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The regulation of inflammatory cytokine secretion in macrophage cell line by the chemical constituents of *Rhus sylvestris*

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ABSTRACT

In our preliminary screening study on the anti-inflammatory activity, eight triterpenes, one sterol, and one chalcone were isolated from the CH_2Cl_2-soluble extract of the stems and leaves of *Rhus sylvestris* Siebold and Zucc (Anacardiaceae). On the basis of their spectroscopic data, these compounds were identified as 10α-cucurbitadienol (**1**), glut-5-en-3-ol (**2**), β-amyrin acetate (**3**), β-amyrin (**4**) and lupeol (**5**), cycloart-24-en-3-one (**6**), cycloart-25-en-3,24-dione (**7**), 24-hydroxycycloart-25-en-3-one (**8**), β-sitosterol (**9**), and 2′-hydroxy-4,4′-dimethoxychalcone (**10**). All of them were isolated from this plant for the first. Furthermore, the compounds in non-cytotoxic concentrations (0–1.0 μM) were tested for their ability to block inflammatory cytokine secretion in the presence of LPS in the murine RAW264.7 macrophage cell line. Among the compounds that were tested, compounds **8** and **9** reduced the LPS-induced secretion of IL-6, as well as TNF-α, in a mouse RAW264.7 macrophage cell line. Moreover, compounds **2**, **3**, **7**, and **10** specifically diminished only the secretion of TNF-α even in 0.01 μM concentrations. It is thus suggested that they are potential therapeutics of TNF-α-related diseases and conditions, such as transplant rejection, type II diabetes, and atherosclerosis.

Rhus sylvestris Siebold and Zucc, which belongs to the Anacardiaceae family, is a deciduous tree that grows in the eastern part of Asia. Its flowers are dioecious and are pollinated by bees. This plant contains toxic substances that can cause severe irritation to some people, and its fresh sap can cause skin blisters. In a previous chemical investigation of this plant, the effects of one megastigmane glycoside and four flavonoids on the function of osteoblastic MC3T3-E1 cells were studied.[1] In continuation of our investigation of biological active compounds from this plant, 10 compounds, including eight triterpenes, one sterol, and one chalcone, were isolated from the CH_2Cl_2 layer of an MeOH extract. Their structures were completely assigned through spectroscopic analysis, using ^1H and ^{13}C NMR, COSY, HMQC, HMBC, and MS spectral data, and were compared with the data in the literature. Many of the triterpenoids[2–4] and chalcone analogs[5,6] were reported to have anti-inflammatory activities by suppressing the secretion of inflammatory cytokines, such as TNF-α or interleukins. The inhibitors of the secretion of these cytokines can be applied to the treatment of chronic inflammatory diseases like rheumatic arthritis, rhinitis, and allergy.

Inflammatory cytokines are produced by innate immune cells, such as macrophage and dendritic cells, during infection. Lipopolysaccharide (LPS) induces very strong inflammation, resulting in the secretion of TNF-α, IL-1, IL-6, and IL-12 in the macrophage cells. Among these, TNF-α activates various cells and induces cell death/survival, differentiation, proliferation, and migration.[7] TNF-α is like a double-edged sword in that it has both beneficial and harmful physiological functions. The beneficial functions of TNF-α are the killing of tumor cells, haematopoiesis, and protection from infection. On the other hand, its harmful functions are tumorigenesis, induction of autoimmunity, and linking with diseases. Increased TNF-α concentrations are co-related with many diseases, such as transplant rejection, rheumatoid arthritis, heart failure, type II diabetes, and atherosclerosis.[8] IL-6 is another potent and pleiotropic cytokine that not only plays a key role in acute inflammation by producing acute-phase proteins but also causes chronic inflammatory diseases, such as rheumatic arthritis, systemic lupus erythematosus, ankylosing spondylitis, psoriasis, and Crohn's disease.[9] Therefore, the reduction of the secretion of these cytokines will greatly help in the treatment or mitigation of chronic inflammatory diseases like infection, rheumatic arthritis, rhinitis, and allergy.[10,11] The effects of the compounds isolated from *R. sylvestris* on the blocking of inflammatory-cytokine secretion in the presence of LPS in a murine RAW264.7 macrophage cell line within non-cytotoxic concentrations (0–1.0 μM) were reported herein.

