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# The effect of lipopolysaccharide-induced obesity and its chronic inflammation on influenza virus-related pathology

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ABSTRACT

Obese individuals show increased susceptibility to infection, low vaccine efficacy, and worse pathophysiology. However, it is unclear how obesity affects these events. The aim of this study was to investigate the effect of obesity-triggered chronic inflammation on immune cells after influenza virus infection. Control and lipopolysaccharide mice, in which an osmotic pump continually released Tween saline or lipopolysaccharide, were prepared and 3 weeks later were infected with pandemic H1N1 2009 influenza A virus. In lipopolysaccharide mice, we found a reduction in macrophage activation markers in the steady state, and reduced production of pro-inflammatory cytokines including tumor necrosis factor-α, interleukin-1β, and interleukin-6, in restimulated peritoneal macrophages. Interestingly, lipopolysaccharide-triggered chronic inflammation exacerbated the severity of pathological symptoms in the lungs after challenge with influenza virus. Taken together, the increased severity of virus-induced symptoms in obese individuals with chronic inflammation may be, at least partially, caused by macrophage dysfunction.



## 1. Introduction

Obesity has become a major problem worldwide and is considered a risk factor for clinically important infectious diseases (Huttunen and Syrjanen, 2013). Obesity is particularly associated with respiratory disorders such as chronic obstructive pulmonary disease, asthma, obstructive sleep apnea, and pulmonary embolic disease (McClean et al., 2008). Recent cohort studies reported an association between obesity and susceptibility to influenza infection (Karlsson and Beck, 2010; Huttunen and Syrjanen, 2013). Obese individuals (body mass index [BMI] ≥ 30 kg/m$^2$) experienced increased hospitalizations and death from influenza infection during the 2009 influenza season (Kwong et al., 2011; Kim et al., 2012a). The Center for Disease Control and Prevention speculated that this was caused by the influence of excess adipose tissue on immune responses in the lung (Centers for Disease Control and Prevention, 2009). Karlsson et al. investigated the impact of obesity on memory cell-mediated defenses against influenza virus infection in a diet-induced obesity (DIO) mouse model (Karlsson et al., 2010). They demonstrated that influenza-specific CD8+ memory T cells and production of interferon (IFN)-γ were decreased in obese mice compared with normal mice. This shows that obesity reduces immune responses against influenza virus. Based on these data, other studies found a correlation between vaccine efficacy and obesity (Kim et al., 2012b; Sheridan et al., 2012; Park et al., 2014). Our previous study showed that the efficacy of influenza vaccine is reduced in a DIO mouse model. In the obese mice, humoral immunity was diminished through a reduction in neutralizing antibodies against influenza virus, indicating that vaccine efficacy was attenuated. In addition, the number of effector memory T cells in the stromal vascular fraction (SVF) of epididymal fat tissue was significantly reduced after infection, whereas inflammation was increased (Kim et al., 2012b). These reports suggest that obesity induces a disorder of immune responses that might contribute to the susceptibility of organisms to pathogen infection, to low vaccine efficacy, and to worse pathophysiology (Kim et al., 2012b; Sheridan et al., 2012; Park et al., 2014). However, the mechanisms by which obesity affects disease outcome are not fully understood.

Obesity, a common metabolic disorder, is associated with accumulated fat and low-grade chronic inflammation in various tissues, which show dysregulation of cytokine production and infiltration of immune cells (Gregor and Hotamisligil, 2011). Several studies have reported that in the obese state, adipose tissue and immune cells, especially macrophages, are altered, with the predominant macrophage type changed from M2 to M1 macrophages, and that

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this results in chronic inflammation (Fujisaka et al., 2009; Martinez-Santibanez and Lumeng, 2014). Chronic inflammation is defined as prolonged inflammation (from weeks to months) caused by tissue injury or disorders of the immune response (Shacter and Weitzman, 2002). In particular, low-level chronic inflammation was associated with the expansion of adipocytes. However, it is unclear which occurs first in the development of obesity: chronic inflammation or adipocyte expansion (Na and Nam, 2012). Cani et al. demonstrated that metabolic endotoxemia induced by lipopolysaccharide (LPS) triggers obesity in mice (Cani et al., 2012). They used an osmotic minipump, implanted subcutaneously, to infuse LPS. Osmotic minipumps are inserted subcutaneously or intraperitoneally and used to deliver drugs, hormones, or other soluble agents (Theeuwes and Yum, 1976). They produce a constant flow rate over a period from 1 day to 6 weeks using the principle of osmotic pressure difference between components of the device. The advantage of this type of pump is that it can continuously infuse the target reagent. Therefore, Cani used this device to maintain a constant concentration of LPS *in vivo*. Interestingly, the results show that metabolic endotoxemia induced by LPS can induce an increase in body mass and epididymal fat tissue ratio even without a high fat diet. Moreover, it can trigger insulin resistance, dyslipidemia, and deterioration of glycemic control *in vivo*. In addition, macrophage infiltration was increased and pro-inflammatory cytokine mRNA levels were upregulated in the adipose tissue (Cani et al., 2012). Thus, experimental chronic metabolic endotoxemia can induce obesity by induction of chronic inflammation. It may also contribute to inactivation of immune cells, as does obesity (Fujisaka et al., 2009; Kim et al., 2009; Strissel et al., 2010). However, the mechanism by which it causes the dysfunction of immune cells is unknown.

