Psychosocial Modulation of Cytokine-Induced Natural Killer Cell Activity in Older Adults

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The objective of this study was to address the cellular and psychological mechanisms underlying previously observed changes in natural killer (NK) cell cytotoxicity associated with chronic stress. We compared 28 current and former spousal caregivers of patients with Alzheimer's disease (AD) and 29 control subjects. NK cells were enriched (E-NK) using a 4-step procedure that resulted in a cell preparation consisting of 88.2% NK cells. These cells were then incubated with either recombinant interferon-γ (rIFN-γ) or recombinant interleukin-2 (rIL-2) for 65 hours. Although an average of over 3 years had elapsed since the death of the patient with AD for the former caregivers, current and former caregivers did not differ in the E-NK cell responses to rIFN-γ and rIL-2. However, the E-NK cell response for the combined caregiver group was significantly suppressed compared with controls, which is consistent with a previous report from our laboratory. Higher E-NK cell responses to each cytokine were associated with heightened levels of positive emotional and tangible social support, independent of levels of depression. Preliminary data suggest that defects of NK cell function in response to rIFN-γ and rIL-2 as a consequence of caregiver stress may be independent of non-NK cells. Finally, our data are consistent with other studies regarding the role of social support in immune modulation.

Key words: chronic stress, social support, NK cytotoxicity.

INTRODUCTION

Although the mental health consequences of caregiving for a family member with Alzheimer's disease (AD) have been well documented (1), the physical health consequences of caregiving have only recently begun to be explored. Longitudinal data from our laboratory suggest that the persistent stress of caring for a family member with AD is associated with poorer immune and physical health outcomes compared with demographically matched, noncaregiving control subjects (2–4). Specifically, compared with matched control subjects, caregivers have impaired proliferative responses to concanavalin A (Con A) and phytohemagglutinin (PHA), as well as higher antibody titers to latent Epstein-Barr virus; each of these results reflects down-regulation of cellular immunity (3). This immunological down-regulation may be clinically important given that caregivers also reported significantly more days of infectious illness, primarily upper respiratory tract infections, compared with their noncaregiving counterparts (4). Moreover, caregivers who reported lower levels of social support at intake into the study showed greater immunological down-regulation 1 year later. Finally, our caregiver model is consistent with other models that have examined the effect of chronic stress on the cellular immune response (5), highlighting the relationship between chronic stressors and immune function.

In addition to cellular immune function, another mechanism believed to link chronic stress and health involves natural immunity and the natural killer (NK) cell. NK cells play an important role in a variety of immune functions, including defense against viral infections and surveillance of tumor cells, and may be particularly important in the control of metastases (6–8). Many studies have shown that interleukin-2 (IL-2) and interferon-γ (IFN-γ) can mediate the augmentation of NK cell cytotoxicity (9–11). Although both IFN-γ and IL-2 activate NK cells, their actions are both quantitatively and qualitatively different. For example, once stimulated with IL-2, NK cells begin to express the IL-2Rα chain and so acquire high affinity receptors. IL-2-stimulated NK cells have enhanced cytolytic activity (i.e., can kill a broader spectrum of tumor targets than can be killed by untreated NK cells) and secrete cytokines, including IFN-γ (12). IL-2 induces NK cells to become lymphokine-activated killer cells (LAK), which are characterized by this enhanced cytotoxicity. In contrast, IFN-γ increases the activity of NK cells, resulting in more efficient lysis of target

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cells (13), as well as enhancement of the recruitment of pre-NK cells and the kinetics of lysis (14, 15).

Current evidence suggests that stress can downregulate NK cell activity (16–18) and modulate IFN-γ and IL-2 synthesis (17, 19). For example, heightened levels of stress have been related to decreased synthesis of IFN-γ *in vitro* by peripheral blood leukocytes (PBL) from healthy subjects (17, 19). In addition, stress reduction interventions have been shown to modulate NK cell activity, T lymphocyte proliferation to PHA, and cytokine synthesis (20, 21). Fawzy and colleagues (21) have shown that a group intervention significantly increased IFN-α-augmented NK cell cytotoxicity in malignant melanoma patients, with parallel decreases in anxiety and depression. Therefore, the literature suggests some interactive relationship between stress and NK cell function that may be mediated, in part, by cytokines.

