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# Characterization of Inflammatory Gene Expression and Chemotaxis of Macrophages Expressing Guanylin and Guanylyl Cyclase-C

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**Abstract:** Depending upon the environment, macrophages can show at least two different phenotypes, including the inflammatory (M1) phenotype and the anti-inflammatory (M2) phenotype. CD11c–positive M1 macrophages produce proinflammatory cytokines such as interleukin (IL) 1β, IL-6, tumor necrosis factor α, and monocyte chemoattractant protein (MCP) 1, which are linked to the development of obesity-associated insulin resistance. Recently, we showed that double-transgenic (dTg) rats overexpressing guanylin (Gn) and its receptor, guanylyl cyclase-C (GC-C), specifically in macrophages did not become obese even when fed a high-fat diet. In the present study, to characterize macrophages expressing Gn and GC-C (i.e., Gn/GC-C macrophages), we analyzed the expression of the M1 and M2 markers of peritoneal macrophages isolated from dTg and wild type (WT) rats. We also examined the chemotaxis of these macrophages after incubation with MCP-1 or fatty acids. The expression of CD11c, an M1 macrophage marker were expressed at a significantly lower level in the peritoneal macrophages of dTg rats than in those of wild-type (WT) rats. In addition, the expression of IL-1, MCP-1 and chemokine receptor 2 were expressed at a significantly lower level in the peritoneal macrophages of dTg rats than in those of WT rats. On the other hand, there were no significant differences in the expression of M2 markers such as CD206, IL10, and arginine 1 between dTg and WT rats. We also found that the chemotaxis of Gn/GC-C macrophages incubated with fatty acids significantly increases compared to the macrophages of WT rats. Our results suggest that the low levels of proinflammatory cytokines and M1 markers in Gn/GC-C macrophages at least in part contribute to the anti-obese phenotype of Gn/GC-C Tg rats. In addition, the accelerated chemotaxis of Gn/GC-C macrophages in response to fatty acids suggests that these macrophages can uniquely react to excess fatty acids.

**Keywords:** Chemotaxis, Fatty Acids, Proinflammatory Cytokines

## 1. Introduction

Circulating monocytes differentiate into macrophages, which can migrate into tissues under steady state conditions or in response to inflammation [1,2]. Macrophages are highly heterogeneous cells found in nearly all tissues of the body [3–5] and can exhibit at least two different phenotypes: the classically activated M1 phenotype and the alternatively activated M2 phenotype [6,7]. CD11c–positive M1 macrophages are characterized by high expression of proinflammatory cytokines such as interleukin (IL) 1β, IL-6, tumor necrosis factor α (TNF-α), and monocyte chemoattractant protein (MCP) 1 [8], which are involved in inflammation in tissues [9–12]. In contrast, mannose receptor CD206–positive M2 macrophages are characterized by high expression of anti-inflammatory cytokines such as IL-10 [13,14] and arginase (Arg) 1 [15,16], which are involved in the repair or remodeling of tissues [4,9,17]. Macrophages are able to modify their phenotype according to their environment. Obesity is strongly associated with a chronic inflammatory state and macrophage infiltration into adipose tissue has been observed in obese mice [18,19]. Most tissue macrophages that infiltrate into the adipose tissue of obese mice are CD11c–positive M1 macrophages responsible for the progress of adipose tissue inflammation [20,21].

Guanylin (Gn) and its receptor, guanylyl cyclase-C (GC-C), are known to be present mainly in the intestine and function to maintain homeostasis of body fluid [22]. Recently, we showed

that double-transgenic (dTg) rats overexpressing Gn and GC-C specifically in macrophages did not become obese even when the rats were fed a high-fat diet [23]. This implies that the Gn/GC-C system in macrophages regulates obesity. From this finding, we expected that Gn/GC-C–expressing macrophages might have a unique balance between the opposing M1 and M2 phenotypes.

Here, to characterize Gn/GC-C–expressing macrophages, we isolated peritoneal macrophages from Gn/GC-C dTg rats, and analyzed the expression of M1 and M2 macrophage markers. We also examined the chemotaxis of these macrophages after incubation with MCP-1 or fatty acids.

