Adiponectin Is a Negative Regulator of NK Cell Cytotoxicity[1]

Kun-yong Kim,* Jae Kwang Kim,* Seung Hyun Han,† Jong-Seok Lim,* Keun Il Kim,* Dae Ho Cho,* Myeong-Sok Lee,* Jeong-Hyung Lee,‡ Do-Young Yoon,§ Suk Ran Yoon,‡ Jin Woong Chung,‡ Inpyo Choi,‡ Eunjoon Kim,¶ and Young Yang[2]*

NK cells are a key component of innate immune systems, and their activity is regulated by cytokines and hormones. Adiponectin, which is secreted from white adipose tissues, plays important roles in various diseases, including hypertension, cardiovascular diseases, inflammatory disorders, and cancer. In this study the effect of adiponectin on NK cell activity was investigated. Adiponectin was found to suppress the IL-2-enhanced cytotoxic activity of NK cells without affecting basal NK cell cytotoxicity and to inhibit IL-2-induced NF-κB activation via activation of the AMP-activated protein kinase, indicating that it suppresses IL-2-enhanced NK cell cytotoxicity through the AMP-activated protein kinase-mediated inhibition of NF-κB activation. IFN-γ enhances NK cell cytotoxicity by causing an increase in the levels of expression of TRAIL and Fas ligand. The production of IFN-γ, one of the NF-κB target genes in NK cells, was also found to be suppressed by adiponectin, accompanied by the subsequent down-regulation of IFN-γ-inducible TRAIL and Fas ligand expression. These results clearly demonstrate that adiponectin is a potent negative regulator of IL-2-induced NK cell activation and thus may act as an in vivo regulator of anti-inflammatory functions. *The Journal of Immunology,* **2006, 176: 5958–5964.**

Adipose tissue is currently considered to be a hormonally active system, because adipokines that are secreted from adipocytes are transported into the bloodstream and are associated with various aspects of metabolic control (1). These adipokines, which include IL-6, TNF-α, leptin, resistin, and adiponectin, play important roles in both the immune system and metabolism. IL-6 and TNF-α were first studied as components of the immune system and were later reported to act as adipokines, whereas leptin and adiponectin were isolated in adipocytes, and their roles in the immune system were investigated later (2–5). The role of leptin in the regulation of the immune system has been extensively examined. Leptin enhances GM-CSF production in peritoneal macrophages (6) and mediates both proliferative and antiapoptotic activities in a variety of cell types, including T cells (7) and monocytes (8). Leptin has been proposed to serve as a potential linker between obesity and chronic inflammation (9).

Adiponectin is produced exclusively by white adipocytes, but, paradoxically, its level is lower in obese than in lean individuals (10). It is known that a decrease in adiponectin levels is associated with diabetes, and that recovery of adiponectin level ameliorates insulin resistance through an increase in free fatty acid oxidation and glucose uptake (11). There is a growing body of evidence to suggest that adiponectin also has anti-inflammatory activity. It negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages (12) and inhibits the endothelial NF-κB signaling pathway (13), foam cell formation by reducing the accumulation of lipids in human macrophages (5), and vascular inflammation in adiponectin knockout mice (14). Adiponectin also causes an increase in IL-10 mRNA expression in human macrophages (15). Although its role as an immune regulator has been studied, little is known concerning the function of adiponectin in NK cell activity. The findings presented in this report show that adiponectin suppresses IL-2-induced NK cell cytotoxicity by inhibiting the NF-κB signaling pathway and down-regulating IFN-γ, Fas ligand (FasL),[3] and TRAIL expression. As a result, we report a new function of adiponectin as a regulator of NK activity.

Materials and Methods

NK cell culture

Human NK 92 cells were cultured in α-MEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 10 mM HEPES, and 100 U/ml IL-2, and primary mouse NK cells were maintained with RPMI 1640 medium containing 10% FBS, L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 10 mM HEPES (Invitrogen Life Technologies) at 37°C in a humidified 5% CO_2 incubator.

Purification of recombinant adiponectin

Escherichia coli strain BL21 (λDE3) was transformed with pET28-adiponectin (Novagen) containing adiponectin cDNA without a signal sequence coding region. The expression of adiponectin was induced by treatment with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37°C. After sonication and centrifugation, the inclusion body was solubilized in guanidine buffer (50 mM Tris (pH 8.0) and 7 M guanidine). The refolding conditions for adiponectin were determined according to the Pro-Matrix protein refolding guide (Pierce). Refolded adiponectin in refolding buffer (55 mM Tris, 21 mM NaCl, 0.88 mM KCl, 1 mM DETA, 2 mM reduced glutathione, and 0.2 mM glutathione disulfide (pH 8.2)) was dialyzed

*Laboratory of Molecular and Cellular Biology, Department of Life Science, Sookmyung Women's University, Seoul, Korea; †Department of Oromaxillofacial Infection Immunity and Dental Research Institute, School of Dentistry, Seoul National University, Seoul, Korea; ‡Korea Research Institute of Bioscience and Biotechnology, Daejon, Korea; §Department of Bioscience and Biotechnology, Konkuk University, Seoul, Korea; and ¶Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejon, Korea

Received for publication October 12, 2005. Accepted for publication February 27, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant R01–2004-000-10648-0 from the Basic Research Program of the Korean Science and Engineering Foundation, a Korea Research Foundation Grant KRF-2005-003-C00134 funded by the Korean Government (Minisry of Education and Human Resources Development, Basic Research Promotion Fund), and the Science Research Center program of Korea Science and Engineering Foundation (R11–2005-017-01002-0).

[2] Address correspondence and reprint requests to Dr. Young Yang, Laboratory of Molecular and Cellular Biology, Department of Life Science, Sookmyung Women's University, Seoul 140-742, Korea. E-mail address: yyang@sookmyung.ac.kr

[3] Abbreviations used in this paper: FasL, Fas ligand; AICAR, 5-aminoimidazole-4-carboxamide-1β-4-ribofuranoside; AMPK, AMP-activated protein kinase; DN, dominant negative; Luc, luciferase.