Previously, we demonstrated that caregiving for a spouse with AD was associated with a significantly poorer response of NK cells to recombinant IFN-γ (rIFN-γ) and recombinant IL-2 (rIL-2) *in vitro* compared with control subjects who did not have any caregiving responsibilities (2). This effect was independent of both the percentage of NK cells and the cell surface marker CD56 receptor density in PBL samples. Moreover, former caregivers, that is, those caregivers whose AD spouse had died an average of 2 years earlier, did not differ from continuing caregivers (22). The present study sought to explore the cellular and psychological mechanisms that might explain our previous findings. We hypothesized that, if the previously observed differences in NK cell activity were due to cellular factors produced by one or more subpopulations of white blood cells, removal of non-NK cell populations would affect the NK cell response to one or both cytokines. Second, depression and social support have been shown to be associated with immune modulation in previous studies, so we hypothesized that the observed differences in NK cell activity would be explained, in part, by these variables.

METHODS

Subjects

Subject Recruitment. Subjects for this study were part of a larger longitudinal study of caregiver stress, health, and immune function (3, 23). At intake, all caregivers were caring for a spouse with AD or a related dementing illness. For study entry, caregivers had to be providing 5 or more hours of care per week. Subjects were recruited from multiple sources in the Columbus, Ohio area, including local dementia evaluation centers in area hospitals, neurologists' referrals, the city's Alzheimer's Association (AA) support groups, the monthly AA newsletter, respite care programs, and governmental caregiver support programs.

Control subjects were recruited through newspaper advertisements, senior citizen centers, area newsletters, church groups, university alumni publications, and referrals from other participants; potential control subjects who reported any caregiving activities were excluded. All subjects gave written informed consent for participation after the procedures were explained.

Subject Characteristics. The first 57 subjects scheduled for their 6th annual appointment from the larger study comprised the sample for this study; the 57 subjects included 11 current caregivers, 17 former caregivers whose impaired spouse had died within the last 6 years, and 29 control subjects. The majority (90%) were Caucasian. The average annual family income, between $20,000 and $30,000, did not differ among the groups. The majority of the subjects had at least some college education. There were no significant differences between the current and former caregivers and controls in education [$F < 1$, race, $\chi^2(2) = 0.21$; gender, $\chi^2(2) = 0.92$]. However, current caregivers (mean = 71.5 years, SEM = 2.97) were older than former caregivers (mean = 61.9 years, SEM = 3.20), and neither differed from controls (mean = 68.9 years, SEM = 1.51; $F(2, 54) = 3.68$, $p < .05$).

Current caregivers, defined as those caregivers who remained active in the caregiving role, included four men and seven women. Current caregivers had been providing care for an average of 9.6 years at the time of this study. They reported currently spending an average of 4.2 hours/day in caregiving activities, with approximately half (45.5%) of the patients living with the caregiver and the rest (54.5%) residing in nursing homes. Former caregivers, those whose relatives had died in the interval between intake into the study and Year 6, included six men and 11 women. The average length of time since the death of their impaired relative was 36.6 months (SEM = 4.46). The control group was comprised of seven men and 22 women. Comparison group subjects did not have any kind of caregiving responsibilities. Although some subjects participated in both studies (20%), subjects in the present study were recruited, blood was drawn, psychological assessments were given, and immune assays were performed independently from that of our previous study (2).

Psychosocial Measures

Depression. The severity of depressive symptoms in subjects was assessed using the Beck Depression Inventory-Short Form (24, 25). The Beck is sensitive to mild to moderate levels of depression (26). The 13 items correspond to 13 symptom-attitude categories associated with depression, such as mood, pessimism, body image changes, sense of failure, and dissatisfaction (24). Studies show that the Beck is an internally consistent, stable, and valid depression measure (25, 27), including for the elderly (28).

The *Perceived Stress Scale* (29) is a 10-item scale that was designed to measure the degree to which individuals appraise situations in their life as stressful and includes items that assess perceptions of daily life as unpredictable, uncontrollable, and overloading and dimensions of particular concern in caregiving. In addition, there are norms for a variety of age groups.

