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Concanavalin A–Induced T-Cell–Mediated Hepatic Injury in Mice: The Role of Tumor Necrosis Factor

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Concanavalin A activates T lymphocytes *in vitro* and causes T-cell–dependent hepatic injury in mice. T lymphocytes were previously identified as effector cells of concanavalin A–induced liver injury. Here we report that hepatic injury is characterized by apoptotic cell death. On concanavalin A challenge, the cytokines tumor necrosis factor-α (TNFα), interleukin-2, granulocyte macrophage–colony stimulating factor, and interferon-γ were detectable in the circulation of the mice. Pretreatment of mice with anti-mouse TNF-α antiserum protected them from concanavalin A–induced liver injury. Nude mice failed to release TNF-α or interleukin-2 after concanavalin A challenge and were protected from liver injury. Lymph node cell transfer from responder mice to resistant nude mice resulted in susceptibility of the latter towards concanavalin A, i.e., to induction of cytokine release and hepatotoxicity. These experiments suggest that immunocompetent T cells play a pivotal role in concanavalin A–stimulated TNF-α release *in vivo*. After intravenous administration of fluorescein isothiocyanate-labeled concanavalin A to mice, the most fluorescence was found within the liver. *In vitro*, concanavalin A stimulation of separate cultures of mouse lymph node cells or nonparenchymal liver cells induced the release of minute amounts of TNF, whereas stimulation of cocultures of these cells resulted in production of substantial amounts of TNF-α. These findings may explain the hepatotropic effect of concanavalin A. In conclusion, T-cell–dependent concanavalin A–induced apoptotic liver injury in mice is related to immunological and cytokine-mediated disorders and possibly to autoreactive hepatic processes.

Abbreviations: IL, interleukin; IFN-γ, interferon gamma; GM-CSF, granulocyte macrophage–colony stimulating factor; TNF, tumor necrosis factor; OKT3, orthoclone T3 antibodies; Con A, concanavalin A; SCID, severe combined immunodeficiency syndrome; FITC, fluorescein isothiocyanate; NMRI, Naval Medical Research Institute; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; LNC, lymph node cell; ALT, alanine aminotransferase; Ig, immunoglobulin; EDTA, ethylenediaminetetraacetic acid; NPC, nonparenchymal cell; BCG, bacille Calmette-Guérin; EAE, experimental allergic encephalomyelitis.

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In mammalian organisms suffering from viral infection, allograft rejection, or autoimmune diseases, T lymphocytes serve as effector cells of the immunostimulatory processes. With respect to human liver disease, viral hepatitis, and at least three autoimmune syndromes, i.e., primary biliary cirrhosis, chronic active hepatitis, and primary sclerosing cholangitis are clinically relevant.[1] T-cell activation results in production of cytokines such as interleukin (IL)-2, interferon gamma (IFN-γ), granulocyte macrophage–colony stimulating factor (GM-CSF), and tumor necrosis factors (TNF). This cytokine production maintains the immunostimulatory and inflammatory processes and may evoke acute toxicity.[2] For instance, administration of T-cell–activating orthoclone T3 antibodies (OKT3) directed against the signal transducing CD3 molecule of T cells to allograft recipients results in clinical symptoms, such as fever, chills, tremor, dyspnea, chest pain, wheezing, and gastrointestinal complications,[3] before subsequent T-cell inactivation and depletion. Pharmacological suppression of TNF production abrogated the side effects of OKT3 administered to patients who underwent renal transplantation.[4,5] In an experimental mouse model a monoclonal anti-CD3 antibody induced massive systemic release of cytokines, such as TNF, IL-2, and IFN-γ, and led to severe sinusoidal congestion of the liver, hepatocyte vacuolization and focal hepatic necrosis.[6] Another group of extremely potent T-cell–activating molecules are enterotoxins of gram-positive bacteria.[7] These molecules, also termed superantigens, account for at least 45% of food poisoning[8] and may cause toxic shock[9] and autoimmune diseases.[10,11] Enterotoxins were shown to induce the release of cytokines *in vivo*. Anti-TNF antibodies protected against enterotoxin-induced lethal shock in D-galactosamine–sensitized mice.[2]

