Original article

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Association of overtime work with cellular immune markers among healthy daytime white-collar employees

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Objective Even though overtime work has been suspected to be a risk factor for ill health, little research has been done to determine the underlying immunological mechanisms. This study investigated the association between overtime work and cellular immunity among Japanese white-collar workers.

Methods A total of 306 healthy, full-time, non-shift, daytime employees (165 men and 141 women), aged 22–69 (mean 36) years, provided a blood sample for the measurement of circulating immune [natural killer (NK), B, and T] cells and NK cell cytotoxicity (NKCC) and completed a questionnaire survey including overtime/month. Blood samples were collected between 09.00–11.00 hours during working days and participants completed the questionnaire within the two weeks prior to the blood sampling. Stepwise linear regression analyses controlling for confounders were carried out to examine the relationship between overtime work and immune markers.

Results Overtime work was mainly related to short sleep duration, increased weight, and reduced job satisfaction, and it was more prevalent among men than women and among younger and married employees. Amount of overtime was inversely associated with NK (CD3-CD56+) cell counts (ß=-0.145; P =0.032) but was not associated with NKCC, NKCC/NK cell ratio, or T or B cells.

Conclusions The NK cell is a lymphocyte that possesses killer activity against tumor and virus-infected cells and constitutes a major component of the innate immune system. A decrease of NK cell counts from overtime work suggests a dampened innate immune defense. However, the finding needs to be further validated with a well-designed study using objective overtime measures.

Key terms immune system; natural killer cell; occupational health; overtime work; work condition.

Long work hours and extended overtime have been, and continue to be, of enormous concern for the health and well-being of working people (1, 2). Studies have reported that long work hours and extended overtime is associated with all-cause mortality (3), cardiovascular disease (4), hypertension (5), diabetes (6), fatigue (7), psychological symptoms (8), sleep deprivation (9), and depression (10), although the evidence has not always been consistent (11). According to the reviews focused on long work hours and health (11–13), contradictory findings across studies are related to several methodological shortcomings as represented by under-adjustment of potential confounders, small sample sizes, reliance on self-reported health outcomes, and/or selection bias, which limit the generalizability and replicability of the findings. These reviews also emphasize that research on the health impact of work hours lacks information on the underlying immunophysiological mechanisms. Thus it seems desirable to conduct research that fills those voids.

As mentioned above, there is only limited research linking the relationship between work hours and immune outcomes. A study in Denmark observed that adults who worked ≥41 hours/week had twice the risk of an acute infection from *helicobacter pylori* (*H pylori*) than those who worked ≤40 hours/week, as confirmed by an increase of *H pylori* specific immunoglobulin (Ig) M, whereas

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the study did not observe chronic infection (as measured by specific *H pylori* IgG) associated with extended work hours (14). In a study of 142 male Japanese workers, those working 55–<65 hours/week and ≥65 hours/week had a significantly decreased percentage of CD56+ natural killer (NK) cells as compared to those working <55 hours/week (15). Similarly, a study of 291 middle-aged male Japanese workers reported that those working ≥12 hours/day (including commuting) had a significant decrease of mitogenic response to phytohemagglutinin-A (PHA) but the levels of interferon (IFN)-γ and interleukin (IL)-4 were comparable to those working <12 hours/day (16). A series of studies assessing the immunological impact of good versus poor health practices (work hours, smoking, drinking, sleeping, exercise, nutrition, stress, and eating breakfast) revealed that working ≥10 hours/day as compared to <10 hours/day was associated with a significant decrease of NK cell cytotoxicity (NKCC) (17), while it was not associated with lymphokine-activated killer (LAK) cell cytotoxicity or lymphocyte subsets (NK, CD4+ T, CD8+ T, perforin, granulyin, and granzymes A/B-expressing cells) (18, 19).

