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Hepatic stellate cells (HSC) cell line (LX-2) and Toll-like receptors – phenotypic/mRNA expression and *in vitro* functional studies

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Abstract

Background: *HSC constitute a small portion of liver cells, but fulfill significant functions. LX-2 cells, a human cell line of HSC, are a model for the study of HSC. HSC express Toll-like receptors (TLRs) and some of them participate in the promotion of liver fibrosis. Little is known about other TLRs expression and function of HSC. Aims of this study were to search the expression and function of all TLRs known in LX-2 cells.*

Material and methods: *Cells were subjected to immunocytochemistry, cell RNA was searched for mRNA specific for respective TLRs. Cells were treated with anti-TLR1-10 antibodies and flow cytometry (FC). They were cultured with TLR ligands and assessed by FC for TLR expression. Supernatants were examined for cytokines content and cells for the percentage of those in a S-phase of cell cycle.*

Results: *All TLRs, both, protein and mRNA were expressed in these cells. TLR ligands had negligible effect on TLR expression. Culture of LX-2 with ligands resulted in the secretion of some cytokines, but did not increase the number of cells in S-phase.*

Conclusions: *To conclude, LX-2 cells express all known TLRs. Their activation has only modest effect on their function.*

Key words: *hepatic stellate cells, LX-2 cells, Toll-like receptors, TLR function.*

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Introduction

Hepatic stellate cells are considered to play a pivotal role in the formation of hepatic fibrosis. They may be traced in the liver by means of several techniques. HSC are located in the subendothelial compartment, between hepatocytes and sinusoidal endothelial cells and comprise about 15% of nonparenchymal resident cells in normal liver [1]. They are stimulated *in vivo* during chronic liver disease by profibrotic cytokines. HSC under influence of the above mentioned cytokines transform into large matrix – producing myofibroblasts. Some other cytokines play anti-fibrotic role in the liver. Several inflammatory cell subsets are usually seen in the sections of liver in the course of various types of hepatitis prior the induction fibrogenesis [2]. It has been shown that some of them and in particular CD8+ T lymphocytes and NK cells have a regulatory and monitoring role for profibrotic and anti-fibrotic effects on HSC respectively [3, 4]. On the other hand, it is known that some other factors, apart from cytokines and inflammatory cells may induce liver fibrosis, as it happens in metabolic and toxic liver injuries. HSC were recently shown to act as the regulatory cells due to the secretion of vitamin A metabolite, retinoic acid (RA). The latter promotes TGF-β dependent induction of Treg cells and inhibits maturation of Th17 ones [5].

HSC are known to express Toll-like receptors (TLRs), such as TLR2, TLR4 and TLR9. Some of them appear to trigger liver fibrosis. TLR4 was found to stimulate TGF-β signaling, the cytokine well known for its fibrogenic activity [6]. Hepatitis C HCV core protein may stimulate HSC via TLR2, resulting in the secretion of profibrogenic proteins such as procollagen type 1a and others [7].

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Apoptotic hepatocyte's DNA inhibits HSC chemotaxis via TLR9, what has been linked to the upregulation of collagen production by these cells [8]. Exposure of murine HSC to ligands of Toll-like receptors (TLRs) such as LPS or lipoteichoic acid, triggered the release a variety of proinflammatory cytokines apparently involved in the liver damage [9].

The aim of the current study was to aquire some more data about the expression and function of TLRs known, in human HSC cell line. We also wished to search LX-2 response to TLRs respective ligands in *in vitro* culture. This might provide some clue, whether TLR ligands might be used to control unwanted effects of HSC *in vivo*.

Material and methods

LX-2 cell line

The cells were generously provided by dr R. Safadi. Cells were cultured in RPMI 1640 medium with HEPES and L-Glutamin supplemented with 10% heat-inactivated fetal bovine serum- FBS and Pen/Strep/Fungizone 10K/10K/25 µg. The cell line was maintained in a humidified incubator at 37°C in a 5% CO_2 environment. Cells were collected at > 90% confluence by 0.25% trypsin with EDTA digestion, centrifuged for 5 min at 1500 rpm, resuspended in RPMI medium and counted using 0.4% trypan blue. Cell viability was 100%. LX-2 cells were cultured in tissue culture flasks with and without FBS.

