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Heat shock protein-GP96 as an innate sensor of damage and activator of autoreactive NKT and regulatory T cells during liver regeneration

Ines Mrakovčić-Šutić*, Hrvoje Jakovac*, Marija Šimin, Damir Grebić, Mira Čuk, Zlatko Trobonjača and Biserka Radošević-Stašić

Department of Physiology and Immunology, Medical Faculty, University of Rijeka, Rijeka, Croatia

*These authors equally contributed to this work

Summary. Tissue disintegration after injury leads, in the endoplasmic reticulum (ER), to activation of adaptive pathways known as the ER stress response. It is directed to the correction of unfolded proteins and to the activation of proteasome-dependent ER-associated degradation of the misfolded proteins, but induces also a rapid activation of natural and adaptive immunity, since a ER resident heat shock protein-gp96 acts not only as a molecular chaperone, but also as a strong adjuvant, able to cross-present the antigenic peptides onto MHC class I or MHC class II pathways.

Analyzing its potential role in processes of normal growth, in mice subjected to 1/3 partial hepatectomy (pHx) we determined the tissue expression of gp96 protein and mRNA in regenerating liver, thymus and spleen, determining simultaneously the phenotypic profile and spontaneous cytotoxic activity of intrahepatic and splenic mononuclear lymphatic cells (MNLC) against NKT- and NK-cells sensitive targets (syngeneic thymocytes and YAC-1) in wild, perforin and FasL deficient mice.

The data have shown that pHx induces fast overexpression of gp96 protein and mRNA in hepatocytes, spleen and thymus, with accumulation of $CD3^{intermediate}/NK1.1^{+}/CD69^{+}$ cells (liver) and $Foxp3^{+}CD4^{+}CD25^{+}$ cells (liver and thymus). Simultaneously, intrahepatic MNLC acquired the FasL-dependent cytotoxic potential against NKT-sensitive targets and both, intrahepatic and splenic MNLC, acquired the perforin-dependent cytotoxic potential against NK-sensitive targets, implying that during the disturbance of morphostasis gp96 serves as a natural adjuvant for chaperoning antigenic self peptides into the immune surveillance pathways, resulting in activation of autoreactive NKT and regulatory cells, as well as NK cells. Moreover, cell cycle analysis revealed that G2+M phase of regenerating hepatocytes in PKO mice was translocated from the 1st to the 7th p. o. day, as well as that hepatocytes from FasL deficient mice were arrested in G0/G1 phase.

Key words: Endoplasmic reticulum resident heat shock protein-gp96, Partial hepatectomy, liver regeneration, NKT cells, regulatory T cells

Offprint requests to: Biserka Radošević-Stašić, Medical School, University of Rijeka, B. Brancheta 22, 51000 Rijeka, Croatia. e-mail: biserr@medri.hr

Introduction

Liver regeneration that follows after injury or surgical resection of hepatic lobes is a well-known model for investigating normally regulated growth and mechanisms connected with the maintenance of morphostasis. The process has been successfully dissected on a molecular and genetic level and presented in several recent reviews (reviewed by Fausto, 2004, 2006; Taub, 2004; Michalopoulos and Khan 2005; Pahlavan et al., 2006; Michalopoulos, 2007). They show that liver growth is characterized by a fascinating replicative capacity of the remaining hepatocytes that constitute the first line of response to injury or resection, as well as by replication of the progenitor (stem or oval) cells that function as a reserve compartment, activated whenever the hepatocyte proliferation is inhibited (Fausto, 2004). Activating and inhibiting signals in the form of cytokines and growth factors come, however, from nonparenchymal cells in the liver, such as Kupffer cells, endothelial and stellate cells (tumor necrosis factor-TNFα, interleukin (IL)-6, hepatocyte growth

factor), as well as from the peripheral organs such as gut (lipopolysaccharide), pancreas (insulin), duodenum or salivary gland (epidermal growth factor), adrenal gland (norepinepherine and glucocorticoids), thyroid gland (triiodothyronine), or thymus, showing that regeneration of the liver is regulated by cooperative signals from various sources (Mocchegiani et al., 1997; Fausto, 2004; Taub, 2004; Michalopoulos and Khan 2005; Milin et al., 2005; Fausto, 2006; Pahlavan et al., 2006; Michalopoulos, 2007). Moreover, widespread evidence suggests that in the control of reparatory processes may directly participate the autoreactive clones of lymphocyte with regulatory and protective effects, which may have a strong influence on reestablishment of self-tolerance after disturbance of morphostasis and may be involved in the control of a variety of autoimmune diseases, tumor growth and infection (Mocchegiani et al 1997; Abo et al., 2000; Kronenberg and Rudensky, 2005; Milin et al., 2005). Prominent among these cells are natural killer T (NKT) cells that express a semi-invariant T-cell receptor (TCR) specific for conserved self ligands (Abo et al., 2000; Godfrey et al., 2000; Hammond and Kronenberg, 2003; Kronenberg and Rudensky, 2005; Seino and Taniguchi, 2005; Van Kaer and Joyce, 2005; Molano and Porcelli, 2006), as well as $CD4^+CD25^+$ T regulatory (TReg) cells, which have a dedicated suppressor function in the control of the immune response (Sakaguchi, 2004; Cava et al., 2006). Besides, there are complex mechanisms of reciprocal regulation between cells of the innate immunity, such as natural killer (NK) cells and the autoreactive T cells, since NK cells may both promote and inhibit the generation of autoreactive T cells, influencing the activation, differentiation and clonal expansion of naive T cells by several pathways (Shi and Van Kaer, 2006). The damage or inflammation may, thus, result in NK-cell recruitment to potential targets of autoimmunity, where through the production of pro-inflammatory or anti-inflammatory cytokines or through the lysis of dendritic cells (DC) or T cells they may regulate the development of autoimmunity and inflammation (Shimamura et al., 2005; Strominger et al 2003; Shi and Van Kaer, 2006). Similarly, it was found that during liver regeneration the liver-resident NK cells regulate the intensity of liver growth (Vujanovic et al., 1995; Sun and Gao, 2004; Huang et al., 2006; Oishi et al., 2006), as well as that in the regenerating liver accumulate particularly self-reactive clones of NKT cells that probably mediate cytotoxicity against superfluous regenerating hepatocytes, or against abnormal self-cells, such as malignant tumor cells or microbially infected hepatocytes (Matsushita et al., 1999; Abo et al., 2000). In these events, the triggering of NK- cell cytotoxicity versus the absence of killing depends on a delicate balance between activating and inhibiting signals delivered by cell-surface receptors belonging to various families, which on the surface of target cells recognize constitutively expressed ligands, or ligands induced by cellular transformation or stress (Janeway and Medzhitov, 2002; Yokoyama, 2004; Shi and Van Kaer, 2006). Similarly, autoreactive NKT cells might be activated by self-glycolipid antigens presented by CD1d molecules (Godfrey et al., 2000, 2006; Hammond and Kronenberg, 2003; Kronenberg and Rudensky, 2005; Seino and Taniguchi, 2005; Van Kaer and Joyce, 2005; Molano and Porcelli, 2006), by endogenous lysosomal glycosphingolipid-isoglobotrihexosylceramide (Zhou et al., 2004), or by exogenous antigens such as alpha-galactosylceramide (alpha-GalCer) and bacterial toxins (Godfrey et al., 2000; Hammond and Kronenberg, 2003; Kronenberg and Rudensky, 2005; Seino and Taniguchi, 2005; Van Kaer and Joyce, 2005; Molano and Porcelli, 2006). Furthermore, as suggested by "the danger theory of immunity" (Gallucci and Matzinger, 2001; Matzinger, 2007) endogenous danger signals, such as heat-shock proteins, nucleotides, reactive oxygen intermediates, extracellular-matrix breakdown products and neuromediators might represent the necessary co-stimulatory signal for activation of naïve T cells on antigen presenting cells (APC), serving not only to alert the immune system to the death of a cell under stress, but functioning also as protein carriers that allow the immune effectors to survey the peptides released by this stressed cell and carried by the stress protein (Gallucci and Matzinger, 2001; Radons and Multhoff, 2005; Matzinger, 2007).

In the light of these complex interactions, pointing to integral role of immune system in wound healing and reparatory processes, in this study we examined the tissue expression of endoplasmic reticulum resident heat shock protein gp96 in the regenerating liver, spleen and thymus, and tested the phenotypic and cytotoxic properties of intrahepatic and splenic mononuclear lymphatic cells (IHMNLC and SMNLC, respectively) isolated from wild-type (WT), perforin knock out (PKO) and FasL-deficient (generalized lymphoproliferative disease; gld) mice subjected to partial hepatectomy (pHx) or to sham operation, trying to enlarge previously reported data about the role of immune system in the normal growth (Vujanovic et al., 1995; Mocchegiani et al.,1997; Matsushita et al, 1999; Abo et al., 2000; Sun and Gao, 2004; Milin et al., 2005; Shimamura et al., 2005; Fausto, 2006; Huang et al., 2006; Oishi et al., 2006), and elucidate the role of perforin and Fas/FasL-dependent pathways in these events.

The data have shown that pHx increases the tissue expression of gp96 in regenerating liver and in the thymus and leads to accumulation of NK1.1+ CD3intermediateCD69+, CD4+ and T-regulatory (TReg) cells in the liver. Moreover, intrahepatic and splenic MNLC acquired the capacity to lyse NKT- and NK-sensitive targets, using FasL-dependent (liver) or perforin- and FasL-dependent (spleen) mechanisms, suggesting that during normal growth gp96-chaperoned self peptides may participate in the recruitment and activation of autoreactive clones of cells, which probably control the reparatory processes. The hypothesis seems to be supported by findings of augmented number of

apoptotic cells in the thymus, as well as by the observation that the progression of hepatocytes through the cell cycle was significantly changed in perforin and FasL-deficient pHx mice.

