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Endoplasmic reticulum resident heat shock protein-gp96 as morphogenetic and immunoregulatory factor in syngeneic pregnancy

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Summary. The severe remodeling of endometrial stroma during blastocyst adhesion and trophoblast invasion initiates at maternal-fetal interface the reaction of evolutionary old heat shock response, in which heat shock proteins, as molecular chaperons, monitor the configurations of newly synthesized proteins and prevent the formation of functionless aggregates of misfolded proteins, targeting them to degradation by a the ubiquitin-proteasome system. In addition, the endoplasmic reticulum (ER)-resident HSPs, such as gp96/GRP94 may, after binding to CD91 and TLRs, elicit antigen-specific and antigen-unspecific immune responses, owing to its peptide-chaperoning capacity and ability to activate APCs.

Considering these properties, we examined tissue expression of gp96 at the maternal-fetal interface and in the maternal liver and spleen on the 16th day of undisturbed syngeneic pregnancy and after the treatment with peptidoglycan monomer linked with zinc (PGM-Zn). The data showed that in undisturbed pregnancy the gp96, CD91 and TLR2 were markedly expressed on extravillous and villous trophoblast. PGM-Zn enhanced these findings, as well as the number of uterine natural killer cells and local NFκB immunoreactivity. Gp96 expression arose also in the maternal spleen and liver, where an accumulation of NKT cells or γδT lymphocytes was seen. The data point to roles of gp96 in maintenance of proteostasis and local and systemic immune balance in pregnancy complicated by infection.

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Introduction

During pregnancy the bi-directional communication between the mother and the fetus ensures the maintenance of gestation and growth of the fetus, leading to blastocyst adhesion, trophoblast differentiation and decidualization that permit normal angiogenesis, placental development and maternal-fetal exchange of nutrients. The processes are highly dependent on the activation of molecular pathways that regulate the differentiation and functions of various trophoblast and adjacent cell subtypes that fuse into a syncytium to increase the surface area for nutrient transport and maternal blood flow (for reviews see Croy et al., 2002; Cross, 2005; Le Bouteiller and Tabiasco, 2006; Harris, 2010). Simultaneously, at the maternal-fetal interface numerous mechanisms are activated that create immunological tolerance, which ensures that the semi-allogeneic fetus is not rejected by the maternal immune system (Szekeres-Bartho, 2002; Laskarin et al., 2007; Riley, 2008; Clark et al., 2010).

Recent evidence shows that during these processes important functions are also performed the phylogenetically conserved family of heat shock proteins (HSPs), which participate in cellular functions that regulate the processes of proliferation, differentiation and apoptosis in reproduction (Walsh et al., 1999; Neuer

et al., 2000; Esfandiari et al., 2007; Yuan et al., 2009). As molecular chaperons HSPs control the processes of folding and assembly of newly synthesized secretory proteins and prevent the aggregation of unfolded and incompletely folded proteins. Besides, after a high ER stress they may induce degradation of misfolded and incompetent proteins (Ma and Hendershot, 2004; Nishikawa et al., 2005; Wu and Kaufman, 2006; Yang et al., 2007), or apoptosis of cells (Morishima et al., 2002; Lai et al., 2007). Importantly, some members of the HSP family, such as hsp60, hsp70, hsp90 and gp96 may stimulate the innate immune system acting as «danger»-signaling molecules (Gallucci and Matzinger, 2001; Wallin et al., 2002; Lai et al., 2007) or as adjuvants that elicit potent antigen-specific immunity to bound peptides of mammalian or microbial origin (Srivastava, 2002; Tsan and Gao, 2004). In such a way, the presence or absence of HSP might also influence various aspects of reproduction in many species (Neuer et al., 2000; Esfandiari et al., 2007; Yuan et al., 2009).

To address some aspects of this issue we investigated the expression of ER-resident heat shock protein gp96 (HSP90b1, glucose-regulated protein-GRP94, endoplasmin) at the maternal-fetal interface and in the maternal liver and spleen in undisturbed pregnancy and in pregnant mice treated with PGM-Zn. The latter was originally prepared by biosynthesis from culture fluids of penicillin-treated Gram positive bacteria-Brevibacterium divaricatum (Kegleviç et al., 1979: Radosevic-Stasic et al., 1995) and used herein as an exogenous agonist of TLR2 and as an agent that may increase the intensity of ER-stress and imitate the situation of pregnancy complicated by infection. At the placental bed the expression of gp96 was correlated with the expressions of CD91 and TLR2, as receptors that mediate the uptake of gp96-peptide complexes on antigen-presenting cells (Basu et al., 2001; Binder et al., 2001) and induce the antigen-unspecific activation of APC, respectively (Vabulas et al., 2002). Simultaneously, the changes in local and systemic immunity were tested by determination of uNK cells and NFκB immunoreactivity and by determination of phenotypic profile of hepatic mononuclear lymphatic cells (MNLC). To restrict the investigations on physiological processes involved in the maintenance of normalcy and integrity of tissue architecture and to compare the data with our previously reported findings (Mrakovcic-Sutic et al., 2003) all experiments were performed in the syngeneic murine pregnancy, where trophoblast did not express foreign MHC.

The data clearly showed that in the late phase of pregnancy gp96, CD91 and TLR2 were present at the maternal-fetal interface as a part of systems that regulate morphostasis and proteostasis, and also that PGM-Zn markedly enhanced these findings, leading to the activation of local immune response. Furthermore, both syngeneic pregnancy and PGM-Zn upregulated the gp96 expression in maternal liver and spleen, suggesting that it contributed to accumulation of NKT cells and γδT lymphocytes in the liver.

Material and methods

Mice

Experiments were done on non-pregnant, virgin female C57BL/6 (H-2b) mice and timed pregnant C57BL/6 mice, mated with syngeneic partner, at the age of 2-4 months. Day of detection of vaginal plug was considered day 0 of gestation. All mice were provided by Animal Cure facility of Medical School, Rijeka, whose ethical committee has approved all experiments.

Treatment with peptidoglycan monomer linked with zinc (PGM-Zn)

Peptidoglycan monomer (PGM; Pliva, Croatia) (GlcNAc-MurNAc-*L*-Ala-*D*-iso-Gln-meso-diamminopimelic acid (w-NH2)-*D*-Ala-*D*-Ala) was prepared from peptidoglycan of *Brevibacterium divaricatum* NRRL-2311, as an apyrogenic, water-soluble substance devoid of any toxic effects (Keglevic et al., 1979). However, owing to our previous finding that the immunostimulatory activities of PGM might be improved by addition of Zn (Radosevic-Stasic et al., 1995; Mrakovcic-Sutic et al., 2002), in this study we treated gravid C57BL/6 (N=8) and non-gravid mice (N=8) with PGM-Zn (Pliva, Zagreb, Croatia) dissolved in phosphate-buffered saline (PBS). Treatment started on day 0 of gestation and lasted 6 days (10 mg/kg intraperitoneally (i.p.) every 48h). Corresponding control groups consisted of gravid (n=8) and non-gravid mice (n=8) identically treated with PBS. To compare the results with our previously reported findings (Mrakovcic-Sutic et al., 2003), animals were sacrificed on day sixteen of pregnancy. Organs (uterus, liver and spleen) were removed for immunohistochemical analysis. Hepatic and splenic mononuclear lymphatic cells (MNLC) were isolated for the flow-cytometric analysis.

Isolation of hepatic MNLC

Isolation of hepatic MNLC was obtained by a modification of the method of Seglen as we previously described (Mrakovcic-Sutic et al., 2003). 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 μm gauze. Each sample was laid on 10 ml of Ficoll-Hypaque and MNLC were isolated by density gradient centrifugation, 20 min at 800xg. Cells were harvested from the middle layer, washed twice in PBS and counted in hemocytometer (Neubauer).

Immunofluorescent staining and flow cytometry.