Although most of these studies did not exclusively focus on work hours and immunity, it seems prolonged work hours are associated with reduced immune functioning to some extent. However, it still remains uncertain whether long work hours or extended overtime is detrimental to immune functioning, because some studies have suggested that overtime work may be even more harmful than merely working long hours if there is less control over working time (20). Several epidemiological studies have shown that having control over work time (ie, control over the starting and ending times of a workday) is protective of workers' health (21, 22). Therefore, this study focused on the association of overtime with immune measures. We hypothesized that overtime is mediated by personal, occupational, and behavioral factors influencing the immune system. As indicated in figure 1, the theoretical model for this study presumed that overtime is associated with negative occupational factors (ie, poor interpersonal relationships, reduced job satisfaction) and unfavorable heath behaviors (ie, short sleep, reduced physical exercise) affecting immune function through intermediary conditions (ie, high depressive symptoms, obesity) consequently leading to physical disorders. Studies have indicated that overtime work is associated with high work-related stress and poor health behaviors (9, 23). Poor health behavior such as sleep deprivation and sleep loss is directly connected to a reduction of NK cells and increased inflammatory responses (24–27) as well as depressed mood, stress symptoms, and obesity (10, 28, 29). Meanwhile, job stress has been shown to exert a negative impact on immune outcomes (30–34), and nocturnal sleep is known to be disturbed by overtime and job stress (23, 35–37). Based on this model, our study was undertaken to examine the association between overtime and cell-mediated immune markers, taking the potential mediating factors into account by stepwise analyses.

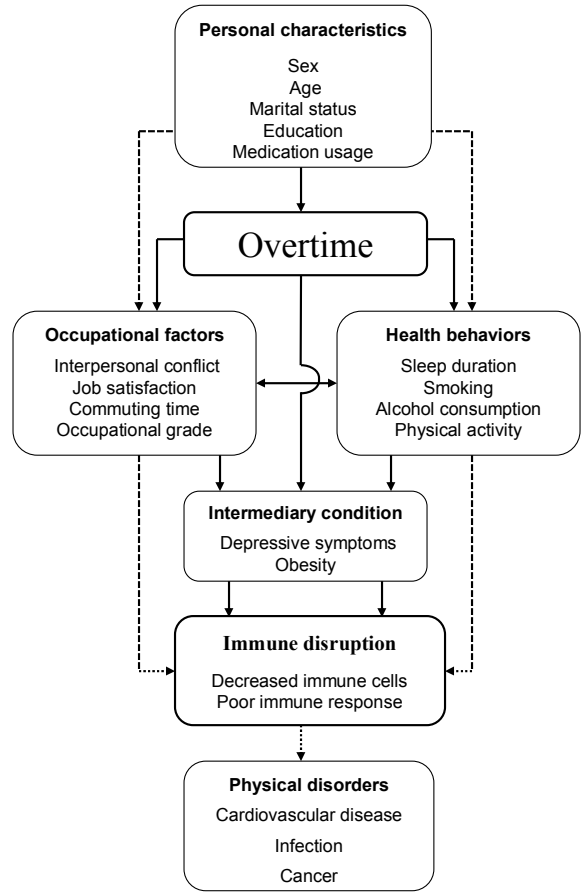

Figure 1. Conceptual framework for the relationship between overtime work and immunity.

Methods

Study participants and procedure

The study design was cross-sectional and data were collected with a self-administered questionnaire at a pharmaceutical company and a trading company in Japan. The study was conducted as a part of occupational health examinations during April–June 2002. All employees in both companies were full-time, white-collar, daytime Japanese workers (working between 09.00–17.00). Employees were neither engaged in any type of shift work (eg, rotating, night, evening) nor working on weekends unless employees had particular reasons to work on weekends/non-workdays. A total of 643 employees were initially recruited for this study. The survey questionnaire,

including purpose, instruction, and informed consent was given to a total of 626 employees [17 employees could not be reached because they were out due to sickness (mostly because of psychiatric illnesses) or maternity leave] two weeks prior to the blood sampling. The completed questionnaires were returned prior to or on the day of the blood sampling. Four hundred and four employees agreed to participate in the questionnaire survey and blood test, replying with a signed consent form (response rate 64.5%). Of these 404 employees, 37 were excluded because of missing data for one of the study parameters. An additional 61 employees reporting physical/mental disorders were excluded (see covariates section for detail), which resulted in a sample size of 306 participants (165 men and 141 women). Participants were not exposed to known hazardous chemicals (ie, benzene, benzidine, chromates, lead, mercury, organic solvents) that could affect immunological outcomes. The Institutional Review Board of the National Institute of Occupational Safety and Health, Japan, and the Ethical Committee of the Kyushu University reviewed and approved the study protocol.