0022-1767/06/$02.00

against 20 mM Tris-HCl (pH 8.0), filtered, loaded onto a Q-Sepharose column, then eluted with 0.5 M NaCl in a stepwise manner. Fractions containing pure adiponectin were dialyzed against 20 mM Tris-HCl (pH 8.0), and endotoxin contaminants were removed by extraction with Triton X-114. The level of endotoxin was determined with a *Limulus* amebocyte lysate QCL-1000 kit (Cambrex). The purity of the adiponectin exceeded 95%, as evidenced by SDS-polyacrylamide gel staining, and the level of endotoxin was <0.128 endotoxin units/mg in the purified adiponectin.

Purification of NK cells using MACS

For NK cell isolation, female C57BL/6 mice were purchased from Polars International. An NK cell isolation kit (Miltenyi Biotec) was used to separate NK cells from splenocytes. Briefly, isolated splenocytes were washed twice with BSA-PBS (0.5% BSA and 0.2 mM EDTA in PBS), incubated with a biotin-Ab mixture (anti-CD4, -CD5, -CD8a, -CD19, -Ly-6G, and -Ter^{119}) at 4°C for 10 min, then incubated with the microbeads conjugated to a monoclonal anti-biotin Ab at 4°C for 15 min. After washing twice, the labeled cells were applied to a MACS column (CS$^+$; Miltenyi Biotec). The column was rinsed with 30 ml of BSA-PBS to flush out unbound NK cells. For analysis of the purity of the cells using flow cytometry, negatively isolated NK cells were stained with goat anti-mouse NK1.1-FITC. The purity of the NK cells exceeded 95%.

^{51}Cr release cytotoxicity assay

After the stimulation period, NK cells were harvested and washed twice. Viable NK cells were counted using the trypan blue dye exclusion method, and an equal number of viable NK cells was used as effectors in a ^{51}Cr release assay. K562 and YAC-1 target cells were incubated with 1.5 μCi of ^{51}Cr for 1 h at 37°C, allowing ^{51}Cr to enter target cells. The labeled target cells were then washed twice and dispensed at a concentration of 5000 target cells/well in a round-bottom, 96-well plate. The effectors were distributed in triplicate at E:T cell ratios from 200:1 to 1:1 and incubated for 4 h at 37°C in 5% CO_2. After the incubation, the supernatants were harvested, and the released radioactivity was counted with a scintillation counter (RACTOBETA; LKB Instruments). The percentage of specific activity was calculated using the following equation: % specific release = ((experimental release − spontaneous release)/(maximum release − spontaneous release)) × 100%.

Gel mobility shift assay

The dsDNA oligonucleotides containing the NF-κB element (5′-agttgagggggactttcccaggc-3′) were labeled with [γ-^{32}P]ATP (ICN) using T4 polynucleotide kinase (New England Biolabs) and purified using a Sephadex G-50 column (Roche). Ten micrograms of nuclear extracts was incubated with ^{32}P-labeled NF-κB oligonucleotides in the binding buffer (100 mM KCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) for 15 min at 4°C. To reduce nonspecific bands, 1 μg of poly(dI-dC) was added to the reaction. DNA binding activity was observed by separating the unbound probe on a 4.8% polyacrylamide gel in 0.5× TBE (250 mM Tris (pH 8.5), 1.92 M boric acid, and 10 mM EDTA). After the electrophoresis run, the gel was dried, then exposed to an x-ray film.

Measurement of luciferase activity

The pNF-κB-luciferase (pNF-κB-luc) gene reporter vector was purchased from BD Clontech. The pCMV-*Renilla* luciferase (pCMV-RL) vector was purchased from Promega. Constitutively active AMP-activated protein kinase-1α (AMPK-α1) and dominant negative AMPK-α1 (DN-AMPK-α1) expression plasmids were gifts from Dr. D. Carling (Medical Research Council Clinical Sciences Center, London, U.K.). Human embryonic kidney 293T cells were plated in 12-well plates 1 day before the experiment, and transient transfection of the indicated genes was conducted using the calcium phosphate method. The next day, the cells were treated with adiponectin and 5-aminoimidazole-4-carboxamide-1β-4-ribofuranoside (AICAR), purchased from Toronto Research Chemicals, for a period of 24 h. The constitutively active pCMV-*Renilla* plasmid, which expresses *Renilla* luciferase, was used to normalize for transfection efficiency. The cells were washed twice with PBS and lysed in 300 μl of reporter lysis buffer (Promega). The two luciferase activities were measured using a dual luciferase assay kit (Promega) on a microplate luminometer (Tuner Designs).

Western blot analysis

Total cell lysates were prepared 24 h after treatment, separated on a 12% SDS-polyacrylamide gel, then transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk in TBS (20 mM Tris-HCl (pH 7.5) and 137 mM NaCl) for 30 min, then incubated with phospho-AMPK and phospho-IκB Abs (Cell Signaling Technology) overnight. After washing three times with TBST (0.05% Tween 20, 20 mM Tris-HCl (pH 7.5), and 137 mM NaCl) for 15 min, the membrane was incubated with the anti-rabbit IgG secondary Ab conjugated with HRP. The signal was detected using the ECL system (Amersham Biosciences).

Real-time RT-PCR analysis

Total RNAs from splenic NK and human NK 92 cells were isolated using RNAzol B (Tel-Test). For the real-time PCR analysis, 3 μg of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) at 37°C for 1 h. PCR for the amplification of various cytokines was then performed. Each amplification cycle consisted of 10 s of denaturation at 94°C, 15 s of annealing at 55°C, and 20 s of extension at 72°C, which was performed on a Rotor-Gene 3000 real-time cycler (Corbett Research). β-Actin was used as the control in the comparative cycle threshold method.

Flow cytometric analysis

IL-2-, adiponectin-, AICAR-, and compound C- (Calbiochem) treated primary NK cells were stained with anti-FasL and anti-NK1.1 (PK136) mAbs (BD Pharmingen) for 20 min at 4°C. NK cell viability was assessed using an annexin V kit (BD Pharmingen). Briefly, NK cells were washed twice with PBS and stained with annexin V-FITC for 15 min at room temperature in the dark. The stained cells were analyzed by flow cytometry. The resulting data were analyzed using the WinMDI software program.

