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# Crosstalk between different family members: IL27 recapitulates IFNγ responses in HCC cells, but is inhibited by IL6-type cytokines

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Interleukin-27 (IL27) is a type-I-cytokine of the IL6/IL12 family predominantly secreted by activated macrophages and dendritic cells. In the liver, IL27 expression was observed to be upregulated in patients with hepatitis B, and sera of hepatocellular carcinoma (HCC) patients contain significantly elevated levels of IL27 compared to healthy controls or patients with hepatitis and/or liver cirrhosis. In this study, we show that IL27 induces STAT1 and STAT3 phosphorylation in 5 HCC lines and 3 different types of non-transformed liver cells. We were especially interested in the relevance of the IL27-induced STAT3 activation in liver cells. Thus, we compared the IL27 responses with those induced by IFNγ (STAT1-dominated response) or IL6-type cytokines (IL6, hyper-IL6 (hy-IL6) or OSM) (STAT3-dominated response) by microarray analysis and find that in HCC cells, IL27 induces an IFNγ-like, STAT1-dependent transcriptional response, but we do not find an effective STAT3-dependent response. Validation experiments corroborate the finding from the microarray evaluation. Interestingly, the availability of STAT1 seems critical in the shaping of the IL27 response, as the siRNA knock-down of STAT1 revealed the ability of IL27 to induce the acute-phase protein γ-fibrinogen, a typical IL6 family characteristic. Moreover, we describe a crosstalk between the signaling of IL6-type cytokines and IL27: responses to the gp130-engaging cytokine IL27 (but not those to IFNs) can be inhibited by IL6-type cytokine pre-stimulation, likely by a SOCS3-mediated mechanism. Thus, IL27 recapitulates IFNγ responses in liver cells, but differs from IFNγ by its sensitivity to SOCS3 inhibition.

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## 1. Introduction

The primary source of Interleukin-27 (IL27) - a type-I-cytokine - are activated macrophages and dendritic cells. Best described are the effects of IL27 on T-cells and on innate immune cells [1,2]. Although IL27 can have pro-inflammatory effects, other studies also suggest that IL27 is immunosuppressive [3,4]. IL27 was shown to promote TH1 responses via the induction of the transcription factors T-bet, upregulation of IL12Rβ2 and of interferon-γ (IFNγ) production [5–8]. Suppression of the TH2 responses was also reported [7]. However, IL27 is also capable to suppress both TH1 and TH2 responses during infection with a variety of pathogens [9–11]. To date, most studies have focused on the effects of IL27 on immune cells, whereas the action on other cell types is less well studied.

IL27 can be grouped into the IL6/IL12 superfamily of cytokines [5]. The heterodimeric IL27, composed of the two non-covalently linked subunits p28 and EBI3 (Epstein-Barr virus induced gene 3) [5], is related to the heterodimeric cytokines IL12 and IL23, in which the two subunits are, however, covalently linked. IL27 signals via a receptor complex composed of the IL27-specific receptor chain WSX1 [5] and the common receptor subunit of IL6-type cytokines, gp130 [12]. It is thus also closely related to the IL6-type subfamily of cytokines.

IL27 expression was observed to be upregulated in patients with hepatitis B [13–16]. Hepatocellular carcinoma (HCC) patients contain significantly elevated levels of IL27 compared to healthy controls or patients with hepatitis and/or liver cirrhosis [15,16]. HCC is a type of

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tumour that is immunosensitive and TH1 cytokine detection in patients is associated with a good prognosis, with type I interferons, IFNγ and IL12 being crucial for immune rejection of HCC [17]. We previously reported interferon-γ-like antiviral activity of IL27 in hepatic cells [18, 19]. IL27 inhibits the replication of hepatitis B virus (HBV) and hepatitis C virus (HCV) in HCC cell lines [14,20], induces further interferon expression in HCC cells and dendritic cells (DC) [14] and has been described to be produced by plasmacytoid dendritic cells of the liver [21].