Materials and methods

Mice

Wild-type (WT), perforin knockout mice (PKO, H-2^b), or Fas ligand-defective mice (gld) mice of strain C57/BL6 aged 2-3 months were selected for the experiment. Mice lacking the perforin or FasL gene were generated by targeted gene disruption in embryonal stem cells. They were originally kindly provided by Dr. E. Podack, University of Miami and then mated in a pathogen free animal facility-breeding colony of Medical Faculty in Rijeka. During the experiments the mice were housed in groups of 6-8 animals, kept under standard conditions and exposed to a natural day-night cycle. The mice were bred and maintained according to the guide for Institutional Animal Care and used with the approval of the Ethical committee of the University of Rijeka.

Partial hepatectomy

The median liver lobe was removed (1/3 pHx) under ether anesthesia. To avoid possible diurnal variability, all operations were done between 8:00 and 9:00 hours. The animals were sacrificed by bleeding at 1, 2, 6, 24 and 48 h and at 7 days after surgery. In control groups the sham operation was done, consisting of laparotomy and gentle touching of the liver, without the removal of liver lobes. Portions of the liver, spleen and thymus were collected from each mouse for routine histological examination and immunohistochemistry.

Immunohistochemistry

Immunohistochemical studies were performed on paraffin embedded tissues of liver, spleen and thymus using DAKO EnVision+System, Peroxidase (DAB) kit according to the manufacturer's instructions (DAKO Corporation, USA). Briefly, slides were incubated with peroxidase block to eliminate endogenous peroxidase activity. After washing, monoclonal anti-Grp94 antibody (Monoclonal rat Anti-Grp94 antibody, Clone 9G10 (Stressgen, Canada), diluted 1:30 in phosphate-buffered saline supplemented with bovine serum albumin was added to tissue samples and incubated overnight at 4°C in a humid environment, followed by 45 minutes incubation with peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins containing carrier protein linked to Fc fragments to prevent nonspecific binding. The immunoreaction product was visualized by adding substrate-chromogen (DAB) solution. Tissues were counterstained with hematoxylin and 37 mM ammonia water, dehydrated in a gradient of alcohol and mounted with mounting medium. The specificity of the reaction was confirmed by substitution of anti-Grp94 antibody with mouse irrelevant IgG2α immunoglobulin (clone DAK-G05; Dako, USA), used in the same conditions and dilutions as a primary antibody.

RNA isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the liver, spleen and thymus tissue homogenates using Trizol Reagent (Invitrogen, USA) following the manufacturer's instructions. The RNA yield and purity was assessed spectrophotometrically and its integrity was confirmed by 2% agarose gel electrophoresis. 1 µg of total RNA from each sample was reverse-transcribed by oligo-p (dT) primers into cDNA using First Strand cDNA Synthesis Kit – AMV from Roche, Germany, as specified by the manufacturer. cDNA was amplified by PCR using Taq polymerase and PCR buffer containing 6 mM $MgCl_2$. Amplification of housekeeping gene ß-actin mRNA was always involved as a positive control.

The composition of PCR primers and product length was as follows:
gp96 sense: 5'- GAATCTGATGATGAAGCTGC -3'
gp96 antisense: 5'- GCAATCTTCTTGATCATGTCC – 3' (535 bp)
ß-actin sense: 5'- GGACTCCTATGTGGGTGACGAGG -3'
ß-actin antisense: 5'- GGGAGAGCATAGCCCTCGTA GAT -3' (366 bp)
The reactions started with denaturation for 4 min at 94°C and had a final elongation step of 10 min at 72°C. The amplifications were performed using ProGene Techne thermal cycler (Cambridge, UK), at the following amplification conditions: Gp96, 30 seconds at 94°C; 30 seconds at 55°C; 30 seconds at 72°C (30 cycles); ß-actin, 1 min at 94°C; 1 min at 61°C; 1 min at 72°C (28 cycles). The amplified products of the RT-PCR reactions were size-fractionated by 2% agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide.

Isolation of hepatic, splenic and thymic MNLC

MNLC were isolated from intact or regenerating liver, using a modification of the method of Seglen, as we previously described (Mrakovcic-Sutic et al., 2002). For this purpose, *in situ* slow perfusion with 10 ml of PBS was used. The liver was extirpated and carefully shaken in PBS. Cell suspension was then separated from non-suspended tissue debris by filtering through 80 mm gauze. Each sample was laid on 10 ml of Ficoll-Hypaque and MNLC were isolated by density gradient centrifugation, 20 min at 800xg. Eventually, cells were harvested from the middle layer, washed twice in PBS and counted in hemocytometer (Neubauer). A single suspension of spleen MNLCs was prepared in RPMI 1640 medium (Gipco BRL, Basel, Switzerland), after

elimination of erythrocytes by lysing solution.

Immunofluorescent staining and flow cytometry

The surface phenotypes of intrahepatic MNLC, thymocytes and splenocytes were identified by direct immunofluorescence analysis on FACScalibur (Becton Dickinson, Immunocytometry Systems, Mountain View, CA), using CELLQuest Software (Macintosh, Quadra 650). As monoclonal antibodies (mAbs) fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated anti-CD3, anti-NK-1.1, anti-IL2R-ß chain, anti-CD4, anti-CD8, anti-CD25, anti-CD5, anti-CD69, anti-CD80 and anti-CD86 mAbs, purchased from Becton Dickinson Co (Mountain View, CA, USA) were used. Mouse Regulatory T cell Staining Kit (w/PE Foxp3 FJK-16s, FITC-CD4, APC-CD25) (eBioscience, Inc., San Diego, CA, California, USA) was used for determination of regulatory T cells in the liver and thymus. All samples had adequate isotypic controls. Propidium iodide (PI; Sigma, MO) (1 mg/ml) stained dead cells were excluded by electronic gating. Relative fluorescence intensities were expressed in log scale, with $1x10^4$ cells.

Flow-cytometry cytotoxicity assay

Functional NK or NKT cells assays were performed with PKH-26 (orange)-labeled YAC-1, P815 or syngeneic thymocytes as target cells respectively, following the manufacturer's instructions (Sigma Biosciences, St Louis, MO; PKH-26 Red Fluorescent Cell Linker Kit), as previously described (Mrakovcic-Sutic et al., 2002). Briefly, hepatic and splenic MNLC, immediately after their isolation, were incubated for 2 hours with $1x10^5$/ml labeled target cells (YAC-1 cells or syngeneic thymocytes) at different effector to target ratios in a final volume of 200 ml at 37°C in a 5% CO_2 atmosphere. After washing in fluorescence-activated cell sorter (FACS) buffer, 200 ml of propidium iodide (PI; concentration 10 mg/ml) was added and the percentage of killed cells were counted by flow cytometry detecting cells with both-orange (PKH-26) and red (PI) fluorescence.

Cell cycle analysis

Freshly prepared hepatic cell suspension was washed twice in Sample buffer (PBS with 1 g/l glucose and 1 mM EDTA) in concentration of $1-2x10^6$/ml and fixed in ice cold 70% ethanol. After 24 h of fixation cells were again washed twice in Sample buffer and finally resuspended in Sample buffer containing 50 mg/ml propidium iodide and incubated for next 2 hours. Cell cycle analysis of the prepared suspension was performed on flow cytometer (FACSCalibur) using Cel Quest Software. Percentage of the cells in G0/G1, S and G2+M phase was calculated.

In situ apoptotic detection (TUNEL method)

The TUNEL method was used on paraffin-embedded tissue sections of the thymus and the liver using In Situ Cell Death Detection Kit, POD (Roche, Germany) according to the manufacturer's instructions. Briefly, dewaxed and rehydrated slides were treated with 0.3 % H_2O_2 solution to block endogenous peroxidase activity, followed by incubation with Proteinase K solution. After washing, the slides were incubated with the TUNEL reaction mixture, which contains recombinant terminal deoxynucleotidyl transferase and fluorescein-labeled deoxynucleotides that bind to fragmented DNA molecules. Signal conversion was accomplished by incubation with Converter-POD solution containing the Fab fragment of anti-fluorescein sheep antibodies, conjugated with horseradish peroxidase. The reaction was visualized by adding DAB Substrate solution. The tissues were counterstained with hematoxylin, mounted and observed with an optical microscope. To estimate the relative number of apoptotic cells after photographing the stained sections of thymus, under a light microscope we counted the total number of cells and the number of cells stained by TUNEL reaction mixture in 5 randomly chosen fields (magnification 400x, Olympus BX51 Microscope). The analysis was made in 3 mice per experimental group in an evaluator-blinded manner, and the data were expressed as percentage of apoptotic cells, calculated by dividing the fragmented DNA-expressing cells by the number of total cells.

Statistical Analysis

Data were analyzed using the Sigma Plot Scientific Graphing System, Version 6.10. Statistical significance was calculated by Mann Whitney U test. The differences were considered significant for $p<0.05$.

Results

Partial hepatectomy upregulates the tissue expression of Gp96 in the regenerting liver, spleen and thymus

Tissue expression of gp96 protein and mRNA in the liver, spleen and thymus were analyzed by immunohistochemistry and RT-PCR in intact mice and in the early period after pHx. The data revealed that pHx is followed by a high upregulation of gp96 expression in regenerating hepatocytes, as well as in lymphatic organs (Fig. 1). In the liver, the process started in periportal zones, affecting after 6h the whole hepatic lobulus. In the spleen the number of polymorphonuclear and mononuclear cells with high cytoplasmic expression of gp96 increased, while in the thymus, 2h after pHx, an impressive overexpression of g96 was found, particularly in the cortico-medullary junction (Fig. 1).