Stems and leaves of *R. sylvestris* were collected in June 2005 and were identified by Prof. KiHwan Bae, College of Pharmacy, Chungnam National University. A voucher specimen (CNU05002) was deposited at the herbarium of the College of Pharmacy, Chungnam

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National University, Korea. Air-dried stems and leaves of *R. sylvestris* (2 kg) were extracted three times with MeOH (50 °C). The MeOH extract (280 g) was suspended in H_2O (3 L) and partitioned with CH_2Cl_2 (3 L × 3) to give 90 g of a CH_2Cl_2-soluble fraction and 175 g of an H_2O-soluble fraction. This fraction was subjected to silica gel column chromatography using a stepwise gradient of hexane–EtOAc (100:1–0:1; 500 ml each), to yield eight fractions (2A–2M). Fraction 2A was subjected to silica gel CC with a hexane–acetone (200:1) elution solvent to give 4 fractions (42A–42D). Fraction 42C was separated by RPCC with a MeOH–acetone (1:1) elution solvent and gave compound **4** (196 mg) and **7** (15 mg). Fraction 2E was subjected to silica gel CC with a hexane–EtOAc (15:1) elution solvent to give 8 fractions (15A–15I). Fraction 15G was separated by RPCC with a MeOH–acetone (2:1) elution solvent and gave compound **1** (20 mg) and **3** (18 mg). Fraction 2F was subjected to silica gel CC with a hexane–EtOAc (12:1) elution solvent to give 5 fractions (24A–24F). A mixture of **5** and **6** (180 mg) was purified from fraction 24E. Fraction 15G was separated by RPCC with a MeOH–acetone (1:1) elution solvent and gave compound **8** (5 mg). Fraction 2G was subjected to silica gel CC with a hexane–acetone–MeOH (30:10:1) elution solvent to give 9 fractions (28A–28I). Combined fractions 28D–28F was separated by RPCC with a MeOH–acetone (5:1) elution solvent and gave compound **10** (5 mg). Fraction 2H was subjected to silica gel CC with a hexane–EtOAc (10:1) elution solvent to give 7 fractions (26A–26G). Fraction 26B was separated by RPCC with a MeOH–acetone (10:1) elution solvent to give 6 fractions (26A–26G). Compound **2** (165 mg) was purified from fraction 44D. Fraction 44B was subjected to silica gel CC with a CH_2Cl_2–MeOH (80:1) elution solvent and gave compound **9** (10 mg). Ten compounds were identified by comparing their physical and spectroscopic data with those reported in the literature.[12–20]

Table 1
Cytotoxicity of compounds 1–**10** on human cancer cell lines

Compound	IC_{50} value[a] (μM)		
	Hela	MCF-7	SK-Hep-1
β-Amyrin acetate (**3**)	>50.0	26.73	>50.0
Cycloart-25-en-3,24-dione (**7**)	24.84	30.91	15.86
β-Sitosterol (**9**)	46.22	42.10	>50.0
2′-Hydroxy-4,4′-dimethoxychalcone (**10**)	>50.0	45.39	41.73
1, **2**, **4**, **5**, **6**, and **8**	>50.0	>50.0	>50.0

[a] IC_{50} value was defined as a concentration (μM) that caused 50% inhibition of cell growth in vitro. HeLa: human cervical cancer cell line; MCF-7: human breast cancer cell line; SK-Hep-1: human hepatoma cell line. 10α-Cucurbitadienol (**1**), glut-5-en-3-ol (**2**), β-amyrin (**4**), lupeol (**5**), cycloart-24-en-3-one (**6**), and 24-hydroxycycloart-25-en-3-one (**8**).

Cytotoxic activities of compounds **1**–**10** were performed against human breast cancer (MCF-7), human hepatoma (SK-Hep-1), and human cervical cancer (Hela) cells using 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described by Mosmann.[21] Data were shown in Table 1. Among of 10 compounds tested in our experiment, only four compounds such as β-amyrin acetate (**3**), cycloart-25-en-3,24-dione (**7**), β-sitosterol (**9**) and 2′-hydroxy-4,4′-dimethoxychalcone (**10**) showed cytotoxicities against tumor cell lines. The compound of β-amyrin acetate (**3**) showed relatively stronger inhibitory activity on the human MCF-7 breast cancer cell line whose IC_{50} value was 26.73 μM than the cervical HeLa and hepatoma SK-Hep-1 cell lines whose IC_{50} were over 50.0 μM. The compound **3** was also reported to have cytotoxicity against A2780 ovarian cancer cell line with IC_{50} of 12.1 μg/ml which is very similar value of our MCF-7 breast cancer cell line.[22] Cycloart-25-en-3,24-dione (**7**) showed consistently strong cytotoxicities with IC_{50} values in the range of 15.86 and 30.91 μM against three cancer cell lines tested, while its derivatives of cycloart-24-en-3-one (**6**) and 24-hydroxycycloart-25-en-3-one (**8**) had no cytotoxicities on our cell lines tested, indicating ketones at the position of 3 and 24 may be important for the cytotoxicity against our tumor cell lines tested. These results are further confirmed by previous report that they found cycloart-24-en-3-one (**6**) had no cytotoxic activity on any of the human cancer cell lines of Bel-7402, BGC-823, and HL-60.[23] The compound of β-sitosterol (**9**) showed moderate cytotoxicities against HeLa cervical and MCF-7 breast cancer cell lines with their IC_{50} of 46.22 and 42.10 μM, respectively. This compound is one of the phytosterols and has various biological activities in animal cells such as anti-inflammation[24] and immunomodulating[25] activities. As β-sitosterol showed antitumor activity in our human breast cancer cell line, this compound also have been reported to induce