Social Support. The Social Support Interview (3, 30) asks subjects to "list the people in your life who are important to you, with whom you have contact, whether or not you like them" up to a total of 10. Subjects rate the degree to which they perceive each of the relationships to be helpful and upsetting/troubling (0 = not at all, 6 = extremely) with respect to both emotional and tangible

assistance. For each person named, subjects rated the frequency of contacts from daily (5) to less than monthly (1) and closeness from not at all close (0) to extremely close (10).

Health-Related Behaviors

Potential immunomodulatory confounds, such as general health status, medication use, nutritional status, caffeine intake, cigarettes smoked, sleep, and alcoholic drinks consumed, were assessed. Nutritional status was assessed by measuring serum albumin levels as previously described (31). Self-report questionnaires assessed general health status, amount of caffeine and alcohol intake in the past 48 hours, smoking, and amount of vigorous physical exercise in the past week. In addition, major medications that could have significant immunomodulatory effects were assessed. These included β-blockers, diuretics, and analgesics. No subjects had health problems or conditions with an immunological component (e.g., cancer or recent surgery) or were using other drugs that had obvious immunomodulatory effects.

Immunological Assays

NK Cell Enrichment. To control for diurnal variation, blood samples were collected between 8:00 AM and 10:00 AM. Peripheral blood leukocytes were isolated on Hypaque-Ficoll gradients, washed, and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.75% $NaHO_3$, 2 mM L-glutamine, and 25 μg/ml gentamicin. This procedure resulted in the elimination of all but approximately 3% granulocytes and less than 0.5% platelets. Cell suspensions were then enriched for NK cells by a 4-step separation procedure. This involved incubating 50×10^6 cells in serum-free DMEM medium for 45 minutes at 37°C to remove the macrophage population (32) and sequentially incubating the cell suspension with uniform, magnetizable polystyrene beads (Dynal, Great Neck, NY) coated with a primary monoclonal antibody specific for CD4 and CD8 (T lymphocytes) and B4 (B lymphocytes). Cells binding to the polystyrene beads were removed from the cell suspension by a magnet. Determinations of the purity of NK cell preparations were performed by flow cytometry; the percentage and identification of any other cell contaminant were also determined.

Treatment of Enriched Natural Killer Cells with rIL-2 or rIFN-γ. Enriched NK cells (E-NK) were seeded (2.3×10^5 cells/ml) in three replicate wells in 96-well V bottom tissue culture plates. Cells were then provided with either complete RPMI 1640 medium alone, RPMI 1640 medium supplemented with 60 IU/ml rIL-2 (Genzyme Corp, Cambridge, MA), or RPMI 1640 medium supplemented with 250 IU/ml rIFN-γ (Genzyme Corp). These concentrations had been previously determined by standard dose-response relationships in separate experiments in our laboratory, which provided the minimum dose needed to produce a maximal effect (2). Cell suspensions with cytokines were gently mixed and incubated at 37°C in an atmosphere of 5% CO_2 for 65 hours.

NK Cell Assay. A standard microtiter ^{51}Cr release cytotoxicity assay was used to determine NK cell cytotoxicity (2). The target cells used in the assay were K-562 cells, an NK cell-sensitive myeloid cell line. After the 65-hour incubation with rIFN-γ or rIL-2, E-NK cells were washed three times in complete RPMI 1640 medium. Triplicate aliquots of E-NK cells and K-562 cells incubated for 16 hours with ^{51}Cr were placed in wells of 96-well V plates (Linbro, CN), resulting in effector-to-target (E:T) cell ratios of 5:1, 2.5:1, and 1.25:1. Although these ratios were smaller than would be expected using whole blood preparations, the total effector cell ratio represented almost exclusively NK cells, whereas in previous studies the effector ratio represented NK cells as well as all other lymphocytes in the cell preparation (e.g., macrophages, T and B lymphocytes). NK cells make up approximately 10% of the total lymphocyte population (11), so these ratios closely approximated the ratios seen in previous studies with respect to numbers of NK cells in the assay preparation.

