Serum leptin concentrations and markers of immune function in overweight or obese postmenopausal women

Julie A Meyers², Amy Y Liu^{1,2}, Anne McTiernan^{1,2}, Mark H Wener³, Brent Wood³, David S Weigle^{3,4}, Bess Sorensen¹, Zehava Chen-Levy³, Yutaka Yasui⁵, Alanna Boynton^{1,2}, John D Potter^{1,2} and Cornelia M Ulrich^{1,2}

¹Fred Hutchinson Cancer Research Center, Cancer Prevention Program, 1100 Fairview Avenue N., Seattle, Washington 98109, USA

²Department of Epidemiology, ³Department of Laboratory Medicine and ⁴Division of Metabolism, Endocrinology and Nutrition, Department of Medicine, University of Washington, Seattle, Washington 98195, USA

⁵Department of Public Health Sciences, University of Edmonton, University of Alberta, Edmonton, Alberta, Canada T6G 2G3

(Correspondence should be addressed to C M Ulrich at Fred Hutchinson Cancer Research Center; Email: nulrich@fhcrc.org)

Abstract

Experimental studies and case reports suggest a multifunctional role of leptin in immune function. However, clinical studies of leptin in healthy individuals with a comprehensive assessment of immunity are lacking. This study investigated associations between serum leptin concentrations and multiple biomarkers of cellular immunity and inflammation among 114 healthy postmenopausal, overweight, or obese women. Leptin was measured by RIA. C-reactive protein (CRP) and serum amyloid A (SAA) were measured by nephelometry. Flow cytometry was used to measure natural killer (NK) cell cytotoxicity and to enumerate and phenotype lymphocyte subsets. T-lymphocyte proliferation was assessed in response to phytohemagglutinin, as well as to anti-CD3 antibodies by the flow cytometric cell division tracking method. Multiple linear regression analysis with adjustment for confounding factors and log transformation, where appropriate, was used. Serum leptin concentrations were positively associated with serum CRP, SAA, and interleukin 6 (IL6) ($P < 0.0001$, $P = 0.01$, and

$P = 0.04$ respectively), more strongly among women with a body mass index (BMI) $< 30 \text{ kg/m}^2$. The associations were attenuated after adjustment for measured body composition, yet remained significant for CRP and SAA. No statistically significant associations were observed between leptin and NK cytotoxicity, lymphocyte subpopulations, or T-lymphocyte proliferation. This study fills an important gap in knowledge about the relationship between leptin concentrations and immune function in healthy individuals. Findings support an association between serum leptin and the inflammatory proteins CRP and SAA, which appears to be mediated only partly by adipose tissue. Our study does not support a link between leptin and other immune parameters among overweight or obese, but otherwise healthy postmenopausal women, perhaps because such effects are only present at low or deficient leptin concentrations.

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Introduction

Leptin is an adipokine best known for its role as a central regulator of food intake, body weight, and adipose stores. Circulating leptin concentrations are proportional to total adipose mass and decrease after weight loss (Friedman & Halaas 1998, Bastard *et al.* 2000). Although the leptin receptor (LEPR) is expressed in the highest density in the hypothalamus, LEPR expression has also been detected in other tissues including hematopoietic and natural killer (NK) cells (Gainsford *et al.* 1996, Marti *et al.* 1999, Zhao *et al.* 2003). The isolation of the LEPR in immune cells, in addition to the hormone's structural resemblance to cytokines, led to the discovery that leptin also functions in the modulation of immune function.

Animal studies show that leptin modulates starvation-induced immunosuppression (Lord *et al.* 1998, Howard *et al.* 1999) and may also play a role in the proinflammatory T helper 1 (Th1) response (Lord *et al.* 1998, 2002, Sanna *et al.* 2003). Additionally, LEPR-defective (*db/db*) mice exhibit significant reductions of both NK cell numbers and NK cytotoxic capacity compared with lean mice (Li *et al.* 2002), suggesting that there might be a relationship between leptin and NK cells. Recent clinical studies suggest that leptin may also be involved in the stimulation of hepatic acute-phase protein production such as C-reactive protein (CRP) and serum amyloid A (SAA; Bastard *et al.* 2000, Bullo *et al.* 2003, Shamsuzzaman *et al.* 2004). Primarily, *in vitro* studies indicate a link between leptin and other components of the immune system such as tumor necrosis factor α , interleukin 1 (IL1),

IL2, IL6, interferon- γ (INF- γ), NK cells, peripheral blood mononuclear cells (PBMC), and T-cell subsets (e.g., CD4⁺T helper cells and naïve T helper cells) associated with the Th1 proinflammatory response (Lord *et al.* 1998, 2002, Gallistl *et al.* 2001, Zarkesh-Esfahani *et al.* 2001, Zhao *et al.* 2003). Further evidence for leptin's role in cell-mediated immunity is provided by studies showing that leptin deficiency induces thymus atrophy in animals and is protective against T-cell-mediated autoimmune diseases (Matarese *et al.* 2001, Siegmund *et al.* 2002, Sanna *et al.* 2003). Although cell and animal studies suggest a complex role of leptin in immune function (Peelman *et al.* 2004, Matarese *et al.* 2005, Meyers *et al.* 2005, Otero *et al.* 2005), the relationship between leptin and immune activity in humans remains unclear.