The surface phenotypes of intrahepatic MNLC were identified by direct immunofluorescence analysis on

FACScan (Becton Dickinson, Immunocytometry Systems, Mountain View, CA), using CELLQuest Software (Macintosh, Quadra 650). As primary monoclonal antibodies (mAbs) were used anti-CD3, anti-CD4, anti-CD8, anti-NK-1.1, anti-CD122 (IL-2Rß) and anti-TCR γδ mAbs, conjugated with fluorescein isothiocyanate (FITC) or with phycoerythrin (PE) (Becton Dickinson Co, Mountain View, CA, USA). 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 1 x 10^4 cells.

Tissue preparation

Organs (uterus, placenta, liver and spleen) were rapidly removed from PGM-Zn or saline treated non-pregnant and pregnant mice. After fixation in 10% buffered formalin solution for 24h, tissue samples were embedded in paraffin wax. Sections were cut at 4 μm using HM340E microtome, Microtom, Germany. Heat induced epitope retrival was done prior to staining procedures by heating tissue slides in boiled citrate buffer pH 6.0 four times, each 5 minutes, using a microwave steamer.

Immunohistochemistry

As previously described (Mrakovcic-Sutic et al., 2008) immunohistochemical studies were performed on paraffin embedded tissues of the uterus, placentas, liver and spleen using DAKO EnVision+System, Peroxidase (DAB) kit according to the manufacturer's instructions (DAKO Corporation, USA). 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-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 IgG2-immunoglobulin (clone DAK-G05; Dako, USA), used in the same conditions and dilutions as a primary antibody. The same protocol was used for the visualization of NFκB immuno-reactivity, after the application of anti-NFκB p65 antibody (Abcam), at a dilution of 1:100. The slides were examined on an Olympus BX51 photomicroscope (Olympus, Tokyo, Japan).

Immunofluorescent labeling

Immunofluorescent labeling was performed on the paraffin embedded tissue sections. Prior to the staining, slides were deparaffinized by the standard techniques and antigen retrival was carried out by microwave heating in the citrate buffer pH 6.0. Nonspecific binding was prevented by incubating slides with 1% BSA in PBS for 1 hour at room temperature.

Subsequently, tissue sections were incubated with PE-conjugated anti-TLR2 (eBioscience USA) and anti-CD91 (Pharmingen, USA) antibodies diluted 1:50 in blocking solution, at 4°C overnight. To visualize uterine NK cells deparaffinized tissue slides were incubated with fluorescein labeled Dolichos Biflorus Lectin (DBA) (Vector, Burlingame, CA), diluted 1:50 in PBS, for 30 minutes at room temperature. It has been described as a unique marker for uNK cells in mice that contain the N-acetyl-galactosamine-binding lectin (Paffaro et al., 2003; Yadi et al., 2008). Nuclei of the cells were stained with DAPI (Invitrogen) diluted 1:1000 in PBS, after incubation for 5 minutes at room temperature. Finally, slides were washed, mounted with Mowiol (Sigma-Aldrich) and analyzed under fluorescent microscope.

For the detection of gp96 or TLR2 in uNK cells dual fluorescent labeling was performed. For this purpose tissue sections were submitted to heat induced antigen retrival. 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) or by anti-TLR2 antibodies (diluted 1:50) at + 4°C overnight in a humid chamber. Thereafter, sections were rinsed and incubated with Alexa Fluor555 goat anti-rat secondary antibody (Invitrogen) diluted 1:500, for 1 h in a dark and humid environment. After rinsing, the procedure was immediately continued with uNK cell labeling as described above.

Immunohistochemical staining quantification

Immunohistochemical staining quantification was performed using Cell F v3.1 software (Olympus Soft Imaging Solutions). Captured images were subjected to intensity separation.

They were subsequently inverted, resulting in grey scale images with different intensity range, depending on the strength of immunohistochemical signals. Regions of interest were set up to cover the cytoplasm of immunopositive cells to measure grey intensity. Twenty regions of interest were analyzed per field (400 x) in the ten fields per microscopic slide of tissue samples, obtained from saline treated or PGM-Zn treated pregnant mice.

Statistical analysis

Statistical significance was analyzed by Mann Whitney U test and Student's t-test. The differences were

considered significant when $p<0.05$.

Results

Expression of gp96, CD91 and TLR2 at the maternal-fetal interface in undisturbed in pregnancy

Mice were treated with PBS or with PGM-Zn dissolved in PBS (10 mg/kg i.p. every 48h from day 0 to day 6). They were sacrificed on the sixteenth day of gestation. The data showed that in "undisturbed" pregnancy (mice treated with PBS) gp96 immunoreactivity was present in basal and labyrinth zones of extravillous trophoblast (Fig. 1a-c), as well as on some cells in the villous stroma, on cytotrophroblasts (CT) and syncytiotrophoblast (SCTs) and on some cells in the villous stroma, belonging most likely to the Hofbauer cells or fetal macrophages (Fig. 2a-c). In the junctional zone of SCT layer gp96 was expressed on glycogen trophoblast cells (GTCs) (Fig. 3c). In the same areas several CD91 (Fig. 1d-f) and TLR-2 positive cells were also noticed (Fig. 1g-i). In addition, prominent CD91 and TLR2 staining was found in the villous area in the form of a continuous layer of SCT positive cells (Fig. 2d-i).

Expression of gp96, CD91 and TLR2 at maternal-fetal interface of mice treated with PGM-Zn

Treatment of pregnant mice with PGM-Zn, however, markedly enhanced these expressions, augmenting gp96 immunoreactivity in anchoring and invading