To control for spontaneously released radioactivity and maximal lysis, respectively, 6 wells with target cells and either media or detergent (1% sodium dodecyl sulfate) were prepared. Cell suspensions were centrifuged at 1000 rpm (200 × g) for 5 minutes to bring the effector and target cells into contact. Cells were incubated at 37°C in an atmosphere of 5% CO_2 for 5 hours. After the 5-hour incubation, the plates were centrifuged at 1500 rpm (300 × g) for 5 minutes, 100 ml of each supernatant was collected, and cpm was determined in a Beckman 9000 γ counter (Fullerton, CA). Results are reported as the percentage of lysis adjusted for media control values using the formula:

Change in percentage of lysis

$$= \frac{\text{Exp}_c\text{ CPM} - \text{SR}_c\text{ CPM}}{\text{MR}_c\text{ CPM} - \text{SR}_c\text{ CPM}} \times 100 - \frac{\text{Exp}_m\text{ CPM} - \text{SR}_m\text{ CPM}}{\text{MR}_m\text{ CPM} - \text{SR}_m\text{ CPM}} \times 100$$

where Exp_c = experimental $\text{CPM}_{\text{cytokine}}$, SR_c = spontaneous $\text{CPM}_{\text{cytokine}}$, MR_c = maximum $\text{CPM}_{\text{cytokine}}$, Exp_m = experimental $\text{CPM}_{\text{media}}$, SR_m = spontaneous $\text{CPM}_{\text{media}}$, and MR_m = maximum $\text{CPM}_{\text{media}}$.

This procedure permitted the determination of the effectiveness of rIL-2 and rIFN-γ to stimulate E-NK cell cytotoxicity over baseline levels found in the standard NK cell cytotoxicity assay. The data are presented as percentage of lysis rather than lytic units because the models for determining lytic unit values incorporate assumptions and methods of calculation that can result in inaccurate model-predicted cytotoxicity in comparison with actual observed cytotoxicity (33).

Lymphocyte Quantitation. Percentages of NK cells, macrophages, T4 and T8 lymphocytes, and B lymphocytes were determined by standard flow cytometric procedures. In brief, 5×10^5 cells were adsorbed with monoclonal antibody conjugated to FITC against the cell surface marker CD56, MO_2, CD4, CD8, or B4 (NK cells, macrophages, T4, T8, and B cells, respectively) for 30 minutes at 4°C. Cells were then washed, fixed with paraformaldahyde, and analyzed using an EPICS C flow cytometer (Coulter Corp, Hialeah, FL).

Data Analysis

We used repeated measures multivariate analyses of variance (MANOVA) to assess differences among current and former caregivers and controls on the E-NK cell cytotoxic response to cytokines. Additionally, a series of one-way ANOVAs comparing current and former caregiver groups and control subjects on measures of depression and social support were conducted. When gender was included as a second between-subjects factor, neither the main effect for gender nor the gender by group interactions approached significance ($Fs < 1$). Finally, hierarchical multiple regression analyses were conducted to assess the contribution of social support and depression to the E-NK cell response to each cytokine.

RESULTS

Enrichment of NK Cells

To determine the extent to which our enrichment procedure resulted in E-NK cells, the enriched cell suspension was analyzed using flow cytometry to determine the percentages of macrophages and T4, T8, B, and NK cells. The final suspension consisted of 88.2% (SEM = 4.5%) NK cells and 1.7%, 3.9%, 2.3%, and 0.34% macrophages and T4, T8, and B cells, respectively.

Effect of rIFN-γ and rIL-2 on E-NK Cell Activity

Percentage of lysis, adjusted for media control values, was calculated for rIFN-γ and rIL-2 stimulation. A 2 (group membership) × 3 (E:T) repeated measures MANOVA identified significant group differences across the different E:T cell ratios with respect to both rIFN-γ [$F(3, 53) = 2.85$, $p < .05$] and rIL-2 [$F(3, 53) = 2.80$, $p < .05$]. Specifically, current and former caregivers had a significantly poorer response to both rIFN-γ and rIL-2 compared with control subjects, and this effect was significant at each E:T ratio (Fig. 1). Current and former caregivers were compared in a post hoc manner to determine whether former caregivers had "recovered" compared with current caregivers. No differences were found between the current and former caregivers with respect to the response of E-NK cells to rIFN-γ or rIL-2 ($Fs < 1$).

E-NK cell cytotoxicity, without cytokine induction, was measured to examine group differences between the current and former caregiver group and controls. Consistent with our previous study and data from Irwin et al. (34), no significant group differences were observed ($F < 1$) (Fig. 2).