ABC immunocytochemistry (ICC)

LX-2 cytospin cell sediments were incubated overnight in 37°C with anti-TLR moAbs . After thorough washing in cold PBS, sediments were subjected to ABC ICC (Santa Cruz CA, USA) according to the manufacturer instruction. In short, they were then subjected to biotynylated rabbit anti mouse IgG, thereafter to avidin solution, followed by biotynylated horseradish peroxidase (HRP). After subsequent washing substrate solution composed of hydrogen peroxide and diaminobenzidine (DAB) was applied. After washing in tap water cells were counterstained with Meyer hematoxylin. Control cell sediments were incubated with saline instead of primary antibodies.

Flow cytometry

LX-2 cells were subjected to immunofluorescence reaction with fluorochrome labeled anti-TLR Moabs (TLR1-10). For surface staining, cells were incubated with antibodies for 15 min in the dark. For intracellular staining, cells were permeabilized with 0.1% saponine–PBS buffer on ice in the dark. Next, cell suspensions were incubated for 20 min in saponine contained buffer on ice in the dark with anti-TLR Moabs. Following washing with 0.1% saponine-PBS by centrifugation cells were resuspended in 0.1% saponine-PBS and cell acquisition was performed. Samples were acquired by a FACS Canto flow cytometer (Becton Dickinson) and analyzed with FACS Diva Software. In each case, isotypic control was performed. The percentage of positive cells and mean fluorescent intensity (MFI) were assessed. MFI values of isotypic control were subtracted from the values obtained from studied samples.

Assessment of TLR1-10 mRNA

RNA isolation and RT-PCR reaction was carried out similarly to that described elsewhere [10]. GAPDH as a housekeeping gene was used. The sequences of the primers used were those used previously. RT-PCR for studied TLRs genes were performed in 54°C temperature of annealing, except reaction for TLR7 performed in 61°C (RT-PCR reaction for TLR7 performed in 54°C annealing temperature gave no visible line).

Assessment of MFI on resting and stimulated LX-2 cells

Cells were cultured in RPMI medium, either supplemented with 10% FCS or in medium alone over 96 h. A portion of cells was collected in selected time intervals (6, 12, 24, 48 and 96 h), washed and incubated for 30 min with a given anti-TLR MoAb. An aliquot of cells prior the incubation with MoAb was subjected to the action of saponin in order to get cell membrane permeable. Following washing cells undergone an acquisition in flow cytometer as before. MFI was determined as a number of events recorded as a surface or cytoplasmic expression of given TLR respectively.

Expression of TLRs on/in LX-2 cells after incubation with respective TLR ligands

LX-2 cells were cultured in RPMI medium either with the addition of 10% FBS or without it. In either medium respective TLR ligand was added in a quantity recommended by the manufacturer. All TLRs ligands were from Cayla-Invivogen, Toulouse, France. The following ligands were used: LTA-SA (TLR2), *P. gingivalis* LPS (TLR2), Poly (I;C)-LMV (TLR3), *E. coli* LPS (TLR4/2), FLA-BS(TLR5), Imiquimod-R837 (TLR7), ssPolyU/LyoVec™ (TLR8), ODN-2006 (TLR9). After 24 h of culture TLRs ligands were added to LX-2 line. Cells in triplicates, were stimulated with TLR ligands for 24 h. Thereafter cells were released by means of trypsin and counted as above. As a control, cells were cultured in the medium alone with and without FBS for the same time and in the same conditions as cells with TLRs ligands. Following 24 h culture, cells were collected, washed, incubated with respective anti-TLR MoAbs and subjected to an acquisition in BD flow cytometer. Cell MFI and cell percentages were determined both, for surface and cytoplasmic TLR expression in FBS stimulated and unstimulated cells.

Assessment of concentration of the IL-6, IL-8, VEGF and GM-CSF

After incubation with TLRs ligands the IL-6, IL-8, VEGF, GM-CSF content were determined in supernatants from LX-2 cell cultures by BD™ CBA Human Flex Sets tests (BD) as described elsewhere [11]. Briefly, the tested analytes were captured by the special beads, and detection was performed using fluorochrome-labeled antibodies. Acquisition was done in flow cytometer and results were analyzed with FCAP Array™ Software (BD).

