Supplementary Data

Supplementary Materials and Methods

Isolation of splenocytes

Mice were euthanized, and the spleen was aseptically removed and placed into a splenocyte washing medium containing cold RPMI containing 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Spleen homogenates were passed through a 70-μm nylon mesh and centrifuged at 600 *g* for 10 min. The resulting pellet was harvested and resuspended in a red blood cell lysis buffer. After washing with the splenocyte washing medium, the cells were counted.

Isolation of T-cells

Splenic T-cells were purified from splenocytes by an MACS negative selection system using the pan T-cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of T-cells was > 95%, as determined by flow cytometry using PE-labeled Abs to CD3 (BD Biosciences, San Jose, CA).

Measurement of vitamin C levels

Blood was collected from the intraorbital plexus with a heparinized capillary tube, and the plasma was obtained by centrifugation. The total liver was harvested and frozen immediately with liquid nitrogen, and homogenized with a lysis buffer. Splenocytes or splenic T-cells were isolated and homogenized with the lysis buffer. Homogenates were obtained by centrifugation, and the supernatants were used for the measurements. The levels of vitamin C were determined by a colorimetric microtiter plate assay kit (Immundiagnostik AG, Bensheim, Germany) according to the manufacturer's instructions.

Measurement of oxygen reactive species

After concanavalin A injection, mice were anesthetized, and the portal vein was cannulated. The liver was perfused with a prewarmed (37°C) liver perfusion medium, following by collagenase–dispase digestion with a liver digestion medium. The liver was aseptically removed and placed into an L-15 medium. The cells were released by gently mincing and pipetting with a large-bore pipette. The cell suspension was passed through a 100-μm nylon mesh and centrifuged at 50 *g* for 5 min. The pellet was resuspended and washed twice with Hank's balanced salt solution (HBSS). Cells were incubated with 20 μM of 2′7′-dichlorodihydrofluorescin diacetate (DCFH-DA) for 30 min at 37°C. DCFH-DA was purchased from Molecular Probes (Eugene, OR), and 10 m*M* stock solution of DCFH-DA in DMSO was prepared and kept at −20°C in the dark for further experiment. The fluorescent oxidized form of DCFH-DA, 2′7′-dichlorofluorescein (DCF), in the presence of oxygen reactive species was analyzed by FACSCalibur (BD Biosciences), and the data were analyzed by FlowJo software (Tree Star, Inc., Ashland, OR). The excitation wavelength was 488 nm, and the observation wavelength was 530 nm for green fluorescence.

Staining of regulatory T-cells

Freshly isolated liver mononuclear cells (MNCs) were resuspended in ice cold fluorescence activated cell sorting (FACS) buffer containing 0.5% BSA and blocked at 4°C for 10 min with an Fc blocking reagent (Miltenyi Biotec GmbH). Then, the cells were stained with anti-CD4 antibodies (BD Biosciences) on ice for 30 min and washed twice with ice cold FACS buffer. Intracellular FACS staining of Foxp3 was performed according to the manufacturer's protocols (eBioscience, San Diego, CA). Cells were analyzed by FACSCalibur (BD Biosciences). FlowJo software (Tree Star, Inc.) was used for the data analysis.

Flow cytometry analysis

Freshly isolated liver MNCs were resuspended in ice cold FACS buffer containing 0.5% BSA and blocked at 4°C for 10 min with an Fc blocking reagent (Miltenyi Biotec GmbH). Then, the cells were stained with anti-CD11b, CD19, NK1.1, and TCR-β antibodies (BD Biosciences) on ice for 30 min and washed twice with ice cold FACS buffer. Cells were analyzed by FACSCalibur (BD Biosciences), and the data were analyzed by FlowJo software (Tree Star, Inc.).

Statistical analysis

Data were presented as the means ± SDs. Unpaired two-tailed *t*-test was used to compare the two groups [wild-type *vs. Gulo(−/−)* or *Gulo(−/−) vs.* vitamin C-supplemented *Gulo(−/−)* mice]. *p*-values of < 0.05 were considered statistically significant. Statistical tests were carried out using GraphPad InStat version 5.01 (GraphPad Software, Le Jolla, CA).