We recently described an experimental liver injury model in mice that is inducible by concanavalin A (Con A),[12] a plant lectin from jack bean (*Canavalia ensiformis*) known to mitogenically activate T lymphocytes *in vitro*. Lectin-induced lymphocyte proliferation *in vitro* is accompanied by the production of lymphokines and monokines, i.e., by lymphocyte-derived and monocyte-derived cytokines.[13] Our previous results showed that intravenous administration of Con A to mice resulted in T-cell stimulation and IL-2 release *in vivo*.[12] Con A–induced T-cell activation led to induction of a

highly specific single organ failure, i.e., to liver injury, within 8 hours after challenge. Hepatic injury was assessed by electron microscopy of the organ and by determination of plasma transaminases as well as of plasma levels of the liver-specific enzyme sorbitol dehydrogenase. Immunosuppressive drugs, such as Ciclosporin A, FK506 (Fujimycin, Osaka, Japan), or corticosteroids, prevented this experimental liver failure. Immunodeficient mice, i.e., mice with severe combined immunodeficiency syndrome (SCID mice) or athymic nude mice failed to develop hepatitis on Con A challenge. Cell depletion experiments identified $CD4^+$ T cells as well as macrophages as effector cells in this model. We now report that Con A causes systemic release of cytokines such as TNF-α, GM-CSF, IL-2, and IFN-γ. Neutralization of TNF-α abrogated the fatal outcome of Con A challenge. It is concluded that Con A induces hepatic injury mediated by cytokines such as TNF-α. Histological examination gives evidence for apoptosis as a possible mechanism of hepatocyte cell death induced by Con A *in vivo*. Possible explanations of the hepatotropic effect of Con A are suggested by the finding that the majority of intravenously administered fluorescein isothiocyanate (FITC)-labeled Con A was found within the liver and by the outstanding capacity of lymphocyte/nonparenchymal liver cell cocultures to release substantial amounts of TNF-α on *in vitro* stimulation by Con A.

MATERIALS AND METHODS

Animals. Male Naval Medical Research Institute (NMRI) albino mice and male nude NMRI albino mice were from the Hannoversches Zentralinstitut, Hannover, Germany. Male BALB/c mice were from the animal house of University of Konstanz, Germany. All mice were kept at least 1 week at 22°C and 55% relative humidity in a 12-hour day/night rhythm with free access to food (Altromin 1313) and water. Sixteen hours before the experiment food was withdrawn, and mice were not fed overnight. All animals received humane care in compliance with the guidelines of the head of the Regierungsbezirk Freiburg, Germany.

Treatment Schedules. Con A was purchased from Sigma Chemical Co, St. Louis, MO, and 20 mg/kg were administered intravenously in a volume of 300 μL of pyrogen-free phosphate-buffered saline (PBS).

FITC-labeled Con A (Sigma Chemical Co, St. Louis, MO) was injected at the same dose as above (20 mg/kg) into the tail vein. Animals were killed 30 minutes after injection, and livers as well as control organs (kidneys, lungs, heart, bowel, muscle, and pancreas) were removed and immediately frozen in liquid nitrogen. Frozen tissue was divided into sections using a microtome (Leitz, Wetzlar, Germany) and was assessed under a fluorescence microscope (Olympus IMT/2, Olympus, Hamburg, Germany).

Polyclonal sheep anti-mouse TNF-α antiserum was intravenously administered 15 minutes before Con A challenge in a dose of 10 μL per mouse (30 g). This antiserum did not display cross-reactivity to mouse IL-1, IL-2, IL-4, IL-6, IL-10, IFN-γ, G-CSF and GM-CSF as tested in bioassays (see below) or in commercially available enzyme linked immunosorbent assay (ELISA) (Pharmingen, San Diego, CA), respectively.

For transfer experiments, male nude NMRI (nude/nude) mice were injected intravenously (IV) 7 days before Con A challenge with 2.5×10^7 lymph node cells (LNC) isolated from male NMRI albino mice.