# 2. Materials and Methods

### *2.1. Animals*

The Gn/GC-C dTg rat has been described previously [23]. Male wild-type (WT) and dTg rats were housed individually in plastic cages in a room at constant temperature and under a 12:12-h light:dark cycle (lights on, 08:00 to 20:00). The rats were allowed ad libitum access to food and water throughout the study. All rats received standard laboratory chow (3.4 kcal/g) containing 4.6% (w/w) fat, 51% (w/w) carbohydrate, and 25% (w/w) protein (CE-2; CLEA Japan, Fuji, Shizuoka, Japan). All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care. This protocol was approved by the Ethics Review Committee for Animal Experimentation of the Faculty of Medicine, University of Miyazaki.

### *2.2. Isolation of Peritoneal Macrophages*

WT and dTg rats were injected intraperitoneally with 2 ml/kg body weight of 4% thioglycollate. Exudate cells were collected by washing the peritoneal cavity with 0.9% (w/v) saline 4 days after injection. The cells were incubated for 3 h at 37 °C in a humidified 5% (v/v) $CO_2$ incubator, and adherent cells were used as peritoneal macrophages [24]. The macrophages were processed for RNA extraction or chemotaxis analysis as described below.

### *2.3. Quantitative Pcr*

Total RNA from the peritoneal macrophages of WT and dTg rats (n = 5 or 7 per group) was extracted with an RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from the total RNA by using an Superscript III First-Strand Synthesis System kit (Invitrogen, Carlsbad, CA, USA). The resultant cDNA samples were subjected to quantitative PCR for CD11c, IL-1β, IL-6, TNF-α, MCP-1, chemokine receptor (CCR) 2, CD206, IL-10, and Arg1 by using a LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany) with the SYBR Premix Ex Taq mix system (Takara Bio Inc., Shiga, Japan) and the primer sets listed in Table 1. The relative abundance of each reaction product was normalized to the level of ribosomal protein 36B4 mRNA.

### *2.4. Chemotaxis*

Chemotaxis of peritoneal macrophages obtained from WT and dTg rats (n = 5 or 7 per group) was assessed with the CytoSelect 96-well Cell Migration Assay kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's protocol. In brief, 5 × 105 macrophages in serum free RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) were placed in the upper chamber on 8-mm polycarbonate nucleopore filters, and the bottom well was filled with RPMI 1640 medium containing 2% (w/v) fatty acid-free BSA and 200 ng/ml MCP-1 or 600 μM fatty acid mixture (120 μM each of arachidonic, lyric, linoleic, myristic, and oleic acid). After incubation at 37 °C for 24 h, macrophages that had migrated into the lower chamber were lysed in the presence of CyQUANT GR dye, and their fluorescent intensity was measured by using an excitation wavelength of 485 nm, an emission wavelength of 538 nm, and an emission cutoff of 515 nm, with the use of a Flexstation Fluorometer (Molecular Devices, Sunnyvale, CA, USA).

***Table 1.*** *Primers used for quantitative PCR.*

| Gene | Sequence | |
|---|---|---|
| 36B4 | Sense | TCATTGTGGGAGCAGACAATGTG |
| | Antisense | AGGTCCTCCTTGGTGAACACAAA |
| CD11c | Sense | AAGCCCAAGTGTTCCTTCG |
| | Antisense | CACATGAGGTGCAGGGAGT |
| IL-1β | Sense | TGTGATGAAAGACGGCACAC |
| | Antisense | CTTCTTCTTTGGGTATTGTTTGG |
| IL-6 | Sense | CACTTCACAAGTCGGAGGCTT |
| | Antisense | TCTGACAGTGCATCATCGCTG |
| TNF-α | Sense | TGAACTTCGGGGTGATCG |
| | Antisense | GGGCTTGTCACTCGAGTTTT |
| MCP-1 | Sense | TGTAGCATCCACGTGCTGTC |
| | Antisense | CCGACTCATTGGGATCATCT |
| CCR2 | Sense | AAGAAGTATCCAAGAGCTTGATGAG |
| | Antisense | TCACCATCATCATAGTCATACGG |
| CD206 | Sense | TGGGTTTGCTGAAGAAGAGAA |
| | Antisense | CATGTGATAAGTGACAAATGCTTG |
| IL-10 | Sense | AGTGGAGCAGGTGAAGAATGA |
| | Antisense | TCATGGCCTTGTAGACACCTT |
| Arg1 | Sense | CCGCAGCATTAAGGAAAGC |
| | Antisense | CCCGTGGTCTCTCACATTG |