Measurement of IFN-γ production

Mouse IFN-γ production was determined using a mouse IFN-γ ELISA kit (Pierce). Briefly, purified NK cells were treated with the indicated reagents, and the resulting supernatant was used according to the manufacturer's instructions.

Statistical analysis

The data are presented as the mean ± SEM and were statistically analyzed using an unpaired *t* test. A value of $p < 0.05$ was considered a significant difference.

Results

Adiponectin suppresses IL-2-mediated NK cytotoxicity

In the present study the role of adiponectin in NK cytotoxicity was examined. NK cells, purified from splenocytes, were treated with adiponectin and IL-2 for 24 h, washed twice, and cultured with ^{51}Cr-labeled YAC-1 target cells for 4 h, and the released ^{51}Cr activity was determined. Interestingly, adiponectin dramatically suppressed IL-2-enhanced NK cytotoxicity in an E:T cell ratio-dependent manner, but did not affect basal spontaneous NK cytotoxicity in the absence of IL-2 (Fig. 1*A*). To determine whether adiponectin inhibits the cytotoxicity of NK cells that had been pretreated with IL-2, purified NK cells were pretreated with IL-2 for 4 h, followed by washing twice, and then were incubated with adiponectin alone for an additional 16 h. At the end of the culture period, the amount of ^{51}Cr released into the culture medium was determined. Although IL-2 was removed 4 h after treatment, adiponectin inhibited IL-2-induced NK cytotoxicity (Fig. 1*B*), suggesting that IL-2-triggered signal transduction pathways are blocked by adiponectin. We next examined the issue of whether adiponectin affects IL-2-enhanced NK cell viability, because IL-2 enhances rodent NK cell viability. IL-2 increased NK cell viability, as assessed by annexin V staining. Adiponectin treatment decreased IL-2-increased NK cell viability, but had no effect on basal NK cell viability (Fig. 1*C*). Therefore, an equal number of viable NK cells was used to avoid the effect of dead NK cells in the NK cytotoxicity assay.

To examine the dose-dependent effect of adiponectin on the suppression of IL-2-mediated NK cytotoxicity, total splenocytes were treated with various concentrations of adiponectin in the presence or the absence of IL-2 for 24 h (Fig. 1*D*). NK cytotoxicity was

