tumor defence, have been suggested to play a relevant role in the possible PTSD-associated immune impairments (Gotovac et al., 2010; Kawamura et al., 2001; Laudenslager et al., 1998; Mosnaim et al., 1993; Segerstrom and Miller, 2004). NK cells are an important population of cytotoxic cells linking innate and cellular immunities (Campbell and Hasegawa, 2013; Farag et al., 2002; Vivier et al., 2011). They originate from common lymphoid progenitors along with B and T cells, and mature in lymphoid tissues (spleen, bone marrow, tonsil) to express a diverse array of activating and inhibitory receptors (Campbell and Hasegawa, 2013; Farag et al., 2002; Vivier et al., 2011). NK cells can react very quickly upon stimulation, faster than T cells, as one of their primary functions is to kill tumor and virally infected target cells that lack Major Histocompatibility Complex I (MHC I) expression without any need for previous sensitization, antibody binding, or peptide presentation (Campbell and Hasegawa, 2013; Farag et al., 2002; Vivier et al., 2011).

NK cells are traditionally identified as CD3[−], CD14[−], CD19[−] lymphocytes expressing CD56 (neural cell adhesion molecule) and are typically characterized into two main subsets. Approximately 90% of circulating NK cells are defined as CD56^{dim}CD16⁺ NK cells and are considered mature while approximately 10% are defined as CD56^{bright}CD16[−] NK cells and represent immature NK cells (Caligiuri, 2008; Lanier et al., 1986). CD56^{dim}CD16⁺ NK cells predominantly contribute to innate immunity through direct cytotoxicity, although they also influence innate and adaptive immunity through cytokine production (Campbell and Hasegawa, 2013; Camous et al., 2012). CD56^{bright}CD16[−] NK cells predominantly secrete cytokines and are more resistant to oxidative stress and apoptosis (Campbell and Hasegawa, 2013; Camous et al., 2012). A third subset of NK cells, defined as CD56[−]CD16⁺, was originally described as an expanded NK cell population in persons with Human Immunodeficiency Virus type 1 (HIV-1) and other viral infections (Tarazona et al., 2002; Bjorkstrom et al., 2010; Gonzalez et al., 2009; Hu et al., 1995). Recent data also suggest an expansion of CD56[−]CD16⁺ NK cells during the process of normal senescence (Camous et al., 2012). While a recent study suggests CD56[−]CD16⁺ NK cells may represent activated, mature NK cells that have recently encountered target cells (Milush et al., 2013), CD56[−]CD16⁺ NK cells are generally considered to represent an unusual, highly dysfunctional NK cell subset with poor proliferative and cytotoxic capacity, which secrete lesser amounts of cytokines and higher amounts of chemokines (Camous et al., 2012; Bjorkstrom et al., 2010; Hu et al., 1995). Elevated frequencies of CD56[−]CD16⁺ NK cells are postulated to account for impaired function of the total NK cell population in certain conditions, such as HIV (Mavilio et al., 2005).

In comparing patients with or without PTSD, studies of NK cell cytotoxicity have yielded inconsistent results, with increases (Laudenslager et al., 1998), decreases (Gotovac et al., 2010; Kawamura et al., 2001) and non-significant differences in cytotoxicity reported (Mosnaim et al., 1993). However, no studies so far have explored the relative preponderance of the different NK cells subsets in PTSD individuals compared to controls. Determining differences in NK cell subset distribution could reveal a new aspect of

immune dysregulation that contributes to immune dysfunction and immunosenescence in this disorder.

It is possible that the PTSD-associated immune disturbances (Levine et al., 2014) might be explained by an expansion of the dysfunctional (i.e. CD56[−]CD16⁺), rather than the functional (CD56^{bright}CD16[−] and CD56^{dim}CD16[−]), NK cell subsets. In this study, we assessed the frequencies of CD56^{bright}CD16[−], CD56^{dim}CD16[−] and CD56[−]CD16⁺ NK cells in a sample of combat-exposed male war veterans with PTSD in comparison with combat-exposed male war veterans without PTSD (i.e. controls). We hypothesized that veterans with PTSD would show a more dysfunctional profile of NK cells, with higher frequencies of CD56[−]CD16⁺ cells and lower frequencies of CD56^{bright}CD16[−] and CD56^{dim}CD16[−] cells.

