Differential Mobilization of Functionally Distinct Natural Killer Subsets During Acute Psychologic Stress

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Objective and Methods: Two functionally distinct natural killer (NK) subsets can be identified according to surface CD56 expression: $CD56^{lo}$ cells compose the majority of NK cells and function as cytotoxic cells, whereas $CD56^{hi}$ cells have an immunomodulatory function through the secretion of cytokines. These NK subsets also differ in the expression levels of adhesion molecules such as CD62L and CD11a, indicating distinct potentials to migrate to lymphoid and nonlymphoid tissues. We investigated whether NK cell mobilization during acute stress varies according to these functional and phenotypic distinctions. **Methods and Results:** Fifty-three undergraduate students performed a public-speaking task and 21 students participated in a control session. The task increased heart rate and catecholamines. No change was observed for the immunoregulatory $CD56^{hi}$ NK subset, whereas the number of cytotoxic $CD56^{lo}$ NK cells tripled. In line with the observation that NK mobilization is related to cytotoxic function, we found larger increases in NK cells that express higher levels of CD16 (a receptor that mediates antibody-dependent cytotoxicity). Consistent with known subset differences in adhesion molecule expression, we also found larger stress-induced increases for NK cells that were CD62L-negative and $CD11a^{hi}$. Plasma levels of soluble CD62L remained unaltered, suggesting that the increase in CD62L-negative NK cells did not result from CD62L shedding. Regression analyses demonstrated independent contributions of epinephrine and norepinephrine to NK subset mobilization. **Conclusion:** The marked specificity and robustness of these effects support the idea that NK cell mobilization is a functionally relevant response that is aimed at protecting the organism during acutely stressful situations. **Key words:** immunosurveillance, innate immunity, social stress, laboratory stress, L-selectin.

ANOVA = analysis of variance; **CD** = cluster of differentiation; **ECG** = electrocardiogram; **ELISA** = enzyme-linked immunosorbent assay; **Hb** = hemoglobin; **HPLC** = high-pressure liquid chromatography; **Htc** = hematocrit; **NK** = natural killer; **POMS** = Profile of Mood States; **sCD62L** = soluble CD62L; **SEM** = standard error of mean.

INTRODUCTION

There is now good evidence linking psychologic stress with susceptibility to inflammatory and infectious conditions (1–3), and much research is dedicated to specifying the exact pathways responsible for this link. In this quest, the study of the interactions between psychosocial factors and immune system processes has gained particular prominence. As shown in a recent metaanalysis, one of the most replicated and robust findings in human psychoneuroimmunology is the rapid increase in circulating natural killer (NK) cells during acute psychologic stressors (4). This response reflects a release of NK cells from various reservoirs such as the margins of the blood vessels into the blood (5). The essential role of the sympathetic nervous system in mediating this effect has been amply demonstrated, and the magnitude of NK cell mobilization correlates with other indicators of sympathetic nervous system activation (e.g., decreases in cardiac preejection period, increases in heart rate and blood pressure, and the release of catecholamines) (6–10). Hence, NK cell mobilization is considered to be an integrated component of the "fight–flight" response.

The longstanding interest in the effects of psychologic and neuroendocrine factors on NK cells is understandable. NK cells are critical for protection against invading pathogens and may also confer protection against malignant transformations (11,12). These lymphocytes (which are considered part of the innate immune system) are fast-acting frontline defenders that have the capacity to swiftly migrate in and out of tissues, and are among the first immune cells to arrive at sites of tissue damage and infection (13,14). There they have the ability to immediately destroy their targets (e.g., infected cells) and to attract and activate other immune cells. These functions are established through a variety of mechanisms such as the release of cytokines and the destruction of target cells, for example by inducing apoptosis or by antibody-dependent cell-mediated cytotoxicity (11,12,15). Antibody-dependent cell-mediated cytotoxicity denotes a process by which NK cells bind and destroy target cells that are coated with antibody and is mediated by the surface immunoglobulin receptor CD16 (12). Another important function of NK cells, which has only been discovered in recent years, is the modulation of adaptive immune responses (i.e., immune responses that depend on prior exposure to an antigen). This regulatory function is related to the aforementioned ability of NK cells to secrete large amounts of cytokines such as interferon-γ (16,17).

Considering the many protective functions of NK cells and the fact that the mobilization of these cells represents one of the most reliable stress responses in humans, it is surprising that only a few attempts have been made to further characterize this phenomenon (see reference 18). NK cells, which are identified by the expression of the cell-surface marker CD56 and the lack of CD3 (15), can be separated into two functionally distinct subsets according to the cell-surface density of CD56. The majority of NK cells express relatively low levels of CD56 ($CD56^{lo}$) and function as cytotoxic cells. The remaining subset expresses high levels of CD56 ($CD56^{hi}$) and has an immunomodulatory function through the secretion of cytokines (15,17).

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These functionally distinct subsets also differ in the expression of adhesion molecules such as CD62L (higher levels on the immunoregulatory $CD56^{hi}$ NK cells) and CD11a (higher levels on the cytotoxic $CD56^{lo}$ NK cells) (19–21). These surface markers are indicative of the potential of NK cells (and other lymphocytes) to migrate to particular sites in the body. For example, CD62L (also denoted as L-selectin) supports the migration of lymphocytes into the secondary lymphoid tissues. This is because the ligands of CD62L (i.e., the molecules to which CD62L binds) are heavily expressed on the specialized vascular endothelial tissues (high endothelial venules) that form a passage from the blood to these secondary lymphoid tissues. Thus, as one would predict, the immunoregulatory $CD56^{hi}$ NK cells, expressing high levels of CD62L, readily bind to these high endothelial venules and are the major NK subtype found in the peripheral lymph nodes (16,21). The ligands for CD11a, on the other hand, are typically upregulated on endothelial cells in the proximity of inflamed tissues (22), and therefore the cell-surface density of CD11a reflects a potential to migrate into these tissues (a feature that seems particularly appropriate for the tissue migration of cytotoxic $CD56^{lo}$ NK cells) (20,21). Cytotoxic $CD56^{lo}$ NK cells also express high levels of CD16, which enables these cells to seek out targets that are antibody-labeled for destruction (15).