Obesity is associated with abnormal immune function, including elevated leukocyte and lymphocyte subset counts, lower T- and B-cell mitogen-induced lymphocyte proliferation, and possibly also reduced NK cell counts and cytotoxicity (Marti *et al.* 2001). Thus, the relationship between leptin and immune function may be mediated in part by obesity. Women who went through substantial weight loss (53% of excess body weight) significantly reduced their leptin levels (Manco *et al.* 2007). Additionally, since it is well known that several aspects of immunity decline with age (Lesourd & Mazari 1999, Chandra 2002), it is important to investigate predictors of immune function in older individuals. The aim of this cross-sectional study was to investigate the association between serum leptin concentrations and measures of inflammation and immune function, including CRP, SAA, IL6, NK cell cytotoxicity, lymphocyte subpopulations, and lymphocyte proliferation among overweight and obese, but otherwise healthy postmenopausal women. We hypothesized that serum leptin concentrations would be positively associated with circulating concentrations of CRP and SAA, as well as IL6, with some degree of mediation by body fat. Also, we hypothesized that leptin concentrations would be negatively associated with NK cell cytotoxicity and cell count, but positively with T-cell lymphocyte proliferation, and the size of T-cell subsets associated with the Th1 proinflammatory response.

Materials and Methods

Participants

The study was conducted at the Fred Hutchinson Cancer Research Center (FHCRC) and the University of Washington (UW). Participants ($n=114$) were a subset of a study population recruited for an exercise intervention trial (McTiernan *et al.* 1999) who met eligibility criteria for participation in an additional study of immune function described in more detail in Shade *et al.* (2004). Subjects were healthy (without serious comorbidities, including diabetes and cardiovascular disease) sedentary (exercising <2 times/week for 20 min at a level to produce sweating)

postmenopausal women, ages 50–75 years with a BMI between 24 and 40 kg/m² (BMI 24.0–24.9 was included if body fat >33%), who had been weight stable for at least 3 months prior to entering the study (McTiernan *et al.* 1999). Exclusion criteria included current smoking, use of hormone replacement therapy (HRT) within the previous 6 months, alcohol consumption of greater than two drinks per day, or a history of invasive cancer, diabetes, cardiovascular disease, emphysema, asthma, or serious allergies. Additional exclusion criteria included regular (≥ 2 times/week) use of aspirin or other non-steroidal anti-inflammatory medications and of corticosteroids or other medications known to affect immune function. Informed consent was obtained from all participants, and the study procedures were approved by the Institutional Review Board. We used baseline data from the exercise intervention trial for all the data in this cross-sectional analysis of leptin and measures of inflammation and immune function.

Data collection

At the clinic visit, study staff measured body weight to the nearest 0.1 kg using a Detecto balance-beam scale (Jericho, New York, NY, USA) and height to the nearest 0.1 cm using a stadiometer. The average of duplicate measures was used to compute BMI. Total body fat was assessed using DXA (Hologic QDR 1500, Hologic Inc., Waltham, MA, USA). Information including age, education, income, employment status, marital status, race/ethnicity, and smoking history were collected by questionnaire. Dietary and alcohol intakes were assessed using a 120-item food frequency questionnaire designed and validated at the FHCRC. Use of nutritional supplements was determined during a face-to-face interview.

Leptin

Leptin assays were performed at the UW, Harborview Medical Center (DSW). Serum leptin was measured in duplicate after a 12-h fast using a commercially available RIA (Linco Research, St Charles, MO, USA) with lower and upper detection limits of 0.5 and 100 ng/ml respectively. Intra- and inter-assay coefficients of variation were 8.7 and 11.2% respectively.

Immune function measures

Blood specimens for immune assays were drawn at the UW Department of Laboratory Medicine under strict blood draw criteria (Shade *et al.* 2004). All immune assays were conducted at the UW Clinical Immunology Laboratory (MHW). Fasting blood samples were taken between 0730 and 0830 h and processed within 1 h. Prior to the blood draw, participants had adequate sleep (6–9 h), did not exercise or drink alcohol in the previous 24 h, and did not use topical corticosteroids or aspirin in the previous 48 h. In the week

before the blood draw, they had no symptoms of infection nor did they use systemic anti-histamines or corticosteroids. Participants did not have immunizations in the preceding 3 weeks.

