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Increased Natural Killer-Cell Mobilization and Cytotoxicity during Marital Conflict

Joel M. Dopp[1]

Department of Microbiology and Immunology, CIRID, UCLA, Los Angeles, California 90096

Gregory E. Miller and Hector F. Myers

Department of Psychology, UCLA, Los Angeles, California 90024

and

John L. Fahey

Department of Microbiology and Immunology, CIRID, UCLA, Los Angeles, California 90096

Natural killer (NK) cells are reproducibly mobilized into the circulation in response to intense physical exercise or acute psychological stress, and altered expression of adhesion molecules potentially contributes to NK-cell mobilization. Studies of leukocyte mobilization during acute stress have used psychological stressors which facilitate tight experimental control but have limited applicability to everyday life. We therefore used a laboratory model of marital conflict as an experientially meaningful acute stressor to elucidate relationships among conflict, cardiovascular reactivity, and altered leukocyte phenotype and function. Forty-one ethnically diverse, nondistressed, healthy married couples were asked to discuss a specific problem in their marriage for 15 min. Blood pressure and heart rate were measured before, during, and after the discussion, and blood was remotely drawn at the same time points to quantify numbers of specific leukocyte subsets, NK-cell adhesion molecule expression, and NK cytotoxicity. Couples responded to the conflict task with cardiovascular reactivity; increases in the percentages of circulating NK cells and $CD8^+$ T cells and decreases in the percentage of circulating $CD4^+$ T cells; decreases in the percentage of NK cells that express L-selectin; and increases in NK-cell cytotoxicity without a commensurate increase in per-cell cytotoxicity. Rapid downregulation or shedding of L-selectin (CD62L) from NK cells did not contribute to their mobilization during conflict. Instead, $CD62L^-$ NK cells were mobilized while $CD62L^+$ NK cells were selectively retained in the vascular marginating pool and/or in extravascular tissue. From a broader perspective, the data support the hypothesis that altered trafficking of specific leukocyte subsets is an integral component of the fight-or-flight response to an acute stressor.

Key Words: stress; natural killer; leukocytosis; mobilization; adhesion molecule.

INTRODUCTION

Mobilization of natural killer (NK) cells into the circulation is arguably the most reproducible cellular immunological response to acute physical and psychological stressors in humans. For example, numbers of circulating NK cells increase during intense exercise (Maisel, Harris, Rearden, & Michael, 1990; Gabriel, Schwarz, Steffens, & Kinderman, 1992; Murray, Irwin, Rearden, Ziegler, Motulsky, & Maisel,

[1] To whom correspondence should be addressed at UCLA MRRC, 68-17 NPI, 760 Westwood Plaza, Los Angeles, CA, 90024. Fax: 310-206-5061. E-mail: jdopp@mednet.ucla.edu.

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1992), parachute jumping (Schedlowski, Jacobs, Alker, Prohl, Stratman, Richter, Hadicke, Wagner, Schmidt, & Tewes, 1994a), distressing mental arithmetic (Naliboff, Benton, Solomon, Morley, Fahey, Bloom, Makinodan, & Gilmore, 1991), and interpersonal interactions (Brosschot, Benschop, Goodart, de Smet, Olff, Heijnen, & Ballieux, 1992). A concomitant increase in NK-cell cytotoxicity also occurs (Murray et al., 1992; Naliboff et al., 1991), although it is unclear whether this is due to an increased in-well percentage of NK cells in cytotoxicity assays or to an increase in per-cell killing. Cellular mobilization is restricted to NK cells, granulocytes, and, to a lesser extent, macrophages and $CD8^+$ lymphocytes, whereas numbers of circulating B cells are generally unchanged, and numbers of $CD4^+$ lymphocytes often decrease in response to acute stressors (Fleshner, Watkins, Lockwood, Bellgrau, Laudenslager, & Maier, 1992; Gabriel et al., 1992; Murray et al., 1992; Naliboff et al., 1991). Mobilization is generally transient, reaching peak levels in less than 30 min, and may be followed by a decrease in numbers of mobilized cells to levels below baseline (Benschop, Rodriguez-Feuerhahn, & Schedlowski, 1996).

Converging lines of evidence implicate the catecholamines epinephrine (E) and norepinephrine (NE) in stress-induced leukocyte mobilization. First, levels of circulating catecholamines increase in concert with mobilization, and injections of β_2 adrenergic receptor antagonists prior to stress prevent mobilization (Benschop, Nieuwenhuis, Tromp, Goodart, Ballieux, & van Doornen, 1994; Schedlowski, Hosch, Oberbeck, Benschop, Jacobs, Raab, & Schmidt, 1996). Second, intravenous injections of E, NE, or the β_2 agonist isoproterenol cause a rapid efflux of granulocytes (Ernström & Sandberg, 1973) and NK cells (Schedlowski et al., 1994a) which is prevented by prior injection of β blockers. Third, the susceptibility of leukocyte subsets to catecholamine-induced mobilization appears to be related to their magnitude of isoproterenol-induced cyclic AMP accumulation (Mills, Berry, Dimsdale, Ziegler, Nelesen, & Kennedy, 1995).

A potential mechanism through which catecholamines could alter NK-cell trafficking and cytotoxicity is via modulation of adhesion molecule expression. Adhesion molecules (AMs) facilitate leukocyte extravasation (directed movement from the circulation into extravascular tissue) and mobilization by mediating interactions with vascular endothelial cells (ECs). Leukocytes extravasate toward a chemotactic factor in a sequential manner, each step of which is mediated by a specific class of AMs: initial stages of loose attachment and rolling are mediated by selectins; firm attachment is mediated by integrins; and transendothelial migration is mediated by AMs belonging to the immunoglobulin superfamily (Springer, 1994; Liao, Huynh, Eiroa, Greene, Polizzi, & Muller, 1995; Newman, 1997). Conversely, leukocyte mobilization during acute stress probably involves rapid shedding of specific AMs from leukocytes which are transiently attached to vascular ECs while rolling along the walls of capillaries. Although NK cells express multiple AMs, including LFA-1, Mac-1, VLA-4, and NCAM (Timonen, Patarroyo, & Gahmberg, 1988; Robertson, Caligiuri, Manley, Levine, & Ritz, 1990; Pinola & Saksela, 1992), the best candidates for mediating NK mobilization are L-selectin (CD62L), which binds glyCAM-1 on ECs to facilitate NK extravasation across lymph node high endothelial venules (HEV) (Lasky, Singer, Dowbenko, Imai, Henzel, Grimley, Fennie, Gillett, Watson, & Rosen, 1992; Bevilacqua, 1993), and H-CAM (hylauronic acid receptor; CD44), which binds extracellular matrix molecules to facilitate NK homing to mucosal lymphoid tissue

and lymph nodes (Pals, Koopman, Griffioen, Ponta, Herrlich, van den Berg, & Horst, 1993). Both of these AMs mediate the initial stages of NK-cell extravasation.