Immune cells continuously traffic through the body, going from the blood, through various organs and back into the blood. This migration is essential for maintaining an effective immune defense network (22). Characterizing how stress affects this continuous redeployment may thus clarify how stressors influence the efficiency of the immune system to protect its host. It has been shown that laboratory stressors (as well as exercise and catecholamine infusions) cause a predominant increase of CD62L-negative NK cells in the blood (18,23). One possible interpretation is that this reflects a rapid catecholamine-mediated decrease in CD62L expression on circulating NK cells. However, another possibility is that NK subsets, which vary in the expression of CD62L and other markers, are heterogeneous in their mobilization response to catecholamines. The present study investigated this possible heterogeneity in relation to the functional and migratory potential of these immune cells. In addition, we used correlation analyses to assess the independent and combined contributions of epinephrine and norepinephrine to this mobilization response.

METHODS

Participants

Seventy-five university undergraduates (mean age 20 years, range 18–31 years, 37 women) volunteered to participate in the present study as part of a longitudinal study on psychosocial factors and wound healing. Participants were ineligible if they were using prescribed medication (except for oral contraceptives and topicals) or reported health problems indicative of cardiovascular, inflammatory, or infectious disease. Twenty-one participants (10 women) were assigned to a no-stress control condition in which they read magazines. Subjects were assigned to the control condition if they had not participated in all segments of the larger study. Control and experimental subjects received the same advance information about the experiment. Participants received monetary compensation for their participation.

Procedures

In preparation for the study, participants were instructed not to engage in strenuous physical exercise and to refrain from using alcohol or nonprescription drugs 24 hours before the experimental sessions. In addition, participants were instructed to abstain from smoking and caffeine the day of the experiment, to eat breakfast before 10:00 AM, and to eat or drink nothing after that point (except for water). Women were scheduled in the 7 days after their menses. On arrival at 12:00 PM: 1) informed consent was obtained; 2) a 19-gauge indwelling catheter was placed into the antecubital vein of the nondominant arm; 3) participants were served a standardized lunch (350 kcal, containing 9 g of protein, 15 g of carbohydrate, and 16 g of fat) and water ad libitum; and 4) electrodes for electrocardiography were attached. Subsequently, while seated in a supine position, participants filled out several questionnaires and engaged in leisure reading. At 1:30 PM, a baseline blood sample was obtained and the procedure for the laboratory stressor was initiated.

The Public-Speaking Task

The stress task consisted of two back-to-back speeches, each with 2 minutes of preparation and 4 minutes of speech delivery, as described by Bosch et al. (24). To enhance social stress, the speeches were attended by a small audience (consisting of a female nurse, a female research assistant, and a male psychologist) and videotaped (allegedly to have the quality of the performance rated). To standardize timing and instructions for the task, the instructions were presented on a video screen. A blood sample was again obtained during the third minute of the second speech as well as 15 minutes after the task.

Questionnaires

Affective responses were assessed with Profile of Mood States (POMS) (25). Health and lifestyle variables were assessed by self-report questionnaire (except for height and weight). These variables included age, gender, and ethnicity, alcohol consumption, smoking and smokeless tobacco use, caffeine consumption, use of recreational drugs, exercise, body mass index (kg/[height in meters]^2). Sleep quality was measured using the Pittsburgh Sleep Quality Index (PSQI), a 19-item self-report questionnaire that assesses sleep quality, latency, and duration (26).

Endocrine Assessments

Whole blood was collected in EDTA tubes, kept on ice, and spun down within 20 minutes after collection and plasma was stored at −80°C. Plasma catecholamine concentrations were determined by HPLC (ESA, Inc., Chelsford, MA). Standards and chemicals were purchased from ChromSystems (Thermo-Alko, Beverly, MA). Samples of the same participant were analyzed in a single run. Intraassay variation for norepinephrine was 3% (interassay variation 6%). Intraassay variation for epinephrine was 6% (interassay variation 13%). Sensitivity for norepinephrine and epinephrine was 15 pg/mL and 6 pg/mL, respectively.

Flow Cytometry

Flow cytometric analyses were performed on baseline and task samples only. EDTA blood was kept at room temperature and prepared within 2 hours after collection. Specific NK subtypes were identified by immunofluorescent antibody staining of whole blood using four-color flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA). CD56 PE, CD56 FITC, and CD3 PerCP were obtained from Becton Dickinson; CD8 APC, CD16 FITC, CD62L PE and CD11a PE were obtained from Pharmingen (San Diego, CA). Briefly, cell suspensions were incubated with antibody for 25 minutes at room temperature and lysed with FACS Lysing Solution (Becton Dickinson). Subsequently, cell suspensions were washed by centrifugation (600 g, 6 minutes, 4°C), resuspended in phosphate-buffered saline containing 1% formaldehyde, and stored in the dark at 4°C. Preparations were read within 24 hours with 12,000 gated events (using the lymphocyte gate) being acquired from each preparation. Matched antibody isotype controls were used to set negative staining criteria. Data were analyzed using Cell Quest-pro software (Becton Dickinson). $CD56^{lo}$ and $CD56^{hi}$ subsets were identified as described

by Cooper et al. (15). Whole blood counts, hematocrit, and hemoglobin (photometric method) were determined using a Coulter GEN-S hematology analyzer (Beckman-Coulter, Miami, FL).

Soluble CD62L

Soluble CD62L was quantified with a sandwich enzyme-linked immunosorbent assay according to the directions given by the manufacturer (Quantikine kit; R&D Systems, Inc, Minneapolis, MN). The within-assay variability is <5% and between assay variability is <10%.