CRP and SAA were measured by latex-enhanced nephelometry using high-sensitivity assays on the Behring Nephelometer II analyzer (Dade- Behring Diagnostics, Deerfield, IL, USA) with a lower detection limit of 0.2 and 0.7 mg/l for CRP and SAA respectively. Inter-assay coefficients of variation were 5–9 and 4–8% for CRP and SAA respectively.

IL6 was measured in duplicate using solid-phase sandwich ELISA with the Biosource Human IL-6 Immunoassay kit (Biosource, Camarillo, CA, USA). Inter-assay coefficients of variation were ~10%. Lymphocytes were isolated from whole blood using a whole blood lysis technique.

A four-color flow cytometer (XL-MCL, Beckman Coulter, Miami, FL, USA) was used to enumerate subsets of lymphocytes in blood samples, as described previously (Shade *et al.* 2004).

The flow cytometric assay for measuring NK cytotoxicity used by our group has been described previously (Shade *et al.* 2004). To prepare the target cells, K562 cells (in log phase of growth) were washed twice with PBS/BSA and incubated with 3-3'-diiodoacetylcarboxycyanine perchlorate (DiO, Live/Dead cytotoxicity kit #L7010, Molecular Probes, Eugene, OR, USA) at a concentration of 2×10^6 cells/ml for 20 min at 37 °C with 5% CO₂. The cells were then washed twice with PBS/BSA, resuspended in Roswell Park Memorial Institute medium (RPMI) to a concentration of 1×10^6 cells/ml, and filtered through a 35 µm strainer. Mononuclear cells were prepared by Ficoll-Hypaque differential centrifugation of blood effector cells, diluted corresponding to final effector-to-target (ET) cell ratios of 50:1, 25:1, 12.5:1, and 6.25:1, and incubated with the DiO-labeled K562 cell suspension (target cells) for 4 h at 37 °C with 5% CO₂. Following incubation, propidium iodide (PI, 0.03 mg/ml final concentration) was added to each tube to identify dead cells. The percentage of dead target cells (i.e., dual positive for DiO and PI) out of total DiO-identified target cells was used as the measure of NK cytotoxicity. Each assay was performed in duplicate and with appropriate controls. We repeated the NK cytotoxicity assay in 13 study participants who underwent additional blood draws between 1 week and 9 months after the initial blood draw, under identical blood draw criteria. Intra-class correlation coefficients between the initial and repeat blood draw were: $r = 0.84$ (ET 6.25:1), $r = 0.91$ (ET 12.5:1), $r = 0.90$ (ET 25:1), and $r = 0.79$ (ET 50:1).

T-lymphocyte proliferation studies, using cryo-preserved PBMC, were carried out by two methods: tritiated [³H]thymidine incorporation in response to the mitogen phytohemagglutinin (PHA), and the cell division tracking method in response to anti-CD3. PBMC were prepared by Ficoll-Hypaque separation and 1 ml aliquots of $5\text{--}10 \times 10^6$ cells/ml were frozen in 30% fetal calf serum (FCS), 60%

RPMI, and 10% dimethyl sulfoxide (DMSO) (Gibco). Cells from two control subjects were included in every experiment.

For the [³H]thymidine incorporation, 200 µl of 5×10^4 peripheral blood mononuclear cells (PBMN) cells/well were incubated in microtiter plates with 25 µl PHA of 0.1 and 0.5 µg/ml in five replicates each. After incubation for 72 h at 37 °C, cells were pulsed for 24 h with 25 µl [³H]thymidine, harvested, and counted with a β-counter.

For the cell division tracking method, 170 µl of 10^7 cell/ml in RPMI were used for each sample. Carboxy-fluorescein diacetate, succinimidyl ester (CFDA SE; Molecular Probes), a precursor of 5- and 6- carboxyfluorescein diacetate succinimidyl ester (CFSE), was added to the cell suspension at a final concentration of 10 µM. Cells were incubated for 10 min, washed twice, re-suspended in 3.0 ml complete medium and 180 µl were then pipetted into 16 wells of a microtiter plate (100 000 cells/well). Next, 20 µg of 2 ng/ml anti-CD3 antibody (BD Biosciences, San Jose, CA, USA) were added to eight of the wells to specifically stimulate T-lymphocytes. The remaining eight wells were used as control unstimulated cells. Following incubation for 3 days at 37 °C, identical wells were pooled into 5 ml sterile tubes containing 2 ml complete medium and incubated for 3 more days. On the 6th day, cells were harvested and the CFSE-fluorescein isothiocyanate (FITC) intensity of viable lymphocytes was measured with a flow cytometer (XL-MCL, Beckman Coulter).

Statistical analysis

After evaluation for normality, Pearson correlation coefficients and multiple linear regression analysis were used to analyze associations between serum leptin and CRP, SAA, IL6, NK cytotoxicity, size of lymphocyte subpopulations, and T-lymphocyte proliferation. Immune outcomes were log-transformed where appropriate (e.g., CRP, SAA, parent %). Because IL6 values for some of the participants ($n = 16$) were 0 ng/l, these were changed to 0.5 ng/l before log transformation. This change (or exclusion of these participants) did not alter the results. Analysis was performed using SAS version 8.02 (SAS Institute, Cary, NC, USA). A two-sided P value < 0.05 was considered statistically significant.