Stress, Social Support, and NK Cell Response to Cytokines

Differences between current and former caregivers were assessed in a MANOVA that included social closeness, positive emotional and tangible support, upsetting emotional and tangible support, and the number of people in the caregivers' network. The correlation between tangible and emotional social support was very high in our sample ($r = .73$, $p < .001$), so a summary social support measure was computed by summing the values of these two variables. Overall, current and former caregivers reported less social support than controls [$F(4, 52) = 2.55$, $p < .05$]. As seen in Table 1, the results of subsequent ANOVAs indicated that current and former caregivers reported fewer social contacts compared with controls [$F(1, 55) = 5.92$, $p < .01$]. In addition, current and former caregivers reported lower levels of positive emotional and tangible support compared with control subjects [$F(1, 55) = 7.34$, $p < .01$]. Finally, current and former caregivers reported marginally lower amounts of closeness in their relationships [$F(1, 55) = 2.79$, $p < .10$]. No differences were found between the caregiver group and controls with respect to upsetting social support [$F(1, 55) = 2.03$].

Post hoc comparisons of current and former caregivers were conducted to determine whether the absence of caregiving responsibilities was associated with improvements in the social networks of former caregivers. No differences were found between the current and former caregivers on any of the social

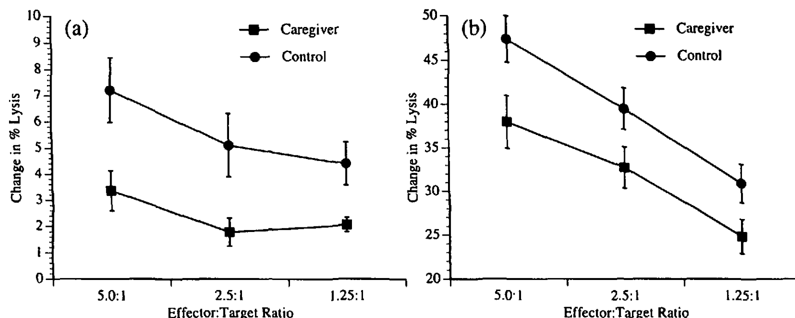

Fig. 1. The response of E-NK cells (±SEM) from current and former caregivers and controls to (*a*) 250 IU/ml rIFN-γ and (*b*) 60 IU/ml rIL-2.

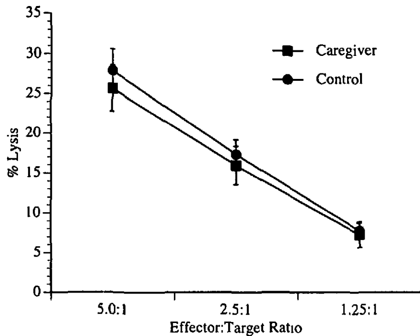

Fig. 2. The response of E-NK cells (±SEM) from current and former caregivers and controls.

TABLE 1. Psychosocial Values Across Caregiver Groups (Mean ± SEM)

	Current Caregivers	Former Caregivers	Control
Closeness	53.64 (4.18)	43.24 (5.61)[b]	56.31 (3.76)**
Contacts (N)	6.55 (0.56)	5.00 (0.56)[b]	7.03 (0.41)*
Positive support[a]	47.64 (4.64)	47.24 (6.05)[b]	61.79 (3.47)*
Upsetting support[a]	21.27 (2.55)	17.35 (2.98)[b]	23.38 (2.36)
Beck	6.27 (1.48)	5.52 (1.77)[b]	4.14 (0.94)

[a] Emotional and tangible social support.
[b] No significant difference between current and former caregivers.
* $p < .01$ between total caregivers and controls; ** $p < .10$ between total caregivers and controls.

support dimensions, that is, current and former caregivers reported similar closeness in their supports [$F(1, 26) = 1.79$] and similar levels of positive and upsetting emotional and tangible support (Fs < 1). Although current caregivers reported marginally greater numbers of social contacts [$F(1, 26) = 3.49$, $p < .07$], most of the current caregivers named their impaired spouse among their network members. Thus, although current caregivers listed an average of one more person than former caregivers, the effective difference in network size is negligible (Table 1).