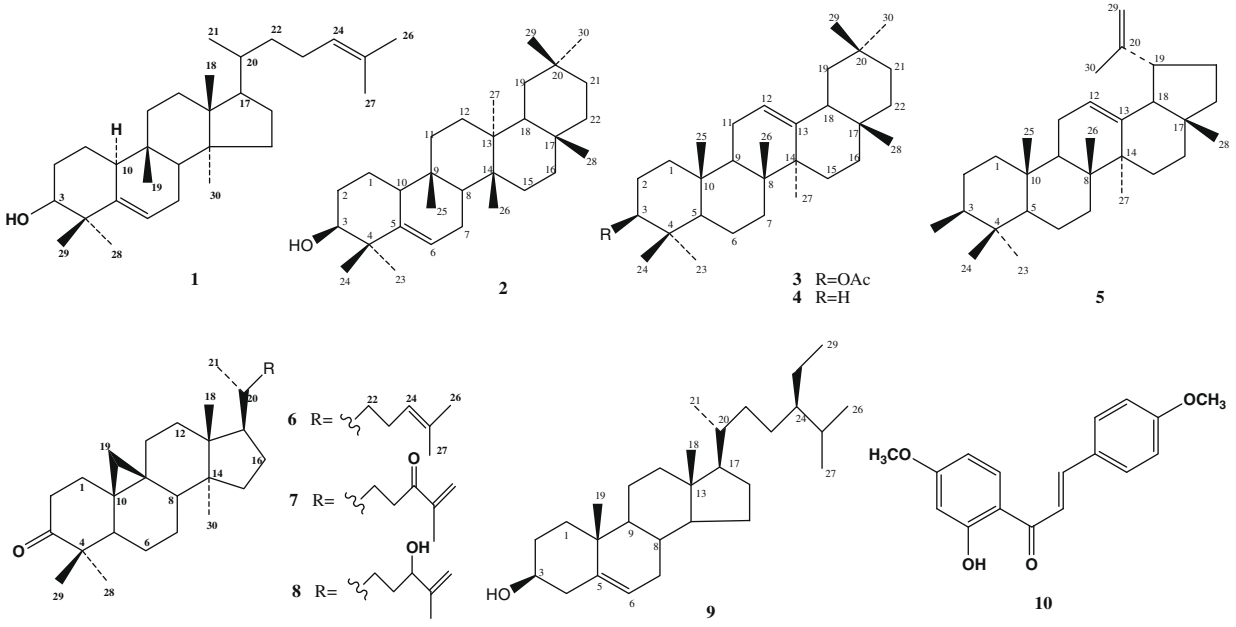

Figure 1. Structures of compounds **1**–**10** isolated from *R. sylvestris*.

apoptosis in hormone-insensitive and metastatic MDA-MB-231 human breast cancer cell line by activating caspase enzymes.[26] The compound of 2′-hydroxy-4,4′-dimethoxychalcone (**10**) inhibited cell growth with IC_{50} values of 45.39 and 41.73 μM in the MCF-7 and SK-Hep-1 cell lines, respectively (Figs. 1–3).