An episode of marital conflict is an acute psychological stressor, encountered in everyday life, that has sympathetic nervous system (SNS), neuroendocrine, cardiovascular, and immunological effects. For example, negative or hostile behaviors during marital conflict are acutely associated with increases in circulating catecholamines, adrenocorticotropic hormone, and growth hormone but not cortisol (Malarkey, Kiecolt-Glaser, Pearl, & Glaser, 1993), concomitant with increased heart rate, blood pressure, and electrodermal conductivity (Gottman & Levenson, 1988; Ewart, Taylor, & Kraemer, 1991; Kiecolt-Glaser, Malarkey, Chee, Newton, Cacioppo, Mao, & Glaser, 1993). In older couples, negative behaviors during conflict are also acutely associated with decreased EBV antibody titers and mitogen-induced lymphocyte proliferation (Kiecolt-Glaser, Glaser, Cacioppo, MacCallum, Snydersmith, Cheongtag, & Malarkey, 1997). In younger adults, these same behaviors are associated with decreased anti-CD3- and mitogen-induced T-cell proliferation and decreased NK-cell lysis of tumor targets 21.5 h after the conflict (Kiecolt-Glaser et al., 1993).

The above seminal studies, while demonstrating that marital conflict can have health-related physiological consequences, raise several questions. First, relationships among individual differences in temperament, emotional responses, and physiological responses to conflict have not been examined. Second, relationships between marital conflict and *acute* changes in leukocyte trafficking, AM expression, and leukocyte functions have not been determined. Instead, analyses have been conducted on blood drawn 1 h before versus 21.5 h after marital conflict, and they have been limited to enumeration of leukocyte subsets and assessment of NK-lytic activity (Kiecolt-Glaser et al., 1993). Because of the rapid kinetics of leukocyte mobilization during acute stress, it is necessary to collect multiple measurements during conflict in order to fully capture mobilization and understand underlying mechanisms.

Building on previous research, we determined whether a 15-min discussion of marital conflict was acutely associated with subjective experiences of mild to moderate stress, cardiovascular reactivity, mobilization of specific leukocyte subsets, and altered NK-cell AM expression and cytotoxicity. By measuring physiological responses before, at several points during, and after marital conflict, we established temporal relationships between conflict and physiological changes that were suggestive of causality. In a separate paper, we describe in detail how individual difference variables and negative emotions relate to neuroendocrine, cardiovascular, and immunological responses during marital conflict (Miller, Dopp, Myers, Felten, & Fahey, 1998).

MATERIALS AND METHODS

Subjects

Forty-one couples, from an initial pool of 113 couples, were recruited from the population near UCLA by responding to a newspaper advertisement and completing a telephone screening interview. Exclusion criteria were any of the following: lack of fluency in English; health problems or current medications which affect the cardiovascular, endocrine, or immune systems; a DSM-IV psychotic, affective, or anxiety disorder within the previous 3 years; consumption of more than 15 alcoholic drinks

per week or use of illicit drugs; or pregnancy. Data on other factors known to affect cells of the immune system (e.g., phase of menstrual cycle, nutrition, sleep, and physical activity) (Kiecolt-Glaser & Glaser, 1988) were also collected. Subjects were 18–55 years old (mean age of females = 30.95 ± 7.45 SD; mean age of males = 31.9 ± 7.68 SD), healthy, English speaking, and from diverse ethnic backgrounds (females: 46.3% Caucasian, 29.3% Latina, 9.8% African-American, 9.8% Asian-American, and 4.9% Middle-Eastern; males: 58.5% Caucasian, 24.4% Latino, 7.3% African-American, 4.9% Asian-American, and 4.9% Middle-Eastern). Couples had been married an average of 3.78 years (SD = 3.91), with a range from 2 months to 14 years, and earned a mean income of $18,200 (SD = $12,950).

Laboratory Protocol

During the 12-h period before testing, couples refrained from eating, drinking anything except water, smoking, and strenuous exercise. Couples arrived at the laboratory waiting room at 8 am and ate a standardized breakfast of 8 oz fruit juice and one muffin per person. During breakfast, subjects (Ss) independently completed questionnaires on their health history; recent consumption of caffeine, nicotine, alcohol, and medication; personality, mood states, coping styles, marital satisfaction, and conflict issues. At 9 am, Ss were escorted to the laboratory conflict room and fitted with an electrocardiographic monitor and matching wrist receiver (Polar, New York, NY) which automatically recorded heart rate every 5 s for subsequent averaging across 5-min intervals. Three blood pressure (BP) measurements per 5-min interval were measured with automated sphygmomanometers (Lumiscope, Trenton, NJ) as follows: subjects were seated facing each other at a 45° angle and fitted with blood pressure cuffs on their outer arms. Each cuff was connected by 4 ft of rubber tubing to a digital display Digitronics Model 300 BP monitor, operated by an experimenter seated 3 ft behind the Ss and separated from them by an opaque sound-deadening curtain. Pre-venipuncture BPs were measured in triplicate (as were subsequent BPs), following which a registered phlebotomist inserted a sterile 21-gauge butterfly needle into the antecubital vein of Ss inner arms. Needles were covered with sterile cotton towels and connected via heparinized polyethylene tubing to separate DakMed Model ML-6-5S3R peristaltic mini-pumps (Buffalo, NY) which drew multiple blood samples without repeated venipuncture. Blood samples were collected into EDTA-containing (1.5 mg/ml blood) tubes and stored on ice for less than 30 min prior to processing. After venipuncture and initial blood collection, subjects read or relaxed without talking for 25 min, following which a blood sample was taken and BP was measured.