RT-PCR analysis has shown that in pHx mice

increases also the expression of mRNA of gp96 not only in the regenerating liver, but also in the thymus (Fig. 2), suggesting that the endoplasmic reticulum–resident gp96 may be involved not only in protein folding in hepatocytes, but also in the induction of self peptides specific and/or antigen- unspecific immune response.

Partial hepatectomy increases the proportion of NKT and regulatory T cells in the liver and upregulates the expression of co-stimulatory molecules on hepatic and splenic dendritic cells

To test the immune consequences of pHx, the surface phenotype of intrahepatic and splenic MNLC was analyzed in intact mice, and those sacrificed at 24 and 48 h and at 7 days after pHx or sham operation. The data showed that on the 2nd p. o. day in the regenerating liver accumulated T and NK cells ($CD3^{high}$ and NK1.1+ cells, respectively), as well as NKT cells, that are $CD3^{intermediate}$/NK1.1+ (Fig. 3). At that time 57,6% of these intrahepatic, NKT co-expressed also the CD69 marker, in comparison with 14,79% of NK1.1+CD3- (NK) cells and 11.71% of $NK1.1^{-}CD3^{high}$ (T cells) (Fig. 4A). The effect was specific for the regenerating liver after pHx, since accumulation of NKT cells was not seen in the spleen (Figs. 3, 4C), while sham operated animals had at that time in the liver only 2.2% of activated NKT cells, and reacted more slowly on laparotomy (Fig. 4C).

Simultaneously, we found that in the early postoperative period (on the 2nd and 6th hour after pHx) in the liver and the spleen significantly arose the proportion of dendritic (CD11c+) cells that express the co-stimulatory molecules CD86 (liver), and CD80 (spleen) (Fig. 5).

Furthermore, in the remaining liver the percentage of CD4+CD25+ FoxP3+ cells rose from 0.62±0.09% to 1.28±0.29% ($p<0.01$), and in the thymus from 0.48±0.02% to 0.96±0.07 ($p<0.001$) (Fig. 6), suggesting that TReg cells are also involved in the control of liver regeneration.

Partial hepatectomy increases the cytotoxicity of hepatic and splenic MNLC against NKT cells-sensitive and NK cells-sensitive targets

To test the functional activities of hepatic and

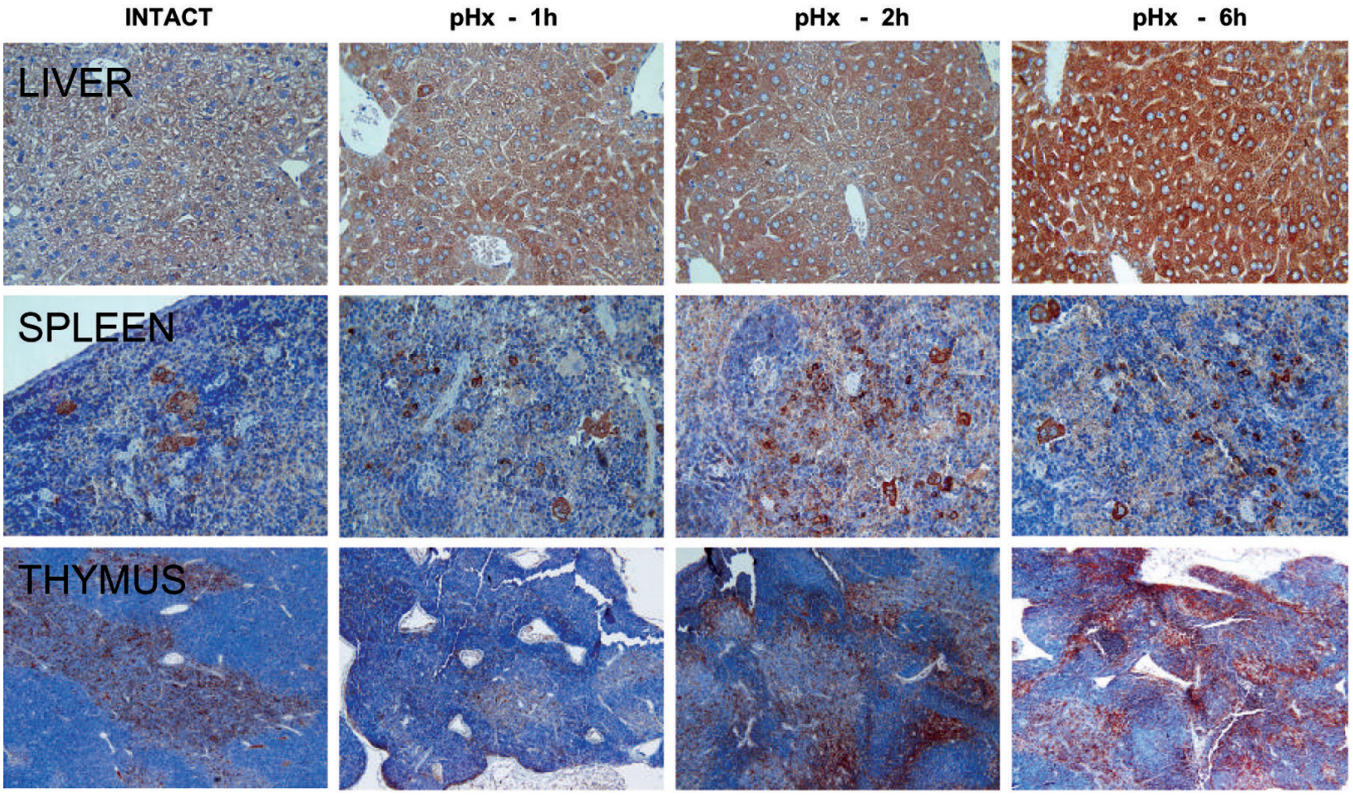

Fig. 1. Immunohistological staining for gp96 protein in the liver, spleen and thymus at various time points after 1/3 partial hepatectomy. One and two hours after pHx the intracytoplasmic gp96 expression was found in periportal zones and after 6h affected the whole hepatic lobulus. In the spleen of intact mice gp96 was constitutively expressed on several mononuclear lymphatic cells (MNLC), but after pHx it was upregulated on some plasma cells and macrophages. In the thymus of intact mice gp96 was found in the medullar zone, while 2h after pHx an impressive overexpression of g96 was found particularly in the cortico-medullary junction. Results are representative findings of 3 intact and pHx mice. x 400

splenic MNLC cells during the various phases of liver regeneration, their cytotoxicity was, immediately after the isolation of cells, tested against syngeneic thymocytes and YAC-1 cell line, sensitive to NKT and NK cells, respectively, at different times after pHx (24h, 48h and 7days). Results were compared with the effects of MNLC obtained in the same time intervals from sham operated mice.

The data showed that after pHx both hepatic and splenic MNLC became highly cytotoxic against syngeneic thymocytes and YAC-1 targets, acquiring the maximal values predominantly 48h after pHx (Fig. 7); the only exception was an earlier increase of cytotoxicity of splenic MNLC against YAC-1 (24h after pHx). Enhancement of all types of cytotoxicity was induced by pHx, since MNLC obtained, in the same time intervals, after the sham operation did not show these characteristics (Fig. 7).

Cytolytic activity of killer cells induced by pHx is FasL and/or perforin-dependent

To elucidate the effector pathways in cell-mediated cytotoxicity after pHx, the cytolytic efficacy of hepatic and splenic MNLC obtained 48h after pHx from PKO and FasL deficient pHx mice was tested against syngeneic thymocytes and against YAC-1 cells (Fig. 8).

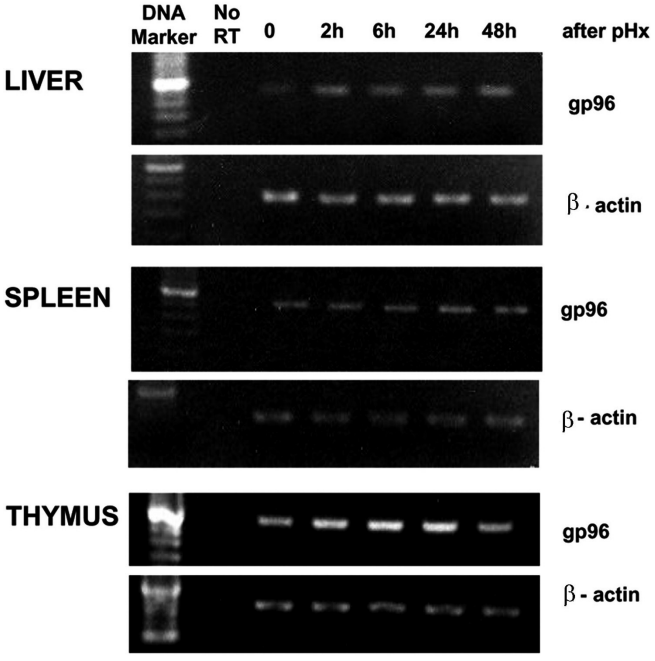

Fig. 2. Gradual upregulation of gp96 mRNA expression in liver, (spleen) and thymus after hepatectomy from 2nd to 48th h after surgery. Beta-actin was used as a loading control. The data are representative of at least two individual experiments.