Measures

Overtime per month. Overtime per month was assessed by an open-ended question: "How many total hours do you work overtime/month including holidays and weekends?" Using a subsample (N=204) of the current study, the test-retest stability over 1 year regarding overtime/month was r=0.750 (P<0.001). Validity was estimated by calculating the correlations between overtime/month with covariates, and the relationships were in the expected direction indicating a high convergent validity (see results section).

Preparation of blood samples. Fasting blood samples were collected between 09.00–11.00 hours from the participants. Among the 306 final participants, 30 were examined on Monday, 71 on Wednesday, 138 on Thursday, and 67 on Friday. Ethylenediaminetetraacetic acid dipotassium was used as an anticoagulant to collect 2 ml of venous blood from subjects for measurement of leukocytes counts and immunofluorescence staining. Similarly, 5 ml heparinized venous blood was collected to measure NKCC. All samples were transported and handled at room temperature (ie, 15–20 °C). Immunofluorescence staining analysis and measurement of NKCC were conducted within 24 and 12 hours of blood collection, respectively. We determined counts of total leukocytes and total lymphocytes by an automated cell counter (Coulter Counter SP-VI, Coulter Electronics, Hialeah, Florida, USA), and lymphocyte subpopulations by flow cytometry analysis (EPICS XL, Beckman Coulter Inc, California, USA), as described in detail elsewhere (30–32).

Cell surface marker analysis. The following sets of monoclonal antibodies were used to perform four-color direct immunofluorescence surface-marker analysis: anti-CD45-FITC / anti-CD56-RD1 / anti-CD19-ECD / anti-CD3-PC5. Anti-CD45-FITC antibody was used to identify and differentiate lymphocytes from non-lymphocytes and debris. A combination of Mouse IgG1-FITC / Mouse IgG1-RD1 / Mouse IgG1-ECD / Mouse IgG1-PC5 was used as the negative control. All monoclonal antibodies were purchased from Beckman Coulter Inc, USA. We calculated the number in each lymphocyte subset by multiplying lymphocyte counts by the percentage of positive cells in each category, as determined by flow cytometer.

With regard to immunoprotective roles of selected lymphocytes, T and B cells bear central roles in cellular and humoral immunity; subsets of T (CD4+ and CD8+) cells control production of immunoglobulins from B cells and secretion of cytokines. NK cells are large granular cells possessing killer activity against certain tumor cells and virus-infected cells without prior sensitization.

Cytotoxicity assay. A standard 4-hour Chromium-51 (^{51}Cr) release assay was used to determine NKCC with effector cells at an effector/target (E/T) cell ratio of 20:1 (38). K562 was used as target cells and labeled with ^{51}Cr-sodium chromate (New England Nuclear, Boston, MA, USA) at 37 °C for an hour, washed and re-suspended at 2×10^5/ml in Roswell Park Memorial Institute-1640 medium containing 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. Labeled target cells were incubated with effector cells at an E/T cell ratio of 20:1 in U-bottomed 96-well plates at 37 °C for 4 hours. Radioactivity in the supernatant was determined by a gamma counter. The assay was performed in quadruplicate. The percentage of specific lysis as cytotoxicity was determined according to the following formula: percentage of specific lysis = [(mean experimental cpm release – mean spontaneous cpm release) / (mean maximal cpm release – mean spontaneous cpm release)]. Reduced NKCC is a significant prognostic indicator of developing infections within 12 months (39). In order to calculate the cytotoxic capacity per cell, NKCC was divided by NK cell counts (NKCC/NK cell ratio).

