

Analysis of cytokine protein levels in mice by enzyme-linked immunosorbent assay. To assess whether C-IgI possessed the ability to activate Th1 cytokines and proinflammatory cytokines, spleens from non-immunized mice were harvested and processed for cell suspensions after red blood cell lysis using the 0.84% NH₄Cl buffer. Spleen cells (2×10^6) were cultured at 37 °C in 5% CO₂ and in 24-well culture plates (Costar) containing RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were stimulated with PBS, 5 µg/ml C-IgI protein or 5µg/ml ConA. After 48 h of incubation, the supernatants were collected and analyzed using a Mouse CK ELISA kit (Quantikine, R&D, USA), which included the following cytokines: IFN-γ, TNF-α, IL-4, and IL-6. Samples were processed following the manufacturer's instructions and measured in pg/ml of supernatant.

Spleen-cell proliferation assay. In the cell proliferation assay, spleens from non-immunized hamsters or mice were harvested and processed for cell suspensions. Spleen cells (2×10^5) were cultured at 37 °C in 5% CO₂ and in 96-well culture plates (Costar) containing RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were stimulated with PBS, 5 µg/ml C-IgI protein or 5 µg/ml ConA. CCK-8 reagents (DOJINDO, Japan) were added to each well at 20 h or 44 h, and optical density (OD) was measured at 450 nm using a microplate reader (Bio-rad, USA) at 24 h or 48 h.