2. Methods

2.1. Ethical statement

The Institutional Review Boards of Icahn School of Medicine at Mount Sinai (ISMMS; New York, NY), the James J. Peters Veterans Administration Medical Center (JJPVAMC; Bronx, New York), New York University Medical Center (NYU; New York, NY), and the University of California, San Francisco, Medical Center (UCSF; San Francisco, CA) approved this study. Study participants gave written and informed consent to participate. The study was conducted in accordance with the provisions of the Helsinki Declaration.

2.2. Recruitment procedures and study participants

This study was developed to test two separate samples of male war veterans in order to limit Type I statistical errors. The first, the “Discovery Sample,” was used to generate hypotheses regarding NK cell characteristics in PTSD. The second, the “Validation Sample,” was used to attempt to replicate and confirm the initial findings. Participating subjects were all Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) male combat-exposed veterans. The Discovery Sample included 42 subjects with current diagnosis of PTSD and 42 non-PTSD controls; 23 subjects in the PTSD group and one subject in the control group were diagnosed with concurrent MDD. The Validation Sample included 25 PTSD subjects and 30 controls; among the PTSD subjects, 16 were diagnosed with concurrent MDD. Participants were recruited by NYU, ISMMS and JJPVAMC. Subjects were recruited from the Mental Health Services of the Manhattan, Bronx and Brooklyn Veterans Affairs Medical Centers, other regional VA medical centers, Veterans Service Organizations, National Guard, reservist agencies and organizations and from the general community. Recruitment methods included flyers, in-person presentations, media advertisements, internet postings (e.g. Craigslist) and referral from clinicians. All subjects had blood drawn and processed at ISMMS or Bronx and Brooklyn Veterans Affairs Medical Centers. Blood was obtained after subjects had rested in the lab, and blood samples were shipped via overnight express mail to the flow cytometry lab at UCSF, where they were assayed immediately upon arrival. Participants were compensated for their participation. Criteria for inclusion were: (a) PTSD subjects were positive for the presence of current combat-related PTSD of at least 3 months duration, as defined by the DSM-IV (First, 1997), and the Clinician Administered PTSD Scale (CAPS) Blake et al., 1990 criteria with a current CAPS score >40; (b) control subjects were also combat-exposed but were negative for lifetime PTSD and had a current CAPS score <20; (c) age between 20 and 60; (d) males; and (e) proficient in the English language. The following exclusion criteria were employed for all subjects: (a) history of alcohol dependence within

the past 8 months; (b) history of drug abuse or dependence (except nicotine dependence) within the past year; (c) lifetime history of any psychiatric disorder with psychotic features, bipolar disorder, or obsessive-compulsive disorder; (d) those who were currently exposed to recurrent trauma or have been exposed to a traumatic event within the past 3 months; (e) subjects with prominent suicidal or homicidal ideation; (f) neurologic disorder or systemic illness affecting central nervous system function; (g) history of hepatitis; (h) history of anemia, recent blood donation in the past 2 months; (i) subjects on medication who were not stable for 2 + months on psychiatric medication, anticonvulsants, antihypertensive medication or sympathomimetic medication; (j) subjects who were classified with a moderate or severe traumatic brain injury (TBI) on the Ohio State University TBI Identification Method—Short Form; and finally (k) subjects who experienced loss of consciousness for >10 min. All study participants experienced combat traumas described in criterion A of DSM-IV PTSD diagnostic criteria. Structured Clinical Interviews for DSM-IV disorders (SCID) First, 1997 were conducted by doctoral level psychologists, and were audio recorded and calibrated weekly with a senior clinician in the PTSD program.

2.3. Psychiatric and psychological assessment measures

The SCID was used to determine DSM-IV Axis I diagnostic criteria (First, 1997). The CAPS was used to determine the severity of current PTSD symptoms (past month; “CAPS current”) and the severity of the most severe lifetime episode of combat-related PTSD (“CAPS lifetime”) Blake et al., 1990.