### *2.5. Statistical Analysis*

We compared groups of data by using Student's t-tests (GraphPad Prism 5, GraphPad, San Diego, CA, USA). P values less than 0.05 were considered significant (two-tailed tests). Data are reported as means ± SEM.

# 3. Results and Disucussion

In this study, to characterize Gn/GC-C–expressing macrophages, we investigated the expression of inflammatory cytokines in peritoneal macrophages isolated from Gn/GC-C dTg and WT rats. CD11c–positive M1 macrophages produce proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, and MCP-1, which are linked to the development of obesity-associated insulin resistance [25–28]. Our results showed that the mRNA expression levels of

CD11c, IL-1, and MCP-1 were significantly lower in the peritoneal macrophages of dTg rats than in those of WT rats (Fig. 1). IL-6 and TNF-α showed the same trend as the other proinflammatory cytokines, but the differences in levels between dTg and WT rats were not statistically significant. The expression of CCR2, the receptor of MCP-1 [29], was also lower in the peritoneal macrophages of dTg rats than in those of WT rats (Fig. 1). In contrast, the M2 macrophage markers (CD206, IL-10, and Arg1) did not differ significantly between dTg rats and WT rats (Fig. 1). These results showed that Gn/GC-C–expressing macrophages derived from dTg rats express low levels of proinflammatory cytokines under steady state conditions.