Covariates

Covariates included age (in years), marital status (unmarried or married), education (in years), smoking (number of cigarettes smoked per day), alcohol consumption (g ethanol per week), leisure-time physical activity, sleep duration, occupational grade (managerial or non-managerial), company type (pharmaceutical or trading), one-way commute time (<30, 30–59, 60–89, 90–119, and ≥120 minutes), depressive symptoms, interpersonal conflict at work, job satisfaction, height, weight, self-reported illness, medication usage, and day of blood sampling (Monday–Friday).

Alcohol consumption was estimated by asking the usual amount of alcoholic drinks consumed per day and the number of occasions in a week that alcoholic drinks were consumed. We converted gross liquor consumption into net ethanol intake. We assessed leisure-time physical activity by calculating the energy expenditure of habitual physical exercise. We asked frequency, type, and length of physical exercise per month and converted these data to metabolic equivalents (MET). Estimated MET were assigned to the physical activities according to their mean intensity levels. One MET corresponds to an energy expenditure of approximately 1 kcal/kg/hour. Weekly leisure-time physical activity was calculated from this questionnaire. Usual daily sleep duration was calculated as a weighted average of weekday and weekend sleep durations (hours) using the following formula: ([usual weekday sleep duration × 5] + [usual weekend sleep duration × 2])/7 (40, 41) and the following question was used: "On average, when do you start sleeping and when do you wake up in the morning on weekdays (workdays) and weekends (non-work days), respectively?" Depressive symptoms were measured by a Japanese version of the Center for Epidemiologic Studies Depression scale (CES-D) (42). The 20-item depressive symptom scale measures the level of depressive symptoms experienced in the past week (43). The internal consistency of the CES-D scale for the study sample was 0.84. We measured interpersonal (intragroup) conflict at work and job satisfaction by the Generic Job Stress Questionnaire developed by the US National Institute for Occupational Safety and Health (NIOSH) (44, 45). Interpersonal conflict at work is an 8-item scale that measures how much the worker feels the relationships within their working group are harmonious, cooperative, and supportive. The internal consistency of this scale was 0.83. Job satisfaction is a 4-item scale, which assesses whether the worker would maintain their current job if given a choice to take a new job or whether they would recommend the job to others. The internal consistency of this scale was 0.68. Information on height (m) and weight (kg) was obtained to assess body mass index (BMI), calculated as weight in kilograms divided by the square of height in meters. For self-reported illness, participants were asked if they had been diagnosed or treated for any of the following symptoms or disorders at the time of the study: hypertension, diabetes mellitus, menopausal syndrome, depression, asthma, allergies, cancer, cardiovascular disease, arrhythmia, angina pectoris, liver disease, cerebrovascular disease, hyperlipidemia, hyperthyroidism, gastric/duodenal ulcer, autonomic imbalance, or other diseases. If the subjects reported "other diseases", they were asked to specify the condition. As a result, participants with the following symptoms or disorders were identified: hypertension (N=18), diabetes mellitus (N=6), menopausal syndrome (N=3), depression (N=4), asthma (N=2), severe allergy (N=12), liver diseases (N=2), gastric/duodenal ulcer (N=4), autoimmune disorders (N=2), hyperlipidemia (N=13), autonomic imbalance (N=2), and the common cold (N=10); the participants reported no other symptoms/disorders (including cancer). In order to eliminate the potential effects of health status on immune parameters, all participants who reported the above disorders as well as women who reported being pregnant were excluded from the analyses (N=61). We also obtained data on the use of the following medications: aspirin (N=38), β-blockers (N=2), acetaminophen (included in pain killers, N=30), corticosteroids (N=1), antidepressants (N=2), and anxiolytic drugs (N=1). After all participants with self-reported illnesses as described above were eliminated, only aspirin (N=35) and acetaminophen (N=30) (over-the-counter medication) users were retained in the subsequent analyses.