2.4. Laboratory methods

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using BD Vacutainer® CPT™ Cell Preparation Tubes. CPT tubes were centrifuged for at 25 °C for 30 min at 1800×g without a brake. PBMC were removed, washed once with phosphate buffered saline (PBS) then treated with ACK (Ammonium-Chloride-Potassium) Lysing Buffer (Lonza Walkersville, Inc) to remove red blood cell contamination. One million PBMC were then stained with the following fluorophore-conjugated antibodies: Phycoerythrin (PE)-Cy5.5-conjugated anti-CD19 (SJ25-C1), PE-Cy7-conjugated anti-CD4 (S3.5), Qdot® 605-conjugated anti-CD8 (3B5), Allophycocyanin (APC)-conjugated anti-CD16 (3G8) and Alexa Fluor®700-conjugated anti-CD45 (HI30) (ThermoFisher Scientific), V450-conjugated anti-CD3 (UCHT1), PE-Cy5-conjugated anti-CD28 (CD28.2) and FITC-conjugated anti-CD56 (NCAM16.2) (BD Biosciences). LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (ThermoFisher Scientific) was added into all stains to exclude non-viable cells. Staining was performed on ice for 30 min, then washed once with FACS buffer (Phosphate-buffered saline containing 0.5% bovine serum albumin and 1 mM Ethylenediaminetetraacetic Acid). Cells were then fixed in 0.5% formaldehyde and data was acquired on a BD LSR II Flow cytometer (BD Biosciences), with ≥200,000 lymphocytes collected for each sample. CS&T beads (BD Bioscience) were used for instrument set up for each run and Rainbow beads (Sphero) standardized instrument settings between runs. FMO controls were also prepared on each sample to check that gates were set consistently between runs. Data was compensated and analyzed in FlowJo V9.8.1 (TreeStar). NK cell subsets were defined after standard lymphocyte, singlet, dead cell exclusion, and CD3 and CD19 lineage negative gates were applied to the data.

2.5. Statistical analysis

The Statistical Package for the Social Sciences (SPSS) was used for statistical calculations. All tests were 2-tailed with an

alpha = 0.05. Significance values between 0.05 and 0.10 are reported as trends. Data are expressed as means ± SD. The frequencies of all cell subtypes and the values of CAPS lifetime subscale were not normally distributed, therefore they were transformed using the Ln transformation. The Mann-Whitney U-test for continuous variables and the chi-square test for dichotomous variables were used to examine participants' baseline between-group differences in each sample.

One-way analysis of covariance (ANCOVA) adjusting for age and body mass index (BMI), due to their known influence on NK cells subsets (Camous et al., 2012; O'Shea et al., 2010), was used to test for inter-group differences in the relative frequencies of NK cell subsets in each sample. In addition to the planned comparisons within the Discovery and Validation samples, we performed a *post-hoc* comparison of subjects with and without PTSD across both samples combined to achieve greater statistical power.

Pearson partial correlation using the same covariates (i.e. age and BMI) was used to determine associations between NK cells subpopulations and the CAPS within PTSD subjects. Further, ANCOVAs were performed to determine whether PTSD subjects with or without comorbid MDD, with or without current antidepressant use, and with or without smoking habit had significantly different NK cells subpopulations.

As written above, our *a priori* analysis plan was to utilize two groups of subjects: a hypothesis-generating “Discovery” sample, and a hypothesis-testing “Validation” sample (based on significance determinations from the Discovery sample). Accordingly, analyses of other available cell types (i.e. CD4 and CD8 T lymphocytes) that did not meet significance criteria in the Discovery sample were performed only in an exploratory manner in the Validation and combined samples.

3. Results

Demographic and clinical characteristics of the “Discovery” and “Validation” samples of subjects are presented in Table 1. Groups were balanced in all sociodemographic and clinical characteristics except for smoking status, time since trauma, years of education, and antidepressants use. As expected, subjects with PTSD compared to controls had significantly higher scores on CAPS current and CAPS lifetime symptom severity scales.

