Cell Injury, Repair, Aging, and Apoptosis

Th17 and IL-17 Cause Acceleration of Inflammation and Fat Loss by Inducing \( \alpha_2 \)-Glycoprotein 1 (AZGP1) in Rheumatoid Arthritis with High-Fat Diet

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Rheumatoid arthritis (RA) is a chronic autoimmune disorder that affects the joints. High-fat diet (HFD) is a risk factor for RA and is related to inflammation but responds minimally to medication. Given the association between HFD and inflammation, it is important to understand the function of inflammation-related T cells in RA with HFD. Collagen-induced arthritis (CIA), a model of RA, was induced in HFD mice by injection of collagen II, and metabolic markers and T cells were analyzed. The metabolic index and IgG assay results were higher in HFD-CIA mice than in non-fat diet—CIA mice. Numbers of inflammation-related T cells and macrophages, such as Th1 and Th17 cells and M1 macrophages, were higher in spleens of HFD-CIA mice. HFD-CIA mice had a high level of \( \alpha_2 \)-glycoprotein 1 (Azgp1), a soluble protein that stimulates lipolysis. To examine the association between Azgp1 and Th17 cells, the reciprocal effects of Azgp1 and IL-17 on Th17 differentiation and lipid metabolism were measured. Interestingly, Azgp1 increased the Th17 population of splenocytes. Taken together, our data suggest that the acceleration of fat loss caused by Azgp1 in RA with metabolic syndrome is related to the increase of IL-17. Mice injected with the Azgp1-overexpression vector exhibited more severe CIA compared with the mock vector— injected mice. (Am J Pathol 2017, 187: 1049–1058; http://dx.doi.org/10.1016/j.amjpath.2016.12.023)

High-fat diet (HFD) has been posed as a risk factor for rheumatoid arthritis (RA); however, still controversial in its contribution to RA because its association with inflammation is not clear. The findings of several previous studies examining the influence of HFD on RA development were also inconsistent.\(^1-4\) Inflammatory rheumatic diseases, such as RA, are sometimes accompanied by metabolic syndrome. RA is associated with cardiovascular disease or atherosclerosis.\(^5-7\) Reciprocally, metabolic factors, such as cholesterol and oxidized low-density lipoprotein and fatty acid, were also related to IL-17 production in immune system.\(^8-11\)

Metabolism-related obesity, rheumatoid cachexia, dyslipidemia, and change in adipokines are related to RA.\(^12\) Although the changes of adipokines differ among patients, inflammatory mediators are related to metabolic changes.\(^12-14\) In fact, adipokines have been implicated in inflammation as mediators of an immune response.\(^15-17\)

Adipokines, such as leptin, adiponectin, tumor necrosis factor (TNF)-\( \zeta \), or IL-6, are related to insulin sensitivity and inflammation.\(^18,19\) TNF-\( \zeta \) as well as IL-6, IL-1\( \beta \), and CCL2 are increased in obese tissue,\(^20-22\) including the liver, pancreas, brain, and muscle.\(^23-26\)

\( \alpha_2 \)-glycoprotein 1 (AZGP1), another adipokine, was first identified from human plasma.\(^27\) The sources of AZGP1 are liver, breast, prostate, and lung epithelium cell,\(^28,29\) where its plasma levels are elevated in patients with breast, prostate, or lung cancer.\(^29-32\) Although the biological role...
of AZGP1 is not fully understood, it has been reported that smoking or cancer cachexia increase its expression, leading to weight loss. Many studies suggested that AZGP1 may involve lipid metabolism with adipose tissue atrophy. In patients receiving hemodialysis, elevated AZGP1 is associated with proatherogenic factors, which is closely related to inflammation and oxidative stress, where AZGP1 progresses lipolysis in mouse epididymal adipocyte in vitro. We proposed the circuit of inflammation and metabolic disorder in which there is positive regulation effects on Th17 cell and Azgp1 reciprocally. This vicious cycle worsens inflammation in rheumatoid cachexia in the HFD-RA mouse model.

Plasma AZGP1 signaling is mediated through the activation of β3-adrenoceptor (β3-AR), which increases intracellular cyclin AMP level. β3-AR or β2-AR affects T-cell proliferation, differentiation, or cytokines secretion. We propose AZGP1 as a cachexia-inducing factor that mediates Th17 cell activation, which intensifies the severity of RA.