In immune cells, IL27 was reported to promote its cellular responses via STAT1 and STAT3 [7–9,22]. We have previously described phosphorylation of both STAT1 and STAT3 upon IL27 stimulation in hepatic cells, although the responses we observed so far are typically associated with a STAT1 response. Thus, we set out to investigate the IL27-induced STAT3 activation and its downstream targets in HCC cells. We compared the IL27 responses with those induced by IFNγ (STAT1 response) or IL6-type cytokines (IL6, hyper-IL6 (hy-IL6) or OSM) (STAT3 response). We find that in hepatocytes IL27 induces an IFNγ-like, STAT1-dependent transcriptional response, whereas a STAT3 response is not observed. In contrast to responses to IFNγ, however, IL27 responses are inhibited by IL6-type cytokine pre-stimulation, through a SOCS3-mediated mechanism.

## 2. Material and methods

### 2.1. Materials and cell culture

All cells were grown at 37 °C in a water-saturated atmosphere at 5% $CO_2$. HepG2, Hep3B, Huh7, PH5CH8, HLE, HLF cells were maintained in DMEM medium (Sigma) supplemented with 10% fetal bovine serum (PAA), 100 μg/L streptomycin and 60 mg/L penicillin (Lonza) and 25 mM HEPES (Lonza). "Upcyte"-hepatocytes (genetically engineered human primary hepatocytes) were cultured in Upcyte hepatocyte medium as recommended by the supplier (Medicyte). SV40 large T antigen-immortalized normal human liver epithelial cells (THLE2) were cultured in LHC-8 medium supplemented with 70 ng/mL phosphoethanolamine, 5 ng/mL epidermal growth factor, 10% FBS, 100 mg/L streptomycin, 60 mg/L penicillin.

The gp130, STAT1, STAT3 SMARTpool siGENOME siRNAs and the siGENOME non targeting siRNA pool #2 were obtained from Dharmacon. WSX1 silencer select siRNA (s18137 and s18138) and Silencer Select Negative Control no.1 siRNA were purchased from Life Technologies. siRNA reverse transfections were performed using Lipofectamine RNAiMAX (Life Technologies) and 10–20 nM of siRNA according to the manufacturer's protocol. Cells were stimulated 24–48 h after transfection.

Cytokines were used according to their molecular weight, so that stimulation occurs at comparable molar concentrations. Cells were stimulated with 20 ng/mL human recombinant OSM, IL6, IFNγ or IFNα (Peprotech), 50 ng/mL IL27 (R&D systems) or 40 ng/mL of hyper-IL6 (a kind gift of Prof. Stefan Rose-John, University of Kiel, Germany) except if stated otherwise in the figure legends.

### 2.2. Cell lysis and Western blot analysis

Cell lysis was performed as described before [23]. Proteins were separated by SDS-PAGE, followed by electro-blotting onto a PVDF membrane (PVDF-PSQ or PVDF-FL, Millipore). The following antibodies were used: STAT3 (BD Biosciences Cat# 610190, RRID:AB_397589), pSTAT1 (BD Biosciences Cat# 612233, RRID:AB_399556), STAT1 (BD Biosciences Cat# 610116, RRID:AB_397522), pSTAT3 (Santa Cruz Biotechnology Cat# sc-32293, RRID:AB_628412), γfibrinogen (Millipore Cat# 05-760, RRID:AB_11211422), actin (Millipore Cat# MAB1501, RRID:AB_2223041), pSTAT3 (Cell Signaling Technology Cat# 9145S, RRID:AB_561305), TAP2 (Cell Signaling Technology Cat# 12259 RRID:AB_2619687), JunB (Cell Signaling Technology Cat# 3753S, RRID:AB_2130002), Vinculin (Abcam Cat# ab18058, RRID:AB_444215). HRP-conjugated secondary antibodies were purchased from Cell Signaling. Signals were detected using an ECL solution containing 2.5 mM luminol, 2.6 mM hydrogen peroxide, 100 mM Tris/HCl pH 8.8 and 0.2 mM para-coumaric acid [24] and a CCD camera system (BioRad) or an Odyssey Infrared Imaging System (Li-COR Biosciences) using IRDye 680RD anti-rabbit IgG and IRDye 800CW anti-mouse IgG. Quantitation was performed on single channels with the analysis software Image Studio Lite V4.0 provided by LI-COR Biosciences (Lincoln, NE, USA). Briefly, the signals to be quantitated were normalized with respect to the loading control for each lane. Each normalized signal was then divided by the mean of all normalized signals of one membrane to adjust for the possible variation of signal intensity between different membranes (each membrane including one biological replicate). The signal intensity was then represented as % of a specific signal as described in the corresponding figure legends.