In this study, we investigated the effect of chronic inflammation on immune cells. To mimic chronic inflammation, we developed a mouse model of metabolic endotoxemia using an osmotic minipump containing LPS, as described previously (Geurts et al., 2013). In this mouse model, we observed dysregulation of activation markers on peritoneal macrophages and splenic T cells in the steady state. LPS-induced chronic inflammation was able to cause immune tolerance in peritoneal macrophages and splenic T cells, and significantly reduced production of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. Taken together, these results suggest that chronic inflammation in the obese state induces the dysfunction of macrophages and T cells. Accordingly, it might contribute to obesity-induced disorders and the severity of virus infection-induced pathology.

## 2. Materials and methods

### 2.1. Animals

Twelve-week-old male C57BL/6 mice (Dae-Han Bio link, Eumseong, Korea) were housed in a controlled environment (inverted 12-h daylight cycle) with free access to food and water. All animal experimental procedures were approved by the Animal Care and Use Committee of the Catholic University of Korea (Cho and Seok, 2013). Mice were fed a normal-fat diet (containing 5% fat) and were simultaneously treated with LPS from *Escherichia coli* 055:B5 (Sigma, St Louis, MO, USA). Body weight and food intake were recorded weekly for 4 weeks.

### 2.2. Surgical procedures

The surgical procedure for insertion of the osmotic pump followed the manufacturer's instructions. Briefly, after the hair was shaved, the back of the mouse was incised and a space made in the subcutaneous tissue. The device was inserted into the space, then the incision was closed using wound clips (7 mm). The mice were implanted with an osmotic minipump (Alzet model 1004; DURECT Corp., Cupertino, CA, USA) that was filled with either Tween-saline (0.9% NaCl and 0.1% Tween 80 in distilled water, Sigma) (normal control [NC] group) or LPS diluted in Tween-saline infused at 300 μg/kg/day for 4 weeks (LPS group).

### 2.3. Influenza infection

Three weeks after implantation of the osmotic minipump, the mice were challenged by intranasal injection with $1 \times 10^7$ plaque-forming units (PFU)/40 μL of influenza A/California/04/2009 (H1N1) virus, which originated from swine influenza H1N1 viruses. The mouse organs and serum were harvested for experiments 7 days postinfection.

### 2.4. Histology

The epididymal fat, liver, lung, and pancreas tissues of osmotic minipump-implanted mice were fixed in 10% neutral formalin. After fixation, these samples were embedded in paraffin and stained with hematoxylin and eosin (H&E).

### 2.5. Magnetic resonance imaging (MRI)

The study used a 7-T MRI system (BIOSPEC 70/20 USR; Bruker-Biospin, Billerica, MA, USA) with a quadrature coil. T1-weighted sections in the axial plane were obtained at the following settings: fast spin echo sequence with time to repetition (TR) 625 ms and time to echo (TE) of 12 ms; 156 × 156 matrix; 30.0 mm × 30.0 mm field of view and average of 10 signals. The section thickness was 1.0 mm with a 0 mm gap.

### 2.6. Isolation of peritoneal macrophages

Mouse peritoneal macrophages were obtained from C57BL/6 mice that had an implanted osmotic minipump. To isolate peritoneal macrophages, the mouse abdominal wall was lifted, and then 8 ml of cold PBS was injected into the peritoneal cavity using a 26-gauge needle and the abdomen was massaged for 30 s. Phosphate-buffered saline (PBS) containing peritoneal exudate cells was withdrawn and washed with red blood cell (RBC) lysis buffer to remove RBCs. After RBC lysis, peritoneal exudate cells were incubated for 2 h and the adherent cells were used as peritoneal macrophages.

### 2.7. Flow cytometry

For measurement of macrophage activation, peritoneal macrophages were stained in 3% fetal bovine serum and 0.02% sodium azide (wash solution) that included fluorochrome-conjugated antibodies. The antibodies used were anti-F4/80 (BioLegend, San Diego, CA, USA), anti-CD86 (BD Biosciences, San Jose, CA, USA), anti-CD80 (BD Biosciences), anti-CD40 (eBioscience, San Diego, CA, USA), and anti-I-A/I-E (to detect MHC class II, BD Biosciences). After staining, the cells were washed twice with wash solution and resuspended in 1% paraformaldehyde. Data for the stained cells were acquired using a flow cytometer (FACS Canto II, BD Bioscience) and analyzed with FlowJo analysis software (FlowJo LLC, Ashland, OR, USA).

### 2.8. Real-time polymerase chain reaction (PCR)

Total RNA was extracted from lung tissue using TRIzol reagent (Invitrogen, Grand Island, NY, USA) and cDNA was synthesized from