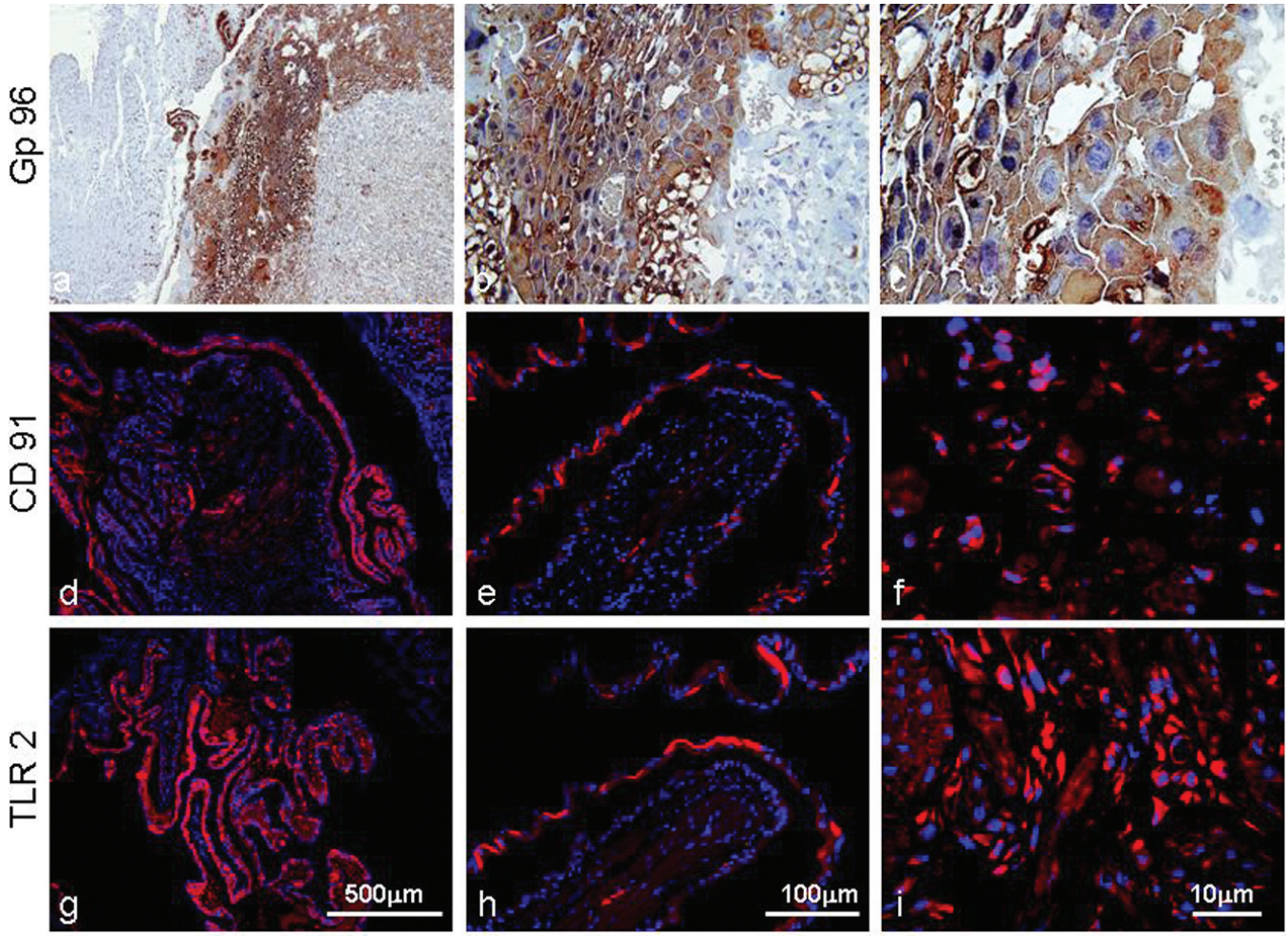

Fig. 1. Expression of gp96, CD91 and TLR2 proteins on 16th gestational day in extravillous part of placenta of C57BL/6 mice, mated with syngeneic partner. Paraffin-embedded tissue sections (4 μm) were immunostained with monoclonal rat anti-Grp94(gp96) antibody. The reaction product was visualized by adding substrate-chromogen (DAB) solution (brown staining). CD91 and TLR2 were detected by direct immunofluorescence, using PE-conjugated anti-CD91 or anti-TLR2 antibodies. Red marks the expression of CD91 and TLR2 and blue marks DAPI staining of nuclei. The results are representative findings of 3 mice.

trophoblasts, affecting the branched labyrinthine layer, vascular endothelium and several mononuclear lymphatic cells (Fig.3a-c). Besides, a high upregulation of gp96 expression was noticed on the cytotrophoblast (Fig. 4a,b). Simultaneously, in these areas a high upregulation of CD91 and particularly of TLR2 immunopositivity were noticed, affecting the outer layer of villous trophoblast (Fig. 4Ac-f). Some TLR2 - positive mononuclear cells were also found in decidual tissue (Fig. 3i). The results, obtained by the technique of cell-based staining quantification (made on comparable slides of placenta obtained from PGM-Zn treated and saline-treated pregnant mice) also showed that the enhancing effects of PGM-Zn on expressions of gp96, CD91 and particularly TLR2 in decidual and in trophoblast sections were statistically significant (Fig. 4B; $p<0.001$).

Uterine NK cells and NFκB immunoreactivity at maternal-fetal unit in pregnant mice treated with saline and PGM-Zn

Owing to the possible immune effects of gp96 and bacterial peptidoglycans on innate immunity, the presence of NK cells and NFκB immunoreactivity was compared in placentas of mice treated with PGM-Zn and those treated with saline. As a specific marker for uterine NK cells the fluorescein labeled Dolichos Biflorus lectin (DBA) was used, which has been described as a very selective and sensitive reagent for discriminating mouse uNK cells from all other lymphocytes. Namely, it is the only lectin that reacts with glycoconjugates, containing N-acetyl-D-galactosamine as terminal carbohydrate moiety, present in NK cells reaching the pregnant uterus (Paffaro et al., 2003; Yadi et al., 2008).

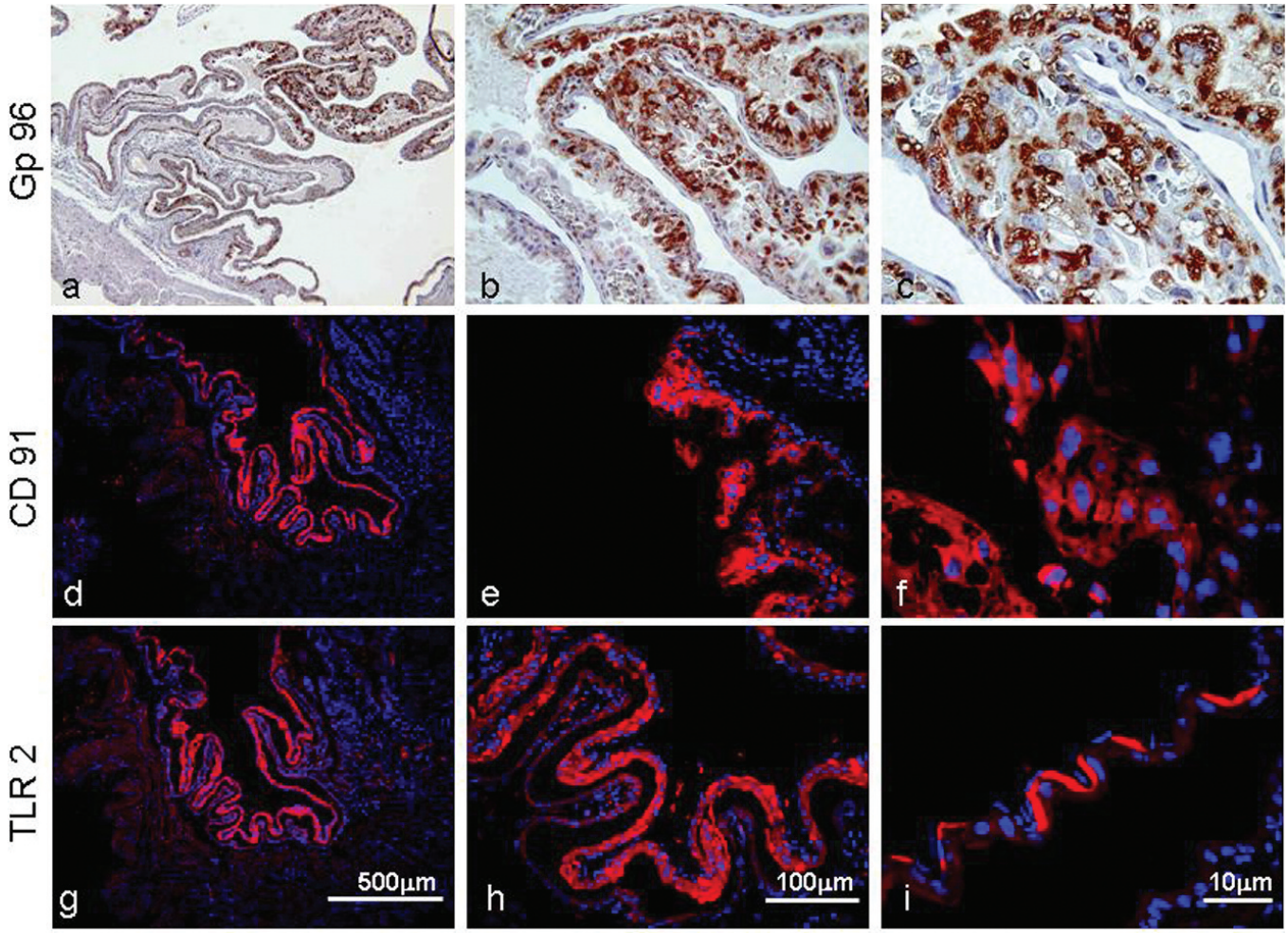

Fig. 2. Expression of gp96, CD91 and TLR2 proteins on 16th gestational day in villous part of placenta of C57BL/6 mice, mated with syngeneic partner. Paraffin-embedded tissue sections (4 μm) were immunostained with monoclonal rat anti-Grp94(gp96) antibody. The reaction product was visualized by adding substrate-chromogen (DAB) solution (brown staining). CD91 and TLR2 were detected by direct immunofluorescence, using PE-conjugated anti-CD91 or anti-TLR2 antibodies. Red marks the expression of CD91 and TLR2 and blue marks DAPI staining of nuclei. The results are representative findings of 3 mice.

