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Thymic alterations induced by partial hepatectomy: upregulation of glycoprotein 96, CD91 and TLR2 and generation of regulatory T cells

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Summary. Glycoprotein 96 (gp96) is an endoplasmic reticulum (ER)-resident heat shock protein. It controls the folding of nascent membrane-spanning and secretory proteins, participates in stress-induced unfolded protein response (UPR) and in pathways leading to proteolysis of damaged proteins through ER-associated degradation pathways and chaperone-mediated autophagy. In addition, gp96 controls the steroid biosynthesis and Ca^{2+} homeostasis and participates in insulin-IGF/signaling pathways. Besides, owing to its peptide chaperone capacity and ability to interact with antigen-presenting cells, gp96 has been implicated in priming of innate and adaptive immunity.

In an attempt to visualize the intensity of ER-stress in thymus and possible participation of gp96 in generation of auto-reactive T cell clones that were detected in regenerating liver, in this study we investigated the dynamics of gp96 expression in partially hepatectomized (pHx) and sham Hx mice. Simultaneously, we detected the thymic expression of receptors responsible for endocytosis of gp96-chaperoned peptides (CD91) and intracellular activation of ER-stress pathways (TLR2), as well as the expression of TGF-β and the distribution of CD4+CD25+FoxP3+ cells. The data have shown that both pHx and sham Hx induced an accelerated apoptosis and hypoplasia in thymus. Partial Hx induced, however, a higher expression of gp96, the translocation of the CD91, TLR2 and TGF-β immunostaining from medulla to cortex and an appearance of Treg cells.

The data show that pHx triggers in thymus the ER-stress and UPR response and suggest that gp96 participates in the generation of natural Treg cells, which might be involved in the control of liver regeneration in the periphery.

Key words: Liver regeneration, Thymus, Glycoprotein 96, CD91, TLR2, TGF-beta, Regulatory T cells

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Introduction

Liver regeneration that follows after partial hepatectomy (pHx) or after different types of hepatic injuries is a well-defined process, which involves the concerted action of extra- and intracellular factors that initiate the growth and replication of differentiated hepatocytes and/or liver progenitor cells and stop these processes at the time when the functional liver mass restores. As described in numerous reviews (Diehl, 2000; Fausto, 2000; Taub, 2004; Michalopoulos, 2007) the process involves: (1) an initial priming phase, starting with expression of a large number of immediate early genes, (2) a proliferative phase, related with growth-factors-induced progression of cells through the G1 restriction point; and (3) a growth termination phase, induced by inhibitory factors, such as transforming growth factor (TGF)-β and interleukin (IL)-1β. The required growth-modulating molecules, which lead to the activation of downstream intracellular signaling pathways, are produced or harbored by hepatocytes and components of the extracellular matrix and epithelial or non-epithelial liver cells, as well as by the resident and

recruited inflammatory cells. Besides, the regenerative process is under a high influence of endogenous- and/or exogenous-derived danger signals that may be released by tissues undergoing stress, damage or abnormal death or elaborated by pathogens (Akira et al., 2001; Gallucci and Matzinger, 2001; Beg, 2002). Depending on the nature of these signals, the innate immune system needs then to activate the several strategies of self/nonself discrimination to avoid the autoimmune attack (Janeway and Medzhitov, 2002; Moudgil et al., 2013; Yates, 2014). The outcome depends on cross-talk between the different hepatic cell populations, on antigen concentration and affinity, as well as on the balance of co-stimulatory and co-inhibitory signals in T cells that may initiate the immune attack or induce the programmed cell death of $CD8^+$ T cells in the liver (Knolle and Gerken 2000; Schurich et al., 2010). Besides, as proposed by Abo and co-workers (Abo et al., 2000), these conditions are often followed by generation of self-reactive, forbidden clones of NKT cells, which may have both beneficial and harmful properties (Kronenberg and Gapin, 2002; Seino and Taniguchi, 2005; Bendelac et al., 2007).

In accordance with this evidence, we previously speculated that in the control of liver growth the important immune functions might have the endoplasmic reticulum (ER) resident, heat shock protein (HSP) - glycoprotein 96 (gp96/glucose-regulated protein 94; HSP 90b1), since in regenerating liver its expression correlated with the accumulation of $CD3^{intermediate}$ $NK1.1^+CD69^+$ cells and with the increased FasL- and perforin-dependent cytotoxicity against the NKT- and NK- sensitive targets (Mrakovcic-Sutic et al., 2008). Moreover, since in both the regenerating liver and the thymus we found increased numbers of $CD4^+CD25^+$ $Foxp3^+$cells, we speculated that during the disturbance of morphostasis gp96 might be involved in processes leading to generation of auto-reactive clones of cells (Mrakovcic-Sutic et al., 2008; Radosevic-Stasic et al., 2012). The hypothesis was based on current knowledge, pointing to the participation of gp96 in the processes of cross-presentation the antigenic self-peptides into MHC class I or MHC class II pathways and in maturation of antigen presenting cells (APC) (Singh-Jasuja et al., 1999; Srivastava and Amato, 2001; Srivastava, 2002), as well as on data emphasizing that different subsets of NKT and naturally occurring $CD25^+CD4^+$ Treg cells might be involved in the maintenance of self tolerance (Sakaguchi and Sakaguchi, 2005; Seino and Taniguchi, 2005; Aschenbrenner et al., 2007; Sakaguchi et al., 2008; Heiber and Geiger, 2012).

In an attempt to expand our previous findings, in this study we investigated the thymic aspects of pHx and sham operation, estimating the expression of gp96 and its relationship to cells bearing CD91 and Toll like receptors (TLR)-2. In addition, TGF-β expression and distribution of Treg cells were tested. The data showed that pHx led to apoptosis in the thymus and upregulation of gp96 and its receptors in thymic cortex, as well as to the appearance of numerous $CD4^+FoxP3^+$ and $CD25^+FoxP3^+$ T-cells, suggesting that ER-stress, induced by pHx might have antigen-specific and/or unspecific influences on selection pathways in the thymus.

Materials and methods

Animals

Mice of strain C57/BL6 aged 2-3 mo were selected for the experiment. They were housed in groups of six to eight 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 approval of the Ethical Committee of the University of Rijeka.

Partial hepatectomy

The median liver lobe was removed (1/3 pHx) under ether anesthesia, as previously described (Mrakovcic-Sutic et al., 2008). To avoid possible diurnal variability, all operations were done between 8:00 AM and 9:00 AM. The animals were sacrificed by bleeding at 24 and 48 h 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 thymus were collected from each mouse for routine histological and immunohistochemical examination.

Immunofluorescent staining and flow cytometry

The surface phenotypes of thymocytes 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 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.

Cell cycle analysis

Cell cycle analysis of freshly prepared hepatic cell suspension was performed on flow cytometer (FACSCalibur) (Becton Dickinson, Immunocytometry Systems, Mountain View, CA), using CellQuest

Software (Macintosh, Quadra 650), as we previously described (Mrakovcic-Sutic et al., 2008). Percentage of the cells in G0/G1, S and G2+M phase was calculated.