Assessment of concentration of the SDF-1a and TGF-b1

The SDF-1α and TGF-β1 content in cell supernatants after stimulation with TLRs ligands were estimated by ELISA assays with a reader at λ = 450 nm. TGF-β1 was activated from latent to active form using the 1 N HCl and 1.2 N NaOH/0.5 M HEPES buffer. As negative control was used RPMI1640 + FBS, and as a blank was used pure RPMI1640.

Determination of S phase of LX-2 cells following culture with ligands

The same ligands were used as in previous experiments. Following culture tumor cells (2×10^6) were resuspended in 0.6 ml of ice cold RPMI 1640 2% FBS containing 1% saponin. After a 30 min incubation, cells were resuspended in 1 ml of PBS containing 10 μg/ml propidium iodide and kept at 4°C for 30 min prior to the measurement. The specimens were subjected to the acquisition in cytometer and the total S phase were analyzed using a FACS Diva software.

Results

Immunocytochemistry (ICC)

LX-2 cell cytospins reacted with TLR antibodies in various way, depending on specificity of antibodies used and the fixation of cell smears. Unfixed specimens manifested worse morphology, but cytoplasmic expression of given TLR was better visible (Fig. 1 A-F). The percent of positive cells ranged from < 10 to < 90 depending on TLR antibody used (results not shown).

Flow cytometry of TLR expression on LX-2 cells

In the case of surface (cell membrane) expressed TLRs, TLR5 predominated, showing almost 100% positive LX-2 cells and MFI = 83042 scores. In the contrary, TLR2 and TLR4 were weakly expressed (TLR2 – 0,8 % positive cells, MFI = 1560, TLR4 – 1,9 %, MFI – 1767). Within TLRs predominantly located in cell cytoplasm, TLR8, 7 and 9 manifested high expression, both, in the percent of positive cells (72,2 – 99,5% respectively) and in MFI (17062 – 101244). Notably, TLR3 has shown relatively modest expression on LX-2 cells (MFI – 4330). There was