Hemoconcentration

Acute stressors cause an acute loss of plasma volume within the intravascular space, which results in nondiffusible blood constituents (i.e., cells and large molecules) becoming more concentrated (27,28). Hence, stress-induced increases in the concentration of blood constituents may, in part, be the result of the same amount of cells and molecules being diluted in less fluid (28). Dill and Costill (29) described a method whereby hematocrit (Hct; the volume of red blood cells) and hemoglobin (Hb) are used to calculate such changes in hemoconcentration. Leukocytes are determined as cell counts per volume of blood, and the change (%) in blood volume (%Δ BV) resulting from plasma loss is calculated as:

$$\%\Delta BV = \left(100 \times \frac{Hb_{baseline}}{Hb_{task}}\right) - 100$$

The number of immune cells during the task can subsequently be adjusted for changes in blood concentration using the formula: adjusted cell numbers = [1 + (%Δ BV/100)] unadjusted cell numbers.

The concentration of plasma proteins is not determined per volume of blood, but per volume of plasma (i.e., the separated noncellular fraction of the blood). The change (%) in plasma volume (%Δ PV) is calculated as:

$$\%\Delta PV = \frac{\left[\left(100 \times \frac{Hb_{baseline}}{Hb_{task}}\right) - \left(Hct_{task} \times \frac{Hb_{baseline}}{Hb_{task}}\right)\right] - (100 - Hct_{baseline})}{(100 - Hct_{baseline})}$$

The task concentration of sCD62L was adjusted for changes in plasma concentration using the formula: adjusted plasma concentration = [1 + (%Δ PV/100)] unadjusted plasma concentration.

Data Analysis

Repeated-measures analysis of variance (ANOVA) was used to determine group (speech, control) × time (baseline, task) interactions for each parameter. This analysis contrasts the changes within the experimental group with the changes within the control group. Subsequent repeated-measures ANOVA were conducted to examine potential time-main effects within each group. Baseline differences between the task and control group were examined by *t* tests for independent groups. Regression analyses were performed to assess the unique, partial, and combined contribution of epinephrine and norepinephrine reactivity to NK mobilization.

The data of one experimental participant (female) was excluded because of unsuccessful attempts to obtain a second blood sample. Occasionally, data were incomplete as a result of incomplete lysis of a blood sample ($N = 1$) or an excluded outlier (z-score >3) (epinephrine $N = 1$, norepinephrine $N = 1$). Degrees of freedom were adjusted accordingly.

RESULTS

Comparisons of Experimental and Control Subjects

Analyses indicated that the two groups were not significantly different in gender composition (control 51% males; experimental 52% male) or ethnicity (control 67% white; experimental 81% white) and were of similar age (mean age controls 20.3, standard error of mean [SEM] 0.3; mean age experimental 19.8, SEM 0.3) (all $p > .2$). Statistical comparisons were also performed on a number of lifestyle factors, including smoking (habitual, past week, past 24 hours), alcohol consumption (habitual, past week, past 24 hours), caffeine consumption (habitual, past week, past 24 hours), hours and quality of sleep (habitual, past 3 nights, past night), body mass index, and exercise. Analyses yielded no significant differences between the two groups ($t[72] < 2.0$, $p > .07$).

Affective Reactions

Analysis of the POMS tension–anxiety and anger–hostility subscales indicated that the speech tasks were perceived as stressful. The group × time interactions were significant for both scales ($F_{[1,72]} > 7.5$, $p < .01$), which were driven by increases during the speech task: tension–anxiety ($M_{baseline}$ 1.9 [SEM 0.4], M_{task} 8.3 [SEM 0.7], $F_{[1,51]} = 74.6$, $p < .001$) and anger–hostility ($M_{baseline}$ 0.6 [SEM 0.3], M_{task} 2.1 [SEM 0.5], $F_{[1,51]} = 7.8$, $p < .01$). No changes in these mood parameters were reported in the control condition ($F_{[1,20]} < 1.1$, not significant [NS]). During baseline the experimental and control groups did not significantly differ on these subscales ($t_{[73]} < 2.0$, $p > .2$).

Plasma Catecholamines and Heart Rate

Replicating prior research, analyses confirmed that the acute psychosocial stressor elevated plasma epinephrine, norepinephrine, and heart rate, whereas no changes were observed in the control group (see Table 1), yielding significant group × time interactions ($F_{[2,140]} > 15.0$, $p < .001$). The control and experimental groups did not differ on these parameters during the baseline measurement ($t_{[73]} < 1.5$, NS). See Table 1 for complete results.

Hemoconcentration

ANOVA demonstrated a significant group difference in blood volume change ($F_{[1,72]} = 17.6$, $p < .001$), which decreased during the speech task by −3.2% (SEM 0.4) ($t_{[52]} = 7.8$, $p < .001$). ANOVA also indicated a significant group difference in plasma volume change ($F_{[1,72]} = 17.3$, $p < .001$), which decreased during the speech task by −5.3% (SEM 0.7) ($t_{[52]} = 8.1$, $p < .001$). No hemoconcentration changes (<0.2%) were observed during the control condition ($t_{20} < 1.1$, NS).

Cellular Responses

The task induced a significant leukocytosis and lymphocytosis (Table 1), as well as a threefold increase in the number of circulating NK cells (see Table 2), yielding significant group × time interaction effects (F-values ranging between 9.3 and 38.4, $p < .001$). Again, no significant changes were observed in the control group (see Tables 1 and 2 for analyses of time-main effects and summary data). The control and experimental group did not differ at baseline ($t_{[72]} < 1.8$, $p > .20$).