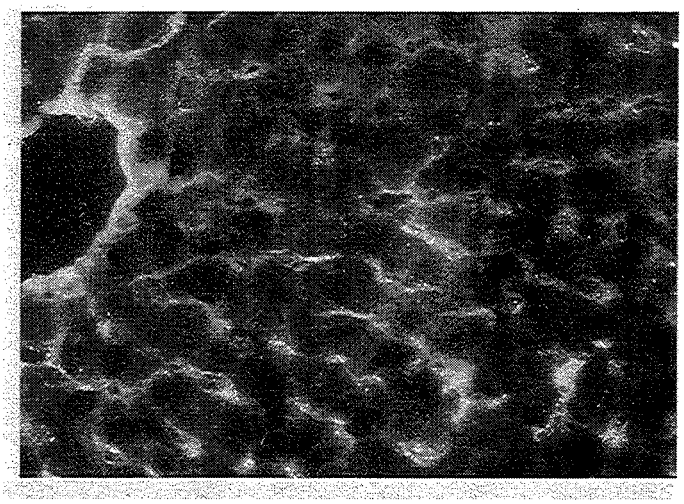

FIG. 1. Binding of FITC-labeled Con A in mouse liver. Frozen tissue section (×800) of a BALB/c mouse liver removed 30 minutes after injection of 20 mg/kg FITC-labeled Con A into the tail vein and assessed under a fluorescence microscope. Linear staining along endothelial surfaces can be observed. No such staining was observed in other organs.

Ciclosporin A (Sandimmune, Sandoz, Basel, Switzerland) was IV administered twice at doses of 130 mg/kg each 15 hours and 1 hour, respectively, before Con A challenge. Mice injected with the corresponding volumes of the diluent (Sandimmune, placebo) were examined as controls.

Blood sampling from the tail vein for TNF-determination or IL-2–determination was performed 1.5 or 4 hours after Con A administration, respectively. Eight hours after Con A challenge, mice were killed by cervical dislocation. Blood was withdrawn by heart puncture into heparinized syringes. The extent of liver injury was assessed by determination of plasma alanine aminotransferase (ALT) activity according to Horder.[14]

For determination of the time courses of cytokine release into the circulation after Con A challenge, mice were killed at the time points indicated, and blood withdrawn by heart puncture was collected in heparinized syringes.

Cytokine Assays. GM-CSF and IFN-γ were determined using a commercially available ELISA kit from Endogen, Boston, MA, and from Gibco RBL, Gaithersburg, MD, respectively. TNF was measured in a bioassay using the murine fibrosarcoma cell line WEHI 164 clone 13 according to Espevik and Nissen-Meyer.[15] IL-2 was determined in a cell proliferation assay according to Tada et al,[16] with the modification of using the mouse cytotoxic T-cell line CTLL-2. IL-1 was determined, according to Hopkins and Humphreys,[17] using a cellular assay based on the IL-1–dependent proliferation of the murine T-helper cell line D10N. Cytokine concentrations determined in the bioassays were calculated by the use of murine standards, specificity was obtained by coincubation with the respective antibodies (monoclonal anti-mouse IL-1α, monoclonal anti-mouse IL-1β, monoclonal anti-mouse IL-2 antibodies, all from Genzyme, Cambridge, MA, and polyclonal sheep anti-mouse TNF-α antiserum from our laboratory).

The polyclonal rabbit anti-human TNF-β antibody (80% immunoglobulin G (IgG), 20% immunoglobulin M (IgM), Gen-