### 2.3. Quantitative PCR

Total RNA was extracted using the NucleoSpin RNA II kit (Macherey Nagel) according to the manufacturer's instructions. 1 μg of total RNA was reverse-transcribed with the iScript cDNA synthesis kit (BioRad) in a volume of 20 μL according to the manufacturer's instructions. Real-time PCR detection was carried out on a CFX96 Detection System (BioRad) using 200 ng RNA input in a 10 μL reaction volume, 2 x i*Taq* SYBR Green Supermix (BioRad) and 5 pmol gene-specific primer pairs (SOCS3-F (ATGAGAACTGCCAGGGAATC), SOCS3-R (CCCAGGCTCCACAACCA), WSX1-F (GCATCCTATTCTTGTGGGG), WSX1-R (CACTTTGTGCCTTAGGTGGT), gp130-F (TGAAACTGCTGTGAATGTGG), gp130-R (CATCCTTCCCACCTTCATCT), Eurogentec). Specificity of the qPCR primers was assessed by a post-qPCR melting curve analysis. Cq values for mRNAs were normalized to 3 reference genes: TBP (TATA binding protein), HPRT1 (hypoxanthine phosphoribosyltransferase 1) and PPIA (peptidylprolyl isomerase A). Based on the geometric mean of the three reference genes, a normalization factor was calculated for each sample using geNorm [25], a VBA applet for Microsoft Excel. The relative amount of each target in each sample was then corrected by dividing its amount by the corresponding normalization factor. Fold changes were calculated by dividing the normalized relative amount of treated samples with the normalized relative amount of the control sample.

### 2.4. Reporter gene assays

Cells were reverse transfected on a 96 well plate with 0.1–0.2 μg of the respective reporter gene construct using Lipofectamine LTX&PLUS or Lipofectamine 2000 (Life Technologies) transfection reagent for HepG2 or Huh7 cells, respectively. 24 h after transfection, cells were stimulated for 24 h as described in the figure legends. Supernatants were discarded and cells were lysed using 20 μL lysis buffer. Lysis and luciferase assay was carried out as described [26]. Experiments were performed at least in biological triplicates, each with 3 technical replicates. The STAT3-specific luciferase reporters, pXP2d2-rat pancreatitis-associated protein 1 (rPAP1) [27] and pGL3-Hepcidin [18] were described earlier. The pGAS-TA-luc and pISRE-TA-luc plasmids were from Clontech. pcDNA5-FRT-TO-V5-SOCS3 (pcDNA5-SOCS3) was generated by inserting PCR amplified SOCS3 into pcDNA5-FRT-TO-V5 using *Bam*HI and *Nhe*I restriction sites. Empty pcDNA5-FRT-TO-V5 was used as negative control.

### 2.5. Flow cytometry

Cells were re-suspended in cold PBS supplemented with 5% FBS and 0.1% sodium azide (PBS/azide) and incubated with anti-HLA-ABC-PE (MHC1, Immunotools, Cat# 21159034, RRID: AB_2629494), anti-gp130 (BD Biosciences Cat#555757, RRID: AB_396098), anti-WSX-1 (R and D Systems Cat# FAB14791P, RRID:AB_10718687) or the corresponding IgG control antibody for 1 h at 4 °C. Cells were then washed

twice with PBS/azide and analyzed on a FACSCanto II flow cytometer using FACSDiva (BD Biosciences) software. Overlays were created using FlowJo software.