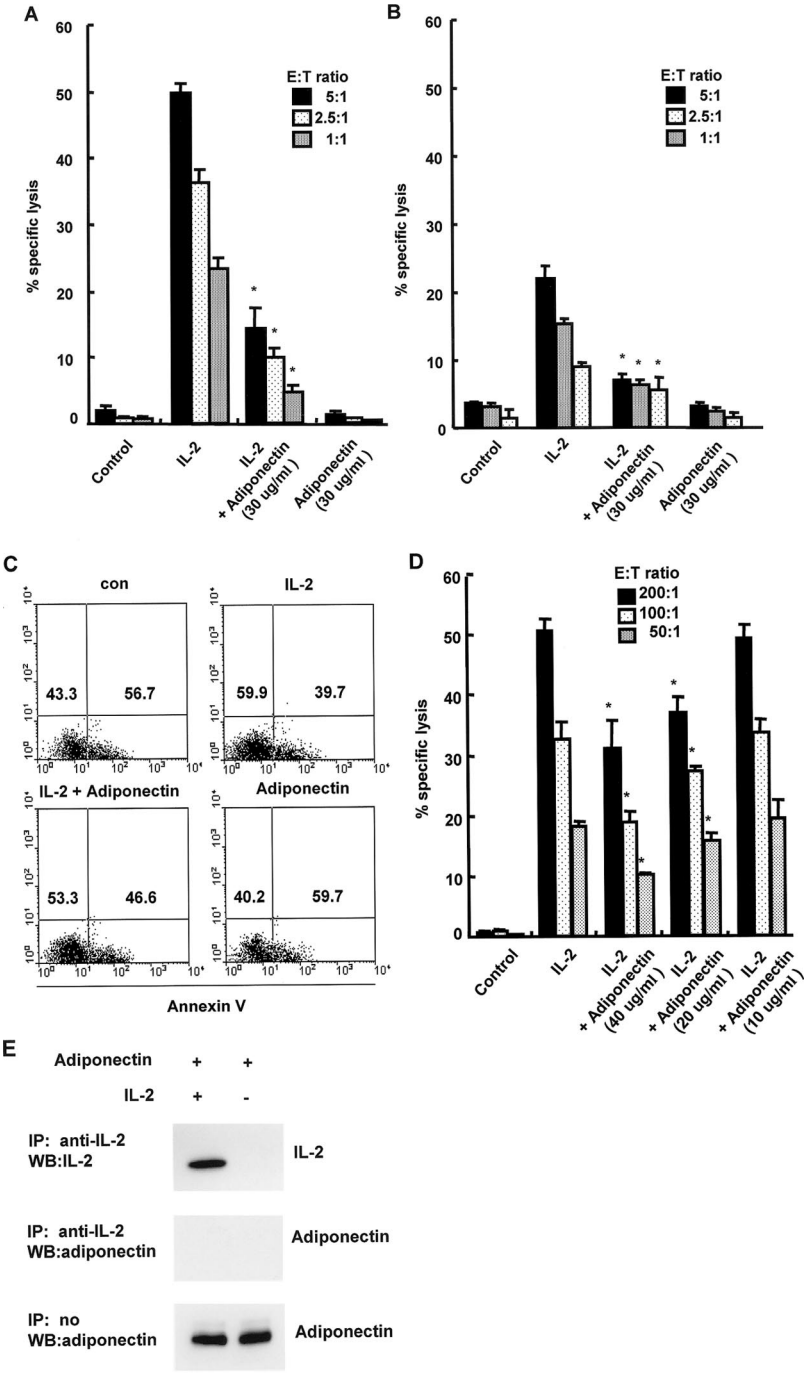

FIGURE 1. Effects of adiponectin on IL-2-activated NK cell cytotoxicity. *A*, Freshly isolated mouse primary NK cells were treated with 100 U/ml IL-2 for 24 h in the presence or absence of 30 μg/ml adiponectin. They were challenged with ^{51}Cr-labeled YAC-1 lymphoma cells at different E:T cell ratios from 1:1 to 5:1. Cytolytic activity against YAC-1 target cells was measured 4 h after the start of the incubation. *B*, Freshly isolated mouse primary NK cells were treated with 100 U/ml IL-2 for 4 h, followed by two washings, then incubated with 30 μg/ml adiponectin for 16 h. Cells were challenged with ^{51}Cr-labeled YAC-1 lymphoma cells at different E:T cell ratios from 1:1 to 5:1. Cytolytic activity against YAC-1 target cells was measured 4 h after the start of the incubation. *C*, Freshly isolated mouse primary NK cells were treated with 100 U/ml IL-2 for 24 h in the presence or the absence of 30 μg/ml adiponectin and were stained with annexin V-FITC. *D*, Splenocytes were incubated with the indicated concentrations of adiponectin in the presence of IL-2 for 24 h, then plated with ^{51}Cr-labeled YAC-1 target cells at a E:T cell ratio from 50:1 to 200:1. *E*, Recombinant IL-2 (100 ng/ml) and adiponectin (80 ng/ml) were mixed and incubated for 1 h. Immunoprecipitation was performed with anti-IL-2 Ab, and the precipitates were subjected to Western blot assay using anti-IL-2 and anti-adiponectin Abs. All values are represented as the mean ± SEM of triplicate determinations. *, $p < 0.01$ compared with the IL-2-treated sample.

suppressed in the physiological concentration range of adiponectin. The cytotoxicity of human NK 92 cells was also suppressed by treatment with human recombinant adiponectin (data not shown). It is possible that adiponectin inhibits IL-2-induced NK cell cytotoxicity through binding to IL-2. To test this possibility, rIL-2 and adiponectin were mixed, incubated for 1 h, then precipitated with anti-IL-2 Ab. The resulting precipitates were subjected to Western blot assay using anti-IL-2 and anti-adiponectin Abs, respectively. No binding was observed (Fig. 1*E*). We also examined adiponectin receptor expression in NK cells using RT-PCR. The expression of adiponectin receptor 1 was higher than that of adiponectin receptor 2 (data not shown). These results indicate that adiponectin inhibits IL-2-induced NK cell cytotoxicity through binding to its specific receptors without interacting with IL-2.

Adiponectin suppresses IL-2-mediated NF-κB activation

IL-2 increases NK cell cytotoxicity and proliferation through activation of the NF-κB signaling pathway. We investigated whether adiponectin has an effect on the IL-2-activated NF-κB signaling pathway in NK cells. Purified NK cells were treated with adiponectin in the presence or the absence of IL-2 for 24 h, and nuclear extracts were isolated. To measure the DNA-binding activity of NF-κB to its respective recognition element, a gel EMSA was performed. Adiponectin inhibited IL-2-mediated NF-κB activation (Fig. 2*A*). The specificity of the shifted NF-κB bands was verified by means of a supershift assay. The shifted NF-κB bands were supershifted with anti-p65 Ab and anti-p50 Ab, indicating that NF-κB bands are composed of p65 and p50. To confirm that

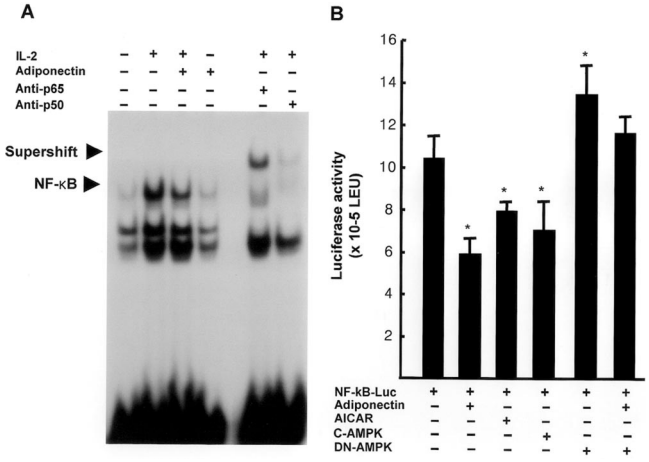

FIGURE 2. Effects of adiponectin on the IL-2-mediated NF-κB signaling pathway. *A*, Purified mouse NK cells were treated with 100 U/ml IL-2 in the presence or absence of 30 μg/ml adiponectin for 24 h. The immediately isolated nuclear extracts were subjected to gel mobility shift assay and supershift assay with ^{32}P-labeled oligonucleotides containing the consensus NF-κB binding sites. *B*, pNF-κB-luciferase reporter vector was transiently cotransfected with constitutively active AMPK-α1 (C-AMPK) or DN-AMPK in human embryonic kidney 293T cells. After overnight recovery, the transfected cells were treated with 30 μg/ml adiponectin or 250 μM AICAR for 24 h, and luciferase activity was measured. All values are represented as the mean ± SEM of triplicate determinations. ∗, $p < 0.01$ compared with the vector control.

the decreased NF-κB activity was associated with the suppression of NF-κB target gene expression, a pNF-κB-luc vector was transfected into 293 cells. Luciferase activity was significantly decreased as the result of adiponectin treatment (Fig. 2*B*). It is known that AMPK is an important signaling component in the adiponectin signal pathway and inhibits NF-κB activation in cultured HUVECs (16). Thus, the effects of constitutively active AMPK, DN-AMPK, and AICAR, which is an AMPK activator, on the suppression of NF-κB activity by adiponectin were examined. As expected, constitutively active AMPK and AICAR inhibited NF-κB luciferase activity, as did adiponectin, whereas DN-AMPK alone increased NF-κB activity through suppression of endogenous AMPK activity. Treatment of DN-AMPK transfectants with adiponectin indicated the control level of NF-κB luciferase activity, because DN-AMPK prevented the adiponectin-mediated suppression of NF-κB luciferase activity. These data indicate that adiponectin inhibits NF-κB activation through the activation of AMPK.