An experimenter then entered the room and assisted the couple in selecting one of three topics identified by their questionnaires as the highest ranking areas of conflict in their relationship. The following script was used by the experimenter:

> ''Most couples have ongoing disagreements over issues such as household management and communication. Judging by your responses to the Inventory of Marital Problems, its seems that you both feel that _____ is an important topic of disagreement. Unless you would prefer to discuss another topic, I would like you to spend the next 15 min describing this problem and trying to find some mutually satisfying resolution.''

Couples were instructed to talk continuously for the next 15 min, even if this required switching topics. For their own safety, couples were asked to refrain from gesticulat-

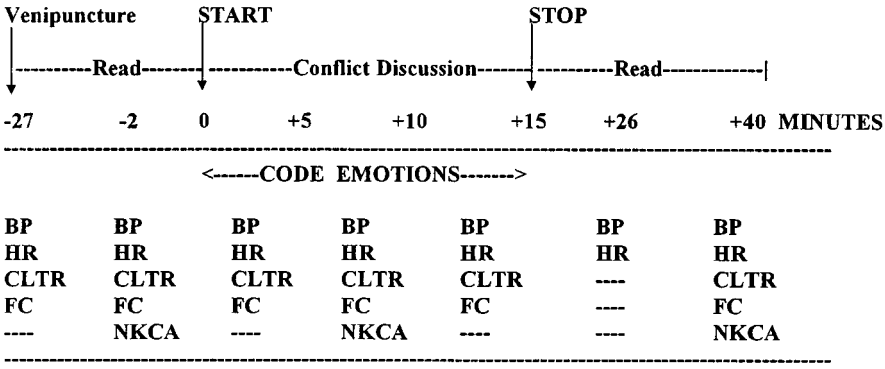

FIG. 1. *An overview of the laboratory protocol.* Abbreviations used: BP, systolic and diastolic blood pressure; HR, heart rate; CLTR, Coulter three-part differential; FC, flow cytometry; NKCA, natural killer cell assay.

ing during the discussion; following these instructions, the experimenter left the room. BP measurements and blood samples were obtained continuously during minutes 0–5, 5–10, and 10–15 after the start of the conflict, and conflict discussions were videotaped for subsequent quantification of overtly expressed emotions (Fig. 1). Following blood collection at 15 min, couples ended their discussion and read or relaxed without talking for 25 min, during and after which blood samples and BPs were obtained. The timing and number of baseline and intraconflict blood samples were designed to ensure that SNS activity had returned to baseline levels prior to marital conflict and to adequately capture the kinetics of leukocyte mobilization during acute stress. Following the final sample collection, blood-pressure cuffs and venipuncture lines were removed and couples were debriefed and paid $60 for their cooperation. To ameliorate any lasting effects of the conflict discussion, couples were offered on-site counseling and informed how to access additional counseling at UCLA; to our knowledge, no couples sought additional counseling. The above protocol was approved by the UCLA Human Subjects Protection Committee.

Coulter and Flow Cytometric Analyses

To quantify leukocyte subsets and assess AM expression, 400 μl of EDTA (1.5 mg/ml)-treated whole blood per sample was aliquoted and mixed mechanically for 15 min. Three-part CBC analyses were performed using a Microdif 16 Coulter Counter (Palo Alto, CA). For flow cytometry, 400 μl of EDTA-treated whole blood per sample was incubated for 15 min in the dark at 21°C with one of the following combinations of mouse monoclonal antibodies (mAbs), at empirically determined optimum concentrations: FITC-conjugated anti-CD4, PE-conjugated anti-CD8, and PerCP-conjugated anti-CD3; FITC-conjugated anti-CD62L (L-selectin), PE-conjugated anti-CD45RO (leukocyte common antigen), and PerCP-conjugated anti-CD8; or FITC-conjugated anti-CD62L, PE-conjugated anti-CD16 ($F_cR_{\gamma III}$) and anti-CD56 (NCAM), and PerCP-conjugated anti-CD3 (all from Becton–Dickinson; San Jose, CA). A subset of samples was alternatively stained with FITC-conjugated antibodies against CD19, CD31 (PECAM), CD44 H-CAM, or CD54 (ICAM-1) (all from Pharmingen, San Diego, CA). To determine background staining due to nonspecific F_{ab}

and F_c-mediated antibody binding, samples in each assay were stained with equivalent concentrations of IgG_1 and IgG_{2a} isotype control mAbs against KLH (Becton–Dickinson). Erythrocytes were lysed by the addition of ammonium chloride (155 mM NH_4Cl, 11 mM $KHCO_3$, and 99 μM EDTA in PBS; pH 7.2) to whole blood, and cells were washed twice in PBS and stored at 4°C in PBS containing 1% BSA, 0.1% sodium azide, and 1% paraformaldehyde for less than 1 week prior to analysis.

Flow cytometric data were acquired and analyzed with a Becton–Dickinson flow cytometer in conjunction with Version 1.0 of LYSYS II software (Becton–Dickinson). Ten, twenty, or forty thousand events/sample were acquired to analyze lymphocyte subsets, $CD8^+/45^+$ lymphocytes, and $CD44^+$ or $CD62L^+$ NK cells, respectively. NK cells were phenotypically defined as $CD3^-/CD16^+/CD56^+$.