### 2.6. Whole genome microarray analysis

HepG2, Hep3B, Huh7, HepG2-shScr and HepG2-shSTAT3 cells were left untreated or were stimulated with hyper-IL6, OSM, IFNγ or IL27 for 24 h. Total RNA was extracted using the using the miRNeasy kit (Qiagen). For each treatment, two independent biological replicates were analyzed. Only high quality RNAs were used with a ribosomal RNA ratio >1.9 and no evidence of degradation, as evaluated using the Agilent Bioanalyzer 2100 RNA 6000 nanoassay. All samples had a RIN number >8. Gene expression profiling experiments were performed using the Affymetrix HTA v.2.0 arrays according to the GeneChip® WT PLUS Reagent Kit, Manual Target Preparation for GeneChip® Whole Transcript (WT) Expression Arrays P/N 703174 Rev.2 2013 protocol. 100 ng of total RNA was used as starting amount for microarray experiments.

Microarray data were normalized and summarized to gene level by RMA [28] algorithm with GC correction in Partek® Genomics Suite® software, v. 6.6. In order to remove uninformative features, only genes with a $\log_2$ expression level above 5 in at least one sample were considered for further analysis. Statistical analysis was performed in R [29] and using linear models in an empirical Bayesian approach implemented in the R/Bioconductor package *limma* [30]. A global linear model accounting for cell lines (HepG2, Huh7, Hep3B or HepG2-shScr, HepG2-shSTAT3) and treatments (IL27, IFNγ, hy-IL6, OSM) was fit, and then *p*-values were estimated for various contrasts. P-values were adjusted for multiple comparison by Benjamin-Hochberg's FDR method [31] and genes with FDR < 0.05 were selected. Volcano plots and heatmaps were drawn using the R package *ggplot2* [32].

### 2.7. Statistical analysis

Representative data are shown and are expressed as the mean ± standard deviation. For statistical analysis (described in detail for the different figures below) the error probabilities <0.05 were considered to be significant, ***P < 0.001, **P < 0.01, *P < 0.05.

For Fig. 4, one-way ANOVA with Dunnett's post-test was performed using GraphPad InStat version 3.10 (GraphPad Software, San Diego California USA, www.graphpad.com).

To test for statistical significance in Fig. 6B we used two-samples *t*-tests to compare all conditions. To adjust for unequal variances, the Welch correction was applied. For 9 data points per sample, the expected number of degrees of freedom is 16, the Welch corrected degrees of freedom (df) are reported below. Finally, correction for multiple testing was done using the *p.adjust* R base function with default parameters, *i.e.* Benjamini & Hochberg method. Computation was performed using *R* [29] with the packages *tidyverse* [33] and *broom* [34]. Luciferase activity is significantly upregulated upon IL27 stimulation in all conditions and cell lines (P < 0.001). This is not indicated in Fig. 6B. On the other hand, the analysis shows that the reduction of luciferase activity due the SOCS3 expression is highly significant compared to the empty vector control. The results of this latter analysis are indicated in Fig. 6B.

## 3. Results

### 3.1. In HCC cell lines and hepatocytes, IL27 induces a STAT1/STAT3 phosphorylation pattern which is more similar to responses generated by interferons than to those generated by IL6-type cytokines

Since it is unclear if the STAT3 phosphorylation elicited by IL27 leads to an upregulation of proteins associated with a STAT3 transcriptional response in hepatocytes, we set out to investigate this potential activity of STAT3 upon IL27 stimulation.