Statistical analysis

The CES-D scale score was logarithmically transformed to achieve a more normal distribution. Intercorrelations between overtime, covariates, and immune markers were calculated by Pearson product-moment correlation coefficients. Multiple linear regression analysis was used to examine the relationship between overtime (dependent variable) and covariates (independent variables). Stepwise multiple linear regression analysis was performed to test the relationship between immune markers (dependent variables) and overtime/month and covariates. In step 1, we entered overtime, sex, age, education, marital status, medication usage, and blood sampling day (model 1). In model 2, we added health behavior (sleep duration, alcohol consumption, smoking, and physical activity) as covariates in addition to model 1 variables. In model 3, we added occupational factors (interpersonal conflict, job satisfaction, commuting time, occupational grade, and company type) as covariates in addition to model 1 variables. And finally, we included all covariates (personal, health behavior, and occupational factors) including intermediary conditions (depressive symptoms and BMI) into the model (model 4).

The independent variables of medication usage, occupational grade, company type, and sampling day were treated as categorical variables while the remaining variables were treated as continuous variables. The significance level for all statistical analyses was $P<0.05$ (two-tailed test). We analyzed the data using SPSS version 17.0 (SPSS, Inc, Chicago, IL**,** USA).

Results

Sample characteristics

Characteristics of the study participants and distribution of overtime are shown in table 1: 54% were men, 48%

were married, 19% used over-the-counter medication, and about half were from a trading company. More than 89% worked overtime and 17.4% worked >60 hours of overtime per month. On average, participants worked 40 hours of overtime per month, were an average of 36 years old, and had been educated for 15 years. They smoked six cigarettes per day, consumed 100 g of ethanol per week, expended 5.6 MET per week, and slept 7 hours per day. Average BMI was 21.8 and CES-D score was 13.2 for this sample.

Relationship between overtime work, covariates, and immune markers

Overtime was positively associated with education, alcohol consumption, BMI, NKCC, and NKCC/NK cell ratio, while it was inversely related to sleep duration and commuting time (table 2). NKCC was positively correlated with education, alcohol consumption, BMI, and job satisfaction while it was inversely associated with CES-D and interpersonal conflict. NK cell counts were related to an increase of alcohol consumption and BMI but to a decrease in the CES-D score. B cell counts were positively related to age, cigarettes smoked per day, and BMI. Total T cells were associated with an increase of cigarettes smoked per day.

Association between overtime work with covariates

The relationships between overtime work and the covariates are shown in table 3. Overtime was more prevalent among men than women and inversely correlated with age, sleep duration, and job satisfaction. Married employees and participants from the pharmaceutical company tend to work more overtime. Overtime was positively associated with BMI.

Relationship between overtime work with immune markers

The relationship between overtime and immune markers is shown in table 4. The stepwise multiple linear regression analysis revealed that overtime work was inversely correlated with NK cell counts but not with NKCC, NKCC/ NK cell ratio, or B or T cells.

Table 1. Characteristics of study participants [a] (N=306). [SD=standard deviation; MET=metabolic equivalents; NK=natural killer; NKCC=NK cell cytotoxicity; CES-D= Center for Epidemiologic Studies Depression scale.]

	N	%	Mean	SD	Range
Male gender	165	53.9			
Married	146	47.7			
Medication usage	59	19.3			
Managerial occupational grade	44	14.4			
Employee of trading company	156	51.0			
Overtime/month:			39.6	31.4	0–160
No overtime	35	11.4			
0.1–20	84	27.5			
21–40	75	24.5			
41–60	59	19.3			
61–80	28	9.2			
>80 hours	25	8.2			
Age (in years)			36.0	10.5	22–69
Education (in years)			15.4	1.6	12–21
Smoking (cigarettes smoked/day)			5.7	10.0	0–60
Alcohol consumption (g ethanol/week)			100.0	124.2	0–805
Leisure-time physical activity (MET/week)			5.6	9.3	0–52.5
Usual daily sleep duration (hours/day) [b]			7.0	0.9	3.9–10.5
Interpersonal conflict at work [c]			19.3	5.5	8–40
Job satisfaction [d]			9.5	1.5	5–13
One-way commuting time [e]			2.3	0.8	1–5
Depressive symptoms (CES-D scale score) [c, f]			13.2	6.1	0–36
Body mass index (kg/m^2)			21.8	3.0	15.4–32.8
Immune markers					
NKCC (% cytotoxicity)			44.1	17.8	4–77
NK (CD3-CD56+) cells (cells/mm^3)			283	187	38–1438
NKCC/NK cell ratio			0.203	0.143	0.02–1.55
B (CD19+) cells (cells/mm^3)			231	129	18–911
Total T (CD3+CD56-) cells (cells/mm^3)			1.181	363	353–3208