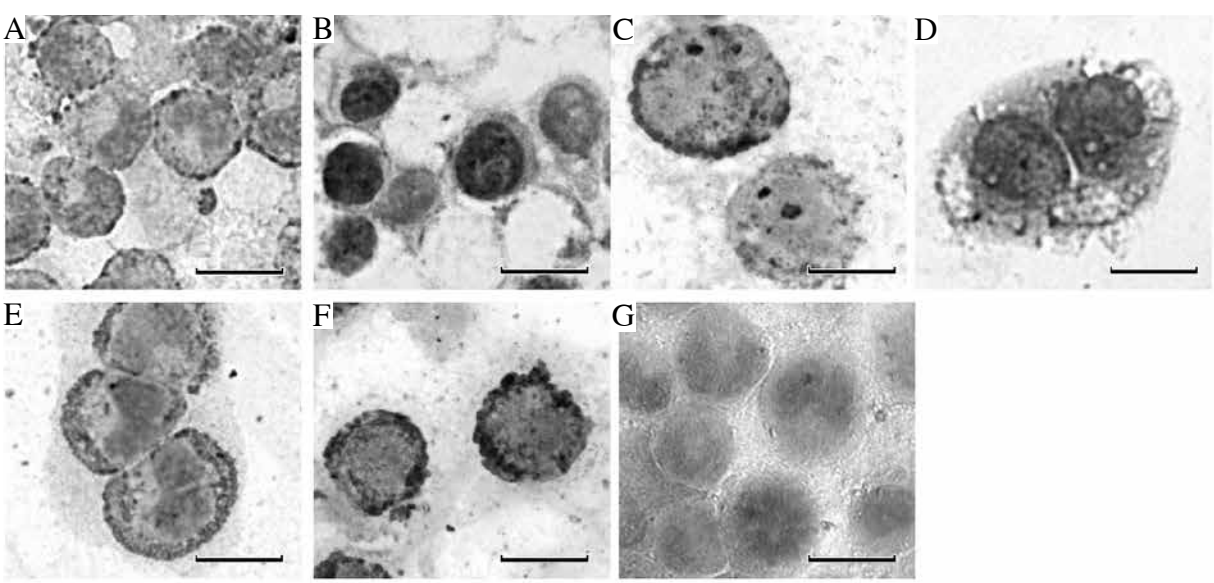

Fig. 1. ABC immunocytochemistry of LX-2 cell smears using anti-TLR Moabs of given specificity: A – anti-TLR 2; Fixed smear. Distinct cell membrane (surface) reaction; B – anti TLR 3; fixed. Cytoplasmic reaction; C – anti TLR 6; fixed. Strong cell surface reaction; D – anti TLR 7, unfixed. Distinct cytoplasmic reaction; E – anti TLR 8, fixed. Mixed surface/cytoplasmic reaction; F – anti TLR 9, unfixed. Mixed cytoplasmic/surface reaction; G – control reaction. PBS instead of primary antibody. No positive staining. Bar in all = 50 μm

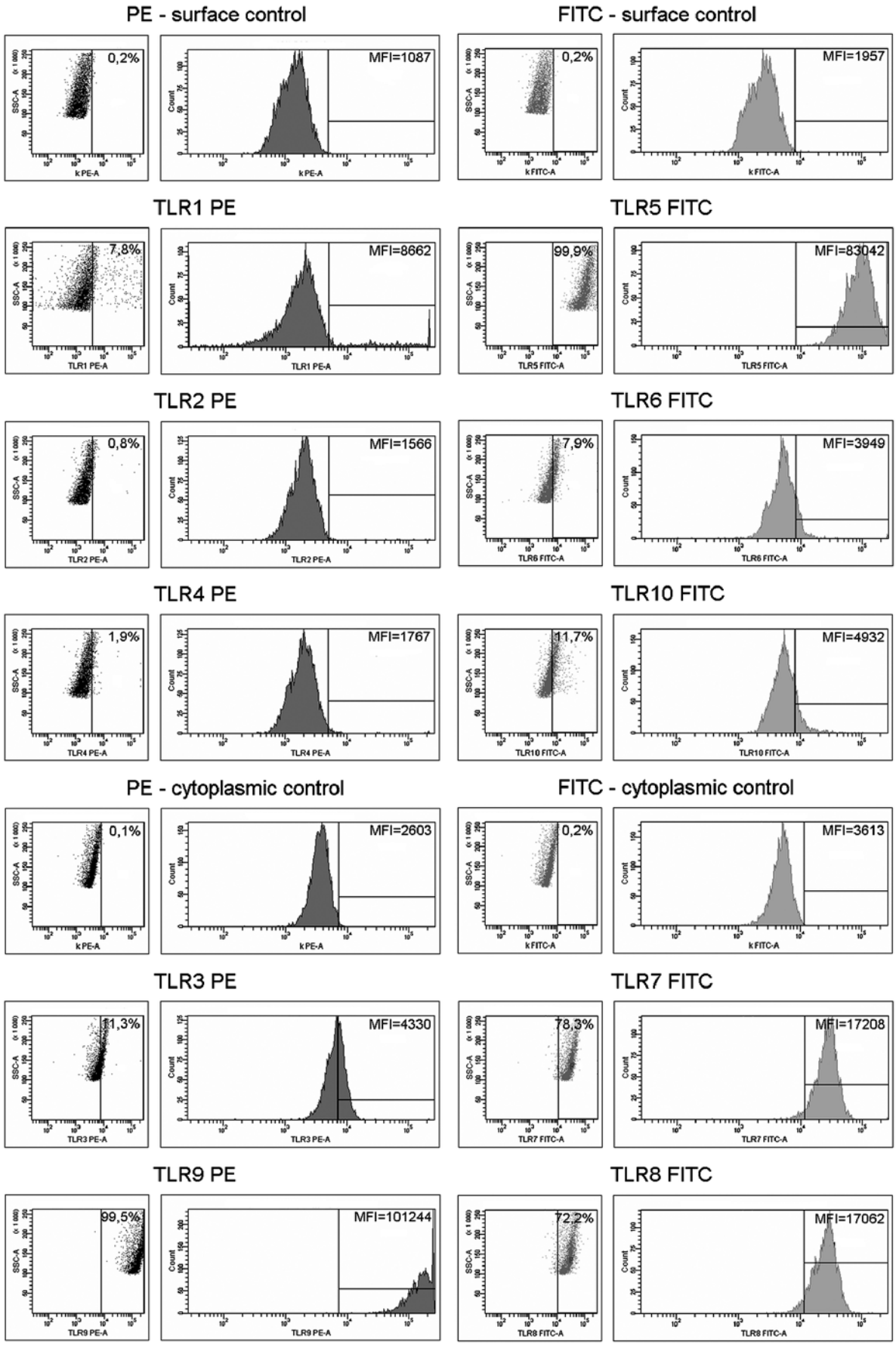

Fig. 2. TLRs expression on LX-2 cells as revealed by flow cytometry

usually the correlation between percent value and MFI of given TLRs (Fig. 2).