The data showed that hepatic MNLC from gld/gld pHx mice lost their cytotoxic properties against syngeneic thymocytes (Fig. 8; $p<0.001$ in comparison with the WT control), while those obtained from PKO were still effective (Fig. 8; n.s. in comparison with the control), suggesting that self reactive clones of NKT cells, that accumulate in the liver, use predominantly FasL-dependent pathway to kill the targets. In contrast, the cytotoxicity of splenic MNLC against syngeneic thymocytes, as well as the cytotoxicity of both hepatic and splenic MNLC against the YAC-1 target, was found to be both perforin and FasL-dependent (Fig. 8; $p<0.001$). Testing with MNLC obtained from sham operated mice, showed no difference in cytotoxicity against syngeneic thymocytes regarding the source of effectors (not shown), confirming that NKT-cytotoxicity was induced by pHx.

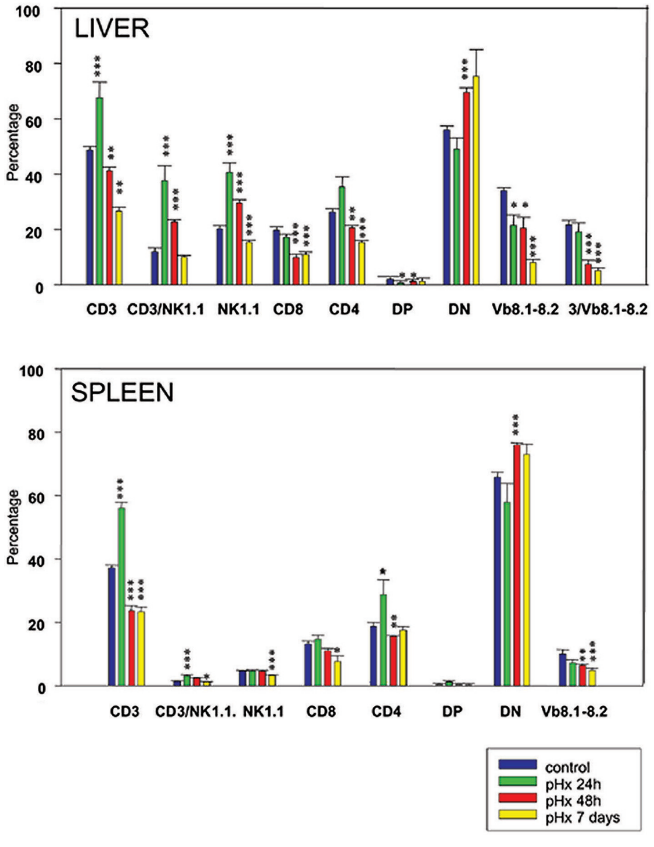

Fig. 3. Phenotypic changes found in the liver and in the spleen after 1/3 pHx in C57BL/6 mice. Flow cytometric analysis was made on freshly isolated hepatic and splenic MNLCs isolated on the 1st, 2nd and 7th day after 1/3 pHx of C57BL/6 mice. Lymphocyte gate was defined by side-scatter and forward-scatter dot plots and single or two color staining was made by mAbs against for CD3, NK1.1, Vß8.1-.8.2, CD4 and CD8. Proportions are shown as mean ± standard errors (n = 7). *$p<0.05$; **$p<0.01$, *** $p<0.001$ in comparison with intact control (Mann- Whitney U test).

Cell-cycle kinetics of regenerating hepatocytes is changed in perforin and FasL-deficient mice

To prove that perforin and Fas/FasL-dependent pathways might be involved in the control of liver regeneration cell cycle analysis of regenerating hepatocytes was made in WT and in perforin- and FasL-deficient C57/BL6 pHx mice. The data have shown that remaining parenchymal hepatocytes after 1/3 pHx enter the cell cycle, elevating maximally the percentage of G2+M phase after 24h (Fig. 9; pHx in control mice). Hepatocytes from both, PKO and Fas/L-deficient mice, were, however, blocked in their progression through G1 phase during the first 24h after pHx ($p<0.05$ and $p<0.001$, respectively, in comparison with wild type mice). This G0/G1 arrest persisted in hepatocytes of FasL-deficient mice, while those obtained from PKO mice continued to grow entering in G2/M phase, but the peak of this phase was translocated from the 1st on the 7th day after pHx (Fig. 9; $p<0.01$ in comparison with the control).

Partial hepatectomy increases the number of apoptotic cells in the thymus

Owing to the finding of high upregulation of gp96 in the thymus (Fig. 1), which implied its possible involvement in the processes of positive and negative selections of thymocytes, we examined also the number of thymic cells that during liver regeneration underwent into the programmed cells death. As is shown in Fig. 10, massive apoptosis was found particularly in the cortex and at corticomedullary junction, and the relative number of apoptotic cells at 24h, 48h and 72h after pHx

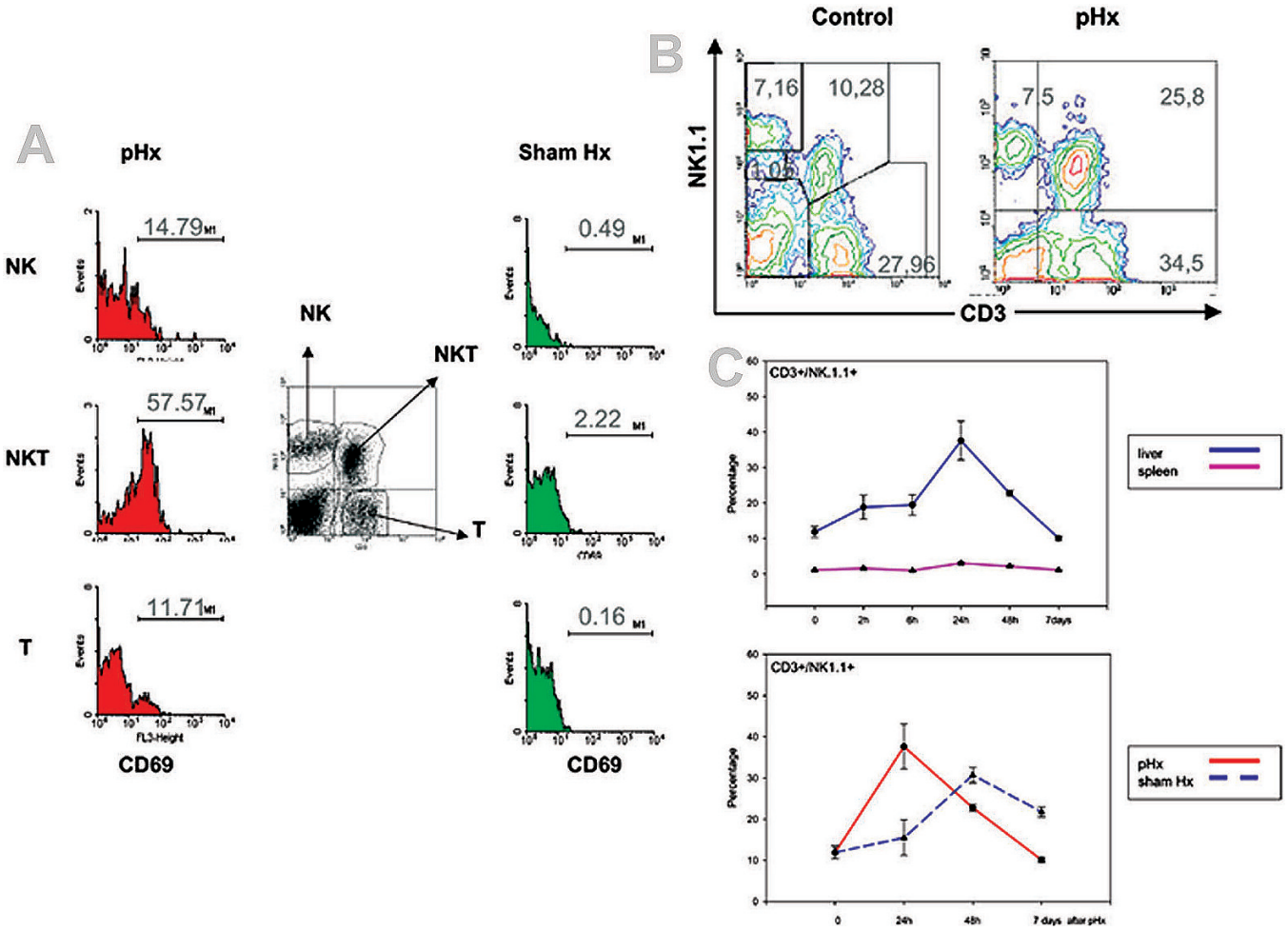

Fig. 4. Augmentation and activation of NKT cells in the liver after 1/3 pHx. **A.** The expression of CD69 on NK, NKT and T cells, respectively, in regions R1 ($CD3^-NK1.1+$ cells), R2 ($CD3^{intermediate}$ $NK1.1^+$ cells), and R3 ($CD3^+NK1.1^-$ cells) in the liver of pHx or sham Hx mice. **B.** FACS profiles of two-color immunofluorescence staining of hepatic NKT ($CD3intNK1.1^+$) cells after pHx, shown as a representative result of four isolated experiments. **C.** Dynamics of hepatic versus splenic NKT cells after pHx and hepatic NKT cells activated by pHx versus NKT cells activated by sham Hx. Data are means ± standard errors from 6 mice in each group.

arose from 1.9% (intact mice) to 37.6%, 47.6% and 63%, respectively ($p<0.001$).