Immunohistochemistry

Immunohistochemical studies were performed on paraffin embedded tissues of the thymus, using DAKO EnVision+System, Peroxidase (DAB) kit according to the manufacturer's instructions (DAKO Corporation, USA), as previously described (Mrakovcic-Sutic et al., 2008). Slides were incubated with peroxidase block to eliminate endogenous peroxidase activity. After washing, 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-rat/mouse immunoglobulins containing carrier protein linked to Fc fragments to prevent nonspecific binding. The immunoreactions 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 same protocol was used for the visualization of CD91, TGF-β and TLR-2 immunoreactivities after the application of anti-CD91 (Pharmingen, USA), anti-TGF-β and anti-TLR-2 monoclonal antibodies, in the dilutions of 1:50, 1:100 or 1: 1000, respectively (Abcam Inc; Cambridge, MA). The specificity of the reaction was confirmed by substitution of specific antibodies with mouse irrelevant IgG1 kappa immunoglobulins, irrelevant IgG2-immunoglobulin (clone DAK-G05; Dako, USA), used under the same conditions and dilutions as primary antibodies. The slides were examined on an Olympus BX51 photomicroscope (Olympus, Tokyo, Japan).

Immunofluorescent labeling

For dual fluorescent labeling of cells the tissue sections were submitted to heat induced antigen retrieval. The nonspecific binding was blocked by one-hour incubation with 1% BSA in PBS at room temperature. Tissues were then incubated with anti-gp96 (diluted 1:30), with anti-TLR2, with anti-CD91 antibodies (diluted 1:50) or with anti-caspase-3, active form (BD Pharmingen) (diluted 1:200), at +4°C overnight in a humid chamber. Thereafter, sections were rinsed and incubated with Alexa Fluor555 goat anti-rat and with Alexa Fluor488 goat anti-rabbit secondary antibodies (Invitrogen) diluted 1:500, for 1 h in a dark and humid environment. T regulatory cells labeling was performed using PE-conjugated anti-FOXP3 antibody, APC-conjugated anti-CD25 antibody and FITC-conjugated anti-CD4 antibodies (eBioscience). Finally, slides were washed, mounted with Mowiol (Sigma-Aldrich) and analyzed under fluorescent microscope.

In situ apoptosis detection

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end-labeling (TUNEL) assay was performed on 4 μm paraffin-embedded sections using *In Situ* Cell Death Detection Kit, POD (Roche Diagnostics) 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, slides were incubated with TUNEL reaction mixture containing recombinant terminal deoxynucleotidyl transferase and fluorescein labeled deoxynucleotides that binds to fragmented DNA molecules. Signal conversion was performed by incubating with Converter-POD solution containing Fab fragment of anti-fluorescein sheep antibody, conjugated with horse-radish peroxidase. Reaction was visualized by adding DAB Substrate solution. Tissues were counterstained with hematoxylin, mounted and analyzed under light microscope.

DNA ladder assay

DNA from thymic tissue was isolated by standard phenol-chloroform method. Formation of DNA ladder was assessed by DNA electrophoresis in 2% agarose gel containing 1.0 μg/ml ethidium bromide and Tris/acetate acid/EDTA buffer. Electrophoresis was performed for 45 min at 90 Volt. Upon electrophoresis DNA fragments were visualized by UV illumination and captured with digital camera.

Statistical analysis

Statistical significance was calculated by Mann Whitney U test. The differences were considered significant for $p<0.05$.

Results

Characteristics of liver regeneration after 1/3 pHx in C57/BL6 mice

In an attempt to compare the results with our previously published data (Mrakovcic-Sutic et al., 2008) C57/BL6 mice were subjected to 1/3 pHx. According to the cell cycle analysis it triggered fast liver regeneration, since 24h after the operation the fraction of liver cells in G2+M phase of cell cycle increased (Fig. 1A). Besides, calculation of regenerating index (from removed and remaining liver weights) showed that restoration of liver mass was the greatest during the first day after pHx (Fig. 1B). Simultaneously, as we reported previously, in regenerating liver augmented both the percentage of natural killer T cells (NK1.1+/CD3+) and Treg cells

(CD4+CD25+FoxP3+) and the cytotoxicity of hepatic mononuclear lymphatic cells (MNLC) against NKT cells-sensitive and NK cells-sensitive targets, pointing to the presence of auto-reactive T cell clones that might be involved in the control of liver growth (Mrakovcic-Sutic et al., 2008). The changes were statistically different from those found in sham Hx mice, but in a later time interval, the fraction of NKT cells increased also in sham Hx mice (Fig. 1C), similarly to the cytotoxicity of hepatic MNLC (not shown), pointing to the effects of laparotomy and/or surgical stress on these events.

Partial hepatectomy reduces the thymic cellularity and increases the number of apoptotic cells

As was expected both pHx and sham Hx reduced the thymic weights and cellularity (Fig. 2A,B). Partial Hx induced, however, on the second postoperative day, statistically greater elimination of CD4+CD8+ cells (Fig. 2E,F) and a higher apoptosis in thymus than that found