Natural Killer Subsets

Functional Subsets: CD56 and CD16

Figure 1 presents the mean numbers of the functionally distinct $CD56^{lo}$ and $CD56^{hi}$ subsets. Repeated-measures

TABLE 1. Mean (Standard Error of Mean) for Catecholamines, Heart Rate, and Leukocyte Numbers (Corrected for Blood Concentration), as Well as Results of Repeated-Measures Analysis of Variance for Time Main Effects[a]

		Baseline	Task	+15 Min		
Epinephrine (pg/mL)	Speech	20.7 (1.6)	45.1 (4.0)	25.6 (2.0)	$F_{(2,102)} = 42.3$	$p < .001$
	Control	21.3 (2.7)	23.0 (3.0)	20.2 (2.5)	$F_{(2,38)} = 1.6$	NS
Norepinephrine (pg/mL)	Speech	295.2 (11.8)	450.0 (20.4)	335.7 (13.0)	$F_{(2,102)} = 69.1$	$p < .001$
	Control	306.2 (21.1)	335.0 (20.9)	334.3 (25.3)	$F_{(2,38)} = 1.4$	NS
Heart rate (beats/min)	Speech	70.0 (1.3)	88.6 (2.3)	71.1 (1.3)	$F_{(2,104)} = 108.2$	$p < .001$
	Control	69.2 (2.5)	70.3 (2.2)	68.8 (2.2)	$F_{(2,40)} = 1.4$	NS
Leukocytes (10^3 cells/μL)	Speech	6.04 (0.19)	6.75 (0.21)	6.18 (0.19)	$F_{(2,104)} = 27.6$	$p < .001$
	Control	5.81 (0.29)	5.81 (0.27)	5.86 (0.27)	$F_{(2,40)} = 0.1$	NS
Lymphocytes (10^3 cells/μL)	Speech	1.65 (0.06)	2.00 (0.06)	1.74 (0.06)	$F_{(2,104)} = 69.4$	$p < .001$
	Control	1.67 (0.12)	1.61 (0.10)	1.65 (0.10)	$F_{(2,40)} = 1.2$	NS

NS = not significant.
[a]Group × time interactions (all $p < .001$) are described in the 'Results' sections.

TABLE 2. Means (Standard Error of Mean) of Total Natural Killer (NK) Cell Numbers and Numbers of NK That Differ in CD16 Expression, as Well as Results of Repeated-Measures Analysis of Variance for Time Main Effects[a]

		Baseline	Task		
Total NK cells (cells/μL)	Speech	105.1 (6.5)	321.3 (18.6)	$F_{(1,52)} = 197.2$	$p < .001$
Corrected for blood concentration			311.5 (18.4)	$F_{(1,52)} = 191.9$	$p < .001$
	Control	121.0 (12.8)	125.0 (11.7)	$F_{(1,20)} = 0.2$	NS
$CD16^{neg}$ NK cells (cells/μL)	Speech	16.6 (0.9)	22.9 (2.2)	$F_{(1,52)} = 11.1$	$p < .01$
Corrected for blood concentration			22.2 (2.0)	$F_{(1,52)} = 9.7$	$p < .01$
	Control	17.9 (2.2)	17.0 (1.9)	$F_{(1,20)} = 0.5$	NS
$CD16^{lo}$ NK cells (cells/μL)	Speech	17.6 (1.2)	40.4 (3.1)	$F_{(1,52)} = 95.8$	$p < .001$
Corrected for blood concentration			39.1 (3.1)	$F_{(1,52)} = 87.1$	$p < .001$
	Control	16.1 (1.8)	17.3 (1.7)	$F_{(1,20)} = 0.8$	NS
$CD16^{hi}$ NK cells (cells/μL)	Speech	70.8 (5.4)	257.6 (21.7)	$F_{(1,52)} = 190.6$	$p > .001$
Corrected for blood concentration			250.0 (15.9)	$F_{(1,52)} = 185.4$	$p < .001$
	Control	83.4 (10.7)	86.4 (10.3)	$F_{(1,20)} = 0.3$	NS

NS = not significant.
[a]Group × time interactions were all statistically significant.

ANOVA demonstrated a significant group × time interaction effect ($F_{[1,73]} = 70.2$, $p < .001$) for the cytotoxic $CD56^{lo}$ subset, which is clearly driven by the increase in $CD56^{lo}$ NK cell numbers during the speech task ($F_{[1,52]} = 194.4$, $p < .001$, task values corrected for changes in blood volume). In contrast, the number of immunoregulatory $CD56^{hi}$ NK cells yielded a nonsignificant group × time interaction ($F_{[1,72]} = 0.2$, NS). Neither subsets showed a significant change during the control condition ($F_{[1,20]} < 2.0$, $p > .20$).

NK cells can be separated into three subsets based on the expression levels of the immunoglobulin receptor CD16, that is, high expression levels ($CD16^{hi}$), low expression levels ($CD16^{lo}$), and CD16-negative ($CD16^{neg}$). As evident in Table 2, the speech task increased the numbers in all three subsets, albeit to a different extent. The respective group × time interactions for each subset were: $CD16^{hi}$ ($F_{[1,72]} = 67.5$, $p < .001$), $CD16^{lo}$ ($F_{[1,72]} = 28.0$, $p < .001$), and $CD16^{neg}$ ($F_{[1,72]} = 4.6$, $p < .05$) (based on values corrected for blood concentration). NK mobilization increased in parallel with CD16 expression; whereas the number of circulating $CD16^{hi}$ NK cells increased approximately 300% from baseline, this was approximately 140% for $CD16^{lo}$ NK cells and only approximately 30% for the $CD16^{neg}$ NK subset. Pairwise comparisons, using paired *t* tests, confirmed that these subsets were differentially mobilized (each comparison $t_{[52]} > 7.3$, $p < .001$). Pairwise analyses of raw and residualized difference scores similarly indicated a differential mobilization (each comparison $t_{[52]} > 5.0$, $p < .001$).