Discussion

Multiple cellular and molecular studies of the liver regeneration have elucidated almost all aspects of high capacity of the liver to react on toxic injuries or on surgical removal of its lobes, providing the essential information about the pathways involved in the initiation and termination of normal growth, metabolic processes and the nature of liver progenitor and bone-marrow derived hematopoietic stem cells, what might be directly relevant to clinical problems, such as acute and chronic liver failure, development of cirrhosis or liver transplantation (Fausto, 2004, 2006; Taub, 2004; Michalopoulos and Khan 2005; Pahlavan et al., 2006; Michalopoulos, 2007). It is also emphasized that a specific role in these events might have the components of the innate immune system, since through cytokine cascade they might regulate the priming phase of liver regeneration, while through some cellular mediators might directly recognize the aberrant, infected or superfluous cells (Abo et al., 2000; Fausto, 2006; Michalopoulos, 2007). The triggering events are however, still investigating, as well as the systemic changes in immune homeostasis.

Trying to underline the possible role of evolutionary highly conserved HSPs in these events, we show herein that pHx induces high upregulation of endoplasmic reticulum resident HSP gp96 in regenerating liver and in the thymus (Figs. 1, 2), implying that it might be involved in the control of protein folding in regenerating liver tissue, as well as in the induction of autoreactive clones of cells, such as NKT and TReg cells that accumulated in regenerating liver (Fig. 3), and through Fas/FasL and perforin/granzyme pathways regulate the intensity of liver growth and participate in removal or suppression of activated lymphocytes after antigen-driven expansion of lymphocytes (Figs. 7, 8).

Hypothesis is supported by widespread evidence pointing to cytoprotective (Ma and Hendershot, 2004; Yang and Li, 2005; Wu and Kaufman, 2006) and immunoregulatory effects of gp96 (Basu et al., 2000; Jolly and Morimoto, 2000; Srivastava and Amato, 2001; Multhoff, 2006; Warger et al., 2006). Thus, during

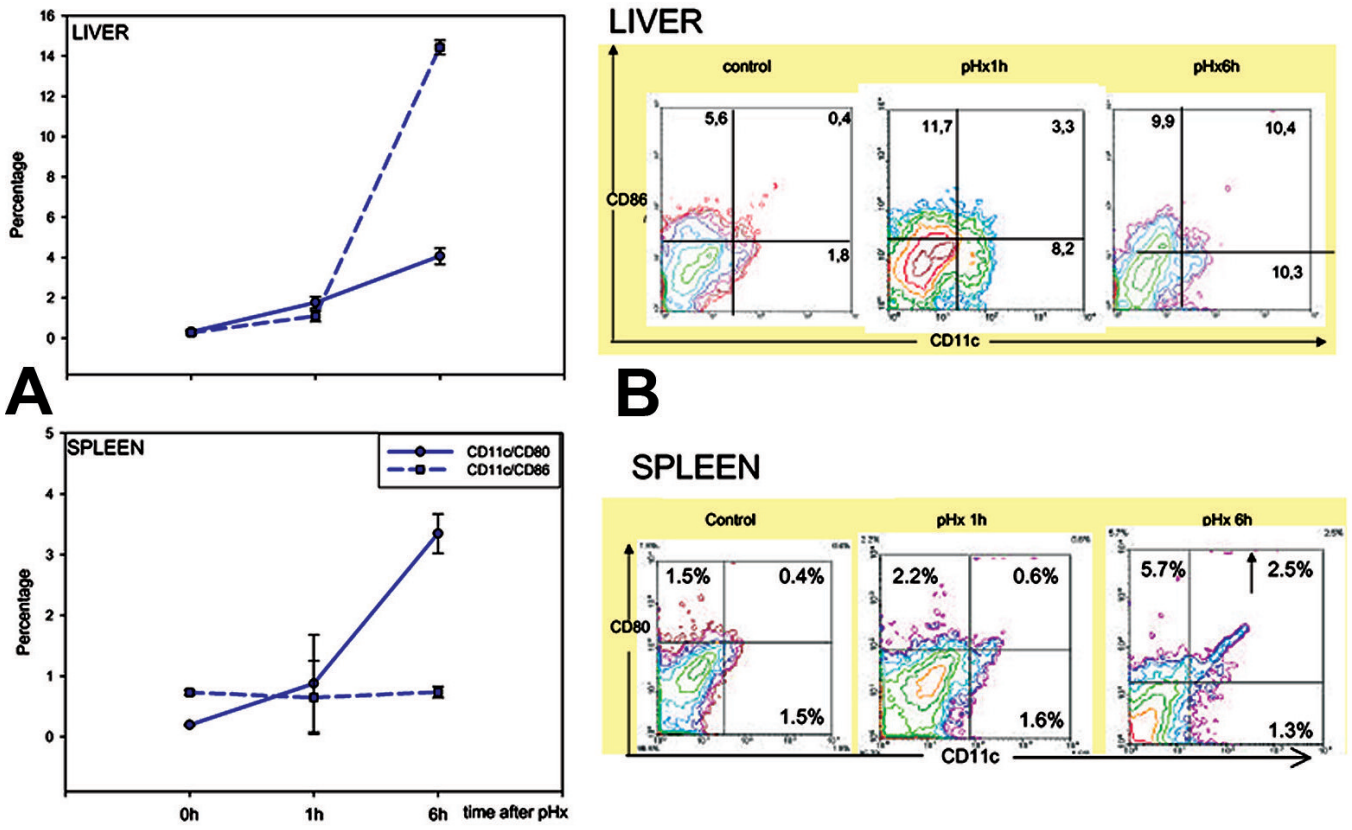

Fig. 5. Partial hepatectomy upregulates the expression of co-stimulatory molecules on hepatic and splenic dendritic cells. **A.** The proportion of CD11c+/CD80+ and CD11c+/CD86+ cells in the liver and spleen in the early phase of liver regeneration, presented as a means ± standard errors from 6 mice. **B.** Representative contour plots show that after pHx hepatic DCs upregulate primarily CD86, while splenic DCs upregulate mostly CD80 molecules. The data are representative of 6 independent experiments with similar results.

normal physiological conditions and following stress stimuli, such as nutrient deprivation, alterations in the oxidation–reduction balance, changes in calcium concentration, failure of post-translational modifications, or simply during the increases in secretory protein synthesis, intracellularly located gp96 functions as a molecular chaperone that guides the normal folding, intracellular disposition and proteolytic turnover of many of the key regulators of cell growth, differentiation and survival factors, promoting cell survival by repairing or by degrading damaged proteins (Ma and Hendershot, 2004; Yang and Li, 2005; Wu and Kaufman, 2006). Moreover, both-physiological processes, such as cell cycle and cell differentiation, and pathological states, like ischemia, fever, inflammation and infection, also transcriptionally regulate the genes encoding HSPs, showing that they may affect multiple checkpoints in major cellular pathways that regulate cell growth and apoptosis (Basu et al., 2000). Besides, the membrane-bound and exported gp96 has strong immunological effects, having an important role in antigen presenting, anti-cancer immune response and specific antitumor immunotherapy (Basu et al., 2000; Jolly and Morimoto, 2000; Warger et al., 2006). Namely, gp96 is a potent carrier of antigenic peptides derived from tumor, bacterial and viral antigens, which, following internalization of gp96-peptide complexes by antigen presenting cells (APCs), may enter the MHC class I and class II-enriched compartment, facilitating the in vivo cross-presentation of restricted peptides to CD8 and CD4 T cells, respectively, eliciting a peptide-specific response (Basu et al., 2000; Jolly and Morimoto, 2000; Srivastava and Amato, 2001; Warger et al., 2006). Additionally, HSPs per se may provide activatory signals for the innate immune system, since binding of peptide-free HSP70 to APCs via Toll-like receptors (TLRs) might also initiate the secretion of pro-inflammatory cytokines and result in a broad non-specific immunostimulation (Warger et al., 2006), showing that in certain "danger" circumstances, HSPs released in the extracellular environment may induce an immune response and deliver a partial maturation signal to dendritic cells (Basu et al., 2000; Binder et al., 2000; Radons and Multhoff, 2005; Gallucci and Matzinger, 2001; Matzinger, 2007).

Confirming this evidence and supporting the recently published data about the role of HSPs in the liver regeneration (Shi and Van Kaer, 2006), herein we show (to our knowledge for the first time) that pHx upregulates the expression of gp96 not only in the regenerating liver, but also in the thymus (Fig. 1), suggesting that during liver growth ER-resident chaperones may interact with intact or fragmented cytoplasmic autoantigens, leading the transient autoimmunity, similarly as it was proposed for the induction of several autoimmune diseases (Routsias and