Materials and Methods

Animals

DBA/1J male mice (Orient Bio, Gyeonggi-Do, Republic of Korea) were maintained in groups of two in polycarbonate cages in a specific pathogen—free environment. Wild-type group was fed standard mouse chow (Ralston Purina) and water ad libitum. All experimental procedures were examined and approved by the Animal Research Ethics Committee at the Catholic University of Korea.

Induction and Evaluation of Arthritis and HFD

Collagen-induced arthritis (CIA) was induced in DBA/1J mice. Type II collagen was dissolved overnight in 0.1 N acetic acid (4 mg/mL) with gentle rotation at 4°C. Male DBA/1J mice were immunized intradermally at the base of the tail with 100 μg of chicken type II collagen (Chondrex Inc., Redmond, WA) in complete Freund’s adjuvant (Chondrex Inc.).

Mice were boosted with 100 μg of type II collagen emulsified with incomplete Freund’s adjuvant (Chondrex Inc.) and injected intradermally into the base of the tail on day 14 after primary immunization. In addition, the HFD-fed mouse group was fed a HFD (60 kcal of fat) at primary immunization. The arthritis score index for the disease severity was as follows: 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the midfoot (tarsals) or ankle joint; 2, erythema and mild swelling extending from the ankle to the midfoot; 3, erythema and moderate swelling extending from the ankle to the metatarsal joints; and 4, erythema and severe swelling encompassing the ankle, foot, and digits. The maximum possible score per mouse was 16. Two independent...
observers (S.-Y.K.) scored the mice without any knowledge of the experimental and control groups.

**Histopathologic Analysis of Arthritis**

The hematoxylin and eosin (H&E)—stained sections were scored for inflammation and bone erosion. Inflammation was scored according to the following criteria: 0, no inflammation; 1, slight thickening of the lining layer or some infiltrating cells in the underlying layer; 2, slight thickening of the lining layer plus some infiltrating cells in the underlying layer; 3, thickening of the lining layer, an influx of cells in the underlying layer, and the presence of cells in the synovial space; and 4, the synovium highly infiltrated with many inflammatory cells. Cartilage damage was determined using Safranin O and toluidine blue staining. Immunohistochemistry was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame,

**Figure 2**  High-fat diet (HFD) increases the disease severity of rheumatoid arthritis (RA) with destructive cartilage increased proinflammatory cytokines.  

A: Joint tissue samples were acquired from nonfat diet (NFD)—collagen-induced arthritis (CIA) or HFD-CIA mouse on 12 weeks and subjected to staining with hematoxylin and eosin and safranin O to evaluate the severity of inflammation and cartilage damage. B: Inflammation and bone erosion scoring for joint tissue in prism. C: Ankle joint tissue is immunohistochemically stained to NFD- or HFD-CIA mouse with specific antibodies to IL-17, IL-1b, IL-6, or vascular endothelial growth factor (VEGF). D: Positive cells of IL-17, IL-1b, IL-6, and VEGF were counted. Data are expressed as means ± SD of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001. Scale bars: 100 μm (A, right column, and C); 500 μm (A, left column).
CA). Joint tissue samples were incubated with the first primary monoclonal antibodies (mAbs) at 4°C, including goat anti-mouse TNF mAb, rabbit anti-mouse IL-1 mAb, rabbit anti-mouse IL-6 mAb, and rabbit anti-mouse IL-17 mAb. The primary antibodies were detected with a biotinylated secondary linking antibody, followed by incubation with streptavidin–peroxidase complex for 1 hour. The final color product was developed using 3,3-diaminobenzidine chromogen (Dako, Carpinteria, CA). Positive cells were counted, with results expressed as means ± SD.

Measurement of Immunoglobulin Concentrations

The serum concentrations of IgG, IgG1, and IgG2a were measured using mouse IgG, IgG1, and IgG2a enzyme-linked immunosorbent assay (ELISA) quantitation kits (Bethyl Laboratories, Montgomery, TX).