We began by assessing the phosphorylation of STAT factors activated by the cytokines IL6, hyper-IL6 (hy-IL6), OSM, IL27, IFNγ and IFNα by comparative Western blot analyses. The 5 hepatocellular carcinoma (HCC) cell lines (HepG2, Hep3B, Huh7, HLE, HLF) and the non-cancer immortalised hepatocytes (PH5CH8, THLE2, hepatocytes (Upcyte)) all reacted to IL27 with a robust phosphorylation of STAT1 and a phosphorylation of STAT3, the latter being only slightly over background for some of the cell lines (Fig. 1 A/B and Supplemental Fig. 1A). Some of the hepatic cell lines were unresponsive or only weakly responsive to IL6. Since stimulation with hyper-IL6 (hy-IL6), a synthetic fusion protein of IL6 and the soluble form of the IL6Rα (gp80) mimicking IL6/sgp80 responses, restored STAT signalling in the IL6-unresponsive cell lines, we conclude that the IL6 unresponsiveness is due to low expression of the membrane-bound form of the IL6Rα (Supplemental Fig. 1A). Hep3B cells did not respond to OSM stimulation (Fig. 1A). This was due to the absence of the OSM-specific signalling receptor OSMR, as we could show by flow cytometry and as was described by others already [35]. The Jak/STAT signalling pathway was induced in all tested cells stimulated with IFNγ and IFNα.

In general, a qualitative comparison of the signals induced by the different cytokines shows that STAT1 phosphorylation by IL27 is robust, comparable to IFNγ and IFNα which are strong STAT1 activators. On the other hand, phosphorylation of STAT3 upon IL27 stimulation was generally lower than the one induced by strong STAT3 activators such as IL6, hyper-IL6, or OSM and even generally lower than the one induced by IFNα. In addition, we found STAT1 phosphorylation upon OSM and IL6 to be transient over time, while STAT3 phosphorylation was sustained. For IL27 the observed STAT1 and STAT3 phosphorylation both seemed to be both stable over time (Supplemental Fig. 1B/C). IL27 also induced STAT1 protein expression, as did IFNγ, while IL6 or OSM did not show this response (Fig. 1B and Supplemental Fig. 1B/C). Thus, we conclude that in all hepatic cell lines tested, IL27 induces an interferon-like robust STAT1 phosphorylation while STAT3 seems to be phosphorylated to a lesser extent than by IL6-type cytokines (IL6, hy-IL6, OSM). Nevertheless, the STAT3 phosphorylation response upon IL27 is sustained and may lead to gene transcription.

### 3.2. The cytokine receptor system used by IL27 in hepatic cells also consists of gp130 and WSX1

To check that the receptor system used by IL27 is identical to the one described in the hematopoietic system, we used siRNA against gp130 and WSX1. Knock-down of gp130 or WSX1 in HepG2 cells led to an abrogation of IL27 signalling and prevented STAT1 protein upregulation (Fig. 1C/D). We also investigated cell surface expression of gp130 and WSX1 by flow cytometry on HepG2, Huh7 and Hep3B cells. All cell lines showed expression of the receptors on the cell surface (Fig. 1E). Thus, IL27 is using its canonical receptor system composed of gp130 and WSX1 also in hepatic cell lines.

### 3.3. Whole genome microarray analysis of IL27 responses shows little overlap with responses to IL6-type cytokines but close resemblance to an IFNγ response

Our aim was to compare the IL27 response to those of the other cytokines. For this we first focused our transcriptome analyses on comparing the number of genes specifically regulated by each cytokine alone (IL27, IFNγ, OSM, or hy-IL6) and the number of genes commonly regulated by the different treatments (Fig. 2A and Supplemental Fig. 2). For all three cell lines (HepG2, HuH7, Hep3B) there is a minor number of genes regulated by all four or at least three of the treatments. These genes, which obviously are STAT-regulated but have no preference for STAT1 or STAT3, were not considered for our analysis. In general, for all cell lines the number of genes regulated commonly by IFNγ and IL27 was very high and few genes were regulated by IL27 only (0–6). In Huh7 cells, for example, where IL27 and IFNγ regulate an approximatively similar number of genes, the overlap between the