Assessment of TLR gene expression – RT-PCR studies

All TLRs mRNAs tested were expressed in LX-2 cell line. The expression differed depending on the TLR examined. Bands of some RT-PCR were much weaker as compared to others. They included those of TLR-2, TLR-4 and TLR-8 mRNA. RT-PCR for TLR7 mRNA had to be performed in higher temperature (61°C) of annealing (see Materials and methods), what resulted in visible reaction (Fig. 3).

TLR surface and intracellular MFI expression of LX-2 cells cultured from 6 to 96 hrs in medium with FBS and medium alone

The pattern of TLR expression on LX-2 cells was heterogeneous, distinct for each TLR examined and related to the time interval tested. MFI scores for both surface and cytoplasmic fluorescence of given TLR were assessed in the same range of scores. In general, TLRs cytoplasmic expression was much stronger than surface one. Moreover, one can notice that some TLRs expression has been within low MFI values ie. 4000-7000 scores (TLR2, 3, 4 and 10) while other, such as TLR5, 6, 7, 8 and 9) required much higher range (up to 40.000 scores). Differences in the expression between cell culture in a medium with 10% FBS and that without FBS were not apparent in the former group, while in the latter the expression of TLR in cells cultured in FBS+ medium prevailed over FBS- one. The maximal cytoplasmic TLR expression within various time intervals was distinct for each TLR tested, being the shortest for TLR1 (6 h) and longest for TLR4 and 8 (96 h) (Fig. 4).

Cells were cultured for 24 h in triplicates in RPMI medium without FBS, containing 10% FBS, and appropriate TLR ligand. The list of ligands is depicted in the Table 3. Following culture cells were washed, subjected to respective TLR Moab and evaluated by flow cytometry. In general, the culture with ligand resulted in lower MFI expression than in medium alone or with FBS added. ODN2006, TLR9 ligand was the only one, that provided slightly higher MFI value of LX2 cells as compared to medium alone. In all other cases MFI of ligand-treated cells was either equal with control ones or 2-3 times lower. When MFI of TLR cell surface expression was compared to intracellular one of cells treated with saponin, the differences were insignificant with a tendency to higher MFI in saponin treated cells (Results not shown).

Induction of secretion of selected cytokines by LX-2 cells cultured with appropriate ligands

Following 24 h culture of LX-2 cells in the presence of TLR ligands as those used in previous experiments, cell supernatants were collected and screened for some cytokines. The only cytokine, or rather chemokine, produced by LX2 cells in reasonable amounts (up to 800 pg/ml) was stromal cell-derived factor-1 (SDF-1a also known as CXCL12). Its production however appeared to be constitutive feature of LX-2 cells, because it was also secreted by cells cultured without any TLR ligands or even without FBS in medium. Another cytokine, being also a chemokine, that was secreted by cells in tiny but measurable range (53 pg/ml) was IL-8. It was evident, when cells were cultured in medium supplemented with FLA-BS, ligand of TLR5. It was not the case, when other TLR ligands were used. Besides, cells cultured in medium FBS+ (without any ligand) secreted TGF-β1, in amounts of 96 pg/ml (Table 1).

Effect of TLR ligands on the cell cycle progression in LX2 cells

Percentage of cells in the S phase was similar in cells treated with various ligands and ranged from 17 to 22. The only highest percent value was obtained, when cells were cultured without ligands but in the medium FBS+. (Results not shown).