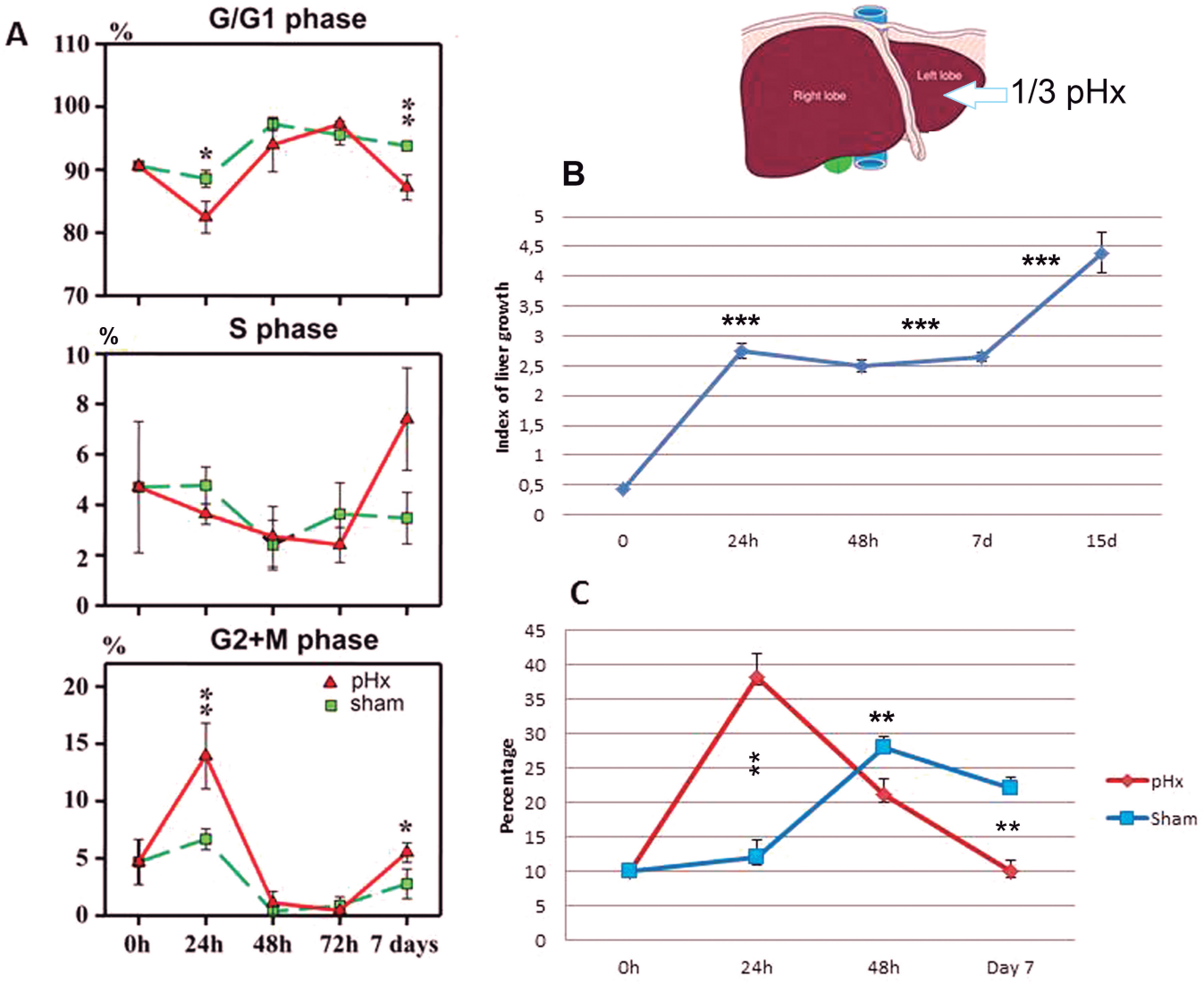

Fig. 1. Characteristics of liver regeneration after 1/3 partial hepatectomy (pHx) in C57/Bl6 mice. **A.** Cell cycle analysis of liver cells isolated 24h, 48h and 7 days after 1/3 pHx or after sham Hx. Analyses were performed by flow cytometer (FACSCalibur) using Cel Quest Software. **B.** Dynamics of liver regeneration, presented as regeneration index, calculated as a ratio of remaining liver weight to removed liver weight. **C.** Dynamics of hepatic NKT cells activated by pHx versus NKT cells activated by sham Hx (reproduced from (Mrakovcic-Sutic et al., 2008) by permission). Data are means ± standard errors from 6 mice in each group. *p<0.05; **p<0.01; ***p<0.001.

in sham Hx (Fig. 2C) and intact mice (Fig. 2D).

Partial hepatectomy induces overexpression of gp96 in the thymus

To visualize the intensity of ER-stress response triggered by pHx in thymus and potential influence of gp96 on the generation of auto-reactive T cell clones we estimated the dynamics of gp96 expression during liver regeneration (Fig. 3). The data clearly showed that pHx is followed by prominent upregulation of gp96 protein and by redistribution of gp96 immunoreactivity in the thymus. Thus, while in intact mice gp96 was expressed in the medulla (Fig. 3Aa-c), in pHx mice the gp96 immunostaining was found particularly in cortical thymic areas, on stromal and epithelial cells and on some lymphatic cells (Fig. 3Ad-f). Changes were visible 6h after pHx (Fig. 3Ae) and became highly expressed 48h after surgery (Fig. 3Af,g), i.e. at the time of high apoptosis and reduction of DP cells in thymus (Fig. 3D,F). Based on these results, the subsequent analyses were done in pHx mice 48h after the operation and these data were compared with findings in sham Hx mice, to separate the effects of pHx from the nonspecific effect of operative stress.

Partial hepatectomy enhances the CD91 and TLR2 expressions in the thymus

Since it was proposed that CD91 and TLR2 might be responsible for uptake of gp96-peptide complexes on antigen-presenting cells (APC) (Binder et al., 2000;