^{51}Cr-Release Assay

Anti-coagulated whole blood (13 ml per sample) was centrifuged for 15 min at 23°C and 500*g*. Plasma was removed and the buffy coat was aspirated and diluted 1:2 in sterile DPBS, layered over Histopaque (Sigma, St. Louis, MO), and centrifuged for 10 min at 23°C and 1000*g*. Mononuclear cells were recovered and washed twice in HBSS, the percentage of viable cells was determined via trypan blue exclusion (always greater than 95%), and cells were counted with a Coulter counter; 2.5×10^6 viable cells/ml were resuspended in RPMI media containing 10% FBS, and effectors were serially diluted in U-bottom 96-well plates (Nunc; Naperville, IL). K562 erythromyeloid cells (2×10^6), maintained in exponential growth phase, were labeled with 100 μCi ^{51}Cr for 1 h, washed three times in HBSS, counted in trypan blue dye (viability always greater than 95%), and resuspended in RPMI media; 5×10^3 K562 cells/well were added to mononuclear cells, yielding effector:target (E:T) ratios of 50, 25, and 12.5:1. Plates were centrifuged for 5 min at 200*g* and incubated for 4 h at 37°C in 5% CO_2 in air, after which 100 μl supernatant per well was harvested, and radioactivity was determined in a gamma emission counter (Bloom & Korn, 1983). The percentage of specific lysis was calculated by the equation

$$\% \text{ specific lysis} = \frac{[\text{cpm, experimental}] - [\text{cpm, spontaneous}]}{[\text{cpm, maximum}] - [\text{cpm, spontaneous}]} \times 100.$$

Statistical Analyses

A series of two-factor (gender by time) ANOVAs, with repeated measures on the second factor, were computed to determine whether marital conflict discussion was associated with changes in neuroendocrine, cardiovascular, or immunological variables. Specifically, 2×3 ANOVAs were used to analyze cytotoxicity and per-cell killing data; 2×6 ANOVAs were used for flow cytometric data; and 2×7 ANOVAs were used for cardiovascular data. Since published literature has shown that immune functions are influenced by gender-related factors (for example, differences in circulating hormones and responsivity to interpersonal conflict), gender served as a blocking factor in all analyses. Statistically significant main effects and interactions were further analyzed with Scheffé planned comparisons ($\alpha = 0.01$). Due to technical difficulties in collecting blood and recording cardiovascular data, subject number and, hence, degrees of freedom occasionally varied among analyses with equal numbers and levels of factors.

To determine whether health behavior and demographic variables contributed to variance in neuroendocrine, cardiovascular, and immunological parameters measured in the experiment, all ANOVAs were recomputed while covarying out the effects of age, socioeconomic status, education, years of marriage, number of previous marriages, marital satisfaction, number of children, phase of the menstrual cycle, sleep efficiency, aerobic and anaerobic exercise frequency, use of alcohol and drugs, and use of medication. No substantive conclusions were altered by the inclusion of these mediators in ANOVAs, suggesting that changes in physiology during conflict were due to the conflict task itself, rather than to spouses' demographic characteristics or health practices.

RESULTS

Though we did not specifically recruit distressed couples, subjects found the conflict task mildly to moderately stressful: when asked to rate the task from 1 (no stress) to 7 (extremely stressful), men gave it a rating of 2.68 (SD = 1.44) and women gave it a rating of 3.05 (SD = 2.55). The task induced a variety of overtly expressed negative emotions including contempt, anxiety, sadness, and anger, but only anger was reliably associated with changes in physiology (discussed fully in Miller et al., 1999).

Consistent with prior reports (Kiecolt-Glaser et al., 1993; Ewart, Taylor, Kraemer, & Agras, 1991), marital conflict was associated with cardiovascular reactivity. In general, heart rate, systolic blood pressure (SBP), and diastolic blood pressure (DBP) decreased following venipuncture, increased during the conflict task, and decreased again after the task. Specifically, women had higher overall heart rates [$F(1,80) = 5.66$, $p < .05$] but both sexes responded similarly over time [$F(6,460) = 1.09$, ns]: following venipuncture, heart rate decreased nonsignificantly during the baseline period, increased significantly in women [$F(6,240) = 22.93$, $p < .001$] and men [$F(6,240) = 11.29$, $p < .001$] during conflict, and returned toward baseline during recovery (Fig. 2A). Men had higher overall SBPs [$F(1,78) = 11.32$, $p < .001$] but, again, both sexes responded similarly over time [$F(6,468) = 0.90$, ns]: SBP decreased significantly following venipuncture, increased significantly in both sexes during conflict [women: $F(6,234) = 17.69$, $p < .001$; men: $F(6,234) = 6.09$, $p < .001$;], and returned toward baseline during recovery (Fig. 2B). Interestingly, while men and women did not differ in overall DBP [$F(1,78) = 0.17$, ns], they did differ in their responses over time [$F(6,468) = 2.01$, $p < .06$]: women's DBPs increased more precipitously than men's during the first 5 min of the conflict [women: $F(6,234) = 18.41$, $p < .001$; men: $F(6,234) = 12.29$, $p < .001$;] (Fig. 2C). The magnitude of changes in SBP and DBP in this study are in accord with those of a similar study of marital conflict in nondistressed couples (Kiecolt-Glaser et al., 1993).

As previously demonstrated for other acute stressors, marital conflict was temporally associated with the redistribution of specific lymphocyte subsets. No differences over time or between genders were observed in total numbers of circulating leukocytes; numbers and percentages of circulating granulocytes, lymphocytes, or monocytes; or numbers and volume of platelets ($n = 82$ for all measures; data not shown). The same held true for numbers and percentages of circulating $CD19^+$ B cells ($n = 10$; data not shown). In contrast, statistically significant changes over time were observed in T cell subsets, NK cells, and NK-cell AM expression. Specifically, a trend toward overall increased numbers of $CD3^+/CD8^+$ lymphocytes in men approached