The data showed that in undisturbed pregnancy, in spite of the high presence of gp96, CD91 and TLR2 at the maternal-fetal interface (Figs. 1, 2) only some senescent uNK might be found (Fig. 5a-c). Furthermore, NFκB immunoreactivity was low and detected only in some TGC, as a cytoplasmic staining (Fig. 5d-f).

On the contrary, in pregnant mice treated with PGM-Zn in decidua basalis the number of uNK cells not only rose, but so did the intensity of DBA staining in these cells (Fig. 6), suggesting that PGM-Zn has contributed both to recruitment of uNK cells and to activation of the resident population of these cells. This speculation seems to be supported also by the morphological aspects of these uNK cells (round cell shape and euchromatic nucleus), which look as less mature cells than those found in undisturbed pregnancy, as well as by findings that some uNK cells were in close contact with trophoblast cells expressing gp96 or TLRs. Besides, the double staining of DBA and gp96 or DBA and TLR2 revealed that several of these uNK cells contained inside the cell the gp96 (Fig. 7a-c) or TLR2 (Fig. 7d-f), implying that they were activated by receptor-ligand mediated interaction. The data also showed that the treatment with PGM-Zn induced a high upregulation of nuclear NF-κB immunoreactivity on multiple cells in chorionic plate and spongiotrophoblast (Fig. 7g-i) supporting the possibility that the expression of endogenous ligand (such as gp96) or exogenous ligand (such as PGM-Zn) had stimulated the signaling triggered by activation of TLRs.

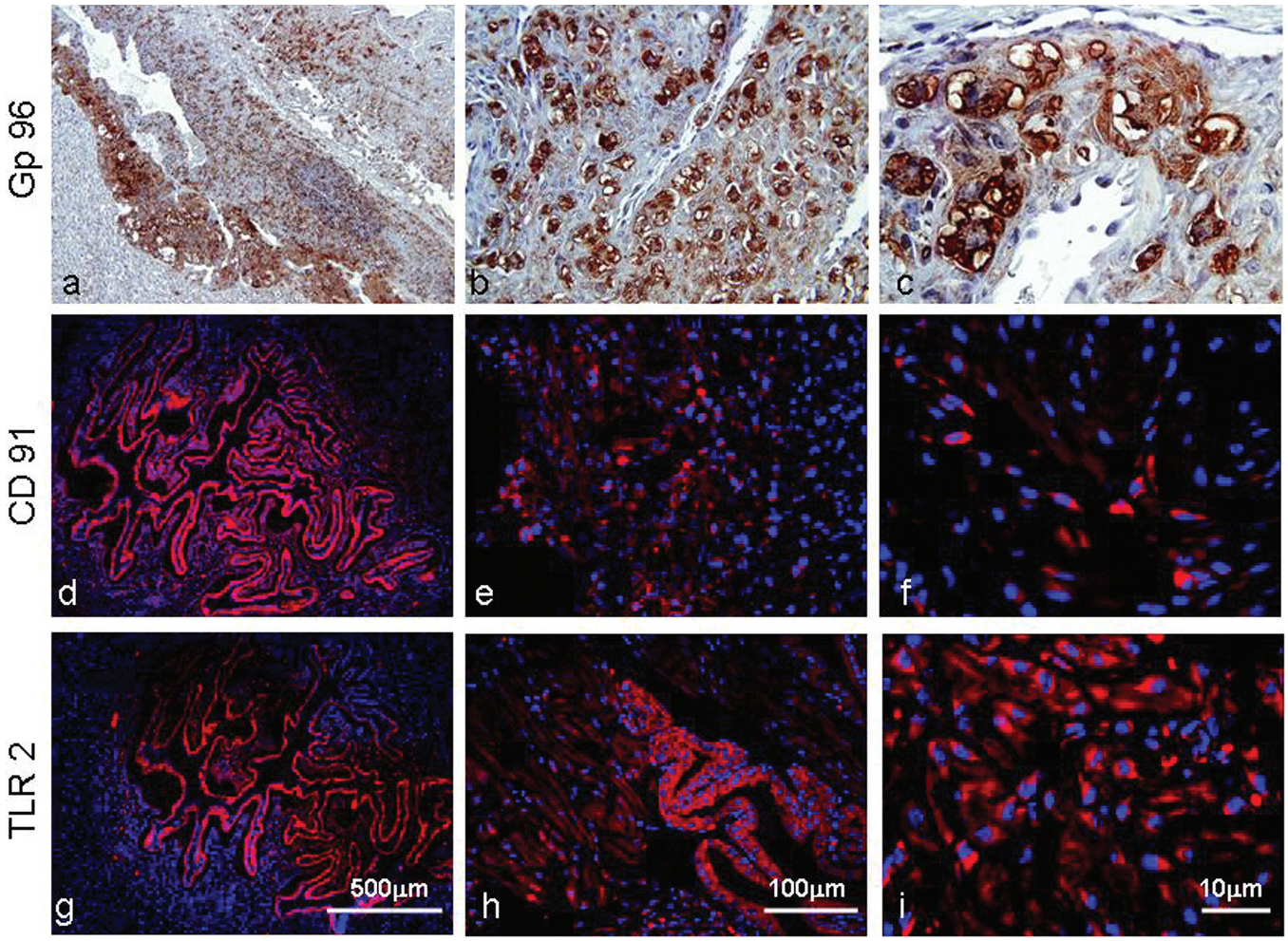

Fig. 3. Expression of gp96, CD91 and TLR2 proteins in extravillous part of placenta during the syngeneic pregnancy after treatment with PGM-Zn. Paraffin embedded tissue sections (4 μm) were immunostained with monoclonal rat anti-Grp94(gp96) antibody. The reaction product was visualized by adding substrate-chromogen (DAB) solution (brown staining). CD91 and TLR2 were detected by direct immunofluorescence, using PE-conjugated anti CD91 or anti-TLR2 antibodies. Red marks the expression of CD91 and TLR2 and blue marks DAPI staining of nuclei. The results are representative findings of 3 mice.

Expression of gp96 in maternal spleen and liver in pregnant mice treated with saline and PGM-Zn. Augmentation of NKT cells and gamma delta T lymphocytes in the liver

Owing to our previous findings that both syngeneic pregnancy (Mrakovcic-Sutic et al., 2003) and PGM-Zn, given *in vivo* (Mrakovcic-Sutic et al., 2002) led to the significant accumulation of primitive T cells in the maternal liver and augmentation of the NKT and NK cell-mediated cytotoxicity in the liver and spleen, in this study we also estimated the expression of gp96 in these organs. Simultaneously, the phenotypic profile of MNLC in the liver was evaluated by flow-cytometry. The data showed that in non-pregnant mice gp96 was abundant both in hepatocytes (Fig. 8) and in paracortical areas of the spleen (Fig. 9). This expression, however, arose during the pregnancy and particularly after the treatment of gravid mice with PGM-Zn, suggesting that gp96 participated in activation of systemic innate and adaptive immunity. This is also supported by significant changes found in the maternal liver, where pregnancy alone increased the proportion of $CD122^+$ (IL-2Rß$^+$), CD3+/$NK1.1^+$, CD3+/$CD122^+$ and $CD4^+$ cells and decreased the proportion of .$CD8^+$ cells. Moreover, treatment of pregnant mice with PGM-Zn additionally enhanced these findings, significantly increasing the proportion of $CD3^+$, $NK1.1^+$, $TCR\gamma\delta^+$ and $CD8^+$ cells (Fig. 10). Besides, supporting the possibility that maternal liver and spleen during pregnancy contained