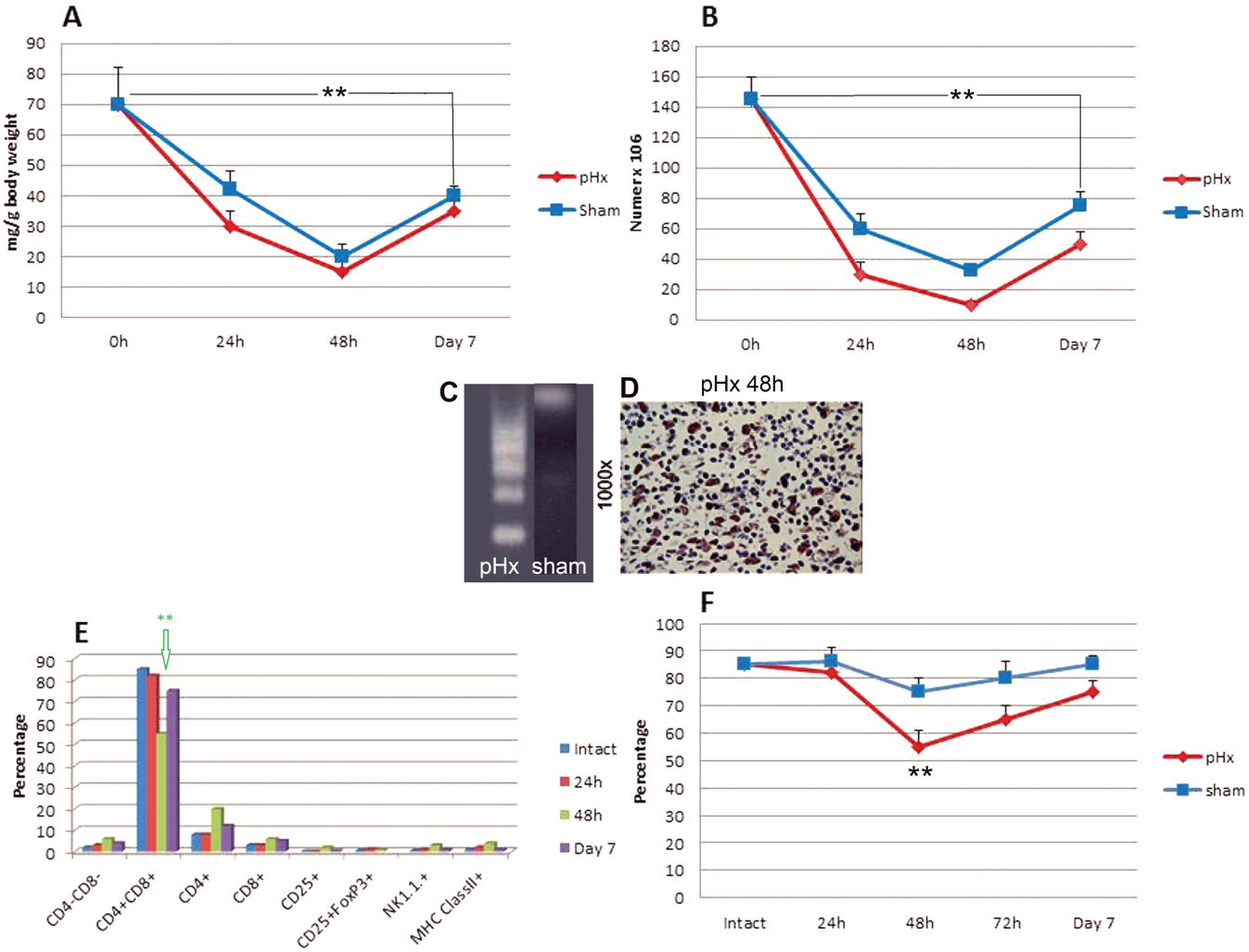

Fig. 2. Thymic changes induced by 1/3 partial hepatectomy. **A.** Changes in dry weights (expressed in mg/g body weight) after pHx and sham Hx. **B.** Changes in cellularity after pHx and sham Hx. **C.** Apoptosis detected 48h after pHx or sham Hx by DNA ladder assay. **D.** Apoptosis detected in intact and pHx mice by TUNNEL assay. **E.** Phenotypic changes detected by flow cytometric analysis on the 1st, 2nd and 7th day after 1/3 pHx. **F.** Dynamics of CD4+CD8+thymocytes during the liver regeneration. Data are means ± standard errors from 6 mice in each group. *p<0.05; **p<0.01, *** p<0.001. D, x 1000

Basu et al., 2001) and for the antigen-unspecific activation of APC (Vabulas et al., 2002) we determined also the expression of these molecules in the thymus. The results clearly showed that in intact (Fig. 4a,b) and sham operated mice (Fig. 4c,d) CD91 was expressed in the medulla, while in pHx mice it was present in cortical areas (Fig. 4Ae,f). Moreover, at the same location we found a high upregulation of TLR2 and numerous loci of TLR2 positive cells (Fig. 4Be,f). Changes were again greater than in intact (Fig. 4Ba,b) or in sham operated mice (Fig. 4Bc,d) and corresponded to the areas where the high expression of gp96 was found (Fig. 3).

Cross talk between the gp96-expressing cells and CD91 and TLR2 positive cells

The presence of gp96 and its link with receptors was visualized by the use of single and dual immunofluorescence staining. The data confirmed that in thymic cortex numerous gp96 positive cells (Fig. 5A,Ba,d), CD91 positive cells (Fig. 5Ab,e) and several foci of TLR2 positive cells were present (Fig. 5Bb,e) and showed that many gp96+ cells co-express CD91 (Fig. 5Ac,f) or TLR2 markers (Fig. 5Bc,f).

Partial hepatectomy enhances the TGF-beta expression in the thymus

On the second day after pHx, in thymic cortex a marked cytoplasmic and nuclear TGF-β expression on numerous stromal cells was also found (Fig. 6e,f). In contrast, in sham operated mice TGF-β was expressed only in medulla (Fig. 6c,d).

Partial hepatectomy enhances the number of Treg cells in the thymus

Since, by the direct immunofluorescence analysis on

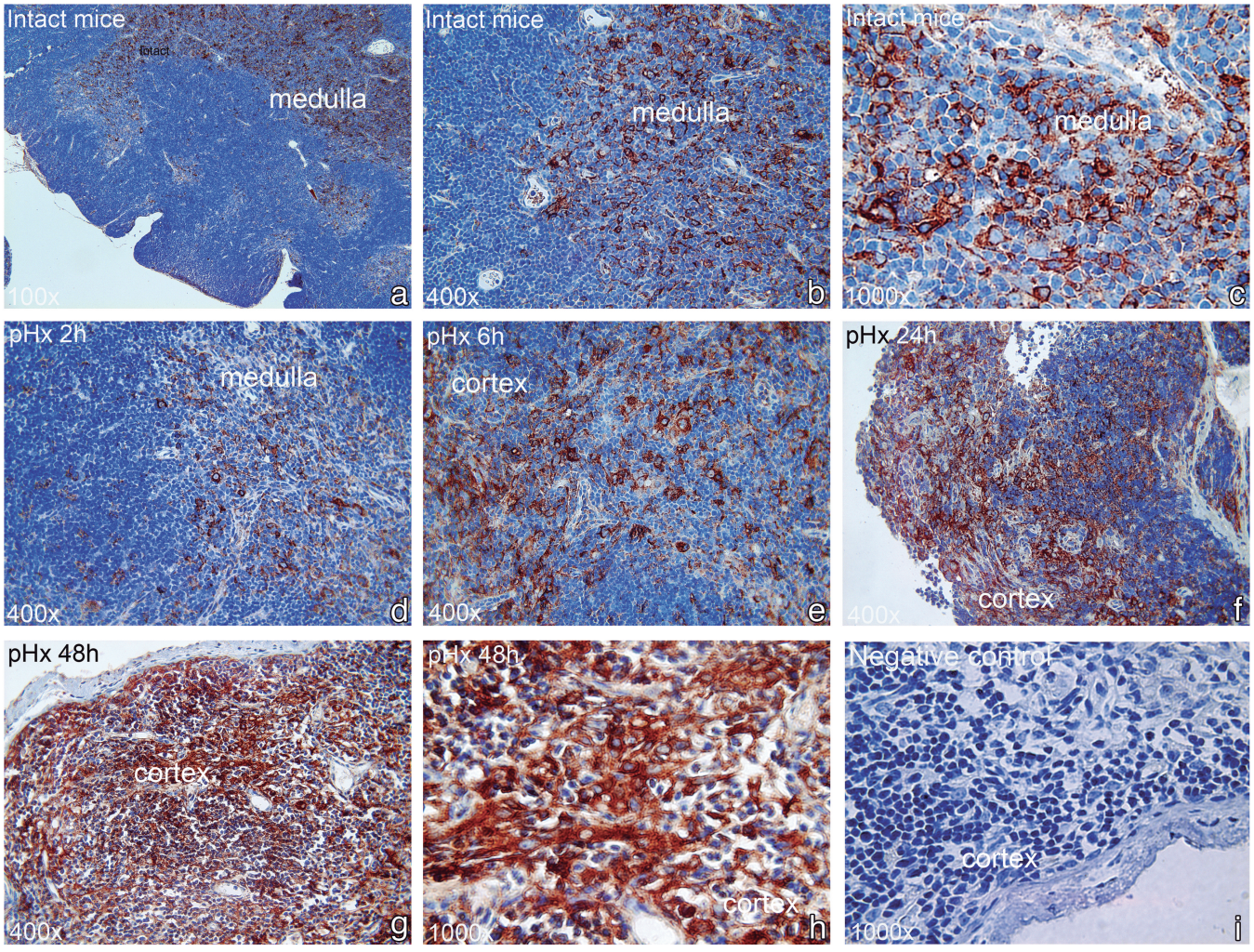

Fig. 3. Expression of gp96 protein in thymuses of C57/BL6 mice, subjected to 1/3 partial hepatectomy (pHx). Gp96 protein was detected by immunohistochemistry. **i.** Shows the negative (IgG1κ isotype) control for gp96 staining on **(h)**. The results are representative findings of 3 mice.