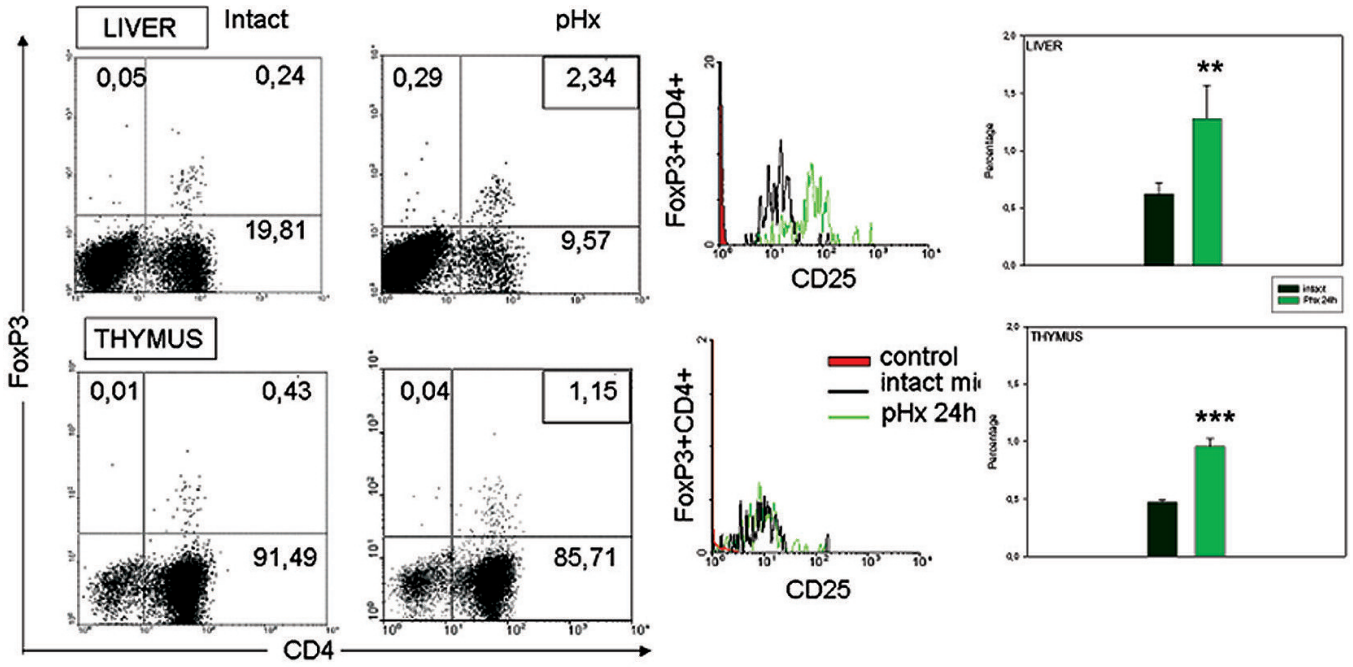

Fig. 6. Partial hepatectomy (after 24h) increases the percentage of regulatory T cells in the regenerating liver and in the thymus. Representative dot plots show the intracellular expression of Foxp3 (FJK-16s) and surface expression of CD4 on freshly isolated IHMNLC and on thymocytes. The histograms show the CD25 expression levels on $Foxp3^{+}CD4^{+}$ cells obtained from control and pHx mice. The red (filled histograms) represent isotype control antibody. The results are representative of three independent experiments. Proportions of $CD4^{+}Foxp3^{+}$ cells found 24h after 1/3 pHx in the liver and in the thymus, are shown also as a mean ± standard error (n = 5). **p<0.01; ***p<0.001.

Tzioufas, 2006). Moreover, there is also the possibility that extracellular gp96, released after secondary necrosis of cells, contributed to the creation of a "proinflammatory milieu" in the liver and in the thymus, acting as a local danger signal which induces functional modification of hepatic and thymic APC, leading to their maturation, expression of co-stimulatory molecules and secretion of cytokines (Basu et al., 2000; Binder et al., 2000). In the thymus this probably affected the APC synapse area, which is comprised of a central cluster of antigenic peptide bound to MHC-encoded molecules (pMHC), together with the ligands capable of interacting with the costimulatory and coinhibitory molecules expressed by the T cell (i.e. CD86 & CD80), which act as critical signal for negative selection (Buhlmann et al., 2003), CD4/CD8 T cell homeostasis (Yu et al., 2000), induction of autoimmunity (Anderson et al., 1999), and the maintenance of TReg cells (Chatenoud et al., 2001). Moreover, since according to the Quantal Theory of Immunity, the maturation of T cells within the thymus depends on the strength of the TCR signal, which is determined by the antigen concentration, as well as by the density of the TCR expressed by an individual cell, and by the duration of the antigen/TCR interaction (Smith, 2006), there is the possibility that an increase in pMHC concentration and triggering of a greater number of TCRs also promoted the processes of positive selection and maturation of TRegs (Sakaguchi 2004; Cava et al., 2006), or the developmental pathways of other 'alternative' thymocyte lineages, such as NKT cells (Ladi et al., 2006). However, although our data show high upregulation of gp96 protein and mRNA in the thymus after pHx (Figs. 1 and 2), the increased proportion of $Foxp3^{+}CD4^{+}CD25^{+}$ T cells in the thymus (Fig. 6), and the presence of massive apoptosis in the thymus during liver regeneration (Fig. 10) this speculation remains to be proven.

Our data, however, clearly show that in the regenerating liver NKT (Figs. 3, 4) and TReg cells accumulate (Fig. 6), as well as that pHx induces

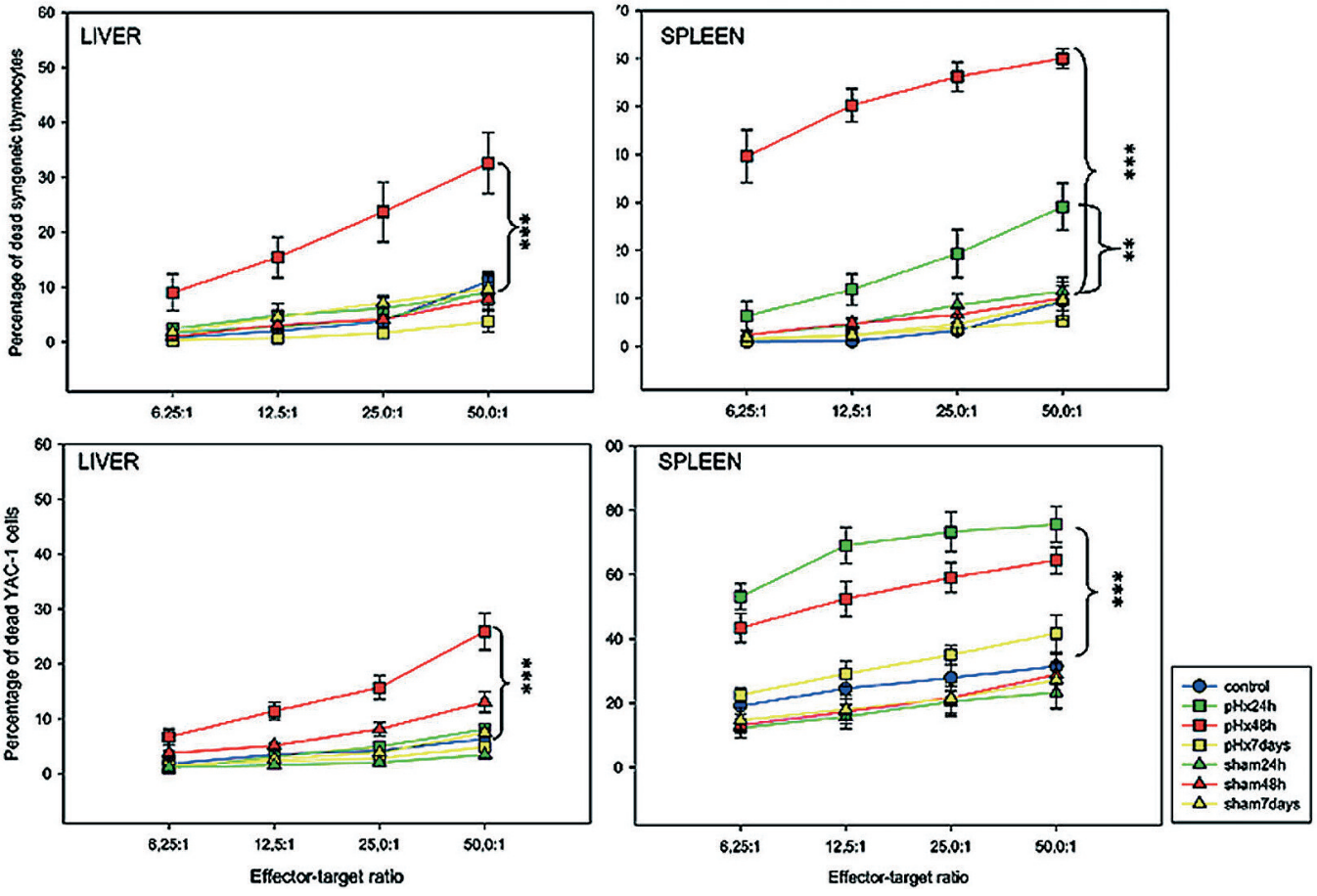

Fig. 7. Time-dependent variation of NKT and NK cytotoxicity of IHMNLC and SMNLC after 1/3 pHx and its comparison with the effects of sham Hx. Effector lymphocytes were obtained from liver and spleen of mice subjected to 1/3 pHx or to sham Hx (24h, 48h and 7 days after the operation), and were used against the syngeneic thymocytes or YAC-1 cells at the indicated target-to-effector cell ratios of 2 h incubation. Results are presented as mean ± standard error (n = 6). *p<0.05; **p<0.01; ***p<0.001.

upregulation of CD86 on hepatic and CD80 on splenic $CD11c^+$ dendritic (Fig. 5), suggesting that gp96 contributed to their generation and/or activation. Furthermore, the data confirm that hepatic NKT cells are autoreactive, cytotoxic cells (Emoto et al., 1997; Abo et al., 2000; Minagawa et al., 2000; Mrakovcic-Sutic et al., 2002), since IHMNLC were able to lyse the syngeneic thymocytes (Figs. 7, 8), i.e. NKT targets (Abo et al., 2000; Oya et al., 2000), suggesting that after pHx these, predominantly CD1d-restricted cells (Emoto and Kaufmann, 2003), might be activated by complexes of gp96-self antigens and/or by other endogenous danger signals - released by regenerating liver tissues undergoing stress, damage or abnormal cell death. Consistent with the previous findings (Emoto et al.,1999) our data (Fig. 8) also imply that cytolytic activity of hepatic NKT cells depends of Fas/FasL pathway, suggesting that these FasL expressing lymphocytes might be responsible for elimination of some Fas-positive cells of liver that undergo apoptosis during the liver regeneration, particularly at the end of DNA synthesis (Sakamoto et al., 1999). Moreover, owing to the interaction of NKT cells or HSPs with other cells and the creation of a specific cytokine milieu, there is also a possibility that subsequent upregulation of FasL on hepatocytes leads to the deletion of activated Fas-positive lymphocytes, since it was shown that IFN-gamma and, to a lesser extent, TNF-alpha can enhance hepatocyte CD95L-mediated cytotoxicity (Guy et al., 2006). The consequences of NKT activation on liver