Migratory Potential: CD62L and CD11a

The distribution pattern of CD62L on lymphocytes allows for a categorization of NK cells into three subsets: high expression levels ($CD62L^{hi}$), low expression levels ($CD62L^{lo}$), and CD62L-negative NK cells ($CD62L^{neg}$). As

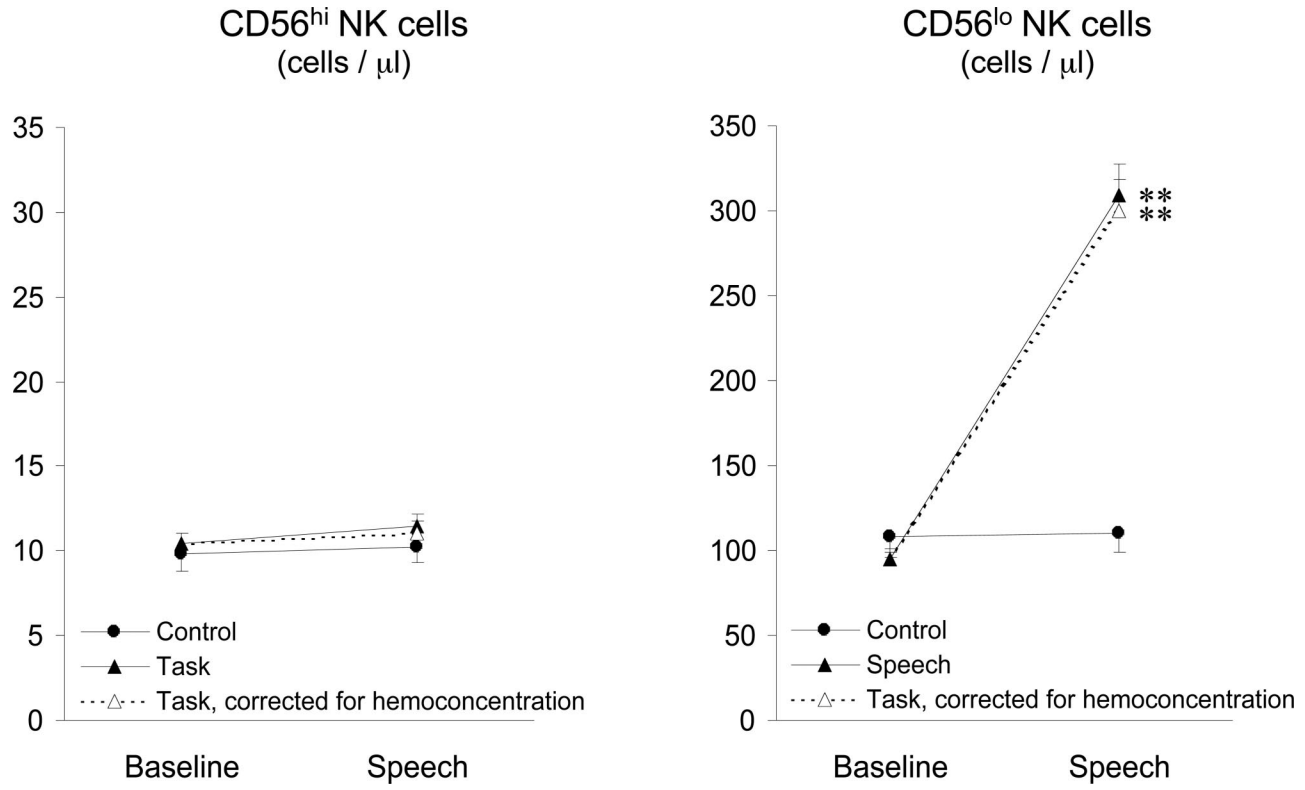

Figure 1. $CD56^{hi}$ and $CD56^{lo}$ natural killer subsets during the control and task conditions. Dots indicate means; vertical bars indicate standard error of mean. Repeated-measures analysis of variance: $^{**}p < .001$.

becomes clear from Figure 2, the speech task increased the numbers of all three subsets. The respective group × time interaction effects were: $CD62L^{hi}$ ($F_{[1,72]} = 27.6$, $p < .001$), $CD62L^{lo}$ ($F_{[1,72]} = 70.8$, $p < .001$), and $CD62L^{neg}$ ($F_{[1,72]} = 64.6$, $p < .001$) (based on values corrected for hemoconcentration). Figure 2 indicates that subset mobilization was inversely related to CD62L expression; whereas the number of circulating $CD62L^{hi}$ NK cells increased by approximately 100%, this was approximately 200% for $CD62L^{lo}$ NK cells and approximately 300% for the $CD62L^{neg}$ subset. Pairwise comparisons (using paired *t* tests) confirmed that these subsets were differentially mobilized (each comparison $t_{[52]} > 8.5$, $p < .001$). Pairwise analyses of raw and residualized difference scores similarly indicated a differential mobilization (each comparison $t_{[52]} > 5.3$, $p < .001$). Subset numbers were unaffected during the control condition ($F_{[1,20]} < 1.5$, $p > .20$). As can be expected on the basis of these results, the fluorescence intensity of CD62L on CD62L+NK cells de-