FACScalibur, we previously showed that pHx led to augmentation of Treg cells, both in the regenerating liver (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) (Mrakovcic-Sutic et al., 2008), in this immunohistochemical study we tried to visualize also the localization of cells that express or co-express the CD4, CD25 and FoxP3 markers. The data revealed that several $CD4^+$ (Fig. 7a) and $CD25^+$ (Fig. 7d) have the same locations in the thymus as $FoxP3^+$ cells (Fig. 7b,e), as well as that many of them were double positive $CD4^+FoxP3^+$ (Fig. 7c) and $CD25^+FoxP3^+$ cells (Fig. 7f), implying that they

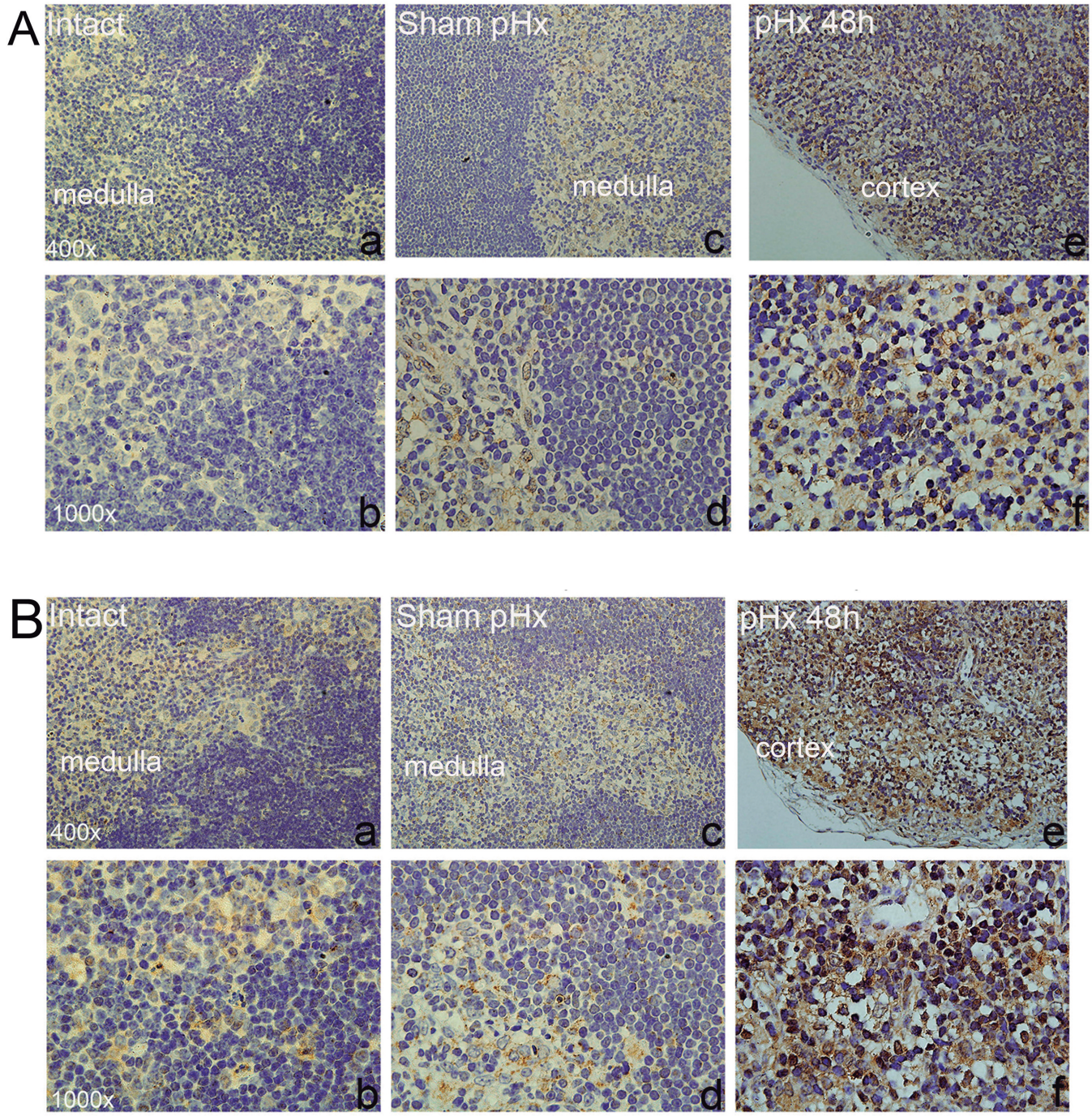

Fig. 4. Expressions of CD91 **(A)** and TLR2 **(B)** in the thymus of partially hepatectomized (pHx) or sham operated C57/BL6 mice 48h after the operations. The results are representative findings of 3 mice. Magnifications are 400 x (first line) and 1000 x (second line).