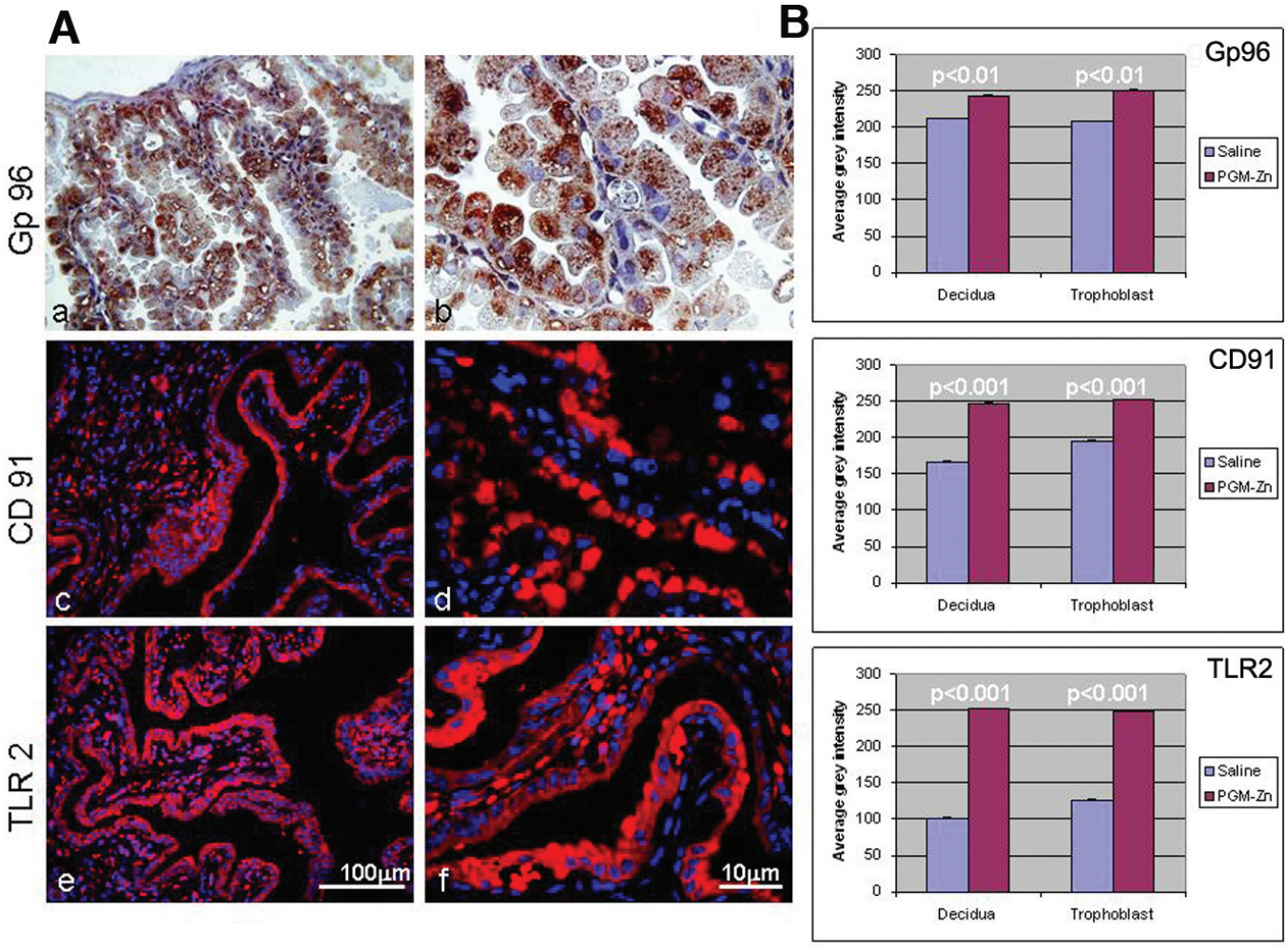

Fig. 4. Expression of gp96, CD91 and TLR2 proteins in villous **(A)** part of placenta during the syngeneic pregnancy after treatment with PGM-Zn. Paraffin embedded tissue sections (4 μm) were immunostained with monoclonal rat anti-Grp94(gp96) antibody. The reaction product was visualized by adding substrate-chromogen (DAB) solution (brown staining). CD91 and TLR2 were detected by direct immunofluorescence, using PE-conjugated anti CD91 or anti-TLR2 antibodies. Red marks the expression of CD91 and TLR2 and blue marks DAPI staining of nuclei. The results are representative findings of 3 mice. **B.** Immunohistochemical, cell-based staining quantification of gp96, CD91 and TLR2 expression in uterus (decidua and trophoblast) of gravid mice treated by PGM-Zn or by saline. Average grey distribution in each group was calculated from twenty regions of interest (magnification per field-400 x) in the ten fields per microscopic slides of tissue samples. Results are mean ±SE.

activated lymphatic cells, we showed that hepatic and splenic MNLC isolated from pregnant mice were more cytotoxic against NKT, NK and LAK-sensitive targets than cells obtained from non-pregnant mice (Mrakovcic-Sutic et al., 2003).

Discussion

The data showed that ER-resident HPS gp96 might be involved both in physiological and in pathological conditions that arose during the maternal infection. Thus, in undisturbed syngeneic pregnancy we found high expressions of gp96 and its receptors CD91 and TLR2 at the maternal fetal interface (Figs. 1, 2), as well as the enhanced expression of gp96 in maternal liver and spleen (Figs. 8, 9). Furthermore, we show herein that these findings might be enhanced by treatment with PGM-Zn (Figs. 3, 4, 8, 9), which was used as a bacterial product that might activate the complex network of host-pathogen interactions and as an adjuvant that might increase the intensity of oxidative stress and enhance the release of HSPs from stressed cells. Moreover, showing that PGM-Zn may also augment the activities of uNK cells and NFκB at the maternal-fetal interface (Figs. 6, 7) and induce an accumulation of NKT cells and CD1-restricted γδ T cells in maternal liver (Fig. 10), we are emphasizing that HSPs and TLRs, as ubiquitous and phylogenetically conserved molecules may contribute to the maintenance and integrity of tissue architecture at the maternal-fetal unit, as well as to the induction of local and systemic immunological disbalances during pregnancy (Esfandiari et al., 2007; Ma et al., 2007; Rindsjö et al., 2007; Koga and Mor, 2008; Miko et al., 2008; Yuan et al., 2009).

The data suggest that the effects may depend on the intensity of ER-stress and integration of the signals directed to the recognition of damage associated molecular pattern (DAMPs) and pathogen-associated molecular patterns (PAMPS), but the mechanisms need to be elucidated.

Thus, in an undisturbed pregnancy it is likely that gp96/GRP94, as an ER-resident HSP, performs its chaperone functions, preventing the proteotoxic damage that may arise at the maternal-fetal interface in highly secretory cells after an increase in protein load or during an ER-stress that disrupts protein folding owing to hypoxia, energy deficiencies, inhibition of protein glycosylation, perturbation of the redox potential and Ca^{2+} depletion (Eletto et al., 2010; Jolly and Morimoto, 2000; Ma and Hendershot, 2004; Nishikawa et al., 2005; Yang and Li, 2005; Wu and Kaufman, 2006; Treglia et al., 2012). Among the client proteins are signal transduction proteins, kinases and transcriptional factors that contribute to physiological processes such as cell