One participant was excluded from all analyses because of an unrealistically low serum leptin concentration giving her high BMI (leptin, 0.43 ng/ml; BMI, 42.0 kg/m²) and much higher leptin concentrations at later time points in the study. A second subject was excluded from the analysis of cytotoxic T cells only also due to an unrealistically low value. From CRP, SAA, and IL6 analyses, we excluded three subjects with elevated CRP concentrations > 10 mg/l, which may be an indicator of an acute infection or inflammatory process (Pearson *et al.* 2003). Results were modestly stronger after exclusion of these individuals, yet statistical significance remained largely identical. Missing data for all assays ranged from $n = 1$ to $n = 3$, except for lymphocyte proliferation by the cell division tracking method where sufficient cells for this novel assay were available for 92 participants.

Potential confounding factors considered included: race (Caucasian, other); regular multivitamin use (yes/no); season during which the blood draw occurred; marital status (married or living with a partner, other); history of smoking (yes/no); and history of previous intentional weight loss of ≥ 10 pounds (yes/no). Any covariate that substantially altered the β -coefficient associated with leptin between a model with and without the covariate was retained in all models. Only age was a confounding factor. We also evaluated the effects of including BMI (continuous and grouped by tertiles: ≤ 28 , 28–31 and > 31 kg/m²) and percent body fat (continuous and grouped: ≤ 45 , 45–49 and $> 49\%$) in the respective linear regression models to assess associations with leptin beyond a link through body fatness. Age-adjusted models were also run with stratification by BMI (overweight (25–29.9 kg/m²) versus obese (≥ 30 kg/m²)).

Lymphocyte subpopulations included the following outcome variables: number and percentage of total T-cells (CD3⁺ and CD45⁺), helper T cells (CD3⁺, CD4⁺, and CD45⁺), cytotoxic T cells (CD3⁺, CD8⁺, and CD45⁺), CD4⁺/CD8⁺ ratio, percentage of naïve helper T cells (CD3⁺, CD4⁺, CD45RA⁺, and CD62L⁺), naïve cytotoxic T cells (CD3⁺, CD8⁺, CD45RA⁺, and CD62L⁺), number and percentage of B cells (CD19⁺ and CD45⁺), and NK cells (CD3⁺, CD16⁺, CD45⁺, and CD56⁺).

NK cytotoxicity was investigated first individually at two of the four original ET ratio dilutions (25:1 and 12.5:1) and subsequently as non-independent repeated measures. These intermediate dilutions were in the linear range of the NK cytotoxicity curve and showed the greatest reproducibility (Shade *et al.* 2004). Generalized estimating equation regression accounted for within-person correlation of the two NK cytotoxicity measures (Zeger & Liang 1986).

PHA-stimulated lymphocyte proliferation was evaluated either directly as counts per minute (CPM-stimulated cells–CPM-unstimulated cells), and/or as a proliferation index (CPM-stimulated cells/CPM-unstimulated cells). A multi-factorial proliferation analysis of flow cytometry results was generated with the ModFit LT Program (Verity Software House LT, Topsham, ME, USA). Parameters of lymphocyte proliferation evaluated by the cell division tracking method included: percentage of parent cells, proliferation index (total number of counted cells/number of parent cells), and precursor frequency (the fraction of original parent cells that went on to proliferate three times and above).

Results

Characteristics of the study participants are described in Table 1. While there is no standard clinical range for serum leptin concentrations, the concentrations of all subjects fell within the 5th and 95th percentile for BMI-adjusted serum leptin as defined by Buettner *et al.* (2002). A wide range in leptin concentrations (about sixfold difference between the lowest and the highest value) was observed. The mean CRP

concentration was 3.6 ± 3.7 mg/l for all subjects and 3.1 ± 2.4 mg/l after exclusion of three subjects with CRP concentrations > 10 mg/l. These results are comparable to the mean CRP concentrations for the general population of women older than 50 (Flegal *et al.* 2002). Overall, we did not observe any immune abnormalities as all the women in the study were found to be within the normal range for the different immune measures. Additionally, statin use was found to be very low ($n = 7$, 6% overall) and did not affect the results (data not shown).

Statistically significant positive associations were observed between serum leptin concentrations and BMI ($r = 0.62$, $P < 0.0001$), percent body fat ($r = 0.63$, $P < 0.0001$), and intra-abdominal body fat ($r = 0.34$, $P < 0.0002$). On average, women classified as obese (BMI ≥ 30 kg/m²) had significantly higher serum leptin concentrations than those classified as overweight (32.5 ± 7.75 vs 23.9 ± 6.84 ng/ml, $P < 0.0001$).