[a] Participants who reported physical/psychological disorders (N=41) at the time of study were excluded to eliminate the potential effects of health status on immune parameters (see text for detail).
[b] Calculated as a weighted average of weekday and weekend sleep durations (hours) using the following formula: ([usual weekday sleep duration × 5] + [usual weekend sleep duration × 2])/7.
[c] Negatively-oriented scale.
[d] Positively-oriented scale.
[e] One-way commuting time (1=<30 minutes, 2=30–59 minutes, 3=60–9 minutes, 4=90–119 minutes, 5=≥120 minutes).
[f] Log-transformed.

Discussion

There are three major findings in this study. First, overtime work was consistently associated with a decrease of NK cell counts even after controlling for various covariates, while it was not associated with other immune indicators (ie, NKCC, NKCC/NK cell ratio, T and B cells). Decrease of NK cell counts by overtime work suggests a dampened innate immune defense. Second, overtime work was associated with increased BMI and reduced sleep hours and job satisfaction. Third, overtime work was more prevalent among men than women and among younger and married employees. Our findings provided some support for the immunologic plausibility of the link between overtime and health and well-being, but needs to be further validated with a well-designed study using objective overtime measures. It is also necessary to reevaluate our findings by measuring other immune indicators such as proliferative response to mitogens, inflammatory markers, and viral reactivation.

Based on our conceptual model (figure 1), it is possible that overtime work reduces sleep duration leading

Table 2. Intercorrelations between overtime, covariates, and immune markers (Pearson product-moment correlation coefficients). **Bold indicates P<0.05**. [MET=metabolic equivalents; BMI=body mass index; CES-D=Center for Epidemiologic Studies Depression scale; NK=natural killer; NKCC=NK cell cytotoxicity.]

Variable	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. Overtime work (hours/month)	-																
2. Age (years)	0.069	-															
3. Education (years)	**0.246**	**-0.299**	-														
4. Smoking (cigarettes smoked/day)	0.102	0.034	0.098	-													
5. Alcohol consumption (g ethanol/week)	**0.280**	0.055	**0.253**	**0.359**	-												
6. Leisure-time physical activity (MET/week)	0.001	**0.120**	-0.054	-0.029	0.097	-											
7. Usual daily sleep duration (hours/day)	**-0.191**	0.080	-0.056	0.075	0.043	-0.043	-										
8. BMI (kg/m^2)	**0.355**	**0.319**	**0.197**	**0.150**	**0.309**	**0.118**	**-0.125**	-									
9. Depressive symptoms (CES-D scale score)	-0.076	**-0.305**	0.033	-0.032	**-0.171**	-0.063	-0.090	**-0.159**	-								
10. Interpersonal intragroup conflict at work	0.013	-0.017	**-0.124**	-0.038	**-0.142**	**-0.115**	-0.045	-0.089	**0.161**	-							
11. Job satisfaction	-0.061	0.055	**0.143**	-0.037	0.043	0.073	0.025	**0.134**	**-0.175**	**-0.337**	-						
12. One-way commuting time	**-0.118**	0.031	**-0.119**	-0.089	**-0.200**	0.031	**-0.209**	**-0.130**	0.014	0.082	0.060	-					
13. NKCC (% cytotoxicity)	**0.197**	0.106	**0.229**	0.029	**0.248**	0.007	-0.031	**0.317**	**-0.113**	**-0.114**	**0.172**	**-0.145**	-				
14. NK (CD3-CD56+) cells (cells/mm^3)	0.021	0.090	0.069	0.019	**0.159**	-0.035	0.042	**0.207**	**-0.148**	-0.082	**0.166**	-0.048	**0.538**	-			
15. NKCC/NK cell ratio	**0.174**	0.002	0.080	0.010	-0.003	0.028	-0.062	0.028	-0.078	0.063	-0.049	-0.048	**0.197**	**-0.662**	-		
16. B (CD19+) cells (cells/mm^3)	-0.039	**0.129**	-0.067	**0.164**	0.054	0.081	0.079	**0.177**	-0.090	-0.029	0.060	-0.088	-0.057	0.079	**-0.127**	-	
17. Total (T CD3+CD56-) cells (cells/mm^3)	-0.095	-0.024	-0.099	**0.192**	-0.030	-0.031	0.046	-0.014	0.060	0.031	-0.033	-0.023	**-0.226**	-0.094	**-0.114**	**0.210**	-