Using a strategy similar to that used in our previous study (2), we used a median split to divide subjects for both rIFN-γ (2.5% increase over media control) and rIL-2 (27.0% increase over media control). This grouping created four groups that were classified as low responders to both cytokines (N = 15), high responders to both cytokines (N = 14), and those subjects who were either low responders to rIFN-γ and high responders to rIL-2 (N = 16) or high responders to rIFN-γ and low responders to rIL-2 (N = 12). Because our current and former caregivers did not differ with respect to any of the social support variables or E-NK responses to either cytokine, current and former caregivers were combined into a single, chronically stressed group. When comparing chronically stressed and nonstressed subjects and their response to the two cytokines in a 4 (low response to rIFN-γ and rIL-2, high response to rIFN-γ and rIL-2, or differential response to both cytokines) × 2 (caregiver vs. control) factorial design, significant differences in distributions were found [χ^2 (3, N = 57) = 7.82, $p < .05$]. Table 2 contains the distribution of subjects along these dimensions, with caregivers highly skewed toward the low rIFN-γ/low rIL-2 end and controls highly skewed toward the high rIFN-γ/high rIL-2 end.

When the caregivers were further divided into either low cytokine responders (i.e., responding below the median to both rIFN-γ and rIL-2) or high cytokine responders (i.e., responding above the median to either or both cytokines), low cytokine responders reported fewer positive emotional and tangible supports (mean = 37.73, SEM = 5.21) compared with the high cytokine responders [mean = 53.65, SEM = 5.30; $F(1, 26) = 4.14$, $p < .05$]. Low and high cytokine responders did not differ on upsetting emotional and tangible supports [$F(1, 26) = 2.92$], numbers of people in their network ($F < 1$), or the level of closeness in their supports ($F < 1$).

Finally, because positive social support and group membership appeared to be important variables in the E-NK cell response to cytokines, we used hierarchical multiple regression analyses to separately analyze their predictive properties. Two regression equations were computed; the first equation evaluated this association with respect to rIFN-γ and the second with respect to rIL-2. With respect to each equation, age was entered on the first step. Group membership was entered on the second step, with current and former caregivers coded as 0 and controls as 1. The summary positive social support scores were entered on the third step. The final variable, entered on the last step, was the interaction

TABLE 2. E-NK Cell Response to rIFN-γ and rIL-2 and Group Membership

	Low rIFN-γ		High rIFN-γ		Total
	Low rIL-2	High rIL-2	Low rIL-2	High rIL-2	
Chronic stress	11	8	6	3	28
Control	4	8	6	11	29
Total	15	16	12	14	57

between positive social support and group membership because previous studies have shown that support might be differentially important between the groups (3).

As seen in Table 3, the overall model with all variables was significant [$F(4, 52) = 3.36, p < .01$] and accounted for 21% of the variance in the E-NK cell response to rIFN-γ. As expected from earlier analyses, the E-NK cell response to rIFN-γ was significantly predicted by group membership, accounting for 14% of the explained variance in the E-NK cell response. Specifically, current and former caregivers showed significantly poorer responses compared with controls. The main effect of social support did not appear to make a significant contribution with respect to the E-NK cell response to rIFN-γ. However, the interaction between social support and group membership was marginally significant and accounted uniquely for 5% of the explained variance in the E-NK cell response to rIFN-γ, reflecting the relatively greater importance of support for caregivers.

A slightly different picture emerged with respect to the E-NK cell response to rIL-2. That is, the overall model with all variables was significant [$F(2, 54) = 2.53, p < .05$] and accounted for 16% of the variance in the E-NK cell response to rIL-2 (Table 4). As with rIFN-γ, the E-NK cell response to rIL-2 was significantly predicted by group membership, accounting for 6% of the explained variance in the E-NK cell response. Specifically, current and former caregivers showed significantly poorer responses compared with controls. In contrast to the effect of rIFN-γ, subjects lower in social support had E-NK cells that responded marginally less well to rIL-2 compared with subjects higher in social support and accounted for 6% of the unique variance in E-NK cell cytotoxicity. Furthermore, the interaction between social support and group membership was not a significant predictor of E-NK cell activity in response to rIL-2.

We used an identical equation with Beck depression scores entered after group membership to assess the possibility that the contributions of social support to immunity simply reflected differences in depression. Although depression did provide a significant contribution to the variance in E-NK cell response to rIL-2 ($t = -2.83, p < .01$), it did not make even a marginal contribution to the variance in rIFN-γ ($t < 1$). However, after controlling for the contributions of depression in each equation, the contributions of social support and the group by social support interaction were unchanged.