A strong positive correlation was observed between serum CRP and SAA concentrations ($r = 0.61$, $P < 0.0001$). As shown in Fig. 1 and Table 2, leptin concentrations were positively associated with CRP and SAA (both log-transformed). The relationship between leptin and acute-phase proteins was stronger among women with a lower BMI (< 30 kg/m²). We then explored whether there was an independent association between serum leptin and the acute-phase proteins after adjusting for markers of obesity. After statistical adjustment for age and BMI or percent body fat, the association between serum leptin and each of the acute-phase proteins was attenuated, yet was still statistically significant. These results were independent of the exclusion of the three subjects with CRP concentrations > 10 mg/l (Table 2).

Serum IL6 was only weakly correlated with CRP (Pearson: $r = 0.20$, $P = 0.03$). IL6 was positively correlated with leptin, although this was statistically non-significant (Pearson: $r = 0.11$, $P = 0.23$). In age-adjusted regression models, serum leptin was positively associated with IL6, although this did not remain significant after adjusting for BMI and percent body fat (Table 2). When using a median split on serum leptin levels, associations were similar yet generally more statistically significant (Table 2).

We observed no statistically significant associations between serum leptin concentrations and absolute T- or B-lymphocyte numbers, T-lymphocyte subpopulations, or NK cells, whether non-transformed or when log-transformed values were considered (data not shown). A statistically non-significant positive association between serum leptin and absolute T-cell count was seen after adjustment for BMI or percent body fat ($P = 0.10$ and $P = 0.07$ respectively).

We did not observe any significant associations between leptin and NK cytotoxicity at ET ratio dilutions of 25:1 or 12.5:1 or when analyzed as non-independent repeated measures (Table 3). No significant association was observed between serum leptin concentrations and T-lymphocyte proliferation as determined by stimulation with PHA at 0.1

Table 1 Characteristics of study participants (*n*=114 women)

Characteristic	<i>n</i>	% of total ^a mean ± s.d. (range)
Demographics		
Age (years)	114	60.7 ± 6.9 (51–75)
Education level		
High school or less	17	14.9
Some college or college graduate	56	49.1
Postbaccalaureate or advanced degree	41	36.0
Body weight		
BMI (kg/m ²)	114	30.2 ± 3.71 (24.1–39.3)
BMI tertiles (kg/m ²)		
≤ 28	37	32.5
> 28, ≤ 31	37	32.5
> 31	40	35.1
BMI category (kg/m ²)		
Normal weight (18.5–24.9)	7	6.1
Overweight (25.0–29.9)	56	49.1
Obese (≥ 30.0)	51	44.7
Percent body fat (%)	114	47.0 ± 4.7 (34.1–58.5)
Ever lost ≥ 10 lb intentionally		
No	38	33.6
Yes	75	66.4
Hormone measure		
Leptin (ng/ml)	114	27.8 ± 8.4 (8.8–50.5)
Immune measures		
C-reactive protein (mg/l)	110	3.1 ± 2.4 (0.2–9.4)
Serum amyloid A (mg/l)	110	5.5 ± 3.4 (1.4–17.8)
Interleukin 6 (ng/l)	110	3.2 ± 3.1 (0–20)
NK cytotoxicity (%)		
25:1 effector-to-target ratio	113	27.1 ± 13.3 (8.8–50.5)
12:5: 1 effector-to-target ratio	113	19.9 ± 12.0 (3.2–59.6)
NK cell counts		
NK cells CD3 ⁺ , CD16 ⁺ , CD56 ⁺ , CD45 ⁺ ^b	114	162 ± 95 (35.1–525)
NK cells (% of lymphocytes)	114	8.6 ± 4.3 (2.4–22.6)
T-cell counts		
T-cells CD3 ⁺ , CD45 ⁺ ^b	114	1304 ± 362 (634–2607)
T-cells (% of lymphocytes)	114	70.6 ± 6.5 (52.3–85.4)
T helper cells CD3 ⁺ , CD4 ⁺ , CD45 ⁺ ^b	113	911 ± 277 (377–1830)
T helper cells (% of lymphocytes)	113	49.3 ± 8.1 (28.8–69.9)
T cytotoxic cells CD3 ⁺ , CD8 ⁺ , CD45 ⁺ ^b	112	374 ± 205 (138–1400)
T cytotoxic cells (% of lymphocytes)	112	20.4 ± 8.4 (9.0–51.4)
CD4 ⁺ /CD8 ⁺ ratio	112	2.9 ± 1.4 (0.6–7.0)
Naïve T helper cells (% of T helper cells)	111	43.3 ± 13.7 (7.0–80.0)
Naïve T cytotoxic cells (% of T cytotoxic cells)	112	30.8 ± 14.9 (6.0–79.0)
B cell counts		
B cells CD19 ⁺ , CD45 ⁺ ^b	114	264 ± 109 (66.0–645)
B cells ^b	114	14.3 ± 4.79 (4.3–27.8)
Lymphocyte proliferation (PHA stimulated)		
PHA (0.1 µg/ml)	109	33 399 ± 19 193 (649–82 116)
PHA proliferation index (0.1 µg/ml)	109	76.7 ± 47.7 (2.1–209)
PHA (0.5 µg/ml)	109	91 344 ± 40 417 (728–178 945)
PHA proliferation index (0.1 µg/ml)	109	207 ± 96.3 (2.4–457)
Lymphocyte proliferation (cell division tracking)		
Proliferation index	92	4.9 ± 2.1 (1.3–11.8)
Precursor frequency	92	0.26 ± 0.12 (0.02–0.51)
Parent %	92	17.1 ± 14.7 (4.4–71.9)
In parent %	92	2.6 ± 0.68 (1.5–4.3)