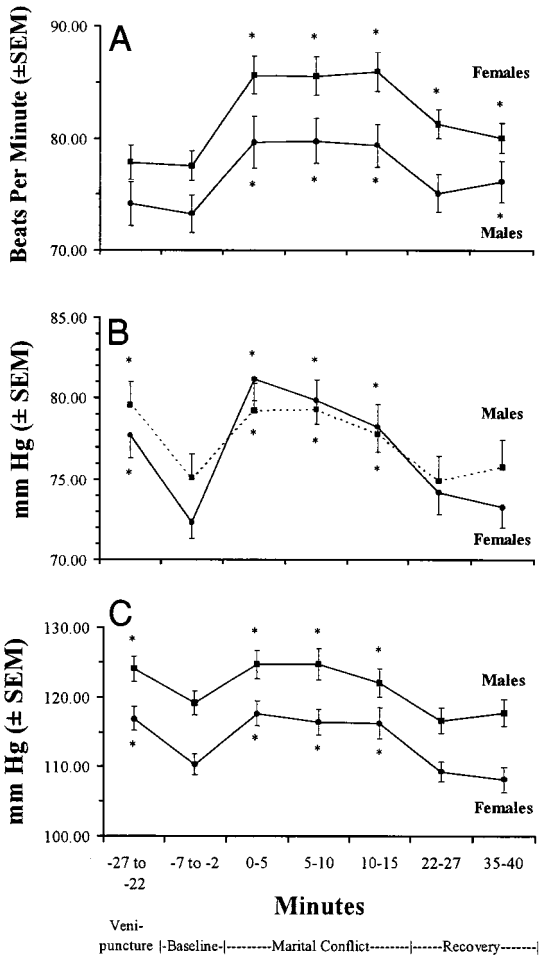

FIG. 2.(A–C) *Marital conflict-induced cardiovascular reactivity.* Heart rate (HR), diastolic blood pressure (DBP), and systolic blood pressure (SBP) were remotely measured before, during, and after marital conflict at the times indicated. Conflict induced significant increases in HR (A), DBP (B), and SBP (C). Compared to men, women showed a significantly more precipitous increase in DBP (B) during the first 5 min of conflict. When comparing across time within a gender, asterisks denote means that differ significantly from the baseline mean (minutes -7 to -2).

statistical significance [$F(1,67) = 2.92$, $p < .09$], but both sexes responded similarly over time [$F(5,335) = 1.78$, ns]: following venipuncture, numbers of circulating $CD8^+$ lymphocytes decreased significantly during the baseline period, increased significantly in women [$F(5,160) = 9.26$, $p < .001$] and men [$F(5,175) = 5.85$, $p < .001$] during conflict, and decreased significantly following conflict (Fig. 3A). Increased numbers of circulating $CD8^+$ lymphocytes, coupled with no change in numbers of circulating $CD4^+$ lymphocytes [women: $F(5,150) = 1.4$, ns; men: $F(5,165) = 0.71$, ns] (data not shown), caused a change in the composition of circulating T cells: the percentage of $CD8^+$ cells increased during conflict (Fig. 3B), whereas the percentage of $CD4^+$ cells reciprocally decreased (Fig. 3C). NK cells showed the

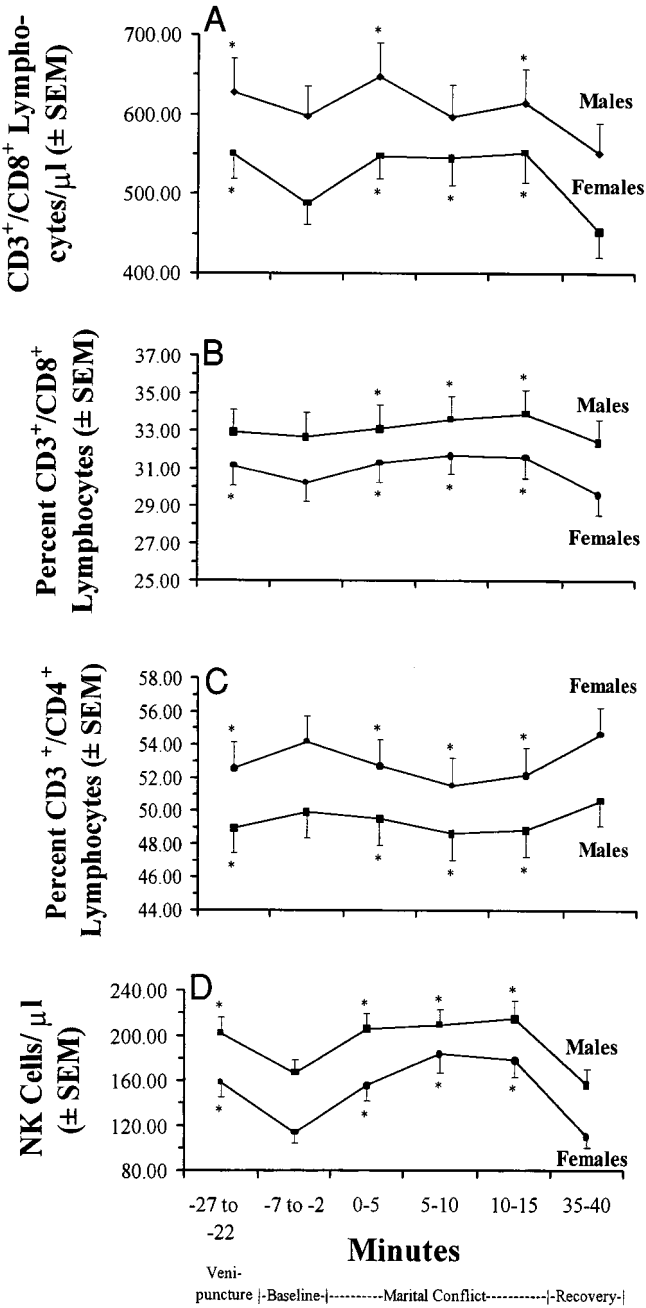

FIG. 3.(A–D) *Trafficking patterns of lymphocyte subsets were altered during marital conflict.* Numbers and percentages of lymphocyte subsets in blood drawn before, during, and after marital conflict were determined by flow cytometry. Marital conflict induced significant increases in numbers (A) and percentages (B) of circulating $CD8^+$ T cells, resulting in a decreased percentage of circulating $CD4^+$ T cells among total T lymphocytes (C). Numbers of NK cells increased markedly during conflict and returned to baseline thereafter (D). When comparing across time within a gender, asterisks denote means that differ significantly from the baseline mean (minutes -7 to -2).