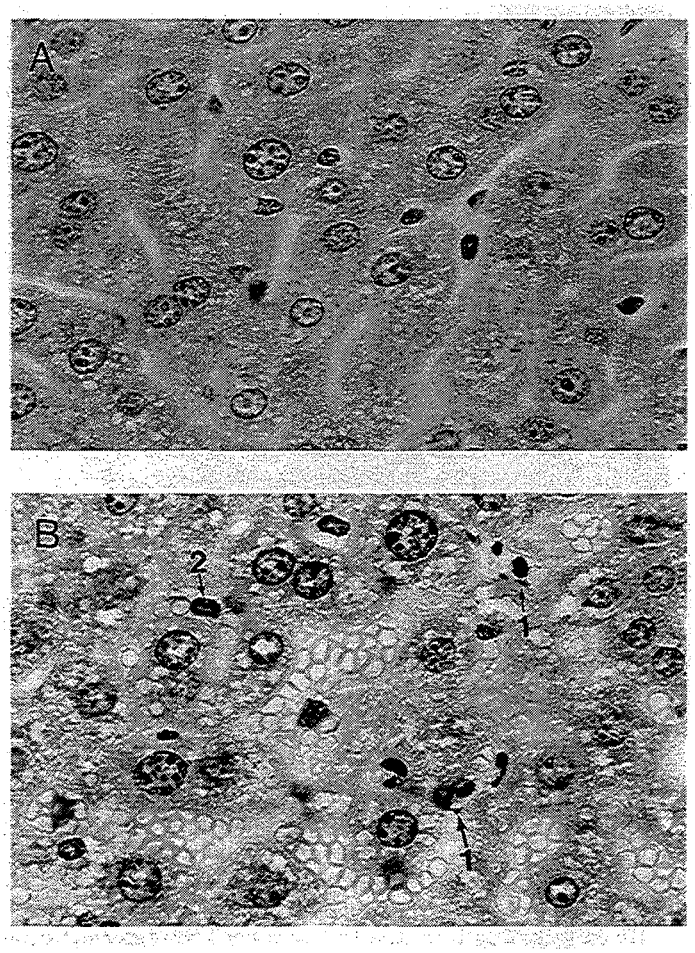

FIG. 2. Histological alterations in mouse livers 8 hours after challenge with 20 mg/kg Con A. A small piece from the large median lobe was fixed by immersion in Carnoy's solution after blood-free perfusion of the liver. Five-μm sections were stained with HE and photographed at 1008× original magnification. (A) Liver from a saline-treated control mouse. No histological abnormalities are detectable. (B) Liver from a Con A–treated mouse. A moderate diffuse cloudy swelling is evident in the hepatocellular cytoplasm. Numerous apoptotic bodies are visible (1). Severe agglutination of erythrocytes accompanied by an infiltration of liver sinusoids with neutrophilic granulocytes (2) is detectable.

zyme, Cambridge, MA) shows approximately 70% cross-reactivity with mouse TNF-β (data provided by the supplier).

Microscopy. Mice were anesthetized by pentobarbital (100 mg/kg). Livers were perfused for 10 seconds with cold buffer (50 mmol/L phosphate, 120 mmol/L NaCl, 10 mmol/L ethylenediaminetetraacetic acid (EDTA) pH 7.4). One liver lobe was excised and fixed for histological examination with Carnoy's solution and imbedded in paraplast. Five-micrometer sections were stained with hematoxylin-eosin (HE) and photographed at 1,008-fold magnification.

Cell Preparations for Coculture Experiments. Spleen or lymph nodes (axillary, brachial, iliac, inguinal, facial, and superior mesenteric) from BALB/c mice were removed and ground through a steel grid (diameter 100 μm) into 5 mL Roswell Park Memorial Institute 1640 medium (Biochrom, Berlin, Germany). *Ex vivo* cytokine determination was carried out directly from the supernatants of spleen cell suspensions after centrifugation at 400*g* for 8 minutes. 10^6 LNC were cocultured with either parenchymal or nonparenchymal liver cells in a volume of 200 μL in 24 well plates and incubated at 37° C, 5% CO_2, and 40% O_2 with or without Con A (2 μg/mL). Supernatants were frozen at −70° C until cytokine determination.

Hepatocytes were isolated from 12-week-old male BALB/c mice by the two-step collagenase perfusion method of Seglen[18] as modified by Klaunig et al.[19] Hepatocytes were plated in 200 μL RPMI 1640 medium containing 10% newborn calf serum (NCS, Biochrom, Berlin, Germany) in 24 well plates at a number of 8×10^4 hepatocytes per well. Cells were allowed to adhere to culture plates for 5 hours before the medium was exchanged for RPMI 1640 medium or RPMI 1640 medium containing LNCs. Incubations were performed for the times indicated in an incubator run at 5% CO_2, 40% O_2 and 55% N_2. Hepatocyte cultures contained 5% Kupffer cells as determined by latex phagocytosis.