Flow Cytometric Analysis of T Cells

Cell pellets were prepared from the spleens of nonfat diet (NFD)—CIA and HFD-CIA mice. To examine the population of Th cells, the cells were stained with peridinin chlorophyll protein complex–conjugated anti-CD4 and allophycocyanin-conjugated anti-CD25 mAb (both from eBioscience, San Diego, CA) and then permeabilized and fixed with CytoFix/CytoPerm (BD Pharmingen, San Jose, CA) according to the manufacturer’s instructions. The cells were then further stained with phycoerythrin–conjugated anti-FoxP3, allophycocyanin-conjugated anti-interferon, phycoerythrin–conjugated anti–IL-4, and fluorescein isothiocyanate–conjugated anti–IL-17. In addition, the population of macrophages were stained with phycoerythrin–conjugated anti-F4/80 mAb and fluorescein isothiocyanate–conjugated anti-CD206 and anti-CD4 and allophycocyanin–conjugated anti-CD11c.

Real-Time PCR

Relative expression of specific mRNA was quantified by real-time PCR using SYBR Green I (Roche Diagnostics, Indianapolis, IN). The following sense and antisense primers were used: for AZGP1, 5′-AGTCTGCATCTGAACACACC-3′ (sense) and 5′-GGTTACCAAGTCCAAGGGG-3′ (antisense); for IL-17 (Il17a), 5′-TGGCTCAATCTCGTCTAGA-3′ (sense) and 5′-GTGGCTACAGTCCAAGG-3′ (antisense).

Glucose Tolerance and Insulin Tolerance Tests

NFD-CIA mice were injected i.p. with 1 U/kg of insulin. For the glucose tolerance test, mice were fasted overnight and then loaded i.p. with 1 g/kg of glucose.

Foam Cell Assay

THP-1 cells were plated in 24-well tissue culture plates (Nunc, Roskilde, Denmark) at a density of 5 × 10⁴ cells/mL and incubated with 160 nmol/L phorbol myristate acetate.
for 24 hours. Then, platelet-activating factor (oxidized low-density lipoprotein) and recombinant IL-17 were added and further cultured for 48 hours (Cayman Chemical, Ann Arbor, MI). THP-1 cells were stained with Oil Red O.

**Staining for Confocal Microscopy**

Spleen tissues were acquired at 12 weeks from first immunization. The tissue was stained using phycoerythrin-conjugated anti-CD4, fluorescein isothiocyanate–conjugated anti–IL-17, fluorescein isothiocyanate–conjugated anti-CD11c, and allophycocyanin-conjugated anti-F4/80 (eBioscience).

**ELISA**

IL-17 antibody was obtained from R&D Systems (Minneapolis, MN). The IL-17 concentration in the splenocyte culture supernatant was measured using sandwich ELISA, according to the manufacturer’s instructions.

**Gene Transfection**

To generate the Azgp1-overexpression vector, a fragment of the mouse gene Azgp1 (https://www.ncbi.nlm.nih.gov/nuccore; GenBank accession number NM_013478) was synthesized at GenScript Corporation, with codon optimization for expression in mammalian cells. Azgp1 was inserted into the vector pcDNA3.1 (Invitrogen, Carlsbad, CA).

**Statistical Analysis**

All data are expressed as means ± SD, representative of experiments performed on three occasions. Statistical significance was determined by the U-test or analysis of variance with Bonferroni’s post hoc test using GraphPad Prism software version 5.01 (GraphPad Software, San Diego, CA). *P < 0.05 was considered statistically significant.

**Results**

**Severity of RA Worsens in HFD Mice with CIA**

To investigate the effect of HFD on arthritis, CIA-induced RA mice were fed the HFD for 12 weeks. These HFD-CIA mice had a more severe state of disease and a higher arthritis score compared with the NFD-CIA (Figure 1A). Interestingly, body weight did not differ between the two groups, which suggested that some weight-consuming mechanism, such as inflammation or lipolysis, occurred in the HFD-CIA mice. There was also a markedly higher incidence of arthritis in HFD-CIA mice. Most of these mice had arthritis within 8 weeks after onset of the diet; NFD-CIA mice had full incidence after 12 weeks on the NFD diet (Figure 1A).

Increased levels of total IgG, IgG1, and IgG2a confirmed that the HFD influenced the severity of arthritis, but IgG2a levels are not significant (Figure 1B). Safranin O and H&E staining revealed increased inflammation and bone erosion with reduced thickness of cartilage, but bone erosion is not significant (Figure 2, A and B). Increased expression of inflammatory cytokine proteins also confirmed the intensified inflammation in joints of HFD-CIA mice (Figure 2C).