Many of the inflammation can be modulated by the cytokines secreted from immune defense cells. Innate immune cells, such as macrophage or dendritic cells, secrete diverse cytokines for defense modulation. During the inflammation stage, some of the cytokines, such as IL-1, IL-6, and TNF-α, are increased, while others, such as IL-10, are decreased by the macrophage cells. To study the anti-inflammatory activity, the regulation of inflammatory cytokine secretion was assayed in non-cytotoxic concentrations within the range of 0–1.0 μM. A murine macrophage RAW264.7 cell line was used for the measurement of secreted inflammatory cytokines of TNF-α and IL-6 in the absence or presence of those compounds isolated from *R. sylvestris* using inflammatory cytokine assay.[27]

When the RAW264.7 cells were cultured with each of the compounds isolated from *R. sylvestris*, the secretion of TNF-α was severely decreased (between 50.5% and 20.9% of that in the LPS-treated control group) by the treatment with compounds **2**, **3**, **7**, **8**, **9**, and **10** even at 0.01 μM concentrations. Compounds **1** and **6**, however, secreted 80.9% and 15.2% TNF-α, respectively, in the LPS-treated control group, at 1.0 μM concentrations. The mixture of compounds **4** and **5**, however, did not reduce TNF-α secretion even at the 1.0 μM concentration. The reduction or neutralization of TNF-α can ameliorate some inflammatory diseases, such as rheumatoid arthritis,[28] atherosclerosis,[29] and graft rejection.[30] In the assay system that was used in this study, compounds **9** and **10** led to the reduction of the LPS-induced secretion of IL-6, as well as of TNF-α, in the mouse RAW264.7 macrophage cell line. Compounds **2**, **3**, **7**, **8**, and **10**, however, specifically and strongly suppressed only TNF-α. Therefore, they are potentially excellent therapeutics for the treatment of abnormally-increased-TNF-α-level-related diseases, such as rheumatoid arthritis, atherosclerosis, and graft rejection. In terms of structure–activity relationship in our experiment, triterpene and chalcone showed most significant inhibitory effects on TNF-α secretion. In the case of triterpene, the glutinane, 3-acetyl oleanane, and 25-en-3,24-dione and 24-hydroxy-25-en-3-one cycloartane types seem to be the key functional elements.

As for the IL-6 inflammatory cytokine secretion assay, compounds **1**, **4**, and **5** (mixture), **8**, and **9** reduced IL-6 secretion to 34.3, 27.6, 54.6, and 54.5, respectively, of that in the LPS-treated con-

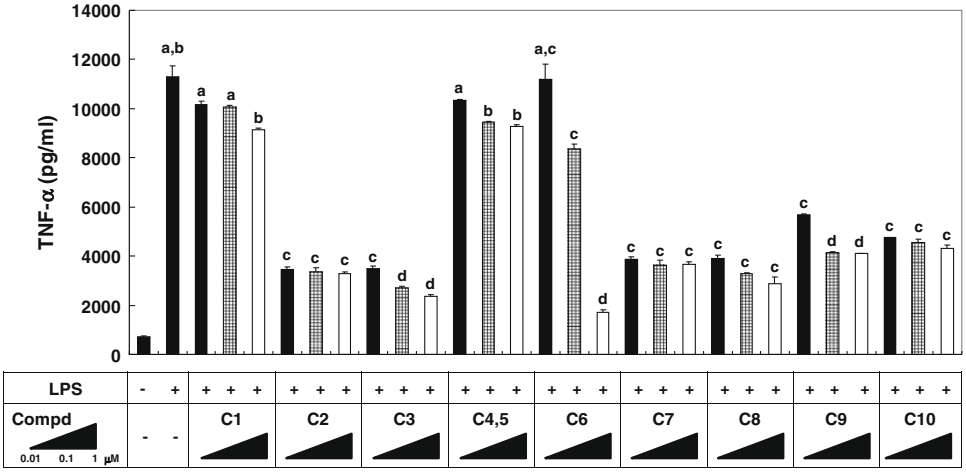

Figure 2. Effects of the compounds isolated from *R. sylvestris* on TNF-α secretion in the murine RAW264.7 macrophage cell line. The cells in triplicate were treated with each of the compounds at the concentrations of 0 (LPS), 0.01 (■), 0.1 (□), and 1.0 (□) μM in the 6-well culture plates for 12 h, in the presence of 100-ng/ml LPS. The control group cells (Cont) were cultured without LPS and the compounds. The alphabets above the bar that do not have a common superscript significantly differ from one another ($p < 0.05$).