Nonparenchymal cells (NPC) were purified by differential

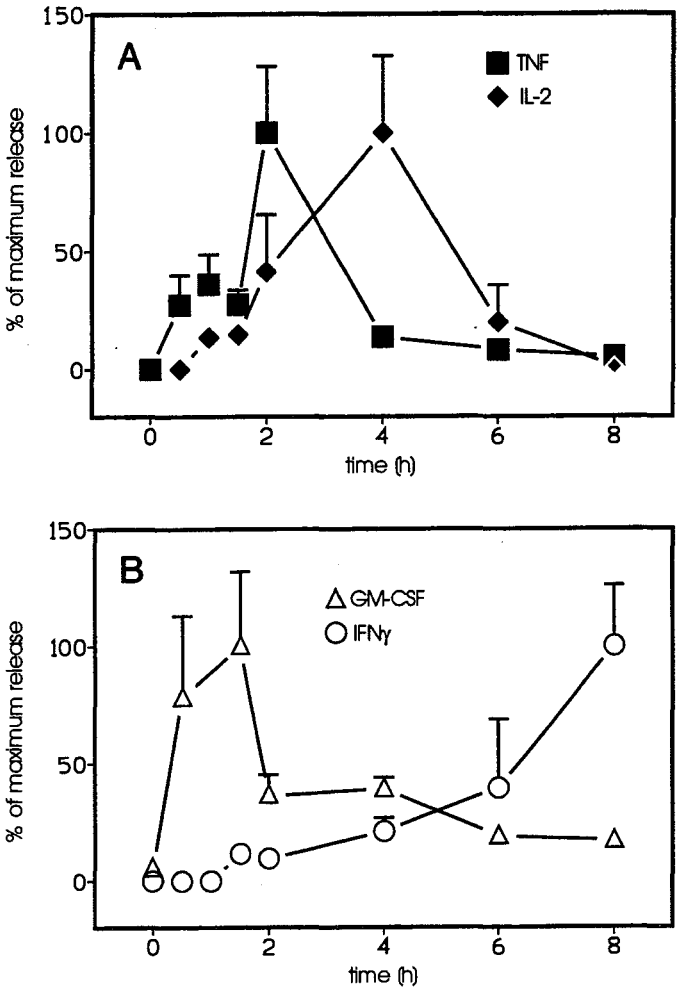

FIG. 3. Time course of cytokine release into plasma of NMRI mice challenged by 20 mg/kg Con A intravenously at time point zero (h). Each time point represents a group of three animals, respectively. Data are given as percentage of the individual maximum concentrations ± SEM. These maxima were (A) TNF, 2,500 ± 690 pg/mL, IL-2, 34,600 ± 11,200 pg/mL, and (B) GM-CSF, 300 ± 90 pg/mL, IFN-γ 2,770 ± 1,200 pg/mL. None of the four cytokines was detectable in plasma of control animals.

centrifugation from the liver cell suspension after collagenase digest according to Hartung and Wendel.[20] Briefly, supernatants from two successive 100*g* centrifugations of hepatocytes were pooled and centrifuged for 8 minutes at 400*g*. The pellet contained the NPC fraction. NPC were washed twice and plated out at 2×10^5 cells/well in 200 μL RPMI 1640 + 10% NCS. After a plastic adherence phase of 3 hours, supernatants were discarded and exchanged for RPMI 1640 or RPMI 1640 containing LNCs. After the adherence step, 1×10^5 NPC stuck to the culture plate, containing 80% to 90% Kupffer cells and no hepatocytes as determined by unspecific esterase stain and latex phagocytosis.

Statistics. The results were analyzed according to the Student's t-test or to the Welsh test. Data are expressed as mean values ± SEM; $P < .05$ was considered to be significant.

RESULTS

***Organotropy of FITC-Labeled Con A* In Vivo.** Thirty minutes after injection of FITC-labeled Con A, significant fluorescence was only noted within the liver (Fig. 1). No specific staining was observed in pulmonary or renal tissue or any of the other organs examined. The fluorescence staining in the liver appeared to be linear along the endothelial surfaces of the liver sinusoids. Uptake of FITC-labeled Con A by hepatocytes was not observed. Therefore, *in vivo* distribution of Con A was specific for the liver.

Mechanism of Hepatocyte Cell Death in Con A–Treated Mice. We wondered which mechanisms are likely to underlie Con A–induced hepatocyte death *in vivo*. Therefore, livers of mice treated with Con A were histologically examined. Infiltration of neutrophils and agglutinated erythrocytes were observed in livers of mice no later than 3.5 hours after treatment with 20 mg/kg of Con A. Eight hours after Con A injection, numerous intracellular DNA-containing apoptotic bodies were detectable as well as karyorrhexis and diffuse cloudy swelling in the hepatocellular cytoplasm (Fig. 2). However, nuclear chromatin condensation was not observed. The formation of apoptotic bodies was noted as early as 3.5 hours after administration of Con A, i.e., at an early point of development of liver injury (not shown). Because only weak signs of necrotic hepatocyte cell death were observed histologically 8 hours after Con A administration and because apoptotic alterations preceded development of hepatic lesions as measured by ALT release (not shown, Table 1), apoptotic mechanisms are likely to be involved in the early events of liver injury induced by Con A.