^aMay not equal 100% due to rounding error.^bCells/µl.

Figure 1 Association between serum leptin concentrations and (A) CRP or (B) SAA.

or $0.5 \mu\text{g/ml}$, whether investigating stimulated cell counts as a direct measure or using the proliferation index (Table 4). We also did not observe an association between serum leptin concentrations and parameters of lymphocyte proliferation as determined by the cell division tracking method using anti-CD3 antibodies (Table 4). A median split on serum leptin levels did not alter associations or significant levels of lymphocyte proliferation or NK cytotoxicity (data not shown).

Discussion

To our knowledge, this is the first study to address the relationship between serum leptin concentrations and

measures of immune function in healthy postmenopausal women. We assessed multiple biomarkers of cellular immunity and inflammation; thus, our study fills an important gap in knowledge about the *in vivo* association between leptin concentrations and immune function in overweight or obese, but otherwise healthy individuals.

As hypothesized, serum leptin concentrations were positively associated with the acute-phase proteins CRP and SAA, as well as IL6. After adjustment for either BMI or percent body fat, we observed an attenuation of the associations between leptin and these markers of inflammation. The relationship between leptin and acute-phase proteins was stronger among women with a lower BMI ($< 30 \text{ kg/m}^2$) for CRP, SAA, and, to some extent, IL6, suggesting a possible threshold effect or due to a smaller sample size for women with higher BMI ($\geq 30 \text{ kg/m}^2$). The possibility of this threshold effect – defined as a weakening association between leptin and the acute-phase proteins with increasingly higher leptin levels – needs to be investigated in future studies. No statistically significant association was observed between serum leptin concentrations and NK cytotoxicity, T-lymphocyte proliferation, or numbers of circulating lymphocytes, including Th1 phenotypes and NK cells.

With respect to the acute-phase proteins, we corroborated the findings of others who reported a positive correlation between serum leptin and CRP concentrations (Bastard *et al.* 2000, Bullo *et al.* 2003, Shamsuzzaman *et al.* 2004). This is the first study to show a similar association for SAA. SAA, an apolipoprotein, has been shown to parallel CRP as a predictor for cardiovascular disease risk in humans, and results from animal studies indicate that it may be involved in atherogenesis (Chait *et al.* 2005). We observed that the association between leptin and CRP and SAA is attenuated by statistical adjustment for parameters of body fat, suggesting that the positive association between leptin and CRP and SAA, in humans, is at least partially mediated via adipose tissue. However, unlike van Dielen *et al.* (2001), we observed that the association between leptin and CRP and SAA remained statistically significant after statistical adjustment for either BMI or percent body fat. These results demonstrate that during non-inflammatory conditions, leptin participates to some degree in the direct modulation of circulating concentrations of the acute-phase proteins, although this relationship is partially explained by a mutual link to adiposity. It is possible that the relationship between leptin and the acute-phase proteins is mediated via the cytokine IL6. IL6 is produced during non-inflammatory conditions in significant amounts by adipose tissue (Mohamed-Ali *et al.* 1997, Bastard *et al.* 1999). The results of clinical studies suggest that in the basal state, serum concentrations of CRP are primarily regulated by adipose IL6 secretion (Banks *et al.* 1995, Bastard *et al.* 1999, 2000). *In vitro* and preliminary human studies provide conflicting findings regarding the association between leptin and IL6 (Laharrague *et al.* 2000, Bruun *et al.* 2002, Vicennati *et al.* 2002), and this remains an area for future investigation. We were not able to evaluate IL6

Table 2 Association between serum leptin concentrations and markers of inflammation