Adiponectin inhibits IL-2 function by preventing IκB degradation

NF-κB activity is regulated by IκB. Upon phosphorylation, IκB proteins are degraded via the proteasome pathway, resulting in the nuclear translocation of NF-κB and, ultimately, the activation of target genes. We examined the effect of adiponectin on the phosphorylation of IκB. IL-2 treatment increased the level of phosphorylation of IκB, but the phosphorylation of IκB was inhibited by cotreatment with adiponectin (Fig. 3*A*). Because it is well known that adiponectin activates AMPK (11), we examined the effect of AICAR, an AMPK activator, instead of adiponectin. As expected, AICAR blocked the IL-2-induced phosphorylation of IκB. To confirm the role of AMPK in the regulation of IκB phosphorylation, compound C, an AMPK inhibitor, was used. The adiponectin-mediated suppression of IκB phosphorylation was recovered in the presence of compound C and IL-2. To determine whether adiponectin and these two compounds affected AMPK signaling pathway, the phosphorylation of AMPK

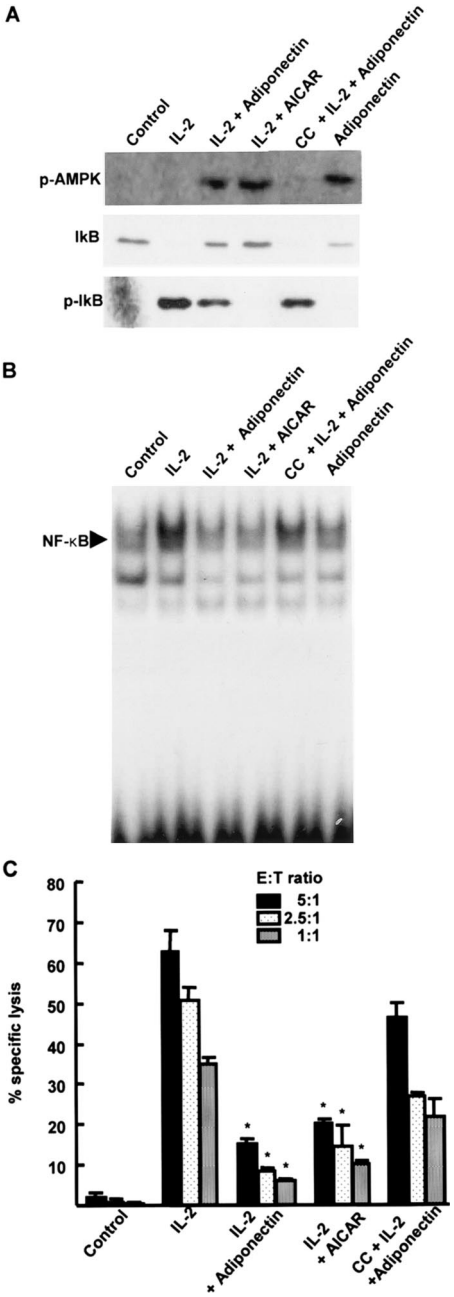

FIGURE 3. Suppression of NK cell activity by adiponectin through the prevention of IL-2-induced IκB degradation. *A*, Human NK 92 cells were serum starved for 24 h, then treated with 30 μg/ml human adiponectin and/or 250 μM AICAR or 5 μM compound C, an AMPK inhibitor. To block adiponectin-mediated AMPK activation, the culture was pretreated with 5 μM compound C for 30 min. After 24 h of incubation for phospho-IκB detection and 15 min for phospho-AMPK, the isolated NK cells lysates were subjected to Western blot analysis. *B*, A gel mobility shift assay was performed with nuclear extracts isolated from the experiment shown in *A*, and ^{32}P-labeled oligonucleotides containing NF-κB binding element were used as probes. *C*, Purified mouse NK cells were pretreated with 5 μM compound C for 30 min, then treated with 30 μg/ml adiponectin or 250 μM AICAR in the presence of 100 U/ml IL-2. After 24 h, NK cells were plated with ^{51}Cr-labeled YAC-1 target cells at an E:T cell ratio from 1:1 to 5:1 for 4 h. All values are represented as the mean ± SEM of triplicate determinations. ∗, $p < 0.01$ compared with the IL-2-treated sample. CC, compound C.

was examined (Fig. 3*A*). The results indicate that adiponectin inhibits NK cell cytotoxicity by suppressing the IL-2-mediated phosphorylation of IκB.

We next performed a gel mobility shift assay to examine the nuclear translocation of NF-κB. Adiponectin inhibited the IL-2-induced nuclear translocation of NF-κB, and the AMPK inhibitor, compound C, reversed the effect of adiponectin on the nuclear translocation of NF-κB, whereas AICAR mimicked adiponectin function (Fig. 3*B*). We next examined whether the AMPK inhibitor and activator had the ability to regulate NK cell cytotoxicity. Purified NK cells were treated for 24 h as indicated in Fig. 3*C*, and the released ^{51}Cr activity was determined. As expected, the AMPK activator inhibited IL-2-mediated NK cell cytotoxicity, similar to adiponectin, and the AMPK inhibitor recovered the NK cell cytotoxicity that had been inhibited by adiponectin.

Adiponectin suppresses IL-2-enhanced IFN-γ production in NK cells

IL-2 stimulates NK cells to produce IFN-γ (17), and the production of IFN-γ is also regulated by the NF-κB element on the promoter of IFN-γ (18). Meanwhile, IFN-γ enhances NK cell cytotoxicity via the induction of FasL and TRAIL expression in NK cells. Thus, we determined whether adiponectin regulates IL-2-enhanced IFN-γ production in NK cells. Purified NK cells were treated with adiponectin in the presence or the absence of IL-2 for 24 h, and the supernatant was used to measure IFN-γ production. Treatment with adiponectin abrogated IL-2-induced IFN-γ production, whereas adiponectin alone had no effect on IFN-γ production (Fig. 4*A*). AICAR, an AMPK activator, also inhibited IFN-γ production, although its effect was not much stronger than that of adiponectin. This suggests that adiponectin exerts its NK inhibitory effect via the suppression of IFN-γ production.