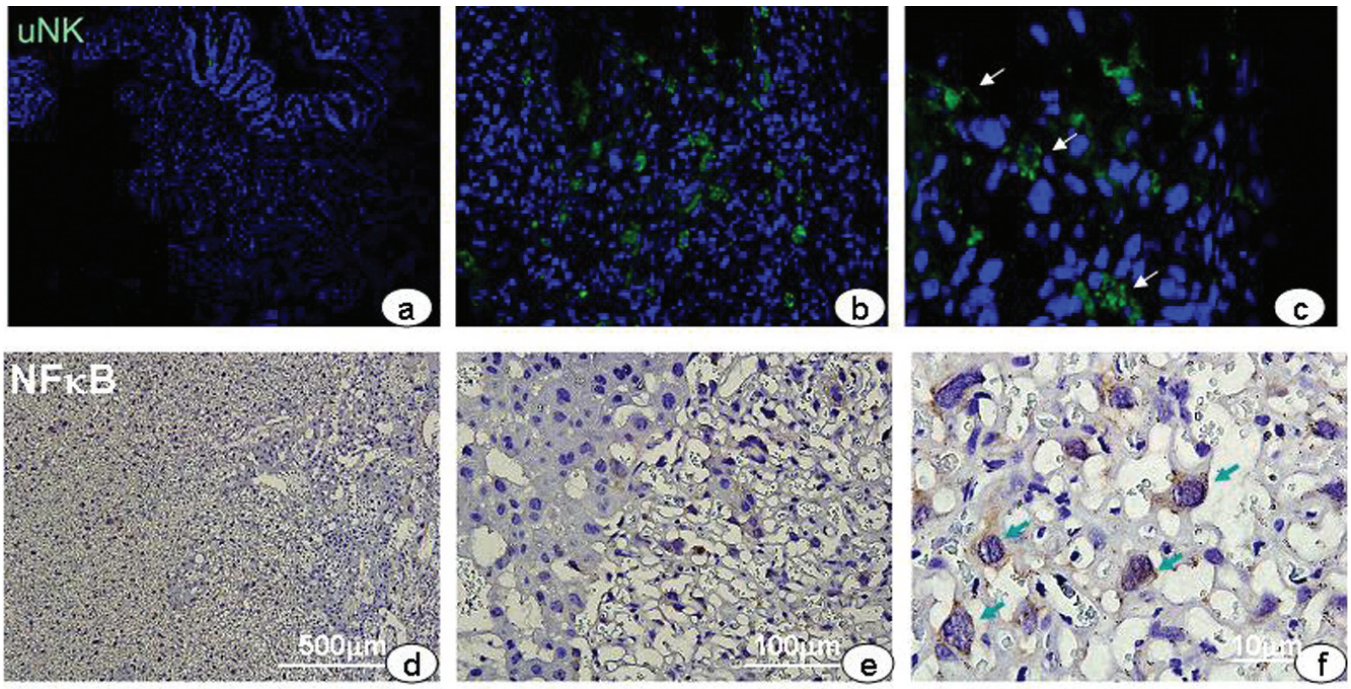

Fig. 5. Uterine NK cells distribution, their relationship to cells expressing gp96 or TLR2 and expression of NF-κB in placenta of C57BL/6 mice treated by saline. **a-c.** Uterine NK cells (green) were detected on paraffin-embedded tissue sections (4 μm), obtained on the 16th gestational day of syngeneic pregnancy by direct immunofluorescence, using FITC-conjugated DBA lectin antibody. Green marks expression of DBA lectin, red marks expression of gp96 or TLR2 and blue marks DAPI staining of nuclei. **d-f.** Expression of NF-κB in placenta of C57BL/6 mice treated by saline detected by the use of monoclonal rat anti-NF-κB p65 antibody (brown staining). Arrows on c and f point to uNK cells with intracellular content of gp96 and TLR2. The results are representative findings of 3 mice.

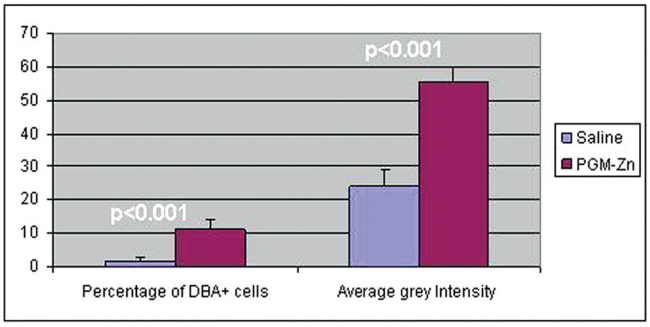

Fig. 6. Percentage of uNK cells and average grey intensity of DBA staining in uNK in PGM-Zn and saline treated pregnant mice. Results are mean ± SE.

cycle regulation, wound healing, cell regeneration and metabolism, but in this context gp96 is more selective than other ubiquitous luminal chaperones, such as PDI, calreticulin or BiP (Elletto et al., 2010; Treglia et al., 2012).

Moreover, it should be emphasized that gp96 may affect also the maturation of insulin-like growth factor (IGF)-I and -II and the functions of multiple TLRs (Yang et al., 2007), which are essential for the expression of specific integrins that regulate lymphatic cell development and ensure the contact of hematopoietic progenitors with stromal cell niche (Staron et al., 2010). Besides, pointing to the impact of gp96 on the processes of tissue remodeling during trophoblast invasion, it was found that Hsp90ß mutant mice failed to form a fetal trilaminar labyrinthine trophoblast (Voss et al., 2000), as