No differences were noted between our groups and levels of perceived stress [$F(2, 54) < 1$]. However, continuing caregivers who were still living with their spouse had significantly lower E-NK cell responses to rIL-2 compared with continuing caregivers who had placed their spouse in a nursing home [$F(3, 7) = 5.98, p < .02$]. No differences were found between these two groups on the E-NK cell response to rIFN-γ although the continuing caregivers who were still living with their spouse tended to have lower E-NK cell values [$F(3, 7) = 1.71$].

TABLE 3. Hierarchical Multiple Regression Analysis Predicting E-NK Cell Response to rIFN-γ (1.25:1 E:T Ratio)

Independent Variables	Cumulative					
	β	r	R²	t	df	p
Step 1						
Age	−.11	−.11	.01	−.84	(1,55)	NS
Step 2						
Group	.36	.34	.14	2 83	(2,54)	.01
Step 2						
Social support	.14	.23	.16	1.00	(3,53)	NS
Step 3						
Social support × group interaction	.77	.41	.21	1.79	(4,52)	.07

TABLE 4. Hierarchical Multiple Regression Analysis Predicting E-NK Cell Response to rIL-2 (1.25:1 E:T Ratio)

Independent Variables	Cumulative					
	β	r	R²	t	df	p
Step 1						
Age	.16	.16	.03	1.24	(1,55)	NS
Step 2						
Group	.26	.28	.09	2.00	(2,54)	.05
Step 2						
Social support	.24	.32	.15	1.79	(3,53)	.07
Step 3						
Social support × group interaction	.45	.36	.16	1.03	(4,52)	NS

Health Behaviors

Current and former caregivers and controls did not differ on health-related behaviors. Pearson correlations, computed among LAK/NK cell cytotoxicity and serum albumin levels, caffeine intake, cigarettes smoked, alcoholic drinks consumed, and sleep, were not significant. No significant differences were found with respect to use of β-blockers, diuretics, analgesics, or antihistamines between current and former caregivers and controls, with all groups reporting minimal usage.

DISCUSSION

To our knowledge, this is the first study to examine the impact of psychological stressors on an

enriched subpopulation of leukocytes in humans. This is significant in that it adds to the understanding of possible mechanisms underlying physiological changes associated with psychological stress. Previous data from our laboratory suggested that the chronic stress of caregiving altered functional parameters of the immune response, including the NK and LAK cell cytotoxic response after incubation with either rIFN-γ or rIL-2; this effect persisted an average of 2 years after the death of the spouse (2). If the previously observed differences in the ability of NK cells to respond to rIFN-γ or rIL-2 were due to extracellular (non-NK cell) factors, removal of non-NK cells should restore the NK cell response to these cytokines. However, consistent with our previous results, the present findings using cell populations significantly depleted of T lymphocytes, B lymphocytes, granulocytes, and macrophages were similar to data from our previous study.

Although an average of over 3 years had elapsed since the spouse's death, current and former caregivers did not differ in the response of E-NK cells to rIFN-γ or rIL-2; together, both caregiver groups had a significantly poorer response to both cytokines compared with controls at each E:T ratio. These data suggest that the previously observed "defect" in the NK cell's response to these cytokines in current and former family caregivers of patients with AD was probably related to direct effects on NK cells.

Consistent with work from our laboratory and others (2, 34), we found no differences in NK cell cytotoxicity between either the current and former caregiver groups or control subjects in the absence of cytokine stimulation. Similarly, animal studies have shown that chronic stress has significant effects on T cell proliferation and IL-2 secretion but no effects on NK cell cytotoxicity (35). These findings suggest that stress-related down-regulation of the NK cell cytotoxic response may be related to physiological changes associated with stressor chronicity.

We continued to find immune down-regulation 3 years after the death of the AD patient, suggesting that the chronic stresses of caregiving may have far-reaching and potentially important physiological implications. Although the relatively small number of former caregivers makes it difficult to draw inferences about possible mediational processes, one mechanism may be social support. In particular, we find that former caregivers have fewer contacts, less closeness, and less positive support compared with controls. In fact, after an average of 36.6 months postbereavement, we found no differences between our former and current caregivers on any social support dimension. Although speculative, this may suggest that, after the death of the AD spouse, former caregivers are not reintegrating with society and are remaining distanced from others around them. This persistent lack of social support has been found previously to sustain chronic stress in older adults by denying the affected individuals the opportunity for social feedback and reassurance of worth and nurturance and by decreasing their self-esteem (36–38).