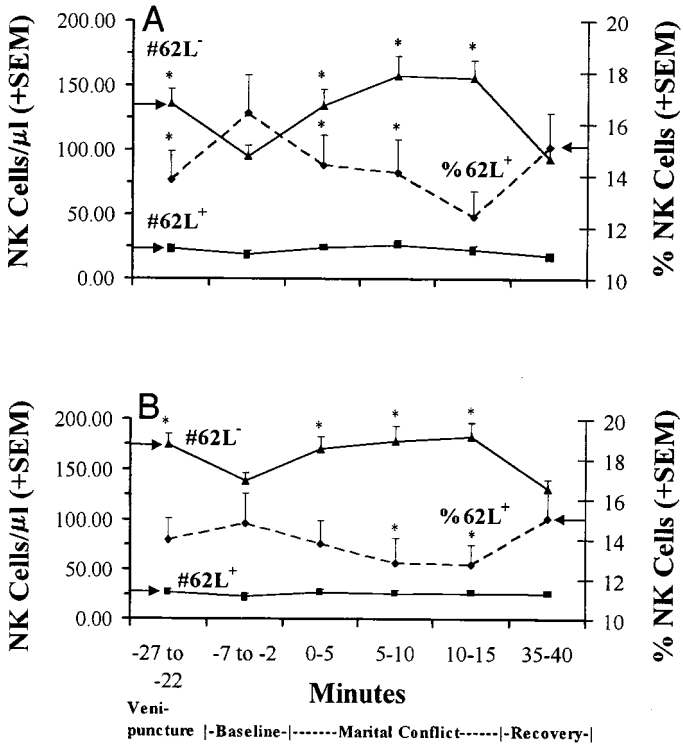

FIG. 4.(A–B) *Decreased percentages of NK cells expressing L-selectin were temporally correlated with NK cell mobilization.* NK cell expression of L-selectin before, during, and after marital conflict was determined by flow cytometry. Marital conflict induced significant decreases in both women (A) and men (B) in percentages of NK cells expressing L-selectin (CD62L; dashed line), concomitant with increased numbers of circulating NK cells (Fig. 3D). Decreased percentages of $CD62L^+$ NK cells were not due to decreased numbers of circulating $CD62L^+$ NK cells (bottom solid line) but, rather, to an influx of circulating $CD62L^-$ NK cells (top solid line). When comparing across time within a gender, asterisks denote means that differ significantly from the baseline mean (minutes -7 to -2).

largest and most consistent changes in relation to marital conflict. Men had higher numbers of circulating NK cells [$F(1,69) = 4.57$, $p < .05$], but both sexes responded similarly over time [$F(5,345) = 1.52$, ns]: following venipuncture, numbers of NK cells decreased significantly during the baseline period, increased significantly in men [$F(5,175) = 12.95$, $p < .001$] and women [$F(6,170) = 20.08$, $p < .001$] during conflict, and decreased significantly following conflict (Fig. 3D).

Because adhesion molecules mediate leukocyte migration into and out of the vasculature, we analyzed AM expression by $CD8^+$ lymphocytes and NK cells to determine whether changes in expression might contribute to the redistribution of these cells during marital conflict. No differences over time or between genders were observed in numbers or percentages of memory ($CD45RO^+$) $CD8^+$ lymphocytes that co-express CD62L, or in NK cells that co-express the hyaluronate receptor (CD44; data not shown). In contrast, the percentage of L-$selectin^+$ NK cells showed an inverse relationship to the number of circulating NK cells during marital conflict: as percentages of circulating $CD62L^+$ NK cells decreased (Figs. 4A and 4B, dashed line), num-

bers of circulating NK cells increased (Fig. 3D). Moreover, men and women did not differ significantly in numbers [$F(1,67) = 0.83$, ns] or percentages [$F(1,67) = 0.03$, ns] or $CD62L^+$ NK cells, and both sexes responded similarly over time [numbers: $F(5,335) = 1.56$, ns; percentages: $F(5,335) = 0.42$, ns]. Further examination of NK-cell subset numbers (Figs. 4A and 4B, solid lines) revealed that decreased percentages of $CD62L^+$ NK cells during conflict were due to an influx of $CD62L^-$ NK cells into the circulation, rather than to a decrease in numbers of NK cells expressing CD62L (e.g., by rapid shedding) or to an extravasation of $CD62L^+$ NK cells out of the circulation. The finding that $CD62L^+$ NK cells did not increase in proportion to the increase in the total number of NK cells suggests that $CD62L^+$ cells were selectively retained in the marginating pool and/or extravascular compartments during acute stress.

To determine whether the increase in numbers of circulating NK cells was associated with a functional change in these cells during marital conflict, we analyzed NK-cell cytotoxicity of the K-562 tumor line. NK cells showed significant increases in cytotoxicity during the marital conflict task. Consistent with higher numbers of circulating NK cells, men approached higher overall NK cytotoxicity than women [E:T = 25:1; $F(1,69) = 3.36$, $p < .07$], but both sexes showed similar changes over time [$F(2,138) = 0.22$, ns] (Fig. 5A). Although NK cytotoxicity increased during conflict, when mathematically adjusted for an increased in-well percentage of NK cells among peripheral blood mononuclear cells (PBMCs), per-cell killing actually decreased nonsignificantly during conflict (Fig. 5B).

DISCUSSION

Using a laboratory model of marital conflict that was perceived as mildly to moderately stressful, we found that conflict discussion was acutely associated with cardiovascular reactivity, the selective mobilization of specific lymphocyte subsets, and the selective retention of others. During conflict, we observed increases in heart rate, blood pressure, circulating $CD8^+$ lymphocytes and NK cells, and NK cytotoxicity, without an increase in per-cell cytotoxicity. $CD62L^-$ NK cells were mobilized but $CD62L^+$ NK cells were not, suggesting that L-selectin might contribute to the retention of subpopulation of NK cells in the marginating pool and/or in lymphoid organs. As a first step in determining individual differences which influence psychological and physiological responses to marital conflict, we analyzed gender. Although when data were collapsed over time, men were lower in heart rate and higher in SBP, NK-cell number, and NK-cell cytotoxicity than women, a significant gender by time interaction was observed only in DBP, where women showed a more precipitous increase than men during the first 5 min of conflict. In related work, which focused on individual differences in the perception of and responses to conflict, we found that cynical mistrust moderated relationships between anger and both cardiovascular reactivity and NK-cell mobilization in men during conflict (Miller et al., 1999).