	<i>n</i>	β Adjusted for age	<i>P</i>	β Adjusted for age and BMI	<i>P</i>	β Adjusted for age and % body fat	<i>P</i>
Immune function outcome							
β -Coefficient predicting immune function outcome for each 1 ng/ml increase in serum leptin level							
ln CRP (mg/l)	110	0.040	0.0001	0.030	0.01	0.029	0.02
BMI <30 kg/m ²	61	0.044	0.006	0.044	0.01	0.034	0.049
BMI $\geq$ 30 kg/m ²	49	0.011	0.43	0.020	0.17	0.026	0.13
ln SAA (mg/l)	110	0.021	0.001	0.017	0.03	0.011	0.15
BMI <30 kg/m ²	61	0.025	0.01	0.022	0.04	0.021	0.06
BMI $\geq$ 30 kg/m ²	49	0.011	0.31	0.012	0.31	−0.003	0.85
ln IL6 (ng/l)	111	0.021	0.04	0.022	0.10	0.010	0.45
BMI <30 kg/m ²	62	0.032	0.07	0.025	0.18	0.014	0.45
BMI $\geq$ 30 kg/m ²	49	0.0072	0.66	0.021	0.23	0.022	0.30
β -Coefficient predicting immune function outcome comparing serum leptin levels $\geq$ 27.79 ng/ml to <27.79 ng/ml (median split)							
ln CRP (mg/l)	110	0.62	0.0001	0.47	0.008	0.45	0.016
BMI <30 kg/m ²	61	0.73	0.001	0.72	0.002	0.61	0.01
BMI $\geq$ 30 kg/m ²	49	0.21	0.35	0.37	0.14	0.46	0.11
ln SAA (mg/l)	110	0.31	0.003	0.23	0.05	0.15	0.20
BMI <30 kg/m ²	61	0.43	0.002	0.1	0.005	0.39	0.009
BMI $\geq$ 30 kg/m ²	49	0.019	0.92	0.0085	0.97	−0.27	0.21
ln IL6 (ng/l)	111	0.30	0.08	0.26	0.18	0.12	0.55
BMI <30 kg/m ²	62	0.27	0.29	0.19	0.46	0.044	0.86
BMI $\geq$ 30 kg/m ²	49	0.31	0.27	0.56	0.06	0.61	0.07

Bold indicates *P* value <0.05.

concentrations directly in the adipose tissue, and serum concentrations tend to be only weakly associated with CRP (Saijo *et al.* 2004, Czarkowska-Paczek *et al.* 2005). Thus, it is not surprising that we observed more modest associations with IL6.

Cell and animal, as well as preliminary human studies suggest that leptin is a modulator of Th1 proinflammatory immune activity. Farooqi *et al.* (2002) reported reversal of depressed circulating CD4⁺T cells as well as reversal of T-cell hyporesponsiveness by administering recombinant leptin to two children with congenital leptin deficiency. Also, leptin-deficient mice exhibit increased infectious morbidity and mortality (Matarese *et al.* 2001, Siegmund *et al.* 2002, Sanna

et al. 2003). Furthermore, *in vitro*, leptin enhances both proliferation of naïve T-cells and their production of pro-inflammatory cytokines while suppressing Th2 response (Lord *et al.* 1998, 2002). However, the links between leptin and lymphocyte proliferation may not be apparent in overweight or obese, postmenopausal women, as we did not observe any association between serum leptin concentrations and specific lymphocyte subpopulations or T-lymphocyte proliferation.

Similarly, animal and *in vitro* studies provide evidence for leptin's role in NK cell activity (Li *et al.* 2002, Tian *et al.* 2002, Zhao *et al.* 2003). Motivala *et al.* (2003) reported that leptin was positively associated with NK cell activity in 27 alcohol-dependent and 34 non-alcohol-dependent men, although

Table 3 Association between serum leptin concentrations and natural killer (NK) cytotoxicity

	<i>n</i>	β^a Adjusted for age	<i>P</i>	β^a Adjusted for age and BMI	<i>P</i>	β^a Adjusted for age and % body fat	<i>P</i>
NK cytotoxicity							
12:1 effector-to-target ratio	113	−0.04	0.77	0.01	0.94	0.15	0.39
BMI <30 kg/m ²	63	−0.26	0.25	−0.13	0.59	−0.03	0.88
BMI $\geq$ 30 kg/m ²	50	0.13	0.59	0.18	0.50	0.21	0.46
25:1 effector-to-target ratio	113	−0.04	0.79	0.04	0.84	0.17	0.40
BMI <30 kg/m ²	63	−0.23	0.36	−0.12	0.66	−0.02	0.94
BMI $\geq$ 30 kg/m ²	50	0.15	0.56	0.21	0.46	0.27	0.39
NK cytotoxicity GEE ^b	113	−0.04	0.78	0.03	0.87	0.16	0.33
BMI <30 kg/m ²	63	−0.24	0.26	−0.12	0.55	−0.03	0.89
BMI $\geq$ 30 kg/m ²	50	0.14	0.53	0.20	0.42	0.24	0.38

^aThe β -coefficient predicting immune function outcome for each 1 ng/ml increase in serum leptin level.

^bGeneralized estimating equation regression, using both E:T 12.5:1 and 25:1 while accounting for within-person correlation (Shade *et al.* 2004).