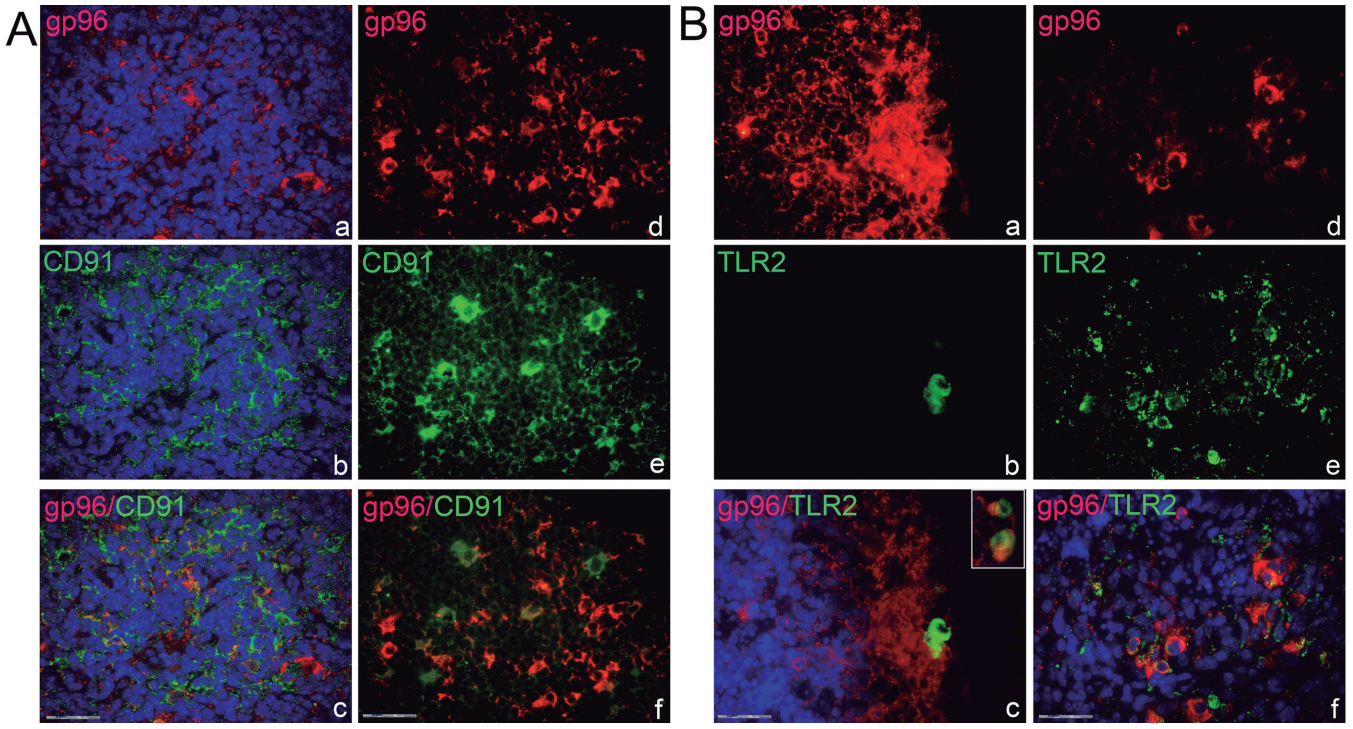

Fig. 5. Distribution of single and double positive gp96 and CD91 cells **(A)** and gp96 and TLR2 cells **(B)** in the thymus of pHx mice. Cells were detected by indirect immunofluorescence using PE-conjugated anti-gp96 and FITC-conjugated anti-CD91 and anti-TLR2 on paraffin-embedded tissue sections (4 µm), obtained 48h after 1/3 pHx from C57/BL6 mice. Red marks gp96+ cells, green marks CD91+ and TLR2+cells, blue marks DAPI staining of nuclei and yellow marks the overlapping of gp96 with other markers. x 1000.

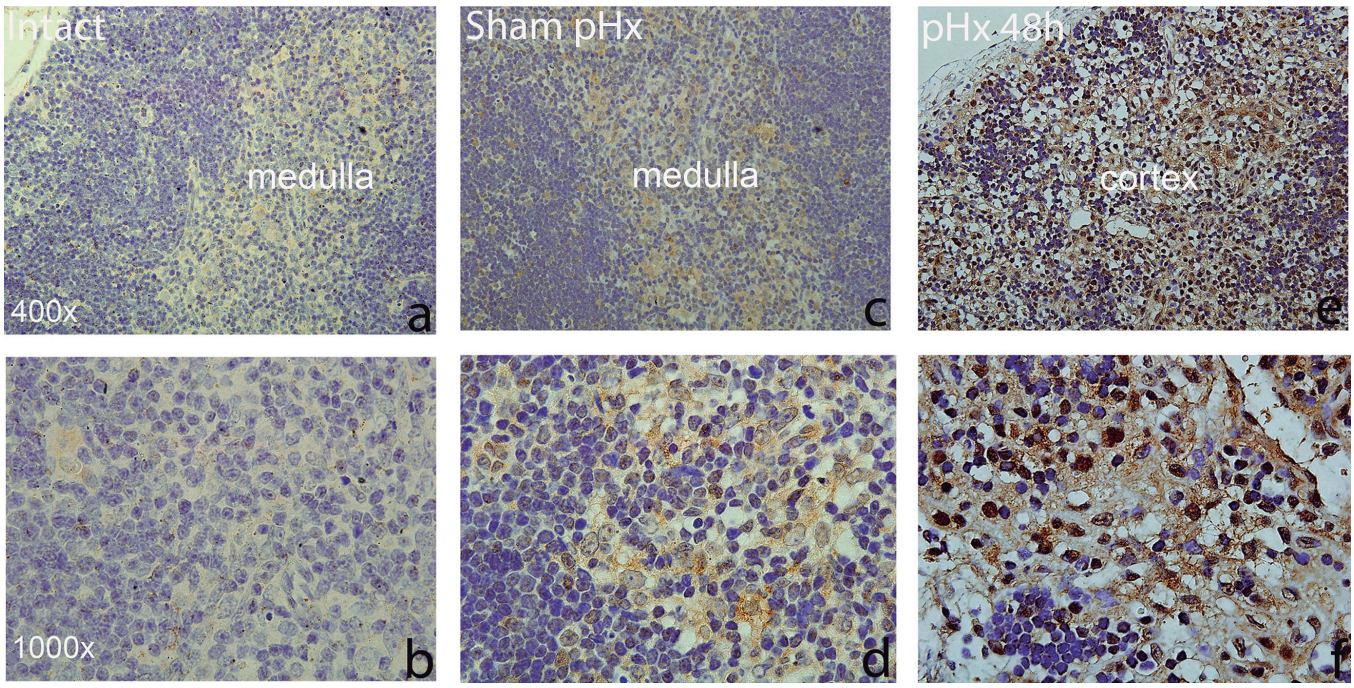

Fig. 6. Expression of TGF--β in thymus of partially hepatectomized (pHx) or sham operated C57/BL6 mice 48h after the operations. The results are representative findings of 3 mice. Magnifications are 400 x (first line) and 1000 x (second line).

belong to the regulatory T cells.

Discussion

In this immunohistochemical study, we show that pHx in mice is followed by upregulation of gp96, CD91, TLR2 and TGF-β, as well as with an accelerated apoptosis and appearance of Treg cells in the thymus. The data enhance our previous findings, showing that pHx induces gp96 overexpression in the liver and in the spleen and marked changes in the peripheral lymphoid system (Mrakovcic-Sutic et al., 2008). The latter were characterized by accumulation of auto-reactive NKT and Treg cells in the liver and increased FasL- and perforin-dependent cytotoxicity of hepatic and splenic NKT and NK cells. We hypothesized that these changes might be related with the tissue repair and prevention of potential autoimmune response against the self-antigens that could be exposed during liver regeneration (Mrakovcic-Sutic et al., 2008). The speculation was in high agreement with the hypothesis, proposed by Abo and co-workers (Abo et al., 2000), which implied that extrathymic T cell generation might be beneficial for the elimination of abnormal self-cells, such as malignant and microbially infected cells and regenerating hepatocytes. It was, however, also underlined that the overactivation or continuous activation of extrathymic T cells might be harmful and responsible for the onset of autoimmune diseases, as well as that the accumulation of "alternative" T cell clones in the liver might be found also in other conditions that suppress the conventional T-cell differentiation in the thymus, such as stress, infection, pregnancy or malignancy (Abo et al., 2000). The high regulatory influences of the autonomic nervous system and cytokines for the switching of the immune system from the thymus to the liver were also emphasized (Abo et al., 2000).