NK cell relative frequencies in the two samples are summarized in Table 2 and Fig. 1. In the Discovery Sample, the mean frequency of CD56⁺CD16⁺ cells in the PTSD group was 10.76 ± 8.24 compared to 8.00 ± 6.15 in the control group ($F(1,81) = 5.080$; $p = 0.027$). In the same sample, the mean relative frequency of CD56^{bright}CD16⁺ cells in the PTSD group was 6.32 ± 3.18 compared to 8.78 ± 6.76 in the control group ($F(1,81) = 3.111$; $p = 0.082$). Also, the mean relative frequency of CD56^{dim}CD16⁺ cells in the PTSD group was 82.75 ± 8.69 compared to 82.96 ± 8.36 in the control group ($F(1,81) = 0.150$; $p = 0.699$).

In the Validation Sample, the mean relative frequency of CD56⁺CD16⁺ cells in the PTSD group was 14.44 ± 8.56 compared to 9.00 ± 9.47 in the control group ($F(1,51) = 9.166$; $p = 0.004$). In the same sample, the mean relative frequency of CD56^{bright}CD16⁺ cells in the PTSD group was 7.00 ± 3.29 compared to 8.67 ± 4.10 in the control group ($F(1,51) = 2.532$; $p = 0.118$). Also, the mean relative frequency of CD56^{dim}CD16⁺ cells in the PTSD group was 78.37 ± 8.19 compared to 82.12 ± 10.23 in the control group ($F(1,51) = 1.413$; $p = 0.240$).

In the two samples combined, the mean relative frequency of CD56⁺CD16⁺ cells in the PTSD group was 12.14 ± 8.49 compared to 8.42 ± 7.66 in the control group ($F(1,135) = 12.838$; $p < 0.001$). In the same sample, the mean relative frequency of CD56^{bright}CD16⁺ cells in the PTSD group was 6.57 ± 3.22 compared to

Table 1
Demographic and clinical characteristics of PTSD subjects and controls in the two cohorts.

	Discovery Sample				Validation Sample			
	PTSD N: 42	Controls N: 42	Mann-Whitney U-test	χ^2	PTSD N: 25	Controls N: 30	Mann-Whitney U-test	χ^2
Age (years, mean $\pm$ SD)	33.07 $\pm$ 7.88	32.93 $\pm$ 8.44	0.60		31.04 $\pm$ 5.87	30.63 $\pm$ 5.75	0.63	
Years of education (mean $\pm$ SD)	13.69 $\pm$ 1.74	15.14 $\pm$ 2.12	<0.01 [*]		13.54 $\pm$ 2.20	14.93 $\pm$ 2.32	0.01 [*]	
Gender	All males	All males			All males	All males		
Smokers (n) ¹	11	3		0.01 [*]	8	5		0.06
BMI (mean $\pm$ SD)	29.87 $\pm$ 5.24	27.85 $\pm$ 3.29	0.11		29.44 $\pm$ 4.79	28.38 $\pm$ 5.44	0.36	
Hispanic/Non-Hispanic (n)	22/21	16/26		0.23	5/12	3/21		0.18
Time since trauma (months, mean $\pm$ SD)	77.33 $\pm$ 25.17	51.42 $\pm$ 24.96	<0.01 [*]		NA	NA		
Taking statins (n)	2	0		0.16	2	0		0.09
Taking antidepressants (n)	12	2		<0.01 [*]	5	2		0.11
Taking NSAIDs (n)	2	4		0.38	1	0		0.24
Taking antidiabetic drugs (n)	1	0		0.32	1	1		0.83
Taking antibiotics (n)	1	1		0.98	0	0		
Clinical hypertension (n)	7	3		0.10	3	6		0.25
Stable angina (n)	1	0		0.36	0	0		
Diabetes (n)	3	0		0.13	1	1		0.87
Inflammatory conditions (n)	1	3		0.31	0	0		
CAPS total current (mean $\pm$ SD)	66.62 $\pm$ 15.31	2.64 $\pm$ 4.21	<0.01 [*]		71.92 $\pm$ 17.97	5.07 $\pm$ 6.24	<0.01 [*]	
CAPS total lifetime (mean $\pm$ SD)	89.29 $\pm$ 12.62	7.69 $\pm$ 7.01	<0.01 [*]		93.76 $\pm$ 16.92	10.13 $\pm$ 9.51	<0.01 [*]	
MDD diagnosis (n)	23	0		<0.01 [*]	16	1		<0.01 [*]

Abbreviations: BMI = Body Mass Index; NSAIDs = Non-steroidal anti-inflammatory drugs; CAPS = Clinician Administered PTSD Scale; MDD = Major Depressive Disorder.
^{*} p < 0.05.