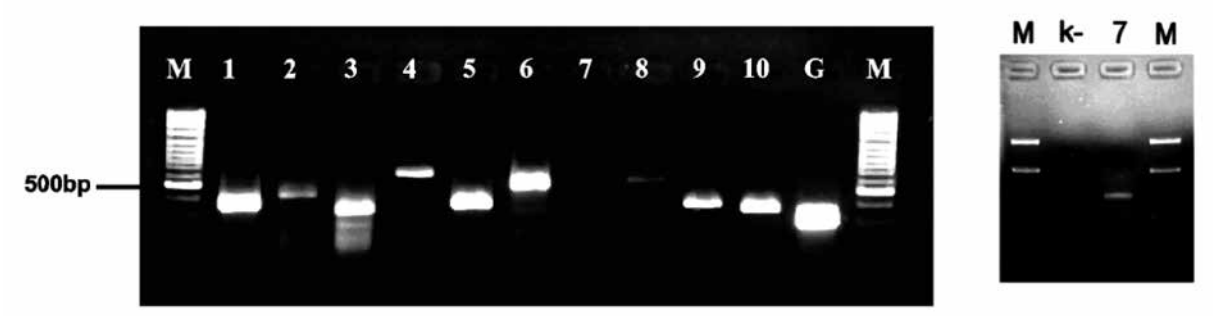

Fig. 3. TLRs 1 – 10 mRNA expression of LX-2 cells

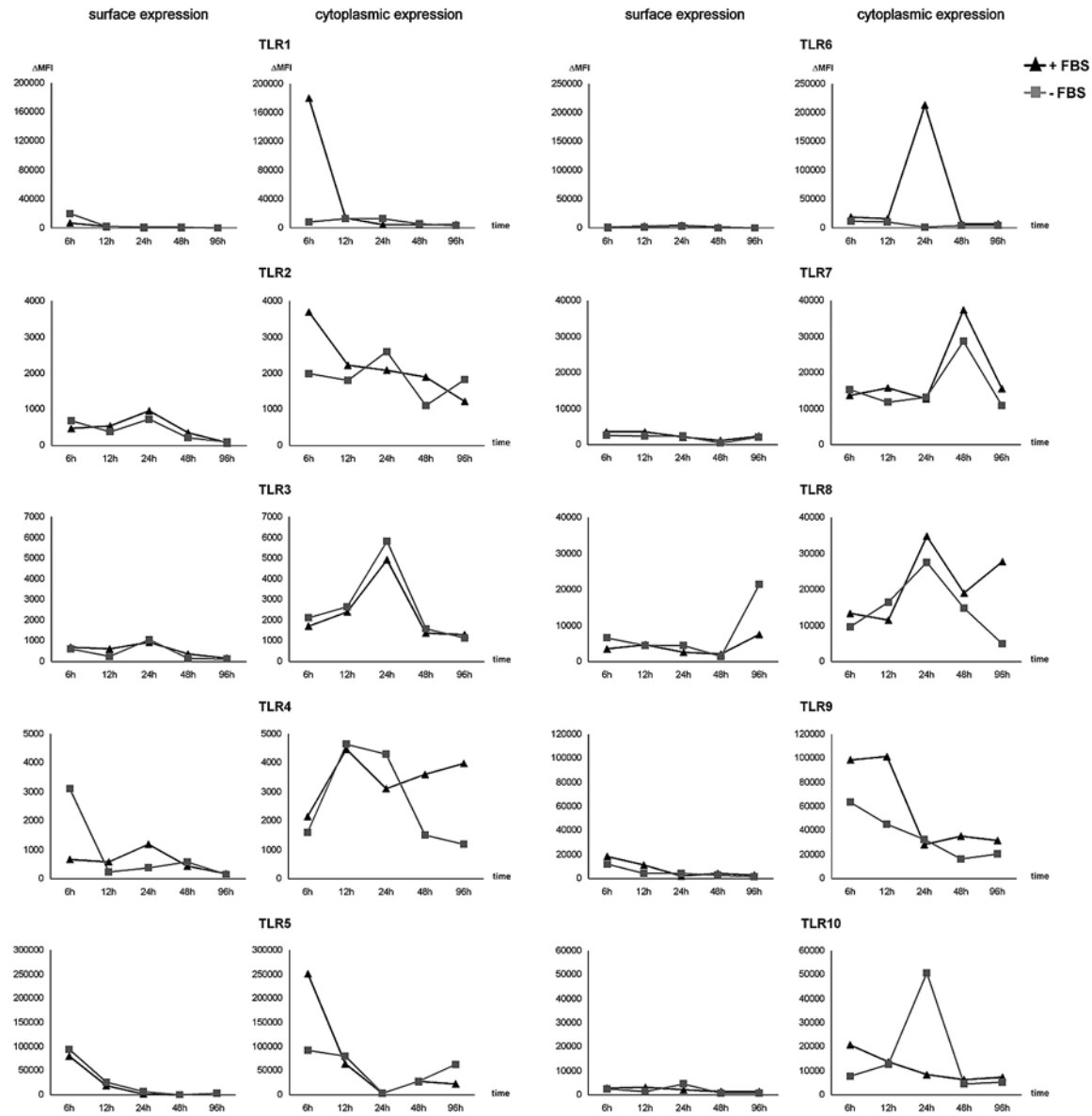

Fig. 4. Mean fluorescent Intensity (MFI) of LX2 cells cultured with or without FBS in various time intervals (6, 12, 24, 48 and 96 h)

Discussion

Results of this study show, that human hepatic stellate cells (HSC), even as established cell line, express practically all Toll-like receptors, both, on a protein and mRNA level. This has been confirmed by various techniques in the current study. Reasons for it are not clear, but it may be related to several functions of HSC in the liver. These cells were found to participate in the liver fibrosis, but also to be the source of some cytokines, to store vitamin A and even to behave as antigen-presenting cells – APCs [12]. Activated HSC are able to mediate the differentiation of macrophages [13]. Such a large array of functions certainly requires the means of contact within liver microenvironment, what may be performed by TLRs. Besides, these cells, in similar way as hepatocytes, are presumably in touch with the antigens of dietary proteins and their microbial contaminants, what may well explain the expression of TLRs sensing pathogen associated molecular patterns – PAMPs. Bacterial products such as Gram-negative li-