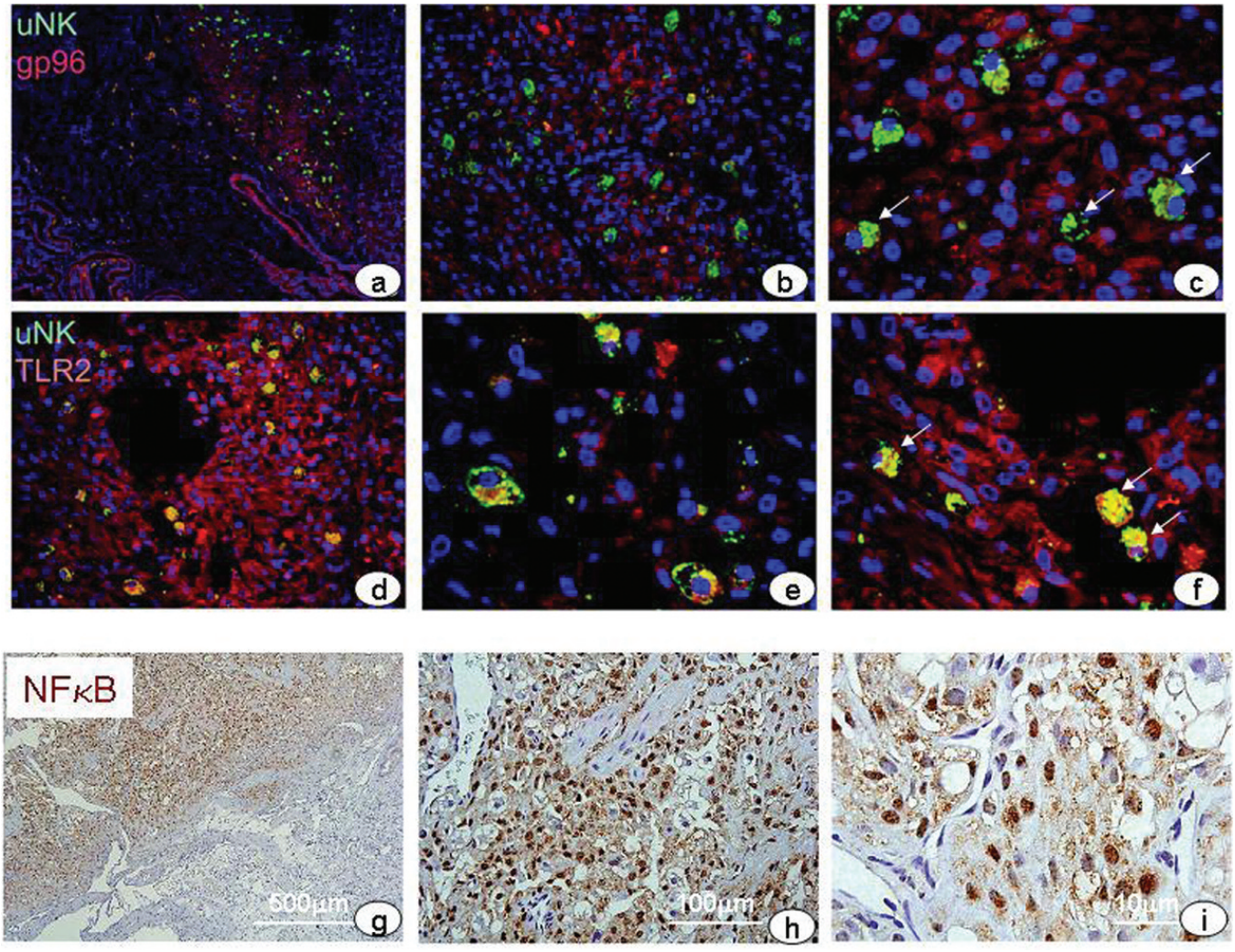

Fig. 7. Uterine NK cells distribution, their relationship to cells expressing gp96 or TLR2 and expression of NF-κB in placenta of C57BL/6 mice treated by PGM-Zn. **a-f.** Double staining with anti-DBA lectin and anti-CD91 antibodies or anti-DBA lectin and anti-TLR2 antibodies. Yellow marks the overlapping of gp96 and DBA **(a-c)** or TLR2 and DBA **(d-f)** in uNK cells. Arrows on c and f point to uNK cells with intracellular content of gp96 and TLR2. **g-i.** Expression of NF-κB in placenta of C57BL/6 mice treated by PGM-Zn, detected by the use of monoclonal rat anti-NF-κB p65 antibody (brown staining). The results are representative findings of 3 mice.

well as that spiral arterial modification and fetal implantation dependent on the interaction between the stress-induced MHC class I-like ligands, such as the products of Rae-1 gene family members and NKG2D receptors on uNK cells (Carayannopoulos et al., 2010). The latter data point also to the high influence of HSPs on the signals transmitted via inhibitory and activating receptors on uNK cells and other cells that at the placental bed regulate tissue remodeling and immune homeostasis (Szekeres-Bartho et al., 2001; Tsuda et al., 2001; Boyson et al., 2008; Horowitz et al., 2012).

In this context, it is emphasized that gp96 particularly might perform a link between innate and specific immunity, owing to its ability to induce the maturation of APC and to carry the peptides released from the necrotic cells from cell to cell (Schild and Rammensee, 2000; Hilf et al., 2002; Srivastava, 2002). During these events CD91 acts as the unique receptor responsible for endocytosis and directing of gp96-chaperoned peptides into the MHC class I Ag re-presentation pathway leading to amplification of cytotoxic T cell activation (Binder and Srivastava, 2004), while the TLRs act as factors responsible for the activation of NFκB-driven reporter genes and mitogen- and stress-activated protein kinases (Vabulas et al., 2002).

Our data showing the presence of gp96, CD91 and TLR2 at the maternal-fetal interface in undisturbed pregnancy (Figs. 1, 2) and upregulation of these findings after treatment with PGM-Zn (Figs. 3, 4A) seems to support these possibilities, suggesting that CD91 and TLR2 in the villous area might be involved in cross-presentation of self peptides chaperoned by gp96 to MHC molecules and in stimulation of maternal immune responses. Moreover, we can speculate that the enhanced expression of gp96 in maternal liver and spleen (Figs. 8, 9) contributed to the accumulation of NKT cells and CD1-restricted γδ T cells, since these cells possess the

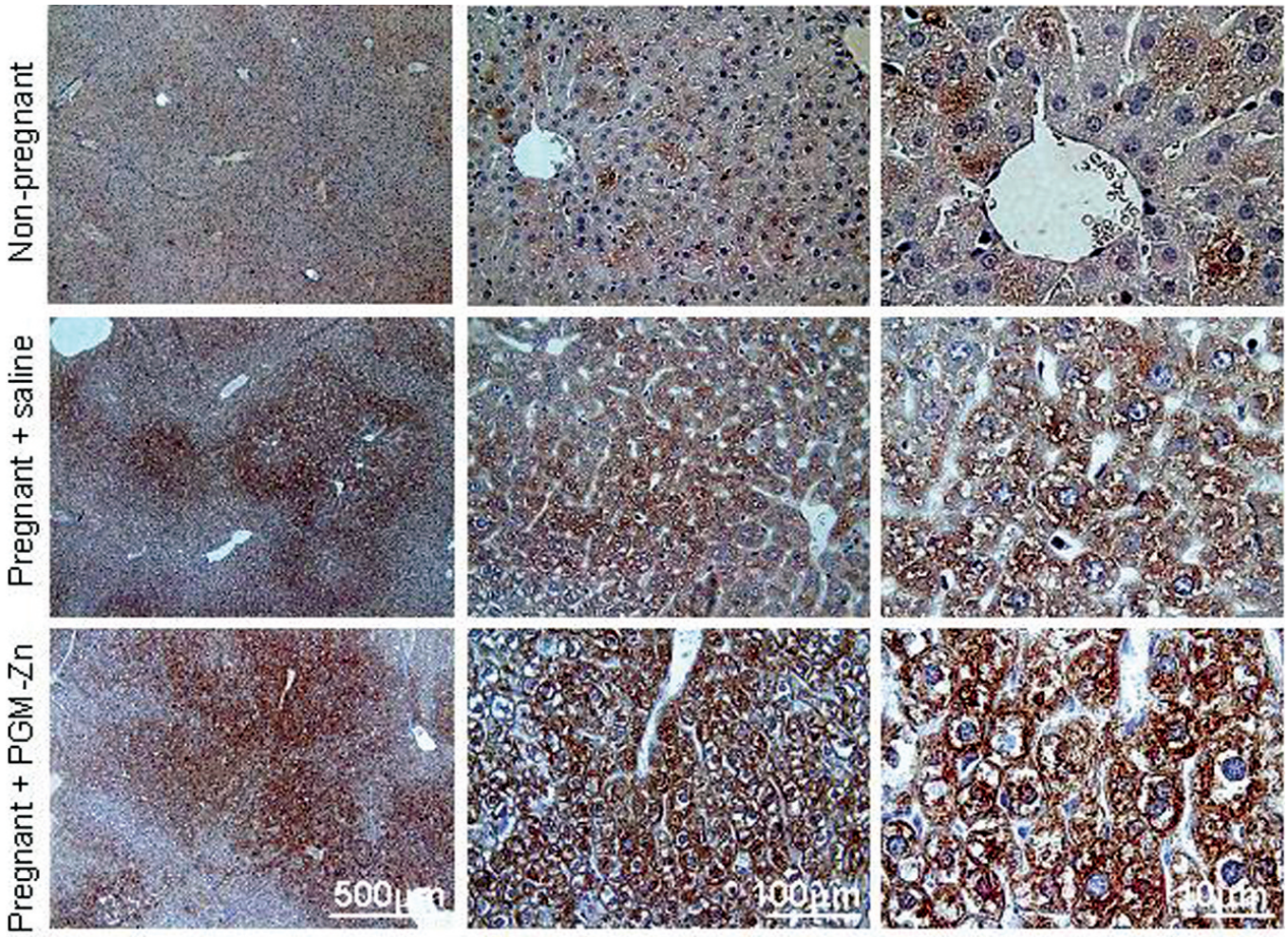

Fig. 8. Expression of gp96 in the liver in non-pregnant mice and pregnant mice treated with saline or PGM-Zn. Paraffin-embedded tissue sections (4 μm) were immunostained with monoclonal rat anti-Grp94(gp96) antibody). The reaction product was visualized by adding substrate-chromogen (DAB) solution. The results are representative findings of 3 mice.

ability to identify stress proteins, such as RAE-1, MICA and MICB, as well as various non-peptide antigens, like lipid and glycolipid components of bacterial cell, α-galactosylceramide and the abnormal self-cells and various metabolites produced by the cells (Constant et al., 1994; Chiu et al., 1999; Shi et al., 2000; Cerwenka and Lanier, 2003; González et al., 2008).