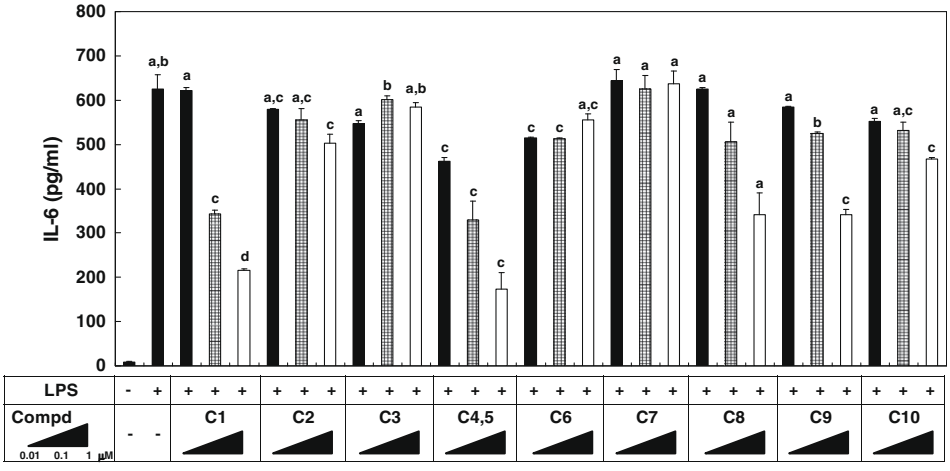

Figure 3. Effects of the compounds isolated from *R. sylvestris* on IL-6 secretion in the murine RAW264.7 macrophage cell line. The cells in triplicate were treated with each of the compounds at the concentrations of 0 (LPS), 0.01 (■), 0.1 (□), and 1.0 (□) μM in the 6-well culture plates for 12 h, in the presence of 100-ng/ml LPS. The control group cells (Cont) were cultured without LPS and the compounds. The alphabets above the bar that do not have a common superscript significantly differ from one another ($p < 0.05$).

trol group, at 1.0 μM concentrations. They reduced IL-6 secretion in a dose-dependent manner. Compounds **2**, **3**, **6**, **7**, and **10**, however, failed to reduce IL-6 secretion in the RAW264.7 cell line even at 1.0 μM concentrations. In terms of structure–activity relationship, triterpene, sterol, and α-tocopherol showed most significant inhibitory effects on IL-6 secretion. Among the active triterpenes, the cucurbitane, oleanane, lupane, and 24-hydroxy-25-en-3-one cycloartane types showed significant inhibitory activity.