Table 1. Quantities of cytokines in culture supernatants of LX-2 cells cultured with TLR ligands

Ligands	TGF-β1		SDF1-α		IL-6		IL-8		VEGF		GM-CSF	
	pg/ml	SD	pg/ml	SD	pg/ml	SD	pg/ml	SD	pg/ml	SD	pg/ml	SD
– FBS	0.0	0.0	816.7	598.3	0.0	–	14.7	12.8	6.2	5.4	0.0	0.0
+ FBS	96.0	121.9	680.6	490.8	0.0	–	20.9	18.2	5.3	4.6	0.4	0.6
LTA-SA	0.0	0.0	645.9	334.8	0.0	–	8.4	7.5	7.3	7.2	0.0	0.0
LPS P. ging.	0.0	0.0	711.6	630.1	0.0	–	8.9	7.7	3.5	3.0	0.0	0.0
poli I:C	7.4	8.2	597.1	339.4	0.0	–	16.7	15.4	5.3	5.5	0.0	0.0
LPS E. coli	0.0	0.0	427.4	199.8	0.0	–	12.7	11.3	7.0	6.2	0.0	0.0
FLA-BS	0.0	0.0	634.9	18.9	0.0	–	52.8	21.5	11.5	12.7	1.4	0.2
Imiquimod	4.1	7.1	534.0	106.8	0.0	–	9.1	7.9	3.2	2.8	0.5	0.7
ssPolyU	0.0	0.0	585.5	201.5	0.0	–	17.3	15.2	7.2	6.2	0.0	0.0
ODN2006	0.0	0.0	607.9	–	0.0	–	6.9	9.7	0.0	0.0	0.0	-

popolysaccharide (LPS) and Gram-negative peptidoglycan (PGN) induce proinflammatory reaction via TLR4 and TLR2 respectively, in activated hepatic stellate cells, what promotes liver fibrosis [14, 15]. HSC express TLR9 that has been shown to be activated by CpG motifs present in bacterial DNA [16]. It has been shown that DNA from apoptotic hepatocytes resulted in the up-regulation of transforming growth factor-β1 (TGF-β1) and collagen mRNA in LX2 cells. It could be blocked by TLR9 antagonists [8]. It suggest significant role of HSC associated TLRs in various metabolic processes of liver.