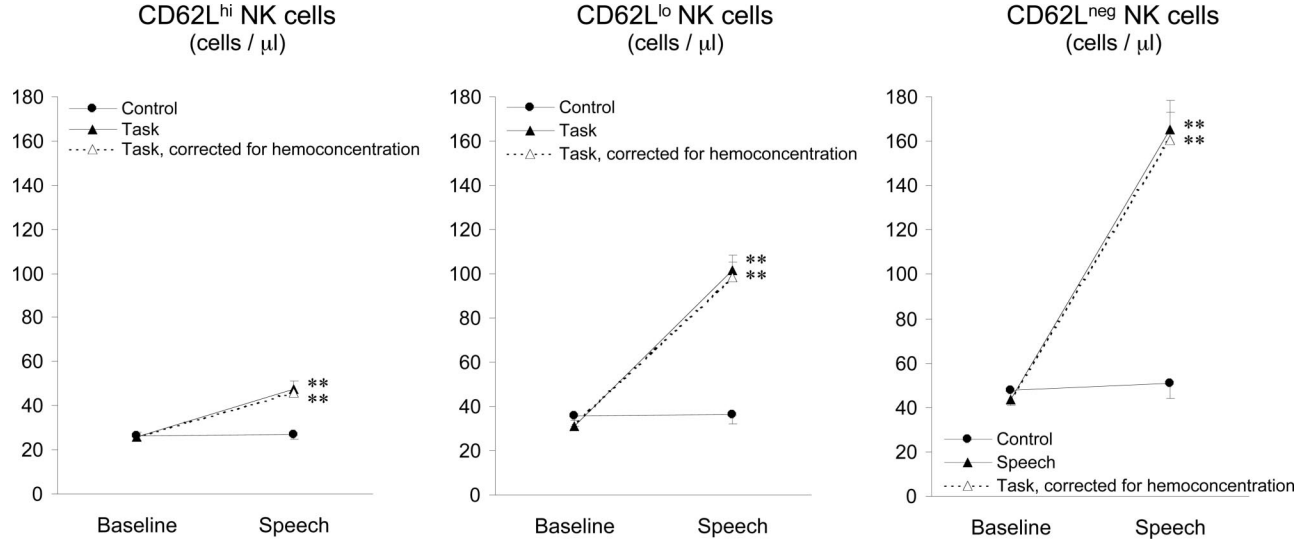

Figure 2. Natural killer subsets, categorized on the basis of CD62L expression, during the control and task conditions. Dots indicate means; vertical bars indicate standard error of mean. Repeated-measures analysis of variance: $^{**}p < .001$ (all time × condition interactions are statistically significant; see text).

creased (see Table 3), indicating lower expression levels on NK cells during the task (group × time interaction, $F_{[1,72]} = 43.4$, $p < .001$).

CD11a exhibits a characteristic bimodal distribution on lymphocytes on the basis of which NK cells were separated into two populations: a low CD11a-expressing subset ($CD11a^{lo}$) and a subset that expressed high levels of CD11a ($CD11a^{hi}$) (note: all NK cells are positive for CD11a, therefore; the fraction $CD11a^{hi}$ cells + fraction $CD11a^{lo}$ cells = 100%). During the task, the fraction of $CD11a^{lo}$ NK cells decreased from 10.1% (SEM 0.8) at baseline to 5.3% (SEM 0.6) ($F_{[1,51]} = 54.3$, $p < .001$). CD11a expression on peripheral blood NK cells increased during the task (group × time interaction $F_{[1,71]} = 10.2$, $p = .001$). No significant changes were seen during the control condition ($F_{[1,20]} = 0.9$, NS), yielding a significant group × time interaction effect ($F_{[1,71]} = 13.8$, $p < .001$). The control and experimental group did not differ in baseline values for any of the subsets described here ($t_{[72]} < 2.0$, $p > .20$).

Soluble CD62L

The serum concentration of soluble CD62L significantly increased during the task and returned to baseline levels 15 minutes posttask (Table 4). No changes were seen during the control condition, yielding a time × condition effect ($F = 4.1$, $p < .05$). However, correction for changes in plasma concentration abrogated this effect (see Table 4).

Gender Differences

Some reports have noted gender differences in NK cell numbers and autonomic stress reactivity (see reference 30). Therefore, exploratory analyses were conducted to identify potential gender differences in catecholamine, hemoconcentration changes, and NK subset distribution. We observed stronger epinephrine increases in males ($\Delta_{[task - baseline]}$ males = 30.9 pg/mL [SEM 5.5]; $\Delta_{[task - baseline]}$ females = 17.5 pg/mL [SEM 2.5] [gender × time interaction $F_{[2,102]} = 2.04$, $p < .05$]). Subsequent analyses demonstrated that this gender effect was largely driven by differences in baseline values (higher levels in males) because analysis of covariance indicated no effect of gender after baseline values were entered as covariate ($F_{[2,100]} = 1.29$, NS). Moreover, analysis of relative change scores (Δ%) yielded no gender difference ($\Delta\%_{[task - baseline]}$ males = 135.0% [SEM 20.1]; $\Delta\%_{[task - baseline]}$ females = 121.8% [SEM 18.6]). No gender differences in reactivity or baseline values were observed for NK subsets or norepinephrine (all analyses $p > .17$).

Catecholamine Responses and Natural Killer Subset Distribution

Regression analyses were performed to determine the unique and combined contribution of epinephrine and norepinephrine to NK subset distribution. Results, presented in Table 5, are expressed as partial and zero-order correlation coefficients; the final column presents the correlation if both catecholamines were entered together in the regression analysis. A partial correlation represents the unique contribution of one catecholamine (i.e., independent of the contributions of the other catecholamine) to individual differences in NK mobilization. The results showed that epinephrine and norepinephrine have unique and additive contributions to NK subset mobilization. Also, the strength of the correlations increased in parallel with the magnitude in which a particular NK subset became mobilized, suggesting subset differences in responsivity to catecholamines.

To maintain a common metric for all parameters, we used Δ% scores in the correlation analyses (which also eliminated the influence of gender differences in epinephrine responses). All analyses are based on hemoconcentration-corrected cell counts.

Examining the Psycho → Neuro → Immune Pathway

The generally held belief is that affective responses drive immune reactions through a neuroendocrine pathway. We sought to test the specific hypothesis that the relation between emotionality and NK mobilization is mediated by the release of catecholamines by conducting a mediational analysis of the experimental data. Mediation requires that 1) all variables in the model (i.e., the dependent, independent, and mediator variables) are intercorrelated, and 2) that the association between the dependent and independent variable becomes significantly attenuated when the association between the mediator and dependent variable is accounted for.

To examine the first condition, correlation analyses were performed. As would be required for mediation, analyses indicated associations between individual differences in affective reactivity (i.e., responses on POMS subscales) and catecholamine and NK reactivity. Epinephrine and norepinephrine reactivity correlated with changes on the subscales tension–

TABLE 3. Changes in Receptor Density (Measured as Median Cell Fluorescence Intensity) on Circulating CD62L+and CD11a + Natural Killer Cells[a]

		Baseline	Task		
CD62L (median CFI)	Speech	284 (13)	152 (8)	$F_{(1,52)} = 136.8$	$p < .001$
	Control	272 (27)	271 (29)	$F_{(1,20)} = 0.0$	NS
CD11a (median CFI)	Speech	1165 (21)	1232 (22)	$F_{(1,51)} = 53.6$	$p < .001$
	Control	1170 (45)	1176 (45)	$F_{(1,20)} = 0.1$	NS

NS = not significant.