¹ Data available for 70 subjects of the Discovery Sample and 48 subjects of the Validation Sample.

Table 2
Inter-group comparisons (PTSD vs controls) of frequency of NK cells subtypes in the different cohorts.

	Discovery Sample				Validation Sample				Combined Samples			
	Mean $\pm$ SD				Mean $\pm$ SD				Mean $\pm$ SD			
	PTSD N: 42	Controls N: 42	ANCOVA ^b		PTSD N: 25	Controls N: 30	ANCOVA ^b		PTSD N: 67	Controls N: 72	ANCOVA ^b	
			F	P			F	P			F	P
CD56 ⁺ CD16 ⁺ ^a	10.76 $\pm$ 8.24	8.00 $\pm$ 6.15	5.080	0.027 [*]	14.44 $\pm$ 8.56	9.00 $\pm$ 9.47	9.166	0.004 [*]	12.14 $\pm$ 8.49	8.42 $\pm$ 7.66	12.838	<0.001 [*]
CD56 ^{bright} CD16 ⁺ ^a	6.32 $\pm$ 3.18	8.78 $\pm$ 6.76	3.111	0.082	7.00 $\pm$ 3.29	8.67 $\pm$ 4.10	2.532	0.118	6.57 $\pm$ 3.22	8.74 $\pm$ 5.76	5.525	0.020 [*]
CD56 ^{dim} CD16 ⁺ ^a	82.75 $\pm$ 8.69	82.96 $\pm$ 8.36	0.150	0.699	78.37 $\pm$ 8.19	82.12 $\pm$ 10.23	1.413	0.240	81.12 $\pm$ 8.71	82.61 $\pm$ 9.13	0.963	0.328

^a Values represent the percentage number of NK cells (mean $\pm$ SD).

^b Using Ln-transformed values, covarying age and BMI.

^{*} p < 0.05.

Fig. 1. Frequency of CD56⁺CD16⁺ NK cells in PTSD subjects and in controls.

8.74 ± 5.76 in the control group ($F(1,135) = 5.525$; $p = 0.020$). Also, the mean relative frequency of $CD56^{\text{dim}}CD16^-$ cells in the PTSD group was 81.12 ± 8.71 compared to 82.61 ± 9.13 in the control group ($F(1,135) = 0.963$; $p = 0.328$).

Across PTSD subjects of both samples combined, CAPS lifetime PTSD severity was near significantly correlated with the relative frequencies of $CD56^-CD16^+$ cells ($r = 0.223$; $p = 0.074$) (Fig. 2) and $CD56^{\text{dim}}CD16^-$ cells ($r = -0.210$; $p = 0.093$), and not significantly correlated with the relative frequency of $CD56^{\text{bright}}CD16^-$ ($r = -0.202$; $p = 0.107$). On the other hand, CAPS current PTSD severity was not significantly associated with the relative frequency of any cell subsets ($CD56^-CD16^+$: $r = 0.157$; $p = 0.213$; $CD56^{\text{bright}}CD16^-$: $r = -0.008$, $p = 0.947$; $CD56^{\text{dim}}CD16^-$: $r = -0.151$, $p = 0.228$).

Across PTSD subjects of both samples combined, the relative frequencies of $CD56^{\text{bright}}CD16^-$, $CD56^{\text{dim}}CD16^-$ and $CD56^-CD16^+$ cells did not significantly differ between subjects with or without comorbid MDD ($p > 0.163$), between subjects who did or did not use antidepressants ($p > 0.445$), between subjects with Hispanic and non Hispanic ethnicities ($p > 0.418$), or between subjects who did or did not smoke ($p > 0.418$). Excluding the four subjects of the Discovery Sample with asthma, allergies or osteoarthritis and the control subject with MDD of the Validation Sample from the analyses did not significantly alter the statistical results.