to insufficient sleep and depressed mood. Overtime work may also be a source of high job stress causing job dissatisfaction and depressive symptoms (28). Decreased NK cells have been found among people with chronic sleep deprivation, depressive symptoms, and job dissatisfaction (25, 26, 31). Physical inactivity and smoking have also been reported as factors disturbing NK cells (18, 19). Thus it is likely that it is not the overtime work per se that causes a decrease of NK cells but rather a combination of work-related stress, sleep deprivation, depressive symptoms, and poor health behaviors triggered by overtime work.

In support of the above reasoning, overtime work was associated with health behaviors (reduced sleep duration), work-related stress (reduced job satisfaction), and intermediary conditions (increased BMI) (table 3). Reduced sleep duration associated with overtime supports the concept of the physiological recovery mechanism: that workers may have less time to recover resulting in suppressed NK cells (46). The results are also supported by the behavioral lifestyle mechanism: that overtime work increases body weight through physical inactivity and possibly a high intake of saturated fat and calories (46). Reduced job satisfaction may be directly related to overtime work.

Table 3. Multiple regression analysis with overtime as a dependent variable and covariates as independent variables (N=306). **Bold denotes significance**. [MET=metabolic equivalents; CES-D= Center for Epidemiologic Studies Depression scale.]

Covariates	Overtime [a]	
	β [b]	P-value
Gender (men=1, women=2)	**-0.270**	**0.001**
Age (years)	**-0.273**	**<0.001**
Marital status (1=unmarried, 2=married)	**0.177**	**0.005**
Education (in years)	0.027	0.658
Medication usage (no=0, yes=1)	0.042	0.439
Smoking (number of cigarettes smoked/day)	-0.053	0.327
Alcohol consumption (g ethanol/week)	0.051	0.868
Leisure-time physical activity (MET/week)	-0.002	0.969
Usual daily sleep duration (hours/day)	**-0.134**	**0.011**
Interpersonal conflict score	0.040	0.441
Job satisfaction score	**-0.116**	**0.030**
Commuting time	-0.032	0.561
Occupational grade (1=non-supervisor, 2=supervisor)	-0.006	0.927
Company type (1=trading, 2=pharmaceutical)	**0.167**	**0.008**
Depressive symptoms (CES-D score)	-0.012	0.828
Body mass index (kg/m^2)	**0.157**	**0.011**

[a] Adjusted R^2=0.278 (P<0.001).
[b] Standardized regression coefficient.

Table 4. Summary of stepwise multiple linear regression analysis for the association of overtime with immune markers (N = 306). **Bold denotes significance.** [NK=natural killer; NKCC=NK cell cytotoxicity.