Because adiponectin suppressed IL-2-induced IFN-γ production in NK cells, it is possible that adiponectin reduces FasL and TRAIL expression by down-regulating IFN-γ production. To confirm this, FasL and TRAIL mRNA expressions were measured by RT-PCR after the various treatments indicated in Fig. 4*B*. As expected, adiponectin dramatically suppressed IL-2-induced FasL and TRAIL mRNA expressions. We also examined FasL protein expression using flow cytometry. Purified NK cells were treated as indicated in Fig. 4*C*. Cotreatment of adiponectin or AMPK activator with IL-2 suppressed the expression of FasL protein, and cotreatment of adiponectin with the AMPK inhibitor in the presence of IL-2 reversed the adiponectin-mediated suppression of FasL protein expression. Collectively, these data indicate that adiponectin inhibits FasL and TRAIL expression via the down-regulation of IFN-γ production induced by IL-2.

Discussion

Previous studies have demonstrated that NK cells are activated by a number of cytokines, including IL-2, IL-12, IL-15, and type I IFNs (19–21), and are inhibited by glucocorticoids at physiological concentrations and by nutrients (22–24). The findings we report demonstrate that adiponectin inhibits IL-2-increased NK cell cytotoxicity without the effect of basal NK cell cytotoxicity and decreases IL-2-induced NK cell viability without the effect of basal NK cell viability. The findings also show that the adiponectin-mediated inhibition of NF-κB activity is an important molecular mechanism by which adiponectin suppresses NK cell cytotoxicity. Adiponectin activated AMPK through its receptor, and the activation of AMPK suppressed IL-2-enhanced NF-κB activity, which is critical in the increase in NK cell cytotoxicity by IL-2 (Fig. 3), consistent with previous reports indicating that AMPK inhibits fatty acid-enhanced NF-κB activity and NF-κB-mediated gene expression by TNF-α in HUVECs (16). This is also supported by previous findings showing that adiponectin inhibits endothelial NF-κB signaling through a cAMP-dependent pathway (13), and

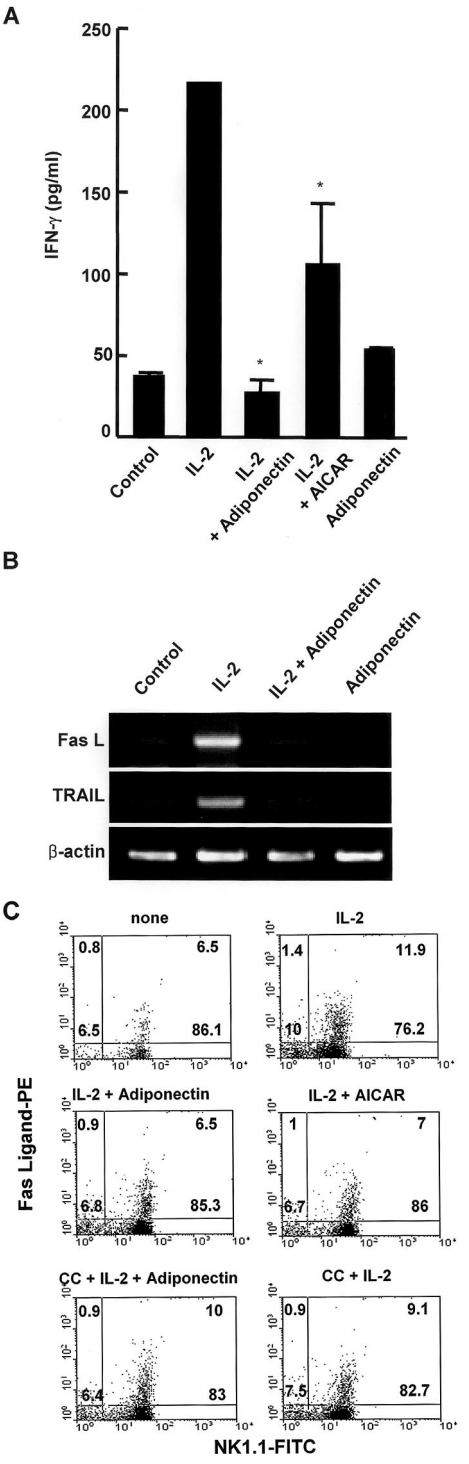

FIGURE 4. Effect of adiponectin on IFN-γ expression in IL-2-activated NK cells. *A*, Freshly isolated mouse primary NK cells were treated with 30 μg/ml adiponectin or 250 μM AICAR in the presence or absence of 100 U/ml IL-2. After a 24-h period of stimulation, IFN-γ production was measured by ELISA. All values are represented as the mean ± SEM of triplicate determinations. *B*, Purified mouse NK cells were stimulated with adiponectin in the presence or absence of IL-2 for 24 h. Total RNAs were subjected to RT-PCR for FasL and TRAIL determinations. *C*, Purified NK cells were incubated at the indicated conditions. To examine the cell surface expression of FasL, treated NK cells were stained with anti-FasL and anti-NK1.1 mAbs for 15 min and subjected to flow cytometric analysis. *, $p < 0.01$ compared with the IL-2-treated sample.

cAMP is able to activate AMPK (25). Experiments involving the use of inhibitors or activators of AMPK confirmed that AMPK plays a role in the adiponectin-mediated suppression of NK cell cytotoxicity. However, the AMPK inhibitor failed to fully recover the adiponectin-mediated inhibition of NK cytotoxicity and NF-κB activity (Fig. 3, *B* and *C*). Thus, the existence of other pathways that regulate NK cell activity or NF-κB activity by adiponectin cannot be ruled out. IL-2 has been shown to activate ERK1/2 and Akt to induce human telomerase reverse transcriptase expression in a human NK cell line NK 92 (26), and the ERK pathway plays a critically positive role in IL-2-induced NK cell activation (27). Therefore, ERK1/2 may be an alternative pathway. In contrast, adiponectin has been reported to suppress ERK1/2 activity (28), leading to the activation of NF-κB (29). Thus, it is possible that IL-2-stimulated NF-κB activation through ERK1/2 activation might be a target signaling pathway that can be blocked by adiponectin.