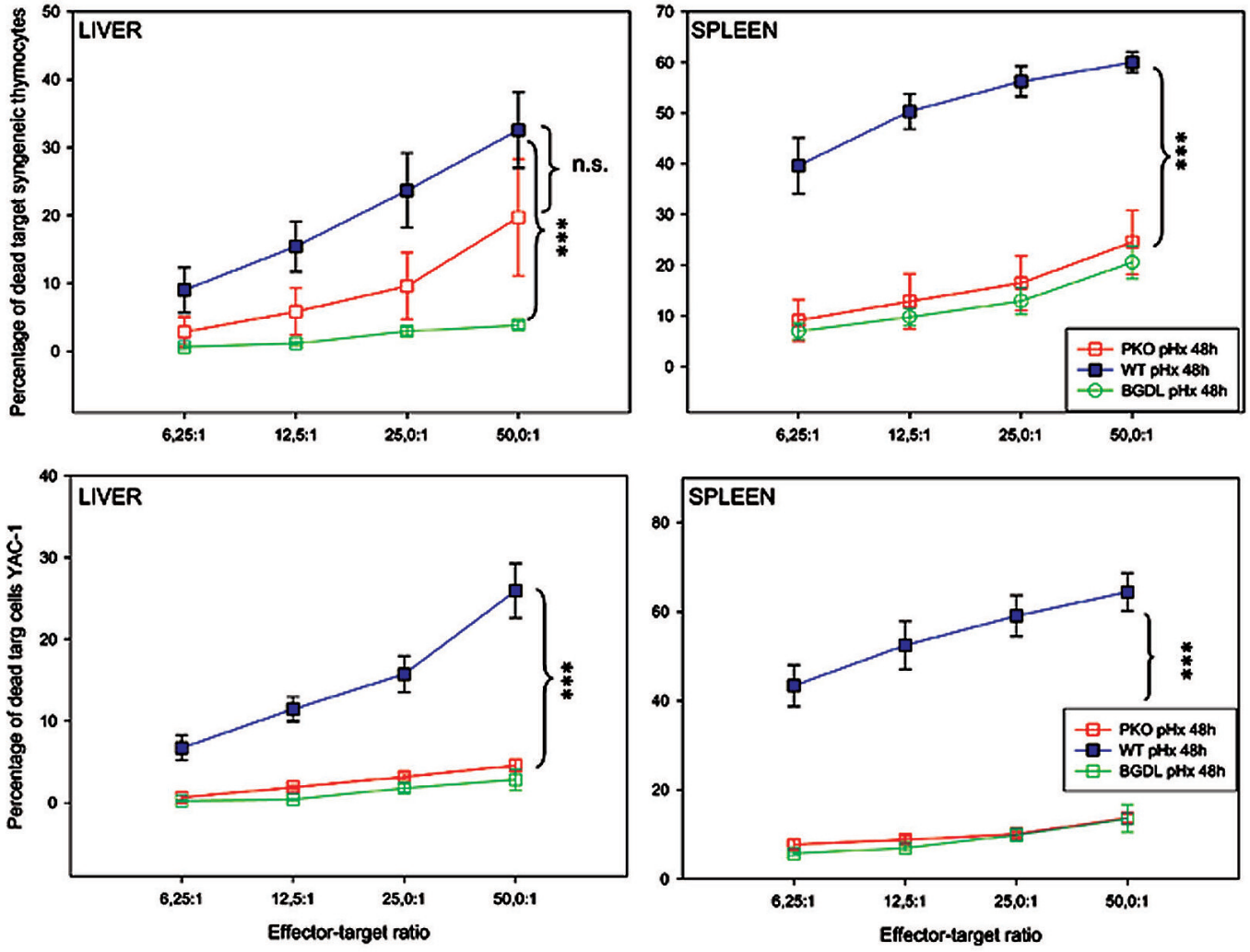

Fig. 8. NKT and NK cytotoxicity in the liver and spleen after pHx and sham Hx in wild, PKO and FasL deficient (gld/gld) C57/Bl6 mice. Effector lymphocytes were obtained from liver and spleens of mice subjected to 1/3 pHx (48h after the operation), and were used against the syngeneic thymocytes or YAC-1 cells at the indicated target-to-effector cell ratios of 2 h incubation. Results are presented as mean ± standard error (n = 6). *p<0.05; **p<0.01; ***p<0.001.

regeneration remain, therefore, often controversial, resulting both in acceleration (Nakashima et al., 2006) and inhibition of liver growth (Matsushita et al., 1999; Ito et al., 2003; Huang et al., 2006), similarly as in the other experimental models, where it was reported that NKT cells might have both protective, as well as aggressive properties, (Godfrey et al., 2000; Hammond and Kronenberg, 2003; Sakaguchi, 2004; Kronenberg and Rudensky, 2005; Seino and Taniguchi, 2005; Van Kaer and Joyce, 2005; Cava et al., 2006; Molano and Porcelli, 2006), emphasizing the multiple pathways of activation of NKT cells and their double–edged sword nature (Mocchegiani et al., 1997; Abo et al., 2000; Godfrey et al., 2000; Hammond and Kronenberg, 2003; Wilson and Delovitch, 2003; Sakaguchi, 2004; Yokoyama, 2004; Zhou et al., 2004; Kronenberg and Rudensky, 2005; Milin et al., 2005; Seino and Taniguchi, 2005; Van Kaer and Joyce, 2005; Cava et al., 2006; Godfrey et al., 2006; Molano and Porcelli, 2006).

In any case, if the hepatic accumulation of activated NKT cells represents an early antigen-specific initiation process', activated by gp96-self peptide complexes via TCR and IL-7R, it may be speculated that NKT cells initially contributed to transitory suppression of NK and LAK-cells mediated cytotoxicities in the early phase of liver regeneration (Vujanovic et al., 1999; Routsias and

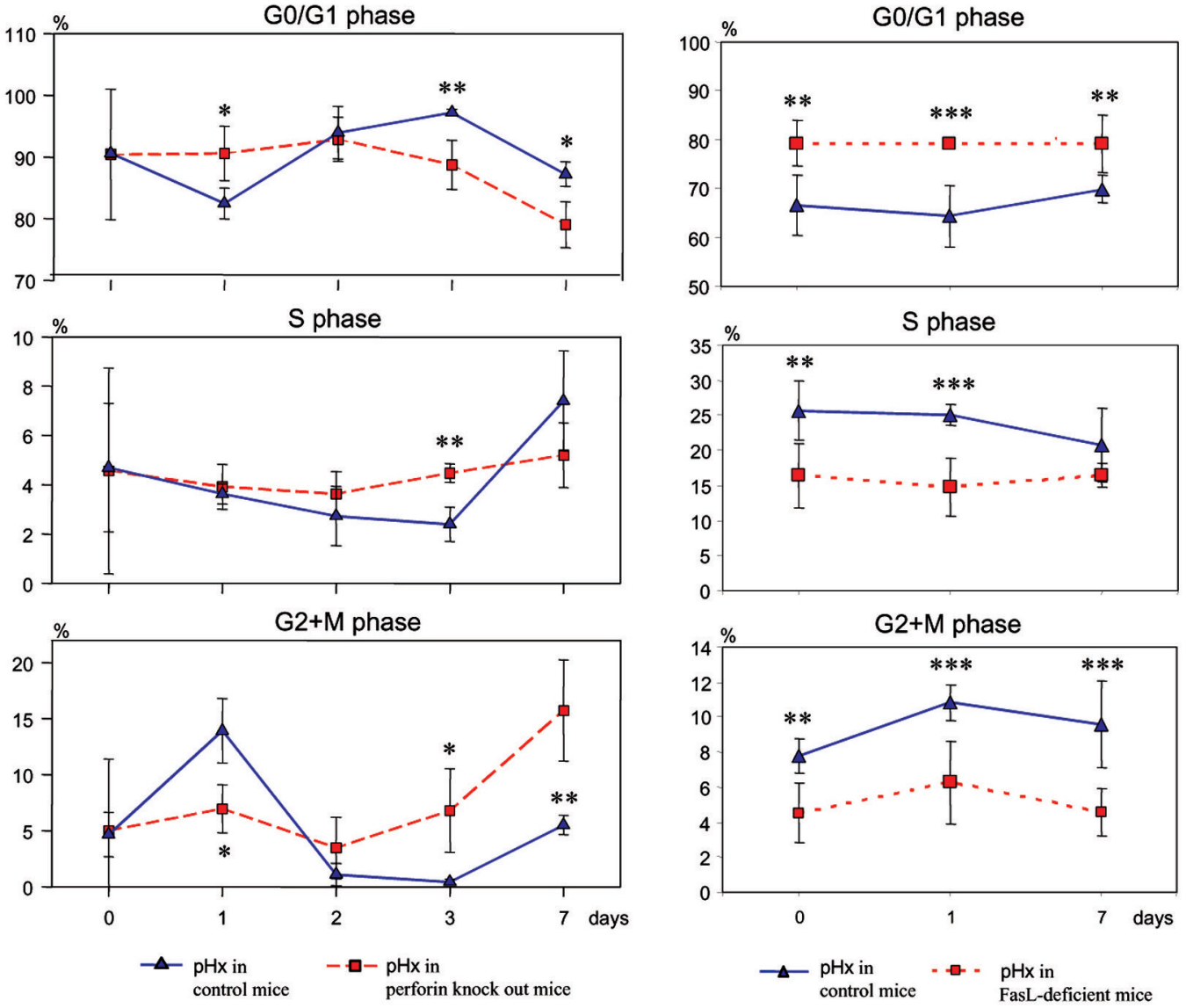

Fig. 9. Cell cycle analysis of regenerating liver cells in wild C57/Bl6, perforin knock out (PKO) and FasL deficient mice. Hepatocytes were isolated 24h, 48h and 7 days after 1/3 pHx and the cell cycle analysis was performed by flow cytometer (FACSCalibur) using Cel Quest Software. Data are the mean values ± SD from 4-6 animals in each group. *p<0.05; **p<0.01, ***p<0.001.