[a]Shown are results for time main effect, because group × time interactions were all statistically significant.

TABLE 4. Means (Standard Error of Mean) for Soluble CD62L Levels (pg/mL) and Results of Repeated-Measures Analysis of Variance, as Well as Results of Repeated-Measures Analysis of Variance for Time Main Effects[a]

Group	Time			
	Baseline	Task	+15 Min	
Speech	1308 (71)	1379 (74)	1298 (69)	$F_{(2,104)}=7.1$ $p<.01$
Corrected for plasma concentration		1307 (71)	1298 (68)	$F_{(2,104)}=1.1$ NS
Control	1280 (80)	1274 (78)	1272 (81)	$F_{(2,40)}=0.3$ NS

NS = not significant.
[a]Group × time interactions were only significant for uncorrected values.

TABLE 5. Associations Between Catecholamine Responses and Increases in Natural Killer (NK) Subset Numbers (Both Calculated as Δ%)[a]

NK Subsets	Epinephrine	Norepinephrine	Catecholamines Combined
Total NK	0.35* (0.45)**	0.38* (0.48)**	0.57**
$CD56^{hi}$	−0.10 (−0.05)	0.17 (0.14)	0.17
$CD56^{lo}$	0.37* (0.47)**	0.42* (0.51)**	0.60**
$CD62L^{hi}$	0.10 (0.21)	0.30 (0.34)*	0.36*
$CD62L^{lo}$	0.26 (0.37)**	0.34* (0.44)**	0.50**
$CD62L^{neg}$	0.49**(0.57)**	0.43**(0.52)**	0.68**

*$p < .01$; **$p < .001$.
[a]Partial correlations (each correcting for the other catecholamine) are presented first; zero-order correlations are between brackets.

anxiety (0.32 and 0.30, respectively, $p < .05$), anger–hostility (0.44 and 0.38, $p < .01$), and vigor–activity (0.25, $p < .05$, for norepinephrine). Two of these POMS subscales also correlated with increases in the stress–reactive $CD56^{lo}$ NK subset: tension–anxiety (0.33, $p < .05$) and anger–hostility (0.29, $p < .05$). A comparable pattern of results was observed for other NK subsets (p values varying between .12 and .001), although no significant correlations were observed for the minimally responsive $CD16^{neg}$ and $CD56^{hi}$ subsets.

We next performed linear regression analyses to test the second condition for mediation. The model and path coefficients are summarized in Figure 3. Entering the two intercorrelated POMS subscales together, regression analyses confirmed a positive association between affective responses and $CD56^{lo}$ NK mobilization ($F_{[2,50]} = 3.22$, $p < .05$, $R^2 = 0.11$). Subsequently entering both catecholamines in the model explained a significant amount of additional variance (R^2-change = 0.25, $F_{[2,48]} = 9.31$, $p < .001$). However, if both catecholamines were entered in the model first, the two POMS subscales did not account for any additional variance (R^2-change = 0.01, $F_{[2,48]} = 0.87$, NS). These results support the hypothesis that catecholamine responses account for (i.e., mediate) the association between task-induced affective responses and NK mobilization.

DISCUSSION

The rapid mobilization of NK cells during acute psychologic stress is a well-established but poorly characterized phenomenon. The present study performed a phenotypical analysis of this response using various cell surface markers to identify NK subsets that differ in functional characteristics and migratory potential. The results demonstrated a marked specificity in the mobilization of functionally distinct NK subsets during acute psychologic stress. We found that the stress-induced increase in circulating NK cells is restricted to the cytotoxic $CD56^{lo}$ subset, whereas no change was seen for the immunoregulatory $CD56^{hi}$ subset. Consistent with the observation that stress-induced mobilization of NK cells is related to cytotoxic potential, we also observed larger increases for NK cells that express higher levels of CD16. CD16 is a receptor essential for antibody-dependent cytotoxicity and

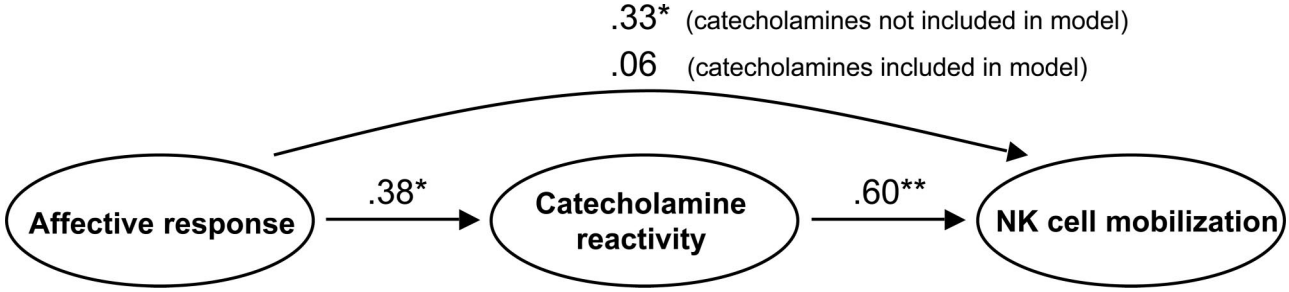

Figure 3. Path model with standardized regression coefficients. Affective response is measured a reactivity on the Profile of Mood States subscales tension–anxiety and anger–hostility. *$p < .05$, **$p < .001$.

has particularly high expression levels on cytotoxic $CD56^{lo}$ NK cells (12,15).

In the psychoneuroimmunology literature, a distinction is often made between functional and enumerative immune changes in which the mobilization of NK cells represents a typical example of the latter. Because of this common distinction, it may become overlooked that leukocyte redistribution is, in fact, a very important functional response; it is a main mechanism through which the immune system ensures that sufficient cells can arrive at locations where they may be needed (17,22,31). Our data showed that a common speech task tripled the number of cytotoxic $CD56^{lo}$ NK cells in the blood (at an average increase of 220 NK cells/μL, this represents over 1 billion additional $CD56^{lo}$ NK cells), providing these newly released cells with ample opportunity to survey the peripheral tissues for signs of damage and infection. Although the health implications of this response pattern are not clear as yet (see the last paragraph for a discussion), our results do indicate that the blood is rapidly modifying its functional potential by selectively increasing the proportion and number of cells that have the ability to immediately destroy infectious and infected cells, and have a strong capacity to migrate into the tissues.