Leptin, an adipocyte-secreted adipokine, plays an important role in regulation of the immune system. Leptin enhances the cytotoxicity of splenic NK cells (30) at physiological concentrations and stimulates proliferation and cytokine synthesis in peripheral human lymphocytes (4, 31). Leptin levels are highly correlated with body fat mass in both lean and obese subjects. Adiponectin also plays a role in the immune system. It inhibits myelomonocytic activity, phagocytic activity, and TNF-α production (12); suppresses lipid accumulation in human monocyte-derived macrophages (5); and prevents the activation of NF-κB in aortic endothelial cells, porcine macrophages, and 3T3-L1 adipocytes (32). In addition to these known anti-inflammatory activities of adiponectin, the findings we present show that adiponectin suppresses cytokine-enhanced NK cell cytotoxicity, indicating that adiponectin may act as a negative regulator, whereas leptin functions as a positive regulator, in the immune system. Meanwhile, contrary to leptin, adiponectin levels are negatively correlated with body fat mass in obese subjects. This might be related to the opposite roles of these two adipokines.

It has been proposed that obesity is a type of low grade chronic inflammation (33), because a higher mass of adipose tissue secretes increased amounts of proinflammatory cytokines, including IL-1, IL-6, TNF-α, and acute phase proteins (34, 35), and macrophages infiltrate the fat mass in obese individuals (36). In addition, the production of adiponectin, which has anti-inflammatory properties, is decreased in obese individuals. Thus, a reduced level of adiponectin may contribute to produce a microenvironment for chronic inflammation in obesity. This chronic inflammation status may be indirectly associated with the development of diabetes.

Several reports have appeared, demonstrating that obesity is associated with altered NK function. For example, the NK cell population among total T lymphocytes in epididymal and inguinal fat tissues is greater than that in the lymph node, and NK cell numbers are decreased in obese mice fed a high fat diet (37). IL-2-induced NK cell-dependent cytotoxicity in PBL of obese individuals is increased with fat and carbohydrate intake (38). Collectively, these results suggest that adipose tissue may elaborate an immune system, including NK cells. Although the physiological role of adiponectin in the regulation of NK cell activity remains to be elucidated, the findings reported in this study demonstrate that adiponectin, an anti-inflammatory adipokine, plays a negative role in the regulation of NK cell activity.

Disclosures

The authors have no financial conflict of interest.

References

1. Fruhbeck, G., J. Gomez-Ambrosi, F. J. Muruzabal, and M. A. Burrell. 2001. The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am. J. Physiol.* 280: E827–E847.
2. Sennello, J. A., R. Fayad, A. M. Morris, R. H. Eckel, E. Asilmaz, J. Montez, J. M. Friedman, C. A. Dinarello, and G. Fantuzzi. 2005. Regulation of T cell-mediated hepatic inflammation by adiponectin and leptin. *Endocrinology* 146: 2157–2164.
3. Lord, G. M., G. Matarese, J. K. Howard, R. J. Baker, S. R. Bloom, and R. I. Lechler. 1998. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* 394: 897–901.
4. Sanchez-Margalet, V., C. Martin-Romero, J. Santos-Alvarez, R. Goberna, S. Najib, and C. Gonzalez-Yanes. 2003. Role of leptin as an immunomodulator of blood mononuclear cells: mechanisms of action. *Clin. Exp. Immunol.* 133: 11–19.
5. Ouchi, N., S. Kihara, Y. Arita, M. Nishida, A. Matsuyama, Y. Okamoto, M. Ishigami, H. Kuriyama, K. Kishida, H. Nishizawa, et al. 2001. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* 103: 1057–1063.
6. Gainsford, T., T. A. Willson, D. Metcalf, E. Handman, C. McFarlane, A. Ng, N. A. Nicola, W. S. Alexander, and D. J. Hilton. 1996. Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. *Proc. Natl. Acad. Sci. USA* 93: 14564–14568.
7. Fujita, Y., M. Murakami, Y. Ogawa, H. Masuzaki, M. Tanaka, S. Ozaki, K. Nakao, and T. Mimori. 2002. Leptin inhibits stress-induced apoptosis of T lymphocytes. *Clin. Exp. Immunol.* 128: 21–26.
8. Najib, S., and V. Sanchez-Margalet. 2002. Human leptin promotes survival of human circulating blood monocytes prone to apoptosis by activation of p42/44 MAPK pathway. *Cell. Immunol.* 220: 143–149.
9. van Dielen, F. M., C. van't Veer, A. M. Schols, P. B. Soeters, W. A. Buurman, and J. W. Greve. 2001. Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals. *Int. J. Obes. Relat. Metab. Disord.* 25: 1759–1766.
10. Arita, Y., S. Kihara, N. Ouchi, M. Takahashi, K. Maeda, J. Miyagawa, K. Hotta, I. Shimomura, T. Nakamura, K. Miyaoka, et al. 1999. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem. Biophys. Res. Commun.* 257: 79–83.
11. Yamauchi, T., J. Kamon, Y. Minokoshi, Y. Ito, H. Waki, S. Uchida, S. Yamashita, M. Noda, S. Kita, K. Ueki, et al. 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat. Med.* 8: 1288–1295.
12. Yokota, T., K. Oritani, I. Takahashi, J. Ishikawa, A. Matsuyama, N. Ouchi, S. Kihara, T. Funahashi, A. J. Tenner, Y. Tomiyama, et al. 2000. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* 96: 1723–1732.
13. Ouchi, N., S. Kihara, Y. Arita, Y. Okamoto, K. Maeda, H. Kuriyama, K. Hotta, M. Nishida, M. Takahashi, M. Muraguchi, et al. 2000. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-κB signaling through a cAMP-dependent pathway. *Circulation* 102: 1296–1301.
14. Maeda, N., I. Shimomura, K. Kishida, H. Nishizawa, M. Matsuda, H. Nagaretani, N. Furuyama, H. Kondo, M. Takahashi, Y. Arita, et al. 2002. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat. Med.* 8: 731–737.
15. Kumada, M., S. Kihara, N. Ouchi, H. Kobayashi, Y. Okamoto, K. Ohashi, K. Maeda, H. Nagaretani, K. Kishida, N. Maeda, et al. 2004. Adiponectin specifically increased tissue inhibitor of metalloproteinase-1 through interleukin-10 expression in human macrophages. *Circulation* 109: 2046–2049.
16. Cacicedo, J. M., N. Yagihashi, J. F. Keaney, Jr., N. B. Ruderman, and Y. Ido. 2004. AMPK inhibits fatty acid-induced increases in NF-κB transactivation in cultured human umbilical vein endothelial cells. *Biochem. Biophys. Res. Commun.* 324: 1204–1209.
17. Jyothi, M. D., and A. Khar. 2000. Interleukin-2-induced nitric oxide synthase and nuclear factor-κB activity in activated natural killer cells and the production of interferon-γ. *Scand. J. Immunol.* 52: 148–155.
18. Sica, A., L. Dorman, V. Viggiano, M. Cippitelli, P. Ghosh, N. Rice, and H. A. Young. 1997. Interaction of NF-κB and NFAT with the interferon-γ promoter. *J. Biol. Chem.* 272: 30412–30420.
19. Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol.* 17: 189–220.
20. Moretta, A., C. Bottino, M. C. Mingari, R. Biassoni, and L. Moretta. 2002. What is a natural killer cell? *Nat. Immunol.* 3: 6–8.
21. Seaman, W. E. 2000. Natural killer cells and natural killer T cells. *Arthritis Rheum.* 43: 1204–1217.
22. Yaqoob, P., E. A. Newsholme, and P. C. Calder. 1994. Inhibition of natural killer cell activity by dietary lipids. *Immunol. Lett.* 41: 241–247.
23. Pedersen, B. K., J. W. Helge, E. A. Richter, T. Rohde, and B. Kiens. 2000. Training and natural immunity: effects of diets rich in fat or carbohydrate. *Eur. J. Appl. Physiol.* 82: 98–102.
24. Rasmussen, L. B., B. Kiens, B. K. Pedersen, and E. A. Richter. 1994. Effect of diet and plasma fatty acid composition on immune status in elderly men. *Am. J. Clin. Nutr.* 59: 572–577.
25. Yin, W., J. Mu, and M. J. Birnbaum. 2003. Role of AMP-activated protein kinase in cyclic AMP-dependent lipolysis In 3T3-L1 adipocytes. *J. Biol. Chem.* 278: 43074–43080.