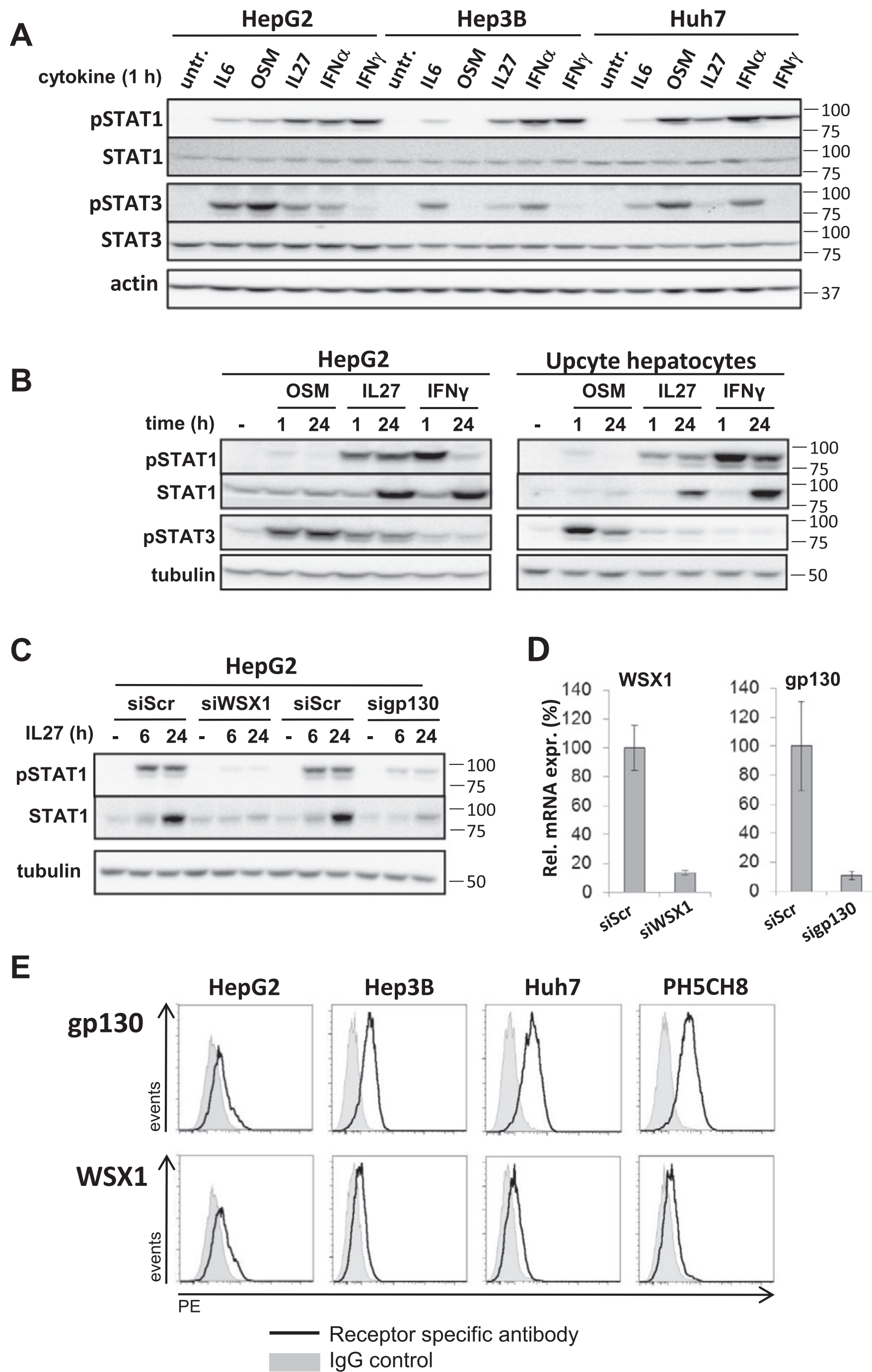


**Fig. 1.** Responses of hepatocellular carcinoma cells and hepatocytes to different cytokine stimuli. A) HepG2, Hep3B and Huh7 hepatocellular carcinoma cell lines were stimulated with cytokines for one hour and lysates were analyzed by Western Blot immunodetection. Phosphorylation of STAT factors (pSTAT3 and pSTAT1), STAT3, STAT1 and actin was monitored. Representative blots of three biological replicates are shown. B) HepG2 and hepatocytes (Upcyte) were stimulated with cytokines as indicated and lysates were analyzed by Western Blot immunodetection for phosphorylation of STAT factors (pSTAT3 and pSTAT1) and changes in STAT1 protein expression. Tubulin detection served as loading control. A representative blot of three biological replicates is shown. C) HepG2 were transfected with siRNA against gp130 or WSX1 and the corresponding scrambled control (siScr). Before lysis, cells were stimulated for 6 or 24 h with IL27. Cells were lysed 48h after transfection. The Western blot was probed with antibodies against pSTAT1, STAT1 and tubulin. A representative blot of three biological replicates is shown. D) The efficiency of down-regulation of WSX1 and gp130 mRNA in HepG2 was assessed by quantitative PCR 48h after transfection of the siRNA. Standard deviations are calculated from three biological replicates. E) HepG2, Hep3B and Huh7 were analysed for gp130 and WSX expression by Flow cytometry. Overlays comparing specific antibody versus control antibody shifts are shown.

two conditions comprises 90 genes compared to the number of genes specifically regulated only by IL27 (0) or IFNγ (23) (Fig. 2A). Thus, the IL27 and IFNγ responses seem very similar. On the other hand, there are very few co-regulated genes when comparing IL27 or IFNγ to IL6 and OSM, while IL6 and OSM again co-regulate many genes (Fig. 2A/B, Supplemental Figs. 2 and 3, see also Supplemental Tables 1–4 for lists of regulated genes). Ingenuity pathway analysis confirmed that IFNγ and IL27 activate the same downstream canonical pathways but did not uncover new functions of IL27 that are different from the ones of IFNγ (Data not shown).

Fig. 2C and Supplemental Fig. 4A show volcano plots in which a set of IFNγ-regulated genes (in blue) and IL6-regulated genes (in red) are highlighted. The list of validated genes is based on previously published studies and is shown in Supplemental Fig. 4B/C [36–41]. The volcano