Collecting data at multiple times before, during, and after marital conflict allowed us to capture the kinetics of physiological responses and extend previous work by others (Kiecolt-Glaser et al., 1993, 1997). First, we showed that alterations in lymphocyte trafficking—increased numbers of circulating NK cells and $CD8^+$ T cells, concomitant with decreased or unchanged numbers of $CD4^+$ T cells and $CD20^+$ B cells—observed in response to a variety of laboratory stressors in humans, occur during marital conflict, an acute psychological stressor encountered in everyday life. Simi-

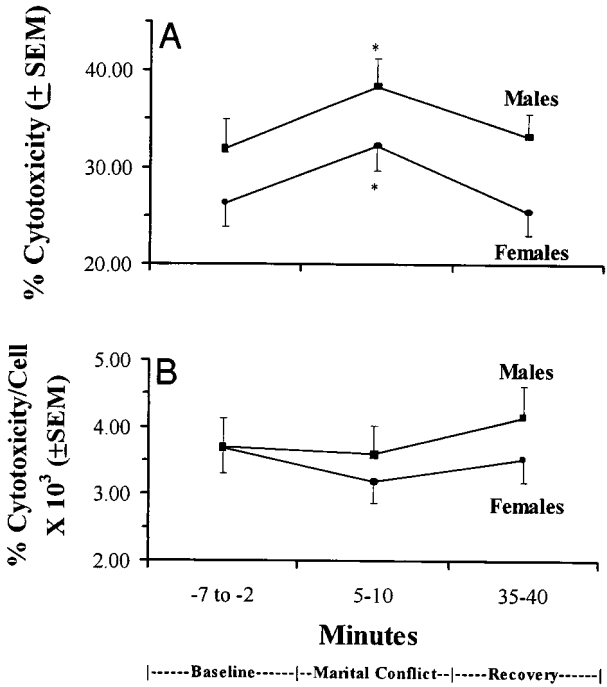

FIG. 5.(A–B) *NK cell cytotoxicity but not mathematically adjusted per-cell cytotoxicity increased during marital conflict.* Mononuclear cells, isolated from blood drawn before, during, and after conflict at the times indicated, were plated in triplicate at 2.5, 1.25, or 0.625 × 10^5/well with 5 × 10^3 ^{51}Cr-labeled K-562 erythromyeloid cells, yielding E:T ratios of 50, 25, and 12.5:1. After 4 h incubation at 37° C, radioactivity in 100 μl of supernatant was determined with a gamma emission counter, and the percentage of specific lysis was calculated. Marital conflict induced significant increases in both genders in NK cell cytotoxicity at all E:T ratios, as depicted by the 25:1 ratio shown (A). However, when mathematically adjusted for an increased in-well percentage of NK cells, per-cell cytotoxicity did not change significantly during marital conflict (B). When comparing across time within a gender, asterisks denote means that differ significantly from the baseline mean (minutes −7 to −2).

larly, we replicated data from a large body of previous studies on acute psychological stress in humans (e.g., Naliboff et al., 1991; Bachen, Manuck, Marsland, Cohen, Malkoff, Muldoon, & Rabin, 1992; Brosschot et al., 1992; Schedlowski, Jacobs, Stratmann, Richter, Hädicke, Tewes, Wagner, & Schmidt, 1993; Benschop et al., 1994; Schedlowski et al., 1994a; Naliboff, Solomon, Gilmore, Fahey, Benton, & Pine, 1995; Benschop, Jacobs, Sommer, Schurmeyer, Raab, Schmidt, & Schedlowski, 1996) and found that NK-cell cytotoxicity increased in concert with NK-cell numbers during marital conflict. To determine whether individual NK cells were actually more cytotoxic on a per-cell basis, we determined per-cell cytotoxicity—mathematically adjusted for an increased in-well percentage of NK cells during conflict—and found that per-cell cytotoxicity did not increase but showed a nonsignificant decrease during conflict. It is important to note that our calculation of per-cell cytotoxicity was entirely mathematical and assumes homogeneous killing among NK cells. This assumption is undoubtedly simplistic, since phenotypically distinct subpopulations of NK cells with heterogeneous killing abilities exist (Nagler, Lanier, Cwirla, & Phillips,

1989; Bennett, Zatsepina, Zamai, Azzoni, Mikheeva, & Perussia, 1996; Jewett & Bonavida, 1996). It is possible that subpopulations of NK cells actually had enhancements in killing mechanisms (e.g., perforin expression, granzyme synthesis, or on/off target rates) during marital conflict, but that such enhancements were diluted in cytotoxicity analyses of unfractionated NK cells. An interesting approach in future assays might be to measure the cytotoxicity of a constant number of NK cells or that of an NK-cell subpopulation, isolated by immunomagnetic beads or fluorescence-activated cell sorting.

In the past decade, significant advances have been made in understanding how circulating leukocytes are recruited toward, and migrate into, an extravascular site of tissue injury or infection. Leukocytes progress through a multistep sequence in interacting with vascular endothelial cells during extravasation. Key steps in this process include loose attachment and rolling toward a chemotactic protein, firm attachment, cellular activation, morphological changes, and transendothelial migration (Springer, 1994). Based on this sequence, we chose to analyze AMs which are both constitutively expressed and mediate the initial stages of leukocyte attachment, reasoning that leukocytes which were unattached or only loosely attached to endothelium would be most susceptible to mobilization. We therefore analyzed the receptor for hyaluronic acid (CD44) and L-selectin (CD62L) and found decreased percentages of NK cells expressing L-selectin during marital conflict. Mills and colleagues also found that leukocyte expression of L-selectin, but not ICAM-1, decreased during a 6-min speaking stressor (Mills & Dimsdale, 1996). They further found that injection of the adrenergic agonist isoproterenol preferentially mobilized $CD62L^-$ versus $CD62L^+$ T cells (Mills, Karnik, & Dillon, 1997). Our data support and extend these findings to NK-cell mobilization during a commonly encountered stressor.