The major progress in study of HSC has been the generation of established cell lines of HSC, namely LX-1 and LX-2 in prof. LS Friedman laboratory. Both these cell lines express crucial receptors involved in hepatic fibrosis and matrix remodeling such as platelet derived growth factor, matrix metalloproteinase (MMP-2), or tissue inhibitor of matrix metalloproteinase (TIMP)-2 [17]. It has been also recently claimed that LX-2 cell line exhibits features of bone marrow-derived mesenchymal stem cells [18], what may well explain HSC versatility. Moreover, HSC were shown to promote the differentiation of embryonic endodermal stem cells into hepatic progenitor ones [19]. The expression of TLRs on LX-2 cells was not however studied by these authors.

In the current study, for quantitative data about the expression of TLRs on LX-2 cells, flow cytometry was performed on cell suspensions, using fluorochrome-labelled anti-TLR Moabs. It pemitted to obtain not only the percent of respective TLR positive cells, but also to get the mean number of given TLR molecules on cell surface, so called Mean Fluorescent Intensity (MFI). The latter was shown to be distinct for various TLRs tested, but TLR7, 8 and TLR9 appeared to express highest MFI on tested cells. This may be the reflection of the ability of these cells acquired in an evolution, to sense viral infections commonly affecting liver, as these aforementioned TLRs "see" viral nucleic acids.

In order to obtain some functional data of LX-2, the cells have undergone a short time culture in various time intervals (6-96 h) with and without fetal bovine serum (FBS), known to be LX-2 stimulant. Again, MFI both surface-bound and intracellular were determined. Individual TLR expression on LX-2 cells during 96 hrs of culture was uneven, distinct for each TLR. In general, FBS+ cells expressed more frequently high MFI values, while cytoplasmic MFI was usually higher than surface one. It suggests, that FBS cell stimulation enhances TLR expression and that the cells may absorb TLR molecules to cell interior. Such phenomenon was observed by us previously, in the case laryngeal carcinoma cell lines [10].

In the next step we wished to learn, what would be the effect of coculture of FBS – negative LX-2 cells with respective TLR ligands on TLR-MFI expression. Unexpectedly, after 24 hrs culture with almost every ligand tested, MFI of the cells was lower as compared to culture with medium alone. The only ligand that resulted in the rise of MFI was ODN 2006, TLR9 one. TLR9 is intracellular and senses viral dsDNA, what again hints for viral susceptibility of LX-2 cells. The lack of reactivity of cells with other ligands is obscure. We have tested only one ligand for given TLR, what does not exclude that other TLR ligands might be more reactive. HSC hyporesponsiveness to TLR ligands was, however, found to correspond to liver dysfunction in chronic viral hepatitis C infection [20].

The culture supernatants from previous experiment were collected and searched quantitatively for the content of six cytokines. Majority of them was not detected and only SDF1a and to lesser degree IL-8 have shown measurable values. Secretion of IL-8 was however, induced exclusively by flagellin, TLR5 ligand. This finding remains obscure, but it is of interest, that surface expression TLR5 was very high on LX-2 cells (Fig. 2). It is worth to mention that cells cultured in medium with FBS alone secreted TGF-β1, while those without FBS did not produce this cytokine. It is in line with well known function of HSC *in vivo* and their role in liver fibrosis. Secretion of SDF-1, stromal cell-derived factor by LX-2 cells deserves special comment. This is a small cytokine belonging to chemokine family (CXCL12), known to be induced by proinflammatory agents such as LPS or some cytokines of these functional features such as IL-1, IL-6 and TNF. Its almost exclusive secretion by LX-2 cells following coculture with TLR ligands tested, implies that this cytokine may be involved in pathogenic events occurring in the liver. It has been shown that SDF-1 is chemotactic for lymphocytes, is active in angiogenesis and plays a role in the recruiting macrophages . All these are relevant in liver pathology [21, 22].

Finally, the attempt was made to see if the coculture with TLR ligands might influence LX-2 cells to enter into S-phase of the cell cycle. It turned out to be not the case and only the culture with FBS resulted in a slight increase of percent of cells in S-phase. It suggests that TLR ligands are not mitogenic for the cells in question.

By and large, these data suggest that TLRs expression in/on LX-2 cells has only limited effect on cell function, but their universal expression on LX-2 hints for other, so far unknown roles of the cells studied.

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