Acknowledgments

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References and notes

1. Ding, Y.; Nguyen, H. T.; Choi, E. M.; Bae, K. H.; Kim, Y. H. *Planta Med.* **2009**, *75*, 158.
2. Cho, J. Y.; Yoo, E. S.; Cha, B. C.; Park, H. J.; Rhee, M. H.; Han, Y. N. *Planta Med.* **2006**, *72*, 1279.
3. Thimmulappa, R. K.; Fuchs, R. J.; Malhotra, D.; Scollick, C.; Traore, K.; Bream, J. H.; Trush, M. A.; Liby, K. T.; Sporn, M. B.; Kensler, T. W.; Biswal, S. *Antioxid. Redox. Signal.* **2007**, *9*, 1963.
4. Vasconcelos, J. F.; Teixeira, M. M.; Barbosa-Filho, J. M.; Lúcio, A. S.; Almeida, J. R.; de Queiroz, L. P.; Ribeiro-Dos-Santos, R.; Soares, M. B. *Int. Immunopharmacol.* **2008**, *8*, 1216.
5. Lee, J. H.; Jung, H. S.; Giang, P. M.; Jin, X.; Lee, S.; Son, P. T.; Lee, D.; Hong, Y. S.; Lee, K.; Lee, J. J. *J. Pharmacol. Exp. Ther.* **2006**, *316*, 271.
6. Hirai, S.; Kim, Y. I.; Goto, T.; Kang, M. S.; Yoshimura, M.; Obata, A.; Yu, R.; Kawada, T. *Life Sci.* **2007**, *81*, 1272.
7. Janeway, C. A.; Travers, P.; Walport, M.; Shlomchik, M. J. *Immunobiology: The Immune System in Health and Disease*; Garland Science: New York, 2005.
8. Aggarwal, B. B. *Nat. Rev. Immunol.* **2003**, *3*, 745.
9. Gabay, C. *Arthritis Res. Ther.* **2006**, *8*, S3.
10. Bradley, J. R. *J. Pathol.* **2008**, *214*, 149.
11. Nishimoto, N.; Kishimoto, T. *Curr. Opin. Pharmacol.* **2004**, *4*, 386.
12. Nes, W. D.; Wong, R. Y.; Benson, M.; Akihisa, T. *J. Chem. Soc.* **1991**, *18*, 1272.
13. El-Seedi, H. R. *Nat. Prod. Res.* **2005**, *19*, 197.
14. Choi, S. Z.; Choi, S. U.; Lee, K. R. *Arch. Pharm. Res.* **2004**, *27*, 164.
15. Ercil, D.; Sakar, M. K.; Del Olmo, E.; San Feliciano, A. *Turk. J. Chem.* **2004**, *28*, 133.
16. Fotie, J.; Bohle, D. S.; Leimanis, M. L.; Georges, E.; Rukunga, G.; Nkengfack, A. E. *J. Nat. Prod.* **2006**, *69*, 62.
17. Cambie, R. C.; La, A. R.; Rutledge, P. S.; Woodgate, P. D. *Phytochemistry* **1991**, *30*, 287.
18. Cabrera, G. M.; Gallo, M.; Seldes, A. M. *J. Nat. Prod.* **1996**, *59*, 343.
19. Hu, J. F.; Kelly, M.; Hamann, M. T. *Steroids* **2002**, *67*, 743.
20. Ilyas, M.; Perveen, M.; Shafiullah; Ahmad, S. M. *J. Chem. Res. Synop.* **2002**, *5*, 231.
21. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55.
22. Chaturvedula, V. S.; Schilling, J. K.; Miller, J. S.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. *J. Nat. Prod.* **2002**, *65*, 1222.
23. Wang, W.; Liu, J.; Han, J.; Xu, Z.; Liu, R.; Liu, P.; Wang, W.; Ma, X.; Guan, S.; Guo, D. *Planta Med.* **2006**, *72*, 450.
24. Garcia, M. D.; Saenz, M. T.; Gomez, M. A.; Fernandez, M. A. *Phytother. Res.* **1999**, *13*, 78.
25. Bouic, P. J. *Curr. Opin. Clin. Nutr. Metab. Care* **2001**, *4*, 471.
26. Park, C.; Moon, D. O.; Ryu, C. H.; Choi, B.; Lee, W.; Kim, G. Y.; Choi, Y. *Acta Pharmacol. Sinica* **2008**, *29*, 341.
27. *Inflammatory cytokine assay*: the cytokines of TNF-α and IL-6 from the compound-treated culture supernatant were measured by sandwich ELISA kit of TNF-α (OptEIA ELISA set, BD Biosciences Pharmingen, USA) and IL-6 (ELISA Ready-SET-Go kit, eBioscience, USA) by the protocols provided by the supplier. The cells were cultured in DMEM containing 10% fetal bovine serum with the compounds in triplicates, at 0, 0.01, 0.1, and 1.0 μM concentrations, in 6-well culture plates. After 12 h incubation, each of the 1-ml culture supernatants was transferred to an Eppendorf tube and was spun down for 3 min at 1,000 rpm and used for cytokine assay. For the cytokine assay using the sandwich method, a capture antibody (1:250 dilution) solution was incubated overnight at 4 °C, in 96-well plates. The plates were washed and blocked with assay diluent (10% FBS in PBS) for 1 h at room temperature, and then 100 μl of the culture supernatants was added to the wells and was incubated at room temperature. After 2 h, the plates were washed and incubated for 1 h with a detection antibody solution (1:250 dilution), biotinylated anti-mouse monoclonal antibody against TNF-α or IL-6, together with a Strepavidin-horseradish peroxidase conjugate solution (1:250 dilution). The plates were washed and incubated for 30 min with a 1:1 mixture of tetramethylbenzidine (TMB) and hydrogen peroxide. A stop solution was then added to the plates, and the optical density of 450 nm was read. The concentrations of the cytokines were calculated using the cytokines' standard calibration curve.
28. Gonzalez-Juanatey, C.; Llorca, J.; Garcia-Porrua, C.; Martin, J.; Gonzalez-Gay, M. A. *Arthritis Rheum.* **2006**, *55*, 150.
29. Lee, J. H.; Oh, G. T.; Park, S. Y.; Choi, J. H.; Park, J. G.; Kim, C. D.; Lee, W. S.; Rhim, B. Y.; Shin, Y. W.; Hong, K. W. *J. Pharmacol. Exp. Ther.* **2005**, *313*, 502.
30. Noel, C.; Copin, M. C.; Hazzan, M.; Labalette, M.; Susen, S.; Lelievre, G.; Dessaint, J. P. *Transplantation* **2000**, *69*, 1102.