Tzioufas, 2006), since stimulation of NKT cells via TCR and IL-7R preferentially induces IL-4 and IL-13 (Smyth et al., 2002). In the second step, however, the increased CD40L expression on NKT cells generally leads to CD40-dependent APC activation and IL-12 production, which triggers a second burst of IFN-γ production by NKT cells and bystander cells, leading to the recruitment of other effector cells (Smyth et al., 2002; Askenase et al., 2004), explaining probably our findings that after pHx in the regenerating liver and in the spleen the crucial killers became NK cells and cytotoxic T cells, which were able to kill the targets by perforin and FasL-dependent pathway (Fig. 8). Moreover, our findings that mice without the perforin had a greater proportion of cells in G2+M phase in the later periods of liver regeneration than cogenic wild pHx mice (Fig. 9), seems to support this hypothesis.

It is, however, likely, that the mechanisms, involve not only the perforin and FasL-dependent cytolytic and apoptotic machinery, but also some non-death functions of Fas and FasL interaction, since in FasL-deficient mice we found that hepatocytes after pHx were permanently arrested in G0/G1 phase (Fig. 9), implying that engagement of the CD95(APO-1/Fas) was necessary for progression of hepatocytes through G1 restriction point. The nature of this influence is still investigating, but the data are in high agreement with the report of Desbarats and Newell (2000), showing that Fas engagement during regeneration accelerates the regenerative response, as well as that 2/3 pHx prevents the systemic lethal effect and apoptotic liver cell death induced by Fas-specific antibodies *in vivo* (Desbarats and Newell, 2000). The later protection was related with increased intracellular expression of FLICE (Fas-associated death-domain-like interleukin 1 -converting enzyme)-inhibitory protein (FLIP) and anti-apoptotic Bcl-2 family members (Desbarats and Newell, 2000), but Fas triggered proliferation might involve also the activation of Jun kinase pathways and NF-1ß, as well as upregulation of the protooncogene, c-fos and protein tyrosine phosphorylation, inhibition of TCR-mediated Ca^{2+} mobilization and TCR-mediated inositol 1,4,5-trisphosphate production (Sakata et al., 1998; Sharma et al., 2000; Wajant et al., 2003; Park et al., 2005). Furthermore, transgenic mice expressing liver-specific dominant negative mutant of the adaptor protein MORT1/FADD had a delayed proliferative response after partial hepatectomy (Schuchmann et al., 2005).

It should be also emphasized that the engagement of CD95 signaling causes the induction of the protein p21cip-1/WAF-1, which is a cell cycle regulator involved in the G1/S and G2/M checkpoints (Arias et al., 2007). In T cells, this activation is essential to induce full susceptibility to CD95-induced apoptosis (Hingorani et al., 2000), but in regenerating hepatocytes it plays a role in governing progression through the cells cycle, acting during the prereplicative (G1) phase (at 12-24h) of liver regeneration as an inhibitor of CDK2 activity, owing to its binding to cyclin D1/CDK4 complexes before and after peak DNA synthesis (Albrecht et al., 1998). Moreover, procaspase 3-p21 complex formation on mitochondria might be essential for the protection of cells from Fas-mediated cell death (Suzuki et al.,1999).

However, although it was shown that p53-dependent

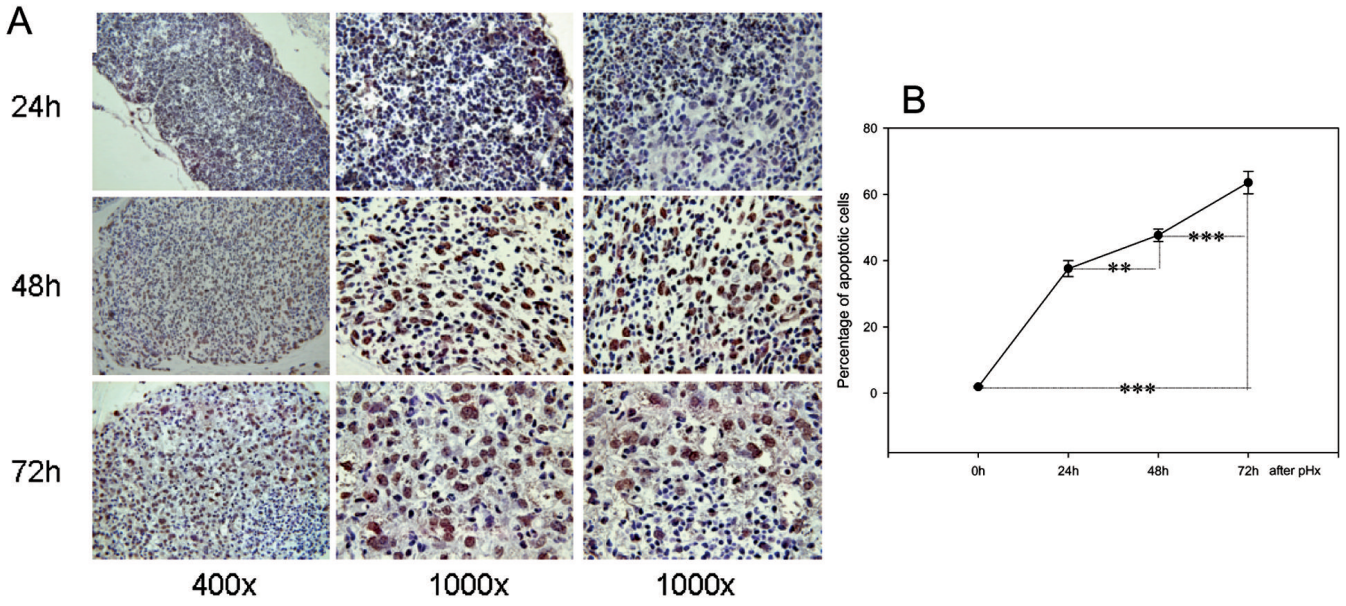

Fig. 10. A. Apoptotic cells in the thymus, detected by TUNEL assay 24h, 48h and 72h after 1/3 pHx. **B.** Relative number of apoptotic cells, found in the thymus after pHx. The data are expressed as percentage of cells (mean ± SEM), calculated by dividing the fragmented DNA-expressing cells by the number of total cells counted in 5 randomly chosen fields under a light microscope (magnification, x 400) in 3 mice per experimental group. ** p<0.01; *** p<0.001.

activation of p21 induced by TGF-beta, IFN alpha, IFN gamma or IL-6 may protect cells from apoptosis, in contrast to activation of p53 without p21 (Gartel and Tyner, 2002), as well as that the engagement of death receptors through CD95 increases also the stability of HSP70 provoking its accumulation in the cells (Concannon et al., 2005), these mechanisms need further investigations. Similarly, the relationship of gp96 to intracellular pathways after pHx is mostly unsolved, although it was proposed that Hsp90 has positive effects on regulation of p53 DNA binding to the promoter sequence owing to its ability to inhibit p53 aggregation or induce the partial unfolding of wt-p53 (Walerych et al., 2004).

Finally, we would to like to emphasize that both endogenous HSP (Wieten et al., 2007), and activated NKT cells, through IL-2-dependent mechanisms (Cava et al., 2006), may contribute to activation of HSP-specific anti-inflammatory TReg, which can dampen inflammation by production of suppressive cytokines such as IL-10, or suppress the proliferation, cytokine release and cytotoxic activity of NKT cells by cell-contact-dependent mechanisms and specifically kill the antigen-presenting cells, using potent granzyme B-dependent, partially perforin-independent mechanisms (Sakaguchi, 2004). Therefore, our data, showing the presence of TReg in the regenerating liver and thymus (Fig. 6), seems to support this hypothesis, suggesting that these autoreactive cells might eventually restrain the immune response induced by pHx, contributing to reestablishment of tolerance. Moreover, it could be speculated that greater presence of CD11c+CD86+ cells in the regenerating liver (Fig. 5) stimulated their accumulation, since it was reported that APC that express CD86, as a costimulatory molecule, preferentially stimulate this type of cells (Yadav et al., 2004).

Taken together our data emphasize that pHx upregulates the expression of endoplasmic reticulum resident HSP gp96 in regenerating liver and thymus, suggesting that gp96-chaperoned self peptides may participate in the recruitment and activation of autoreactive clones of cells (NK1.1+ CD3intermediate CD69+ and Foxp3+CD4+CD25+regulatory T cells), which probably control the reparatory processes, using FasL-dependent and/or perforin- and FasL-dependent mechanisms.

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