Immune marker (dependent variable)	Model 1[a]		Model 2 [b]		Model 3 [c]		Model 4 [d]	
	β [e]	P-value	β [e]	P-value	β [e]	P-value	β [e]	P-value
NK (CD3-CD56+) cells (cells/mm^3)	**-0.161**	**0.013**	**-0.155**	**0.019**	**-0.155**	**0.018**	**-0.145**	**0.032**
NKCC (% cytotoxicity)	0.052	0.400	0.055	0.381	0.038	0.528	0.052	0.408
NKCC/NK cell ratio	0.108	0.106	0.099	0.149	0.098	0.143	0.086	0.220
B (CD19+) cells (cells/mm^3)	-0.029	0.660	0.007	0.913	-0.031	0.645	-0.029	0.665
Total T (CD3 + CD56-) cells (cells/mm^3)	-0.066	0.320	-0.041	0.528	-0.070	0.298	-0.069	0.301

[a] Adjusted for gender, age, education, marital status, medication, and sampling days.
[b] Adjusted for Model 1 variables + sleep duration, smoking, alcohol consumption, and physical activity.
[c] Adjusted for Model 1 variables + occupational grade, company type, one-way commute time, interpersonal conflict, and job satisfaction.
[d] Adjusted for Model 1 variables + sleep duration, smoking, alcohol consumption, physical activity, occupational grade, company type, one-way commute time, interpersonal conflict, job satisfaction, depressive symptoms (CES-D scale score), and body mass index.
[e] Standardized regression coefficients for each variable's unique contribution.

There was a significant positive correlation between amount of overtime and NKCC and NKCC/NK cell ratio when assessed by a simple correlation analysis (table 2) but the significance disappeared after adjustment for confounders (table 4). The results may be relevant to the fact that women had significantly lower NKCC and NK cell counts than men (data not shown); women worked much less overtime than men, suggesting the importance of controlling relevant covariates for this kind of research. It is also important to note that only counts of NK cells were related to overtime work; NKCC and NKCC/NK cell ratio were not related to overtime work. A decrease in counts of NK cells by overtime work may be due to redistribution of NK cells between lymphoid organs and periphery without losing cytotoxic capacity. Alternatively, overtime and related mediating factors may have exerted a decreasing effect on NK cells through the downregulation of β-adrenergic receptors on NK cells (47).

Methodological considerations

The specific strengths of our study are that we controlled for a broad array of potential confounders with a large and relatively homogenous sample. In addition, participants who reported illnesses were excluded to minimize sampling bias (ie, work less overtime as a consequence of health conditions). However, there are a number of limitations to our study. First, information on overtime was collected through self-report, which may introduce various biases (ie, recall, reporting, social desirability). It is desirable to collect absolute overtime with an objective measure to examine the true relationship between overtime and immunological outcomes. Second, the collection of blood samples and questionnaire data were not simultaneous, which may have affected our findings. Also, health behaviors such as sleep duration, eating behavior, and physical exercise immediately before the blood sampling as well as recent travel across time zones, evening and morning types, and late weekend activities may modify immune markers rather than the usual habits captured in the questionnaire. It is also possible that there may be differences in immune responses between the beginning of workweek (Monday) and end of workweek (Friday) that may have affected the results of this study (even though we statistically controlled for the day of blood sampling). Immune function may recover during the weekend through enough sleep and rest and decline towards the end of weekday as a result of fatigue and stress. Thus the timing of the blood sampling should be fixed on a certain day to control for diurnal and weekday–weekend effects. Third, participants were employees from specific occupations and are not representative of the entire Japanese workforce or workers of other racial/ethnic groups. Fourth, since the

study was cross-sectional in nature no causal inference can be made. Fifth, we measured only cellular immune markers, in particular counts of specific immune cells and cytotoxic capacity; cell proliferation and cytokine responses should also be measured simultaneously. Finally, although we considered a variety of confounders, we could not rule out residual confounders (ie, personality traits, genetic components, menstrual phase/oral contraceptive use among women, other work-related factors, and concurrent life stressors such as marital discord, work–family conflict, stress outside work) as well as unknown third factors which could affect both the dependent and independent variables.

Concluding remarks

This study examined the cross-sectional association of overtime work with cell-mediated immunity in a sample of 306 healthy, white-collar, daytime employees. Although there were a number of inherent limitations to our study, the results revealed that overtime work was inversely associated with NK cell counts. Future research should test mechanistic causal associations between objective overtime work, the immune system, and long-term health that could contribute to the well-being of workers.

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