Indeed, consistent with previous research (18,32–34), a marked specificity was also found when NK cells were characterized on the basis of the expression levels of the adhesion molecules CD62L and CD11a. During the speech task, the average density of CD62L (a receptor that supports migration of NK cells into the secondary lymphoid tissues) on circulating NK cells decreased, whereas CD11a levels (a receptor that supports migration of NK cells into the tissues) increased. Such receptor changes are also observed after bouts of exercise and infusion of the beta-adrenergic agonist isoproterenol (23) and can be abrogated after administration of the beta-adrenergic antagonist propanolol (23,34). Moreover, we found positive correlations between the changes in various NK subsets and plasma catecholamines. Clearly, adrenergic mechanisms are largely responsible for the changes in the adhesion molecule densities on circulating NK cells.

The interpretation of these receptor density changes has been a topic of speculation. As for the changes in CD62L expression, two possible interpretations have been proposed (see reference (18), both of which were addressed in the present study. The most common interpretation is that acute psychologic stressors may rapidly downregulate CD62L expression, for example, through the shedding of this receptor from the cell surface. CD62L shedding is a process by which lymphocytes control their movement along, and the attachment to, the vascular epithelium (35). A rapid shedding of CD62L would result in a parallel increase in plasma levels of soluble CD62L (sCD62L). In support of the shedding hypothesis, Redwine and coworkers (36) observed increases in plasma sCD62L during a public-speaking task (in parallel with an increased number of CD62L-negative lymphocytes). We were able to confirm this finding, but also found that the increase in sCD62L disappeared after controlling for simultaneous changes in plasma concentration. Thus, the increased concentration in plasma sCD62L resulted from an increased hemoconcentration (i.e., filtration of fluid out of the intravascular space) rather than an actual increase of soluble receptors in the circulation.[1] Further weakening the shedding hypothesis is that infusion of the beta-adrenergic agonist isoproterenol does not increase plasma sCD62L (although it does increase the number of circulating $CD62L^{neg}$ lymphocytes, including $CD62L^{neg}$ NK cells) (23), and that catecholamines do not affect CD62L expression on lymphocytes in vitro (35). In summary, both our current data and the existing evidence do not support the interpretation that catecholamine-dependent decreases in NK CD62L density are the result of rapid receptor downregulation either through receptor shedding or otherwise.

The second interpretation for the rapid changes in adhesion molecule densities is that acute stressors selectively mobilize NK cells that have preexisting differences in adhesion molecule expression (18). According to this interpretation, the change in average receptor density reflects a change in the cellular composition of the blood rather than a change in receptor density per cell. Our data provided strong support for this hypothesis, because we observed a selective increase in cytotoxic $CD56^{lo}$ NK cells (which are predominantly $CD62L^{neg/lo}$ and $CD11a^{hi}$) in combination with unaffected numbers of immunoregulatory $CD56^{hi}$ NK cells (which are $CD62L^{hi}$ and largely $CD11a^{lo}$) (15,19,21). This phenomenon is also the most likely explanation for the preferential increase in $CD16^{hi}$ NK cells, because high CD16 expression is a characteristic feature of the cytotoxic $CD56^{lo}$ NK subset (15).

The present study also raised some issues that will require further investigation. For example, regression analyses suggested that the differential mobilization of NK subsets reflects a differential responsivity to catecholamines,[2] perhaps resulting from subset differences in adrenergic receptor density and/or sensitivity. This possibility needs to be explored in receptor studies. Also, whereas our analyses demonstrated that the divergent NK response pattern is robust with regard to gender differences, the generalizability of this response pattern to other populations (e.g., elderly, patient groups) is unknown. Also unknown are the effects of the selective NK mobilization on in vitro cytotoxic activity. Most laboratory studies find, however, that in vitro NK cytotoxicity increases in parallel with increasing circulating NK numbers (4). Our results indicate that these changes in NK cytotoxicity are a direct consequence of a selective increase in $CD56^{lo}$ NK cells. Conversely, chronic stressors are associated with decreased in vitro NK cytotoxic activity (4), and future research might thus test whether this decrease is related to changes in the compo-

[1]Note the distinct use of plasma concentration and blood concentration to correct for plasma constituents and immune cells, respectively (see "Methods" for details).

[2]One may argue that the correlation between catecholamines and NK mobilization could be confounded by a third (unmeasured) neuroendocrine variable. This hypothesis is refuted, however, by studies showing that adrenergic blockade abrogates stress-induced NK mobilization (37,38), indicating that nonadrenergic mechanisms explain little additional variance.

sition of the circulating pool of NK cells, i.e., a higher proportion of low cytotoxic CD56^{hi} NK cells (see reference (39).

An important remaining issue concerns the implications of these selective immunologic changes for health. Although the historical literature has often focused on the deleterious effects of stress responses, there is now a growing recognition that acute stressors may, at least in the short run, initiate processes that could promote immune function and health (31). For example, animal studies have shown that acute stressors can lead to a faster resolution of infection (40,41), can enhance both cellular and humoral adaptive immunity (42–44), and accelerate wound healing (45). These protective effects are thought to be mediated in part by the effects of stress hormones on immune-cell trafficking (31,46). NK cell mobilization represents one of the best documented examples of this mechanism in humans (4). Evolutionary pressures may have selected for this facet of the acute stress response because it conceivably enhances immune preparedness in contexts in which tissue damage and infection are more likely to occur. Indeed, acute stress has been found to rapidly upregulate other protective systems as well, including secretory immunity, coagulation, and complement activation (2,47,48). Hence, an obvious potential implication is that acute stressors may enhance, rather than diminish, resistance to infection. This idea has already gained good support from experimental animal studies and warrants further investigation in humans.

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