The frequencies of subtypes of CD4 T lymphocytes (i.e. $CD4^+CD28^-$ and $CD4^+CD28^+$) and CD8 T lymphocytes (i.e. $CD8^+CD28^-$

and $CD8^+CD28^+$), which were available only for 65 PTSD subjects and 71 controls, did not significantly differ between groups in the Validation, Discovery or the combined samples. Details are given in Table 3.

4. Discussion

This is the first study assessing the relative frequencies of three NK cell subsets among veterans with PTSD, while additionally demonstrating correlations with PTSD severity. Our results showed that combat-exposed male veterans with PTSD, compared to combat-exposed male controls without PTSD exhibited: (i) a significantly higher frequency of unusual, dysfunctional $CD56^-CD16^+$ NK cells in the Discovery Sample, which was independently replicated in the Validation Sample; this difference was highly significant in the combined sample. (ii) A non-significantly lower frequency of $CD56^{\text{bright}}CD16^-$ NK cells in the subjects with PTSD in both samples that became statistically significant in the combined sample. (iii) A non significantly lower frequency of $CD56^{\text{dim}}CD16^+$. Further, within subjects with PTSD, lifetime (but not current) PTSD severity measures were near significantly associated with $CD56^-CD16^+$ NK cells (positively) and with $CD56^{\text{dim}}CD16^-$ NK cells (negatively). Among PTSD subjects, the frequencies of NK cell subsets did not differ between individuals with or without comorbid MDD, with or without current antidepressant use, with or without Hispanic ethnicity and with or without smoking habit; therefore, the observed between-group differences are likely not attributable to comorbid MDD diagnosis or antidepressant use or ethnicity or smoking status. Taken together, these results are consistent with the study hypothesis that PTSD individuals exhibit an aberrant profile of NK cells with significantly higher frequencies of an atypical population of $CD56^-CD16^+$ cells and possibly lower frequencies of the functional $CD56^{\text{bright}}CD16^-$ NK cell subsets.

Several previous studies have observed impaired immune responses (Levine et al., 2014; Bauer et al., 2010), NK cell cytotoxicity (Gotovac et al., 2010; Kawamura et al., 2001; Laudenslager et al., 1998; Mosnaim et al., 1993; Segerstrom and Miller, 2004) and pro-inflammatory imbalances (Levine et al., 2014; Bauer et al., 2010; Lindqvist et al., 2014) in PTSD. Our study differs from, and adds to, these previous findings by exploring, for the first time in any psychiatric illnesses, an additional marker of immune function, i.e. the relative frequencies of NK cell subsets.

$CD56^-CD16^+$ NK cells in healthy individuals are rare and represent at most a few percent of total NK cells in the blood (Bjorkstrom et al., 2010), although expansion of these cells has been described in senescent individuals and in patients with certain chronic viral infections. $CD56^-CD16^+$ NK cells, in fact, may represent up to 40% of all NK cells present in peripheral blood of

Fig. 2. Scatterplot of the correlation between the frequency of $CD56^-CD16^+$ NK cells and CAPS total lifetime within PTSD subjects after covarying age and BMI (using Ln-transformed data; $r = 0.223$; $p = 0.074$).

Table 3

Inter-group comparisons (PTSD vs controls) of frequency of T lymphocytes subtypes in the different cohorts.