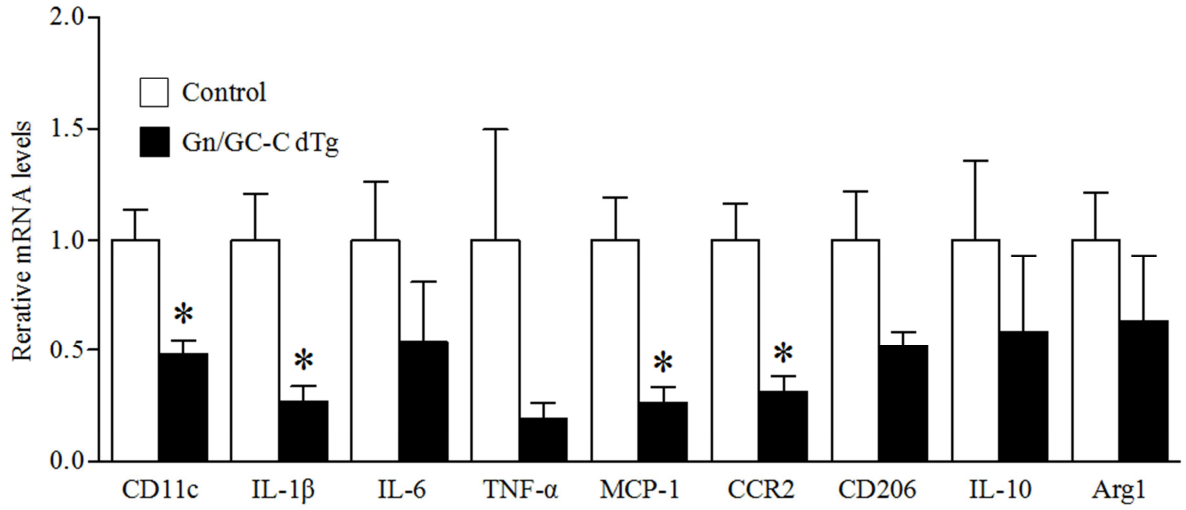


***Fig. 1.*** *Characterization of the markers of inflammatory (M1) and anti-inflammatory (M2) phenotypes in peritoneal macrophages from wild-type (WT) and Gn/GC-C dTg rats. Gene expression of M1 macrophage markers: CD11c, interleukin (IL) 1β, IL-6, tumor necrosis factor α (TNFα), monocyte chemoattractant protein (MCP) 1, and chemokine receptor (CCR) 2. Gene expression of M2 macrophage markers: CD206, IL-10, and arginase (Arg) 1. Data were normalized to 36B4 mRNA levels and are presented as means ± SEM (n = 5 or 7 per group). *, P < 0.05 compared with WT rats.*

Recently, we showed that dTg rats overexpressing Gn and GC-C specifically in macrophages did not become obese even after ingesting a high- fat diet [23]. Taken together, these findings suggest that Gn/GC-C–expressing macrophages have unique "anti-inflammatory" characteristics under conditions in which inflammation would be expected to be induced (e.g., a high fat diet). To clarify how Gn/GC-C system regulates inflammatory state, further investigation will be required.

Fatty acids [30,31] and proinflammatory cytokines including MCP-1 [32,33] are known to recruit monocytes and macrophages to sites of inflammation in adipose tissue. Therefore, we examined the chemotaxis of peritoneal macrophages isolated from Gn/GC-C dTg and WT rats after incubation with fatty acids or MCP-1. The chemotaxis of macrophages after incubation with MCP-1 did not differ between dTg rats and WT rats. However, the chemotaxis of macrophages after incubation with fatty acids was significantly induced in these macrophages of dTg rats than in those of WT rats (Fig. 2). Recently, we found that the mRNA expression of Gn and GC-C in a cell line significantly increased when the cells were incubated with fatty acids (data not shown). This finding indicates that the expressions of Gn and GC-C in macrophages may be induced at least in part by an excess of fatty acids. Together, fatty acids may act as not only a functional trigger of Gn/GC-C macrophages but also an inducer to promote the expressions of Gn and GC-C in macrophages.

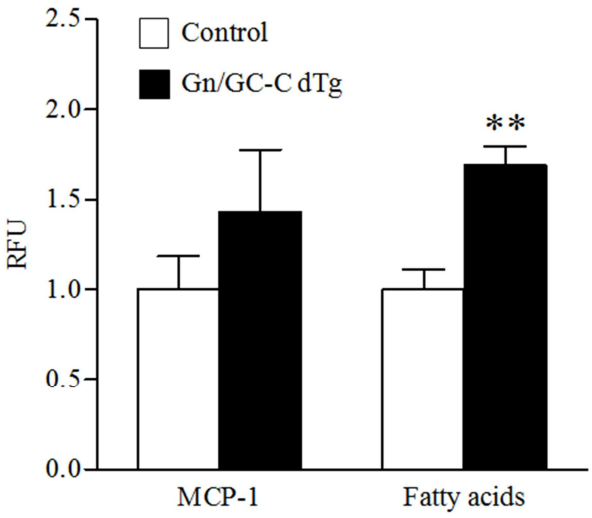


***Fig. 2.*** *Chemotaxis of peritoneal macrophages from Gn/GC-C dTg and wild-type (WT) rats. After 24-h induction with monocyte chemoattractant protein (MCP) 1 (MCP-1; 200 ng/ml) or fatty acid mixture (fatty acids; 600 μM), the chemotaxis of peritoneal macrophages was measured as relative fluorescent units (RFU). The data are presented as means ± SEM (n = 5 or 7 per group). **, P < 0.01 compared with WT rats.*

## 4. Conclusion

Gn/GC-C macrophages have the unique characteristics of low levels of proinflammatory cytokines and M1 macrophage

markers in the steady state and a high migratory ability in response to fatty acids. These characteristics of Gn/GC-C macrophages might contribute to the anti-obese phenotype of Gn/GC-C Tg rats.

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