**Staining of proinflammatory cytokines, including IL-6, IL-1β, and especially IL-17, the main inflammatory cytokine in RA, was markedly greater in the joints of HFD-CIA mice compared with NFD-CIA mice. Staining of vascular endothelial growth factor, which might increase lymphocyte infiltration into joints, was greater in HFD-CIA than in NFD-CIA mice (Figure 2, C and D).**

**Metabolic Disorder and Glycogenesis Accelerate Inflammation in HFD-CIA Mice**

As described above, inflammation increased in HFD-CIA mice, and we next analyzed the inflammation-related...
lymphocytes in HFD-CIA and NFD-CIA mice. Confocal microscopy revealed more Th17 cells and M1 macrophages in the spleens of HFD-CIA mice compared with NFD-CIA mice (Figure 3A). Total splenocytes were isolated from HFD-CIA and NFD-CIA mice and were examined by flow cytometry. The numbers of IL-17 and interferon-γ-expressing cells and cells expressing the M1 macrophage phenotype (F4/80+\(\text{CD11c}^+\)) were increased in HFD-CIA mice (Figure 3B). The numbers of IL-4 and Foxp3-expressing cells increased slightly but not significantly in HFD-CIA mice.

These data suggest that the HFD affected the disease severity in this mouse model of RA and support the concept that inflammation is closely related to HFD or metabolic dysfunction. We next examined the metabolic and glycolytic pathways in HFD-CIA mice. Levels of alanine transaminase in the liver and low-density lipoprotein cholesterol and total cholesterol in the blood were higher in HFD-CIA mice than in NFD-CIA mice (Figure 4A). HFD-CIA mice were more vulnerable in the insulin tolerance test, but the test results did not differ significantly between groups (Figure 4B). Collectively, these data suggest that lipid metabolism is impaired in HFD-CIA mice compared with NFD-CIA mice.

AZGP1 and IL-17 Have Reciprocal Effects in RA Accompanied by HFD

The primary development of cachexia requires inflammation.\(^{37}\) Considering our finding that HFD-CIA mice had weight loss and impaired lipid metabolism, we hypothesized that some inflammation-related factors are related to lipid metabolism under this cachexia-inducing condition. Because AZGP1 is associated with weight loss in cachexia and some chronic diseases, we tried to determine whether Azgp1 is related to IL-17, the prominent cytokine expressed in the HFD-CIA mouse. Both AZGP1 and IL-17 mRNA levels were up-regulated in the liver in HFD-CIA mice compared with NFD-CIA mice (Figure 5B). Histochemical staining and counts of cells expressing Azgp1 protein were analyzed. Azgp1 expression was greater in the liver, joints, and spleen in NFD-CIA mice. Glucose level in the glucose tolerance test did not differ significantly between groups (Figure 4B). Collectively, these data suggest that lipid metabolism is impaired in HFD-CIA mice compared with NFD-CIA mice.
HFD-CIA mice compared with NFD-CIA mice, but spleen Azgp1-positive cells were not significant (Figure 5, C and D), findings that were consistent with the expression of IL-17 in joints of HFD-CIA mice (Figure 2C). The in vitro foam cell assay revealed that treatment with IL-17 increased lipid droplet deposition in THP-1 cells in a dose-dependent manner (Figure 5A).

The experiments described above revealed that increased Azgp1 level was accompanied by metabolic impairment and increased number of Th17 cells in HFD-CIA mice, which suggested a reciprocal influence between Azgp1 and IL-17. We further evaluated the association between Azgp1 and IL-17. We measured Azgp1 expression in response to exposure to IL-17 in THP-1 cells. Treatment with recombinant IL-17 protein increased the Azgp1 mRNA level by approximately three times in THP-1 cells (Figure 6A). The effect of IL-17 on the secretion of Azgp1 was also assessed. Normal mouse total splenocytes were treated with or without recombinant human AZGP1 protein under the Th17 condition, and IL-17 secretion was measured by ELISA in the supernatant of splenocyte. AZGP1 accelerated IL-17 production in the Th17 condition (Figure 6B). This result was confirmed by flow cytometry, which revealed that the percentage of IL-17–expressing cells in the Th17 condition slightly increased from 8.3% to 11.0% after AZGP1 treatment, but these data are not significantly different (Figure 6C). Collectively, our data suggest that Azgp1 and IL-17 have reciprocal influences that lead to dysfunction of lipid metabolism and inflammation and that this vicious cycle worsens the severity of disease in the HFD-CIA mouse. In addition, Azgp1 may increase IL-17 expression but not Th17 cell differentiation on the basis of ELISA and fluorescence-activated cell sorter.