The latter seems to be supported also by our data showing that both pHx and sham Hx were followed by a high reduction of thymic weight and marked depletion of DP thymocytes (Fig. 2A,B), as well as by the appearance of NKT cells in the liver in both groups of operated mice (Fig. 1C). In this context, we can also speculate that the greater expression of gp96, observed particularly in pHx mice (Fig. 3d-i), was connected with the greater stress-induced ER-dyshomeostasis, provoked by anesthesia, surgery, hemorrhages and/or exposure to bacterial endotoxins after removal of the liver. In this scenario, it is likely that gp96 performs in the thymus its chaperon functions, since ER is a stress-sensing organelle, which detects the disequilibrium between ER load and folding capacity and reacts on various proteotoxic conditions, such as hypoxia, inhibition of protein glycosylation, Ca^{2+} depletion and perturbation of the redox potential in ER lumen. The activated UPR, which is facilitated and monitored by ER folding enzymes and molecular

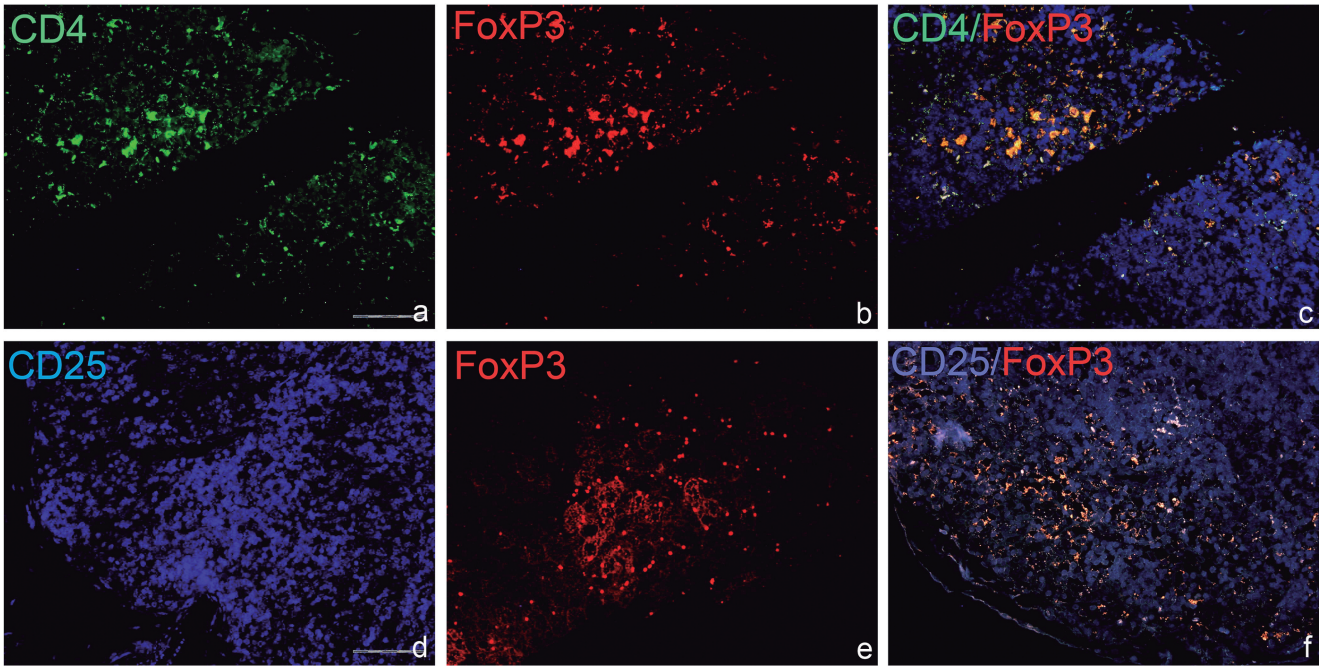

Fig. 7. Distribution of single and double positive CD4, CD25 and FoxP3 cells in the thymus of pHx mice. Cells were detected by indirect immunofluorescence using FITC-conjugated anti-CD4, APC-conjugated anti-CD25 and PE-conjugated anti-FoxP3 antibodies, respectively, on paraffin-embedded tissue sections (4 μm), obtained 48h after 1/3 pHx in C57/BL6 mice. Green marks the expressions of CD4 **(a)**, blue marks the expression of CD25 **(d)** and red marks the expression of FoxP3+ cells **(b, e)**. Yellow marks the overlapping of CD4 with FoxP3 **(c)**. Pink marks the overlapping of CD25 and FoxP3 **(f)**. Magnifications are x 1000

chaperones, transmit then the survival or apoptotic signals, depending on the intensity of stress and its ability to restore the protein homeostasis in ER (Ma and Hendershot, 2004; Martinon, 2012; Treglia et al., 2012). Besides, it should be taken into account that ER is a principal site for the folding and maturation of newly synthesized transmembrane, secretory and ER-resident proteins (Ni and Lee, 2007) and for the elimination of misfolded proteins by ER-associated protein degradation pathway (ERAD) (Lai et al., 2007; Eletto et al., 2010). Moreover, during the loss of homeostasis in ER, gp96 might be engaged in quality control of numerous factors involved in regulation of cell growth and differentiation (Macario and Conway de Macario, 2007; Eletto et al., 2010; Treglia et al., 2012), as well as in those involved in anti-apoptotic (Lanneau et al., 2007) and apoptotic pathways (Lai et al., 2007). Furthermore, evidence shows that gp96 may monitor the synthesis of membrane proteins, integrins and multiple Toll-like receptors that specifically regulate early T and B lymphopoiesis and enable the integration of cells into tissues (Staron et al., 2010). Importantly, gp96 also ensures the generation of antigenic peptides of the right quality and assembly of MHC molecules (Singh-Jasuja et al., 1999) and participates in regulation of ER Ca^{2+} signaling. i.e. in pathways that might have a high influence on differentiation and apoptosis of thymocytes (Luo et al., 2011). In the context of this evidence we can, therefore, speculate that pHx mice were exposed to higher ER-stress than sham Hx mice and that greater synthesis of gp96 in the thymus was related to correction of UPR and restoration of normal thymic functions.