26. Kawauchi, K., K. Ihjima, and O. Yamada. 2005. IL-2 increases human telomerase reverse transcriptase activity transcriptionally and posttranslationally through phosphatidylinositol 3′-kinase/Akt, heat shock protein 90, and mammalian target of rapamycin in transformed NK cells. *J. Immunol.* 174: 5261–5269.
27. Yu, T. K., E. G. Caudell, C. Smid, and E. A. Grimm. 2000. IL-2 activation of NK cells: involvement of MKK1/2/ERK but not p38 kinase pathway. *J. Immunol.* 164: 6244–6251.
28. Wulster-Radcliffe, M. C., K. M. Ajuwon, J. Wang, J. A. Christian, and M. E. Spurlock. 2004. Adiponectin differentially regulates cytokines in porcine macrophages. *Biochem. Biophys. Res. Commun.* 316: 924–929.
29. Lee, S. H., C. W. Lee, J. W. Lee, M. S. Choi, D. J. Son, Y. B. Chung, C. K. Lee, K. W. Oh, D. C. Moon, B. M. Kwon, et al. 2005. Induction of apoptotic cell death by 2′-hydroxycinnamaldehyde is involved with ERK-dependent inactivation of NF-κB in TNF-α-treated SW620 colon cancer cells. *Biochem. Pharmacol.* 70: 1147–1157.
30. Tian, Z., R. Sun, H. Wei, and B. Gao. 2002. Impaired natural killer (NK) cell activity in leptin receptor deficient mice: leptin as a critical regulator in NK cell development and activation. *Biochem. Biophys. Res. Commun.* 298: 297–302.
31. Santos-Alvarez, J., R. Goberna, and V. Sanchez-Margalet. 1999. Human leptin stimulates proliferation and activation of human circulating monocytes. *Cell. Immunol.* 194: 6–11.
32. Ajuwon, K. M., and M. E. Spurlock. 2005. Adiponectin inhibits LPS-induced NF-κB activation and IL-6 production and increases PPARγ2 expression in adipocytes. *Am. J. Physiol.* 288: R1220–R1225.
33. Pickup, J. C., and M. A. Crook. 1998. Is type II diabetes mellitus a disease of the innate immune system? *Diabetologia* 41: 1241–1248.
34. Visser, M., L. M. Bouter, G. M. McQuillan, M. H. Wener, and T. B. Harris. 1999. Elevated C-reactive protein levels in overweight and obese adults. *J. Am. Med. Assoc.* 282: 2131–2135.
35. Lindsay, R. S., J. Krakoff, R. L. Hanson, P. H. Bennett, and W. C. Knowler. 2001. Gamma globulin levels predict type 2 diabetes in the Pima Indian population. *Diabetes* 50: 1598–1603.
36. Bouloumie, A., C. A. Curat, C. Sengenes, K. Lolmede, A. Miranville, and R. Busse. 2005. Role of macrophage tissue infiltration in metabolic diseases. *Curr. Opin. Clin. Nutr. Metab. Care* 8: 347–354.
37. Caspar-Bauguil, S., B. Cousin, A. Galinier, C. Segafredo, M. Nibbelink, M. Andre, L. Casteilla, and L. Penicaud. 2005. Adipose tissues as an ancestral immune organ: site-specific change in obesity. *FEBS Lett.* 579: 3487–3492.
38. Dovio, A., V. Caramello, R. G. Masera, M. L. Sartori, L. Saba, M. Tinivella, P. Prolo, A. Termine, P. Avagnina, and A. Angeli. 2004. Natural killer cell activity and sensitivity to positive and negative modulation in uncomplicated obese subjects: relationships to leptin and diet composition. *Int. J. Obes. Relat. Metab. Disord.* 28: 894–901.