However, owing to the high overlapping between the pathways leading to the recognition of DAMPS and PAMPS (Matzinger, 1998; Gallucci and Matzinger, 2001; Wallin et al., 2002; Marras et al., 2011), we can only speculate about the involved mechanisms. Thus, the activation of local and systemic immune response found after treatment with PGM-Zn might be mediated by recognition of PGM or its metabolic degradation products-PGM and zinc ions, by gp96-mediated pathways, as well as by dual recognition of self and microbial ligands. In any case, since the treatment with PGM-Zn started during the early stage of embryonic development (from days 0-6) and its effects were evaluated in the phase of gestation, it is likely that PGM-Zn had induced the long lasting immunological disbalance in gravid mice.

In this sense, our data imply that the enhanced expression of gp96 in the maternal spleen and liver contributed to the overactivation or continuous activation of extrathymic T cells clones in the liver that might be harmful and be responsible for the onset of complication in pregnancy (Abo et al., 2000). However, in spite of the fact that the multiple pathways of activation of NKT cells and their double-edged sword nature has been repeatedly emphasized (Godfrey et al., 2000; Kronenberg, 2005; Bendelac et al., 2007; Boyson et al., 2008) the link between gp96 and these events remains to be proved. Moreover, the other properties of PGM-Zn should be taken into account, such as its ability to upregulate the expression of MHC cell class II antigens in the liver, to stimulate the antibody production

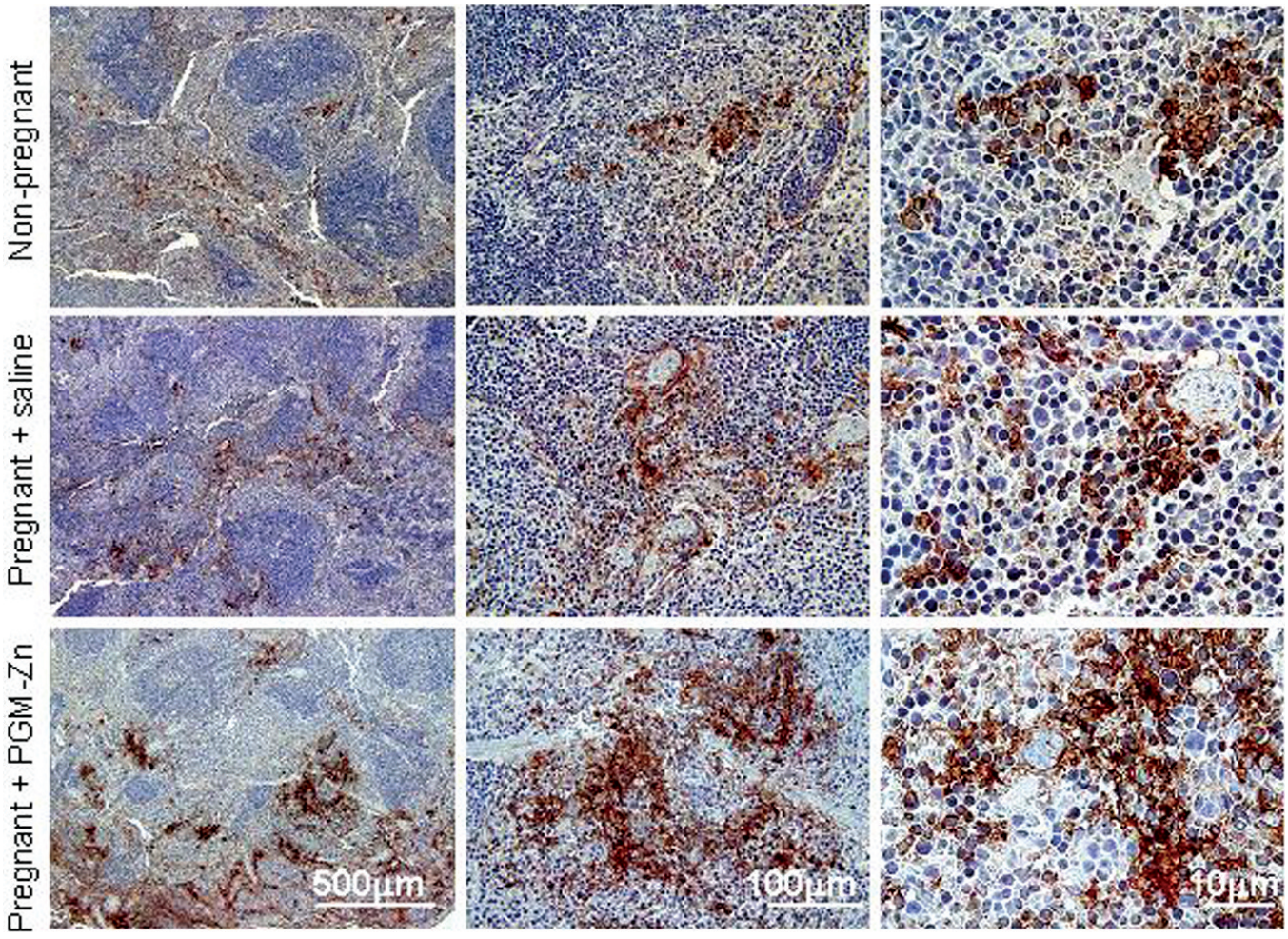

Fig. 9. Expression of gp96 in the spleen in non-pregnant mice and pregnant mice treated with saline or PGM-Zn. Paraffin-embedded tissue sections (4 μm) were immunostained with monoclonal rat anti-Grp94(gp96) antibody). The reaction product was visualized by adding substrate-chromogen (DAB) solution. The results are representative findings of 3 mice.

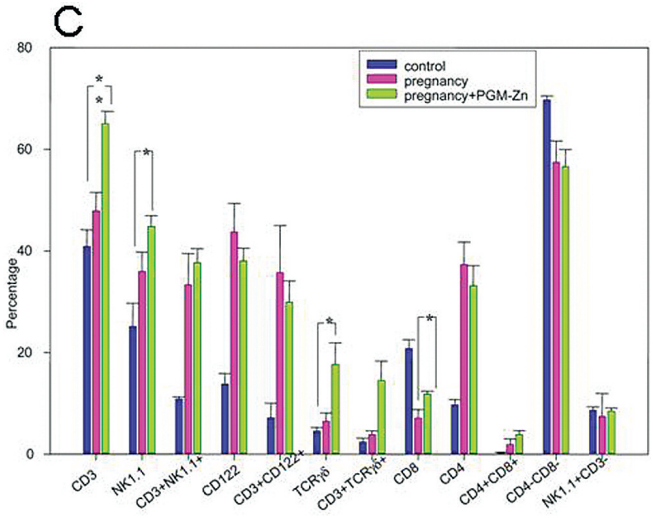

Fig. 10. Phenotypic profiles of hepatic MNLCs isolated from non-pregnant mice or from pregnant mice treated with saline or PGM-Zn. Mean ± standard errors (n = 5) are given. *p<0.05; **p<0.01.

and lymphatic blastogenesis and induce the appearance of peritoneal macrophages with suppressive properties that have been described in our previous reports (Radosevic-Stasic et al., 1995; Mrakovcic-Sutic et al., 2002).

Taken together, our data imply that ER-resident gp96 participates in the regulation of morphostasis and proteostasis in the placental bed and in the maintenance of balance between the protective and aggressive autoreactive clones in maternal immune system. Moreover, supporting the concept of "stress-induced self recognition" (Bauer et al., 1999; Cerwenka and Lanier, 2003; Raulet, 2003; González et al., 2008; Lopez-Larrea et al., 2008) and the "danger theory of immunity" (Gallucci and Matzinger, 2001) our data suggest that in pregnancy complicated by infection the greater ER-stress and the combination of signals activated by DAMP and PAMS have high impact on the activation of local and systemic innate and adaptive immune system in the mother.

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