	Discovery Sample				Validation Sample				Combined Samples			
	Mean $\pm$ SD				Mean $\pm$ SD				Mean $\pm$ SD			
	PTSD N: 41	Controls N: 41	ANCOVA ^d		PTSD N: 24	Controls N: 30	ANCOVA ^d		PTSD N: 65	Controls N: 71	ANCOVA ^d	
			F	P			F	P			F	P
$CD4^+{}^a$	43.68 $\pm$ 9.12	41.26 $\pm$ 9.19	2.253	0.137	43.34 $\pm$ 9.03	43.95 $\pm$ 6.33	0.237	0.629	43.55 $\pm$ 9.02	42.38 $\pm$ 8.18	0.790	0.376
$CD8^+{}^a$	21.64 $\pm$ 24.44	24.44 $\pm$ 7.15	3.492	0.065	23.99 $\pm$ 8.96	24.63 $\pm$ 6.72	0.240	0.626	22.51 $\pm$ 7.85	24.52 $\pm$ 6.93	3.108	0.080
$CD4^+CD28^-{}^b$	1.61 $\pm$ 2.64	1.22 $\pm$ 2.45	0.559	0.457	1.89 $\pm$ 4.47	1.23 $\pm$ 2.27	0.904	0.346	1.72 $\pm$ 3.40	1.23 $\pm$ 2.36	0.055	0.815
$CD4^+CD28^+{}^b$	98.36 $\pm$ 2.64	98.77 $\pm$ 2.48	0.359	0.551	97.70 $\pm$ 4.83	98.76 $\pm$ 2.27	0.875	0.354	98.12 $\pm$ 3.59	98.77 $\pm$ 2.35	1.092	0.298
$CD8^+CD28^-{}^c$	22.73 $\pm$ 14.15	26.15 $\pm$ 15.49	2.041	0.157	29.27 $\pm$ 19.19	20.99 $\pm$ 12.69	1.395	0.243	1.72 $\pm$ 3.40	1.23 $\pm$ 2.36	0.138	0.711
$CD8^+CD28^+{}^c$	76.01 $\pm$ 14.09	73.51 $\pm$ 15.60	1.031	0.313	70.65 $\pm$ 19.26	78.78 $\pm$ 12.85	4.483	0.068	74.03 $\pm$ 16.26	75.74 $\pm$ 14.64	0.226	0.635

^a Values represent the percentage number of lymphocytes (mean $\pm$ SD).

^b Values represent the percentage number of $CD4^+$ lymphocytes (mean $\pm$ SD).

^c Values represent the percentage number of $CD8^+$ lymphocytes (mean $\pm$ SD).

^d Using Ln-transformed values, covarying age and BMI.

patients chronically infected with HIV-1 or Hepatitis C virus (Bjorkstrom et al., 2010). The relatively higher number of CD56⁺CD16⁺ cells observed in PTSD subjects, together with the suggestions of possibly lower CD56^{bright}CD16⁺ and CD56^{dim}CD16⁺ cells, support previous evidence that PTSD may be associated with an impaired innate immune system and help explain the higher incidence of immune disturbances associated with PTSD.

The activity of NK cells is extremely vulnerable to various stressors and psychological changes (Segerstrom and Miller, 2004); therefore, it is not surprising that a psychological trauma, as well as its frequency and timing, might be associated with the distribution of NK cell subsets. A meta-analysis of >300 studies on the impact of psychological stress on the human immune system showed that impaired cytotoxic activity of NK cells is associated with the state of chronic stress (Segerstrom and Miller, 2004). The major pathways linking trauma-associated psychological stress to the immune function likely include hyperactivity of the sympathetic nervous system, alteration of hypothalamic–pituitary–adrenal axis functioning (generally with hypercatecholaminergic and hypo-cortisolemic states in PTSD) and adoption of certain stress-related behaviors (Segerstrom and Miller, 2004; McEwen, 2000; Sherin and Nemeroff, 2011). However, the exact mechanisms linking psychological stress, and trauma specifically, to the redistribution of NK cell patterns are not known and remain to be elucidated. The observed near significant positive correlation between CD56⁺CD16⁺ NK cells and lifetime (but not current) PTSD severity measures raises the possibility that alterations in circulating NK cell subsets may more likely reflect chronic, rather than acute, alterations in trauma-related symptoms and underlying neurobiological changes; further, it is possible that CAPS lifetime may represent the overall sensitivity to experiencing PTSD symptoms severity rather than just the experience of PTSD in the past.