Notably, the stress-induced scenario includes also the stimulation of hypothalamus-pituitary-adrenal (HPA) axis and the nervous fibers in the thymus by peripheral cytokines (Besedovsky and del Rey, 2000) or by other damage associated (DAMP) and pathogen associated molecular patterns (PAMP) that might be released during the resection of the liver. Glucocorticoids (GCs) and catecholamines decrease then the intrathymic cellularity and increase the T cell apoptosis, influencing the generation of the T cell repertoire by setting the TCR avidity window for thymocyte selection (Ashwell et al., 1996; Lechner et al., 2000). Importantly, upregulation of gp96 in these events might be related with the activation of genomic and nongenomic glucocorticoid-receptor (GR)-dependent pathways, since they include the dissociation of the activated GR from HSP 90 and other chaperones in the cytoplasm (Ohta et al., 2004; Stahn et al., 2007; Boldizsar et al., 2010; Bellavance and Rivest, 2014) and the rapid dissociation of a TCR-associated protein complex containing HSP90, LCK and FYN (Lowenberg et al., 2006) leading to the death of thymocytes bearing TCRs with subthreshold avidity for self-antigen (Ashwell et al., 1996) and to the activation of caspase-3, -8, and -9 (Fukuzuka et al., 2000; Wang et al., 2006).

On the other hand, owing to the peptide chaperone capacity of gp96 and its ability to actively interact with professional APC (Gullo and Teoh, 2004; Nicchitta et al., 2004) we cannot exclude either the possibility that during liver regeneration gp96 participated in reestablishment of immunological self-tolerance to tissue-restricted self-antigens. The hypothesis seems to be supported by findings that overexpression of gp96 was related with overexpression of CD91 (Figs. 4Ae,f, 5Ae,f), which is responsible for endocytosis of gp96-peptide complexes and re-presentation of gp96-chaperoned peptides to the major histocompatibility complex (MHC) molecules of APCs (Li et al., 2002) and with overexpression of TLR2 (Figs. 4Be,f, 5Be,f), which might be involved in maturation and functional activation of APCs (Binder et al., 2000; Basu et al., 2001) (Singh-Jasuja et al., 1999; Srivastava and Amato, 2001; Srivastava, 2002; Vabulas et al., 2002). There is a possibility that these pathways also contributed to the appearance of Treg cells (Fig. 7), which in the periphery might be necessary for the suppression of the transient auto-immune reaction to self antigens released from injured hepatocytes, although this highly speculative hypothesis remains to be proven. It seems, however, that it is in agreement with recent explanations of cellular and molecular mechanisms engaged in dominant control of self-reactive T cells, since they show that a highly promiscuous expression of tissue-restricted self-antigens is an inherent property of the thymic stroma and essential for the induction and maintenance of self-tolerance. The issue is covered by excellent reviews in this field, which show that the thymic selection of Treg cells is a multi-step process involving a variety of APC, cytokines and co-stimulatory molecules. They also predict that different outcomes for developing thymocytes depend on affinity and avidity of TCR interactions with MHC-peptide complexes on cortical thymic epithelial cells (cTECs) on the recognition of self-antigens on hematopoietic cells or on autoimmune regulator expressing medullary epithelial cells (mTECs), and on the maturation stages of APC and thymocytes that regulate the "strength" of the individual TCR/MHC-self peptide interaction (Modigliani et al., 1996; Kyewski and Derbinski, 2004; Coutinho et al., 2005; Sakaguchi and Sakaguchi, 2005; Ladi et al., 2006; Aschenbrenner et al., 2007; Sakaguchi et al., 2008; Derbinski and Kyewski 2010; Klein et al., 2011; Klein and Jovanovic, 2011; Lio and Hsieh, 2011; Heiber and Geiger, 2012; Yates, 2014). According to current models of thymocyte selection MHC/self peptide interactions of intermediate "strength" are required for positive selection, whereas very strong interactions lead to negative selection, although many questions about Treg biology are still under investigation, since their generation has been viewed as the consequence of "altered positive selection" of cortical DP thymocytes, as well as the consequence of "altered negative selection" in the thymic medulla (Maggi et al., 2005; Sakaguchi et al., 2006; Klein and Jovanovic, 2011). Gp96 probably affected the pathways that enable the presentation of self-antigens *in situ* by promiscuous gene expression in

mTECs, as well as the processes of antigen-cross presentation on resident thymic DCs, since they are dependent on the TEC-specific MHC/peptidome, on maturation stage of cells and on avidity of mTECs/DCs interactions on different APC (Derbinski and Kyewski, 2010; Klein et al., 2011), but our data based on immunohistochemistry, unfortunately, do not permit further discussion in this direction.

Current knowledge also shows that the complex processes of thymocytes differentiation and selection involve not only the rearrangements of T cell receptor (TCR) genes and the interaction between the TCR/peptide-MHC, but also a high influence of soluble products released by thymic microenvironmental cells (Coutinho et al., 2005; Goldman et al., 2005; Sakaguchi and Sakaguchi, 2005; Ladi et al., 2006; Bodey, 2007; Derbinski and Kyewski, 2010; Lio and Hsieh, 2011) and by the neuro-endocrine system (Besedovsky and del Rey, 2000; Roggero et al., 2011). In this context, it was also shown that TGF-β is necessary for signaling pathways that initiate and maintain Foxp3 expression (Chen et al., 2003; Fu et al., 2004), but in spite of the fact that in pHx mice we found the overexpression of TGF-β in the thymus (Fig. 6), as well as the presence of Treg cells (Fig. 7) the link between these events needs to be confirmed. The hypothesis is, however, supported by reports showing that natural Treg cells may travel from the thymus to the periphery to control the autoimmune, inflammatory and destructive processes in the liver, such as autoimmune hepatitis, viral hepatitis, and hepatocellular carcinoma, participating in the re-establishment of hepatic tolerance towards self antigens (Oo and Sakaguchi, 2013). There is a high possibility that by contact dependent mechanisms and secretion of immunosuppressive cytokines, such as IL-10 and TGF-β, they also suppress the transitory autoimmune reaction during liver regeneration and restrain the fast liver growth induced by pHx, but our preliminary findings require further examinations.

In conclusion, in this immunohistochemical study, we show that pHx is followed by depletion of DP thymocytes, apoptosis in the thymus and by a high upregulation of ER-resident HSP gp96, CD91, TLR2 and TGF-β in the thymic cortex, as well as with the appearance of $CD4^+$ $FoxP3^+$, $CD25^+$ $FoxP3^+$ cells. The data imply that thymic ER-signaling pathways act as stress sensor that detects various types of cellular insults during liver resection and suggest that gp96, by its chaperon-related and immunogenetic properties participates in re-establishment of thymic homeostasis during stress conditions and in maintainace of hepatic tolerance to self antigens. The mechanisms need to be elucidated, but the data seem to be in agreement with reports pointing to the interconnection of signaling pathways emerging from the endoplasmic reticulum and immune responses during stress, inflammation and infection (Martinon and Glimcher, 2011; Martinon, 2012), with the proposal that ER-stress is critically involved in the regulation of immune surveillance (Singh-Jasuja et al., 1999; Hilf et al., 2002; Srivastava, 2002) as well as with the evidence showing that HSP may directly or indirectly stimulate Tregs, via acetylation, TLR, ligation and induction of changes in thymic cytokine microenvironment (Brenu et al., 2013).

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