**Overexpression of Azgp1 Accelerates RA**

Our advanced research reveals that Azgp1 is involved in the progress of RA through the up-regulation of proinflammatory cytokines. To determine whether Azgp1 can accelerate CIA in our mouse model, NFD mice were injected with either a mock vector or a Azgp1-overexpression vector once a week for 9 weeks. Mice injected with the AZGP1-overexpression vector had more severe CIA compared with the mock vector injected mice (Figure 7 A). Safranin O and H&E staining revealed increased inflammation and bone erosion in mice injected with the AZGP1-overexpression vector (Figure 7, B and C).

**Discussion**

Clinically, RA with HFD is more severe than common RA. Unfortunately, anti–TNF-α therapy for RA does not work in obese patients.38,39 Thus, HFD increases the severity of RA, possibly by promoting Th17 cells or IL-17 production, which is the main driving force of RA pathogenicity, which is worse in RA with HFD than in RA alone.40 However, there is little research on the interactions of RA with HFD. Our study suggests that, in this CIA mouse model of RA with HFD, Th17 cells and IL-17 increase the RA severity by stimulating lipolysis through up-regulation of Azgp1. The HFD-CIA mice had increased Azgp1 level and up-regulation...
of inflammation, in particular increased IL-17 production and number of Th17 cells \textit{in vivo}.

The increased inflammation, IgG levels, and histologic data observed in our study indicated that inflammation was worsened in the HFD-CIA mice (Figures 1B and 2). The number of Th17 cells and the IL-17 level increased markedly in the HFD-CIA mice (Figure 3A). Increased numbers of macrophages and interferon-γ–secreting cells also indicated aggravated inflammation in HFD-CIA mice (Figure 3, A and B).

HFD-CIA did not weigh more than NFD-CIA mice, which indicates that the HFD-CIA mice had cachexia (Figure 1A). After observing cachexia and impaired lipid and glucose metabolism in HFD-CIA mice (eg, increased cholesterol concentration and insulin tolerance test result), we focused on \textit{Azgp1} and its association with inflammation. \textit{Azgp1} is secreted from adipose or liver tissues and is closely related to cachexia, a syndrome that involves weight loss and weakness, which can worsen the disease state. In this study, \textit{Azgp1} was up-regulated in the liver, spleen, and joints in the HFD-CIA mice with severe arthritis (Figure 5, B–D).

\textit{AZGP1} is known to be induced by the β3-AR agonist and by itself in adipose tissues.\textsuperscript{11,42} The β3-AR cooperates with the β2-AR, which is also expressed in T lymphocytes and is involved in regulation of T-cell proliferation and differentiation and cytokine production.\textsuperscript{36,43} Therefore, the increased \textit{AZGP1} level found in joints might be produced by T lymphocytes or may reflect diffusion of excess \textit{AZGP1} from its main sources such as the liver. The β2-AR is also an antagonist that attenuates the onset of disease and joint injury in experimental arthritis.\textsuperscript{44} In our study, increased \textit{Azgp1} increased the Th17 cell population and IL-17 secretion in HFD-CIA mice. Reciprocally, IL-17 led to the up-regulation of \textit{Azgp1} \textit{in vitro} (Figure 6A). IL-17 triggered the accumulation of lipid droplets in THP-1 cells, which reveals a general effect of IL-17 on lipid metabolism.

\textbf{Conclusion}

Impaired lipid metabolism caused by IL-17 might stimulate fat lipolysis by up-regulating \textit{AZGP1} expression in adipose
tissue in obese individuals and induce severe inflammation. This vicious cycle of AZGP1 and IL-17 up-regulation might aggravate RA. In an advanced state of this disease, AZGP1 production increases and induces cachexia. This study indicates that AZGP1 is a key factor that can worsen inflammatory disease, such as RA, occurring with HFD. This finding may help to explain the therapeutic complications of HFD, diabetes mellitus, and atherosclerosis in RA or other autoimmune diseases. Our study suggests that controlling AZGP1 may help to end or lessen the effects of the vicious cycle of inflammation and cachexia associated with RA and HFD.

References