Table 4 Association between serum leptin concentrations and measures of T-lymphocyte proliferation in response to anti-CD3 by flow cytometric cell division tracking and to PHA by [³H]thymidine uptake

	<i>n</i>	β ^a Adjusted for age	<i>P</i>	β ^a Adjusted for age and BMI	<i>P</i>	β ^a Adjusted for age and % body fat	<i>P</i>
Lymphocyte proliferation							
Anti-CD3 ^b							
Proliferation index ^c	92	−0.04	0.13	−0.01	0.64	−0.01	0.86
Precursor frequency ^d	92	−0.0003	0.83	−0.0005	0.76	−0.0008	0.68
Parent % ^e	92	0.09	0.62	−0.03	0.91	−0.06	0.80
In parent % ^e	92	0.007	0.41	0.004	0.71	0.002	0.83
PHA ^f							
Proliferation index ^g (0.1 µg/ml)	109	0.17	0.76	−0.30	0.67	0.64	0.38
Proliferation index ^g (0.5 µg/ml)	109	−0.09	0.94	−0.83	0.56	0.43	0.77

^aThe β-coefficient predicting immune function outcome for each 1 ng/ml increase in serum leptin level.
^bT-cells stimulated with anti-CD3 antibodies and measured by flow cytometry. Sufficient cells for flow cytometric assays on lymphocyte proliferation were available on *n*=92 individuals.
^cProliferation index=total number of counted cells/number of original parent cells.
^dPrecursor frequency=fraction of parent cells that went on to proliferate more than three times.
^eParent %=% original parent cells counted.
^fT-lymphocyte proliferation determined by [³H]thymidine uptake after stimulation with phytohemagglutinin (PHA).
^gProliferation index=counts per minute stimulated cells/counts per minute unstimulated cells.

leptin was not found to be a direct stimulator of NK cytotoxicity *in vitro*. Our study may have different results because their study participants had a lower average BMI (27.0 and 24.8 kg/m² for controls and alcoholics respectively) than our subjects (30.2 kg/m²) and because we studied postmenopausal women. Our study is consistent with research by [Dovio *et al.* \(2004\)](#) who did not observe differences in spontaneous NK cell activity between obese subjects versus controls. They showed a correlation between inhibition of NK cytotoxicity by cortisol, yet we did not perform this analysis. In general, our results did not corroborate the evidence from some *in vivo* and *in vitro* studies for a positive association between leptin concentrations and NK cell numbers or cytotoxicity. It is conceivable that this may be a characteristic of the population studied, which had generally higher leptin levels than young adults or males.

One reason why we may not have identified a relationship between leptin and numbers of T-lymphocytes, lymphocyte proliferation, or NK cell activity in our subjects is that our study was limited to overweight or obese, but otherwise healthy individuals. A leptin-immune function relationship may exist only among leptin-deficient individuals or among those with chronic or acute inflammatory conditions. Animal studies that demonstrate links between serum leptin and immune function were based on leptin-deficiency models ([Lord *et al.* 1998, 2002](#), [Howard *et al.* 1999](#), [Sanna *et al.* 2003](#)). Our study suggests that among overweight, or obese women, there is no link between leptin and immune function markers with the exception of the acute-phase proteins, CRP and SAA. For the latter, the association was stronger among leaner women, supporting the notion of a biological threshold effect.

The major strengths of this study include the study size, the homogeneity of the study population, state-of-the-art measurements of both cellular immunity and inflammation,

and strict exclusion criteria, which reduced the potential effects of factors that could influence the relationship between leptin concentrations and immune function measures (e.g., HRT, smoking, alcohol, chronic inflammatory conditions). Our study was limited to women classified as overweight or obese or with percent body fat >33%, and thus our findings may not extend to non-overweight women. Additionally, our study only included sedentary women, potentially explaining the differences between our results and those of other studies. A control group with normal BMI was unfortunately not available in this study, due to funding limitations and timing in assays; we certainly recommend that such a control group be included in future studies. Nevertheless, the study included a very wide range of BMI and percent body fat (24.1–39.3 kg/m² and 34.1–58.5% respectively) as well as serum leptin concentrations that varied nearly sixfold (8.8–50.5 ng/ml). Furthermore, the body mass indexes of the participants in this study reflect about 60% of the general US population in this age group ([Flegal *et al.* 2002](#)).

This study provides evidence for a strong association between serum leptin and the inflammatory proteins CRP and SAA, which is mediated only to some degree by adipose tissue. This suggests a direct link between these markers of inflammation and leptin. Our data provide no evidence for associations between leptin and other measures of immune function among overweight or obese, but otherwise healthy postmenopausal women, which indicates that immunomodulatory effects of leptin on NK or T-cell function may only be apparent during leptin deficiency. Future research should also consider additional immune function assays, such as neutrophil/monocyte counts or the production of pro-inflammatory cytokines by PBMCs.

Declaration of Interest

The authors have no conflicts of interest to declare.

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