

Supporting materials

Diverse Roles of Invariant Natural Killer T Cells in Liver Injury and Fibrosis Induced by Carbon Tetrachloride

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Materials and Methods:

Histology and immunohistochemistry. After routine processing, liver sections of 5 μm thickness were stained with hematoxylin and eosin (H&E) for histological analysis or Sirius red for collagen deposition. Immunostaining for α -SMA was performed on formalin-fixed, paraffin-embedded sections using monoclonal α -SMA primary antibody (clone 1A4, Dako, Carpinteria, CA) and a biotinylated alkaline phosphatase-conjugated secondary antibody. DAB (Zymed, South San Francisco, CA) was used as the chromogen/substrate. The collagen area/portal vein area and α -SMA-positive area/portal vein area were quantified by digital imaging (NIH Scion Image and Adobe Photoshop) using Sirius Red and α -SMA stained sections, respectively.

TUNEL assay. Apoptotic cells in sections of mouse livers were detected using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon International Inc., Temecula, CA) according to the manufacturer's instructions.

Western blotting. Western blotting was performed as described previously.¹ Protein bands were visualized by enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Piscataway, NJ).

Real time polymerase chain reaction (PCR). Real time PCR was carried out as described previously.¹ The sequences of primers for TCR-V α 14 genes are: Forward: 5'-TGG ATG ACA CTG CCA CCT ACA-3'; Reverse: 5'-GCC TCC CTA AGG CTG AAC CT-3'. CD68 primers: Forward; 5'- TTC TGC TGT GGA AAT GCA AG-3' and Reverse; 5'- AGA GGG GCT GGT AGG TTG AT-3'. The sequences for other primers have been previously described.¹

Alanine aminotransferase (ALT). Serum levels of ALT were measured using a kit from Drew scientific Ltd (Barrow-in-Furness, UK).

Serum cytokine levels. Serum cytokine levels were measured using a Cytometry Bead Array (BD Biosciences, San Jose, CA) according to the manufacturer's protocol.

Isolation and in vitro culture of HSC and Kupffer cells. HSCs and Kupffer cells were isolated as described previously.^{1,2} HSCs and Kupffer cells were cultured for 16 h and the supernatant was then collected and used for the measurement of cytokine levels.

Isolation of mouse liver lymphocytes, liver NKT cells, and flow cytometric analysis. Liver lymphocytes were isolated as described previously.³ NK cells, NKT cells, and T cells from mice livers were determined using anti-NK1.1 and anti-CD3 antibodies (BD PharMingen, San Diego, CA). CD1d-restricted NKT cells were also measured using the anti-CD3 antibody and α -GalCer analog-loaded CD1d tetramer (NIAID tetramer facility, NIH). Liver NKT (NK1.1⁺CD3⁺) cells were purified as described previously and the purity of liver NKT cells was greater than 90%.³ NKT cell apoptosis was determined by FACS analysis using anti-CD3 and anti-NK1.1 antibodies and Annexin V.

Cytotoxicity of liver lymphocytes and NKT cells against HSCs. HSCs were isolated by OptiPrep gradient centrifugation as described previously.⁴ [HSCs isolated from injured liver is often contaminated with Kupffer cells. These contaminated Kupffer cells were removed by negative magnetic selection with anti-CD11b antibody \(Miltenyi Biotech, Auburn, CA\) as described previously.⁵ The purity of isolated HSCs was >95% as demonstrated by immunostaining with anti-desmin antibody.](#) Freshly isolated HSCs or 4-day cultured HSCs were used as target cells. Liver lymphocytes or purified liver NKT

cells were used as effector cells. The cytotoxicity assay against HSCs was described previously.⁴ The ratio of effector cells (purified NKT) and target cells (HSCs) was 10:1 or 25:1 when liver lymphocytes were used as effector cells. In some experiments, anti-NKG2D antibody and isotype control IgG (eBioscience, San Diego, CA) were included in the cytotoxicity assay.

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