There are various potential explanations for the higher frequency of CD56⁺CD16⁺ NK cells in persons with PTSD. Even though causality may not be inferred from our cross-sectional data, one possible explanation is that PTSD results in accelerated or premature biological senescence (early or accelerated aging) (Lohr et al., 2015; Lindqvist et al., 2015). Indeed, studies on senescence biomarkers (including leukocyte telomere length, nitric oxide biosynthesis, mitochondrial DNA copy number, and pro-inflammatory markers), as well as studies on the associations between PTSD and age-associated medical illnesses, suggest a model of early biological senescence in PTSD (Lohr et al., 2015; Lindqvist et al., 2015; Bersani et al., 2015, 2016). Of relevance to the present findings, in the elderly there is an increase in number and a redistribution of NK cell subsets, with a decrease in the CD56^{bright}CD16⁺ NK cell and an expansion of CD56⁺CD16⁺ cells (Camous et al., 2012). Therefore, it is possible that the pattern of NK cell distribution observed in this study is an additional evidence of a PTSD-associated biologically senescent phenotype. Alternatively, a recent study indicated that CD56⁺CD16⁺ NK cells represent a mixed population of NK cells and myeloid cells (Milush et al., 2013). Interestingly, the myeloid cell population that best fits the phenotype of CD56⁺CD16⁺ cells is a pro-inflammatory dendritic cell subset referred to as slanDCs. SlanDCs and NK cells have been shown to co-purify together using commercially available NK cell enrichment kits (Costantini et al., 2009) and play an important role in activating NK cells through direct cell-cell contact and the production of cytokines (Wehner et al., 2009).

4.1. Strengths and limits

Limitations of the present study include our use of an all male study sample; future studies, including an ongoing one by our group, will be needed to investigate similar biological parameters

in combat-exposed females with and without PTSD. Another limitation of the study is that, although none of the participants had a diagnosis of acquired immune deficiency syndrome (AIDS), we did not specifically explore the presence of unknown HIV infections, which are known to potentially contribute to the expansion of CD56⁺CD16⁺ NK cell subset; future studies should take this variable into consideration. Since this was a cross-sectional study based on single time-point blood and behavioral measurements, we cannot determine moment-to-moment variability in the measures, and we cannot assess temporal causality. A recent history of alcohol dependence or drug abuse was an exclusion criteria for all subjects; although this strategy may be regarded as a strength of the study, since it seeks to avoid the potentially confounding effects of substance abuse on NK cell populations, it may also be a limitation due to the potentially decreased generalizability to clinical PTSD populations in which substance abuse is common. In the present paper we based the conclusion that the CD56⁺CD16⁺ cells might be dysfunctional on published studies assessing cell phenotype and function under chronic viral infection (Camous et al., 2012; Bjorkstrom et al., 2010; Hu et al., 1995); however, we did not specifically test whether these cells are dysfunctional in our sample. Future studies are needed to replicate our finding of an expansion of this atypical population and to assess its function in PTSD. While a strength of our study is our use of a combat-exposed non-PTSD group, since the non-specific effects of combat exposure are controlled for, we recognize that individuals who did not develop PTSD despite combat trauma exposure may represent especially resilient individuals, relative to normative control subjects; further, it is also true that trauma exposure may have impacted the frequency of NK subsets also in the control group. A major strength of this study is our use of a Discovery Sample and a Validation Sample, which showed replicability of our major finding across two independent samples. Other strengths of the present study are that the sample was clinically well-characterized, information on potential covariates was available (medical illness, BMI, comorbid depression, medication, cigarette usage, time since index trauma event, etc.), all blood samples were drawn fasting and at the same time of day, and all assays were conducted in the same lab. Finally, an additional strength of the present study is its sample of relatively young war veterans, since age-related illnesses can pose significant confounds in studies of psychiatric disorders and in studies of NK cells populations (Camous et al., 2012).

4.2. Conclusion

The present study provides the first evidence that the frequency of CD56⁺CD16⁺ cells is higher in combat-exposed men with PTSD. These findings contribute to shed some light on novel aspects of PTSD pathophysiology that could possibly result in the development of novel treatment strategies focused on the immune system. The results of the study add to the accumulating evidence that PTSD might be associated with alterations of the immune system, and contribute to an expanded comprehensive view of PTSD as being – in addition to a mental illness – an illness with important somatic underpinnings.

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