Time Course of Cytokine Release After Con A Challenge. Our previous work showed that NMRI mice challenged by IV administration of 20 mg/kg Con A released IL-2 into the circulation.[12] IL-2 production was considered to be a consequence of Con A–induced T-cell activation *in vivo*. As shown in Fig 3, the cytokines GM-CSF, TNF, and IFN-γ were also detectable in plasma of mice treated with Con A. The time course of cytokine release into plasma (Fig. 3) showed the following peak concentrations: GM-CSF, 300 pg/mL at 1.5 hours; TNF, 2,500 pg/mL at 2 hours; and IL-2, 34,600 pg/mL at 4 hours after Con A administration. The time course of IFN-γ release showed a steady further increase with time. Maximal concentrations, i.e., 2,770 pg/mL, were measured at the end of the experiment at 8 hours after intervention. Within this time window of 8 hours, Con A-challenged mice developed severe liver injury (Table 1).[12] The cytokine release pattern seems to be typical for a T-cell response, because IL-2 and IFN-γ are typical lymphokines.[21] At present, we cannot define the cellular origin of GM-CSF or TNF (see below). We also reported previously that macrophages serve as effector cells together with $CD4^+$ T cells in liver failure induced by Con A.[12] Antigen-presenting macrophages are known to release IL-1 in order to activate T-helper cells for the respective immune response. Therefore, we measured IL-1 production after *in vivo* treatment of mice by Con A. Because mouse plasma was cytotoxic towards the D10N cells used in the IL-1 bioassay, we were not able to determine circulating IL-1. To overcome this problem we chose a different approach: at different time points after *in vivo* challenge of mice with 20 mg/kg Con A, we prepared spleens and determined cytokine contents *ex vivo* by assaying cytokine concentrations in the supernatants of these cell suspensions. The following maximum concentrations were found: IL-1α, 9,590 pg/mL at 3 hours; GM-CSF, 120 pg/mL at 1 hour; TNF, 3,600 pg/mL at 1 hour; and IL-2, 47,500 pg/mL at 4 hours after Con A challenge.

TABLE 1. Pretreatment of Mice With Anti-Murine TNF-α Antiserum Protects Against Con A–Induced Hepatitis

Treatment	ALT (U/L)	TNF (pg/mL)	IL-2 (pg/mL)
Solvent control	40 ± 20	≤ 20	≤ 100
Con A	1,600 ± 767	175 ± 36	8,010 ± 1,530
Control antiserum + Con A	1,715 ± 653	236 ± 71	6,820 ± 1,850
Anti-murine TNF-α antiserum + Con A	55 ± 20*	≤ 20	9,020 ± 2,870

NOTE. Con A (20 mg/kg) was intravenously injected. Control sheep antiserum or polyclonal sheep anti-murine TNF-α antiserum wa intravenously administered at a dose of 10 μL per mouse in a total volume of 300 μL PBS 15 minutes before Con A. ALT was determined 8 hours after Con A challenge. TNF or IL-2 were measured in plasma of the animals 1.5 hours or 4 hours, respectively, after Con A administration. Data are expressed as mean ± SEM; n = 6.

* $P \leq .05$ vs. disease control.

The Contribution of TNF-α to Con A–Induced Liver Injury. TNF has been identified as a central mediator in experimental macrophage- or T-cell–dependent infectious disease models such as endotoxin shock,[22] gram-negative,[23] and gram-positive sepsis[24] as well as in endotoxin-induced hepatitis in galactosamine-sensitized mice.[25] The capacity of Con A to stimulate TNF release *in vivo* prompted us to investigate the effect of an anti-TNF antiserum on Con A challenge. The results in Table 1 show that pretreatment of mice by polyclonal sheep anti-mouse TNF-α antiserum fully protected

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