Our finding of stable numbers of $CD62L^+$ NK cells—in the face of decreased percentages of these cells—has several mechanistic implications. First, $CD62L^+$ NK cells were not converted during conflict to $CD62L^-$ NK cells by rapid shedding. Had this occurred, we would have seen a decrease in $CD62L^+$ NK cells that was simultaneous with the increase in $CD62L^-$ NK cells. Such a conversion is actually unlikely because CD62L shedding is coordinated with cytoskeletal rearrangements and only occurs as a cell progresses from loose to firm attachment during extravasation. Furthermore, because only about 15% of NK cells express L-selectin, changes in its expression could not solely account for NK-cell mobilization. Second, $CD62L^+$ NK cells did not rapidly extravasate from the circulation. Instead, decreased percentages of $CD62L^+$ NK cells were caused by an influx of $CD62L^-$ NK cells into the circulation during conflict. The fact that $CD62L^+$ NK cells did not increase in proportion to the increase in the total number of NK cells suggests that these cells were selectively retained in the marginating pool, in secondary lymphoid organs, or both. Hence, $CD62L^-$ NK cells were mobilized while $CD62L^+$ NK cells were retained. A potential parallel may be found in memory versus naive T cells, which have dichotomous patterns of adhesion molecule expression. Naive T cells have a distinct phenotype ($CD2^{lo}/CD11a^{lo}/CD44^{lo}/CD45RA^+/CD62L^+$) and utilize L-selectin during extravasation across HEV and entry into lymph nodes (Bradley, Watson, & Swain, 1994); they recirculate continuously from blood to lymph nodes, where antigen is most likely encountered. In contrast, the absence of L-selectin and the expression of other AMs ($CD2^{hi}/CD11a^{hi}/CD44^{hi}CD45RO^+/CD62L^-$) by memory T cells facilitates their continuous recirculation from blood to tissue to lymph nodes via the afferent

lymphatics (Mackay, Marston, & Dudler, 1990). To follow the parallel, $CD62L^-$ NK cells might be presently or recently activated; mobilization of activated NK cells into the circulation could expedite their response (e.g., cytokine secretion) once they extravasate into a wound site (see below). However, unlike T cells, NK cells are neither antigen specific nor MHC restricted and do not need priming for activation.

If the rapid shedding of L-selectin from NK cells does not account for NK-cell mobilization, what are plausible alternatives? First, a $CD62L^-$ NK-cell phenotype indicates only that the cell does not express CD62L and does not indicate which AMs it *does* express. As discussed above, leukocyte/EC interactions involve multiple and redundant AMs, expressed in exquisitely coordinated sequences. While pilot analyses showed no changes in NK-cell expression of PECAM (CD31), H-CAM (CD44), or ICAM-1 (CD54) during marital conflict (data not shown) other candidate AMs, such as VLA-4 and vascular addressin protein-1 (VAP-1; Salmi, Tohka, Berg, Butcher, and Jalkanen, 1997), were not analyzed. Another possibility is that adhesive interactions which occur prior to L-selectin binding were modulated, namely, interactions between chemokines on ECs and chemokine receptors on NK cells. Chemokines are categorized into two families, C-C (e.g., MIP-1α, RANTES, eotaxin) and C-X-C (e.g., IL-8, NAP-2); they function to attract leukocytes to sites of inflammation and loosely tether them to ECs. Given that NK cells express receptors for several chemokines (Imai, Hieshima, Haskell, Baba, Nigira, Nishimura, Kakizaki, Takagi, Nomiyama, Schall, & Yoshie, 1997; Salazar-Mather, Orange, & Biron, 1998), it is possible that loose interactions between these receptors and EC chemokines are modulated by catecholamines during acute stress. It is also possible that changes in shearing force of the blood and/or changes in AM affinity for endothelial ligands (due, for example, to changes in tertiary protein structure), rather than levels of AM expression or numbers AM-positive cells, regulate NK mobilization.

The reproducibility of NK mobilization across disparate acute stressors prompts the question of why trafficking of this particular lymphocyte subset should be so susceptible to the effects of acute stress. It has been hypothesized from a teleological perspective (Naliboff et al., 1991; Dhabhar, Miller, McEwen, & Spencer, 1995; Benschop et al., 1996) that redistribution of specific leukocyte subsets is a previously unrecognized, integral component of the fight-or-flight response which serves to expedite a specific immune response. Specifically, mobilization of nonspecific phagocytic/cytotoxic leukocytes into the circulation is proposed to be an anticipatory response which confers survival value by decreasing the intervals between infection by a pathogen, and ensuing antigen processing and presentation, cytokine secretion, and T- and B-cell-specific immune responses under circumstances in which an organism is likely to encounter bacterial pathogens (while fighting or fleeing during predator/prey interactions). During marital conflict, we observed increases in numbers of circulating $CD8^+$ T lymphocytes and NK cells which, when translated into values for the entire circulatory system, equate to an additional 3×10^7 and 3.6×10^7 circulating cells, respectively. It has been demonstrated that both bacterial DNA and oligodeoxynucleotides containing unmethylated CG motifs (which are 10 times more prevalent in bacterial than vertebrate DNA) induce NK-cell IFN-γ production (Cowdery, Chace, Yi, & Krieg, 1996) and cytotoxic activity (Ballas, Rasmussen, & Krieg, 1996). Furthermore, NK-cell IFN-γ is important in early macrophage activation (e.g., MHC class II upregulation) against infections by intracellular bacteria (Bancroft, Schreiber, Bosma, & Unanue, 1987). Hence, mobilized NK cells could

conceivably extravasate into a wound site, become stimulated by bacterial DNA, and both activate tissue macrophages and induce (via IFN-γ secretion) vascular endothelial cells to recruit additional leukocytes, thus expediting a specific immune response. In addition or alternatively, activated NK cells might expedite an immune response by trafficking back to secondary lymphoid organs and activating lymphocytes through paracrine cytokine secretion.

In summary, we built upon previous studies and demonstrated that marital conflict is acutely associated with cardiovascular reactivity, mobilization of $CD8^+$ T cells and NK cells, and increased NK-cell cytotoxicity, without an increase in per-cell killing. Mechanistically, $CD62L^+$ NK cells were selectively retained in the marginating pool and/or extravascular tissue, whereas $CD62L^-$ NK cells were mobilized into the circulation. Future mechanistic studies might fruitfully focus on alterations in NK-cell expression of chemokine receptors and/or changes in AM tertiary structure, whereas cytotoxicity studies might include analyses of NK-cell subsets and cytotoxicity effector molecules.

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