Previously, we found that caregivers responding below the median to both rIFN-γ and rIL-2 reported less positive emotional and tangible support and rated less closeness in their relationships compared with those caregivers who responded above the median to either or both cytokines (2). This study extends these results by both replicating these previous data as well as by demonstrating the predictive relationship between positive emotional and tangible social support and the E-NK cell response to two cytokines in both current and former caregivers as well as in subjects who have no caregiving responsibilities. Together, these data continue to support evidence of a possible physiological mechanism through which personal relationships may affect health (39).

Subjects higher in depression had lower enriched NK cell cytotoxic responses to rIL-2 but not rIFN-γ. This result is consistent with that of other research demonstrating that depression levels are related to NK cell cytotoxicity (34, 40, 41) and poses an additional mechanism that might mediate such an effect. In particular, depression was only related to rIL-2 stimulation, so depression may be associated with NK cell function through IL-2-mediated pathways of cytotoxicity. Future studies are necessary to better understand this interaction.

We found the same NK cell effects in response to the chronic stress of caregiving and/or bereavement, whether non-NK cells were present or not, and this suggests that non-NK cells played a minimal role in the stress-induced immunosuppression that was observed in our previous study (2). This suggested that certain abnormalities or defects may be present in the NK cell. Although this study was not designed to dissect the NK cell defects but to determine whether the reduced response to cytokines was due to factors from non-NK cells or within the NK cell itself, several hypotheses can be generated to explain the observed NK cell effects across these studies. For example, previous studies have shown that persons experiencing chronic stress have a more activated hypothalamic-pituitary-adrenal (HPA) axis compared with controls as demonstrated by chronically elevated levels of cortisol, epinephrine, and norepi-

nephrine (5, 42–45). This chronic activation may lead to greater concentrations of these stress products in lymphoid organs where NK cells reside. If this is the case, it is possible that receptors on the NK cell may be altered or blocked, leading to poorer binding of specific cytokines necessary for NK cell activation (especially IFN-γ and IL-2). This is supported by evidence that suggests that these HPA products bind to lymphocytes, including NK cells, to alter cellular function (46–48). If the NK cells are not able to efficiently bind these cytokines, their killing efficiency will be greatly reduced, leading to poorer cytotoxicity reactions. Alternatively, cytokines may be able to efficiently bind to NK cells; however, once bound, the receptor may be unable to activate the appropriate biochemical pathways within the NK cell to trigger the activated, or killer, state. These issues may be understood in future studies by examining NK cell receptor integrity by performing competing analyses and following NK cell function as a consequence of the subject's exposure to chronic stress.

Although we can account for most of the PBL, we cannot completely rule out that one or more of the residual subpopulations of PBL are involved in the NK cell response to the cytokines. However, if this were true, then the significant enhancement of NK cell lysis observed would have to be influenced by a very small number of cells. In addition, we would expect at least some modulation of the effect of the cytokines on the E-NK cells in this study compared with NK cells in PBL samples treated with both cytokines in our previous study (2).

In our previous study (2), it was observed that the change in percentage of lysis was much higher in comparison to the present data. This observation is not entirely surprising because the conditions of the two experiments were quite different. That is, in the first study, the PBL containing NK cells were used. This was not the case in the second study. Specifically, the E-NK cells' cytokine response, by definition of the study protocol, was controlled and determined by the exogenous incubation of cytokine. Future studies will need to be performed to determine the nature of the changes induced by stress on NK cell function.

In summary, our major hypothesis regarding reduced E-NK cell cytotoxicity to cytokine stimulation in current and former caregivers was supported by our findings; these data provide some explanation of the NK cell differences (with respect to stress-associated alterations in the response of NK cells to rIFN-γ and rIL-2), which may be independent of interactions with other leukocytes. This hypothesis also suggested that physiological adaptation did not occur; although an average of over 3 years had elapsed since the death of the patient with AD, current and former caregivers did not differ in their E-NK cell response to either cytokine, and their E-NK cell responses as a whole were significantly suppressed compared with controls.

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