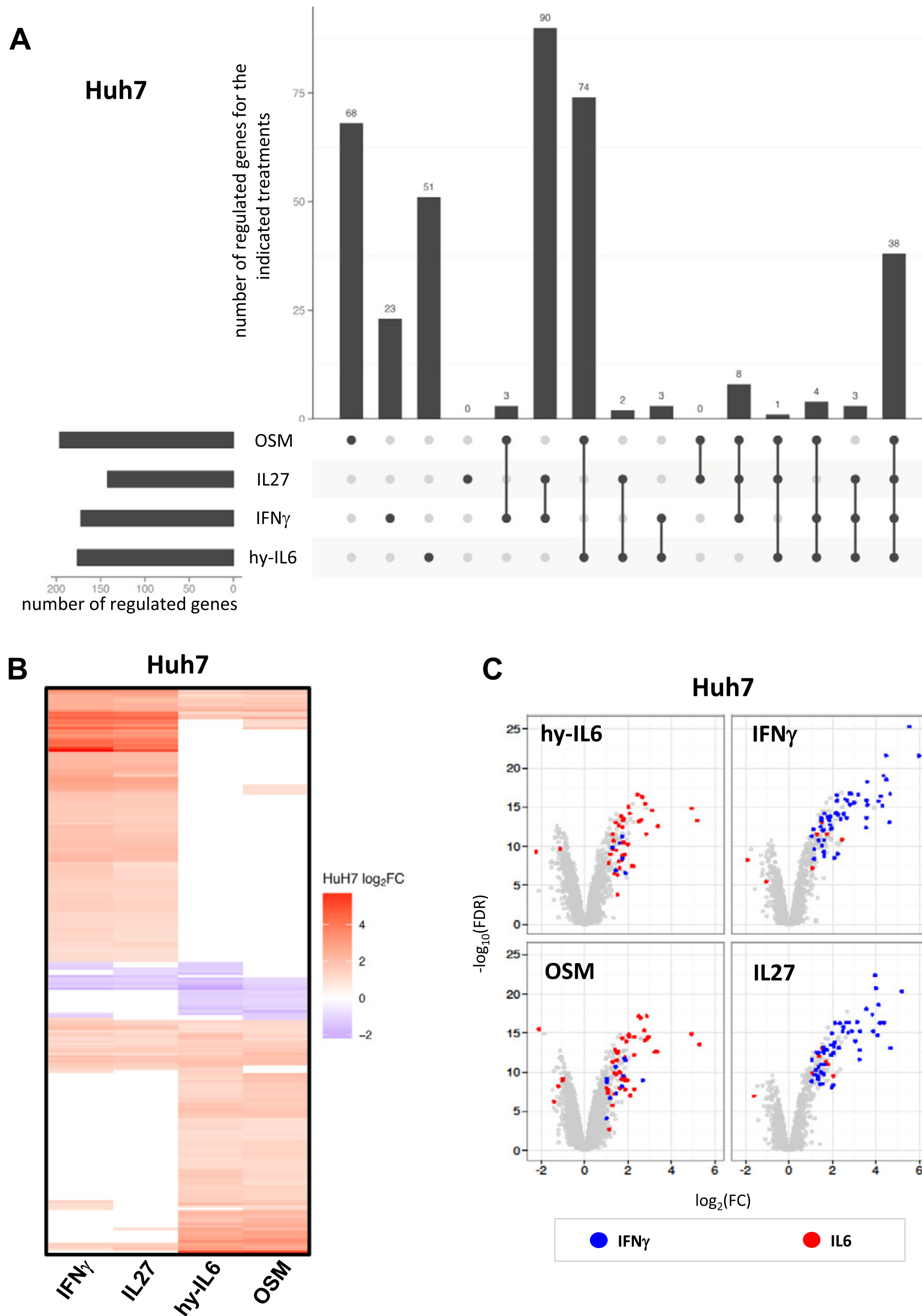


**Fig. 2.** Microarray analysis of hy-IL6, OSM, IL27 and IFNγ responses in Huh7 cells. A) Graph showing the number of differentially expressed genes upon cytokine treatment, as well as the number of differentially expressed genes that are unique for each cytokine, or common for two or more cytokines. The horizontal bars show the number of differentially expressed genes (|logFC|≥1, p-value <0.05) upon OSM, IL27, IFNγ, and hy-IL6 stimulation, as compared to the non-stimulated control. The vertical bars indicate the number of genes that are uniquely regulated by each cytokine (●), and the number of genes that are regulated by two (●–●), three (●–●–●), or all four cytokines (●–●–●–●), and that for all cytokine combinations. As an example, 68 genes are only regulated by OSM, 0 genes are regulated by IL27 alone, and 38 genes are regulated by all four cytokines. B) A heatmap summarizing the microarray analysis for Huh7. Shown are genes that are differentially regulated by at least two of the four cytokines (logFC|≥1, p-value <0.05). C) Volcano plots showing the regulation of a set of validated IL6 (red) and IFNγ (blue) target genes, upon OSM, IL27, IFNγ, and hy-IL6 treatment in Huh7.

plots again show that the IL27 response is very similar to the one of IFNγ, while it shows little resemblance to the one of IL6 or OSM.

### 3.4. On the protein level, IL27 stimulation initiates an IFNγ-like response in HCC cell lines and hepatocytes

A number of STAT1-dependent gene readouts were validated to be upregulated upon IL27 stimulation on the protein level in a number of HCC cell lines (HepG2, Hep3B, Huh7) and in immortalised hepatocytes (hepatocytes from Upcyte, PH5CH8). STAT1, Tap2 and MHCI were clearly upregulated upon IL27 and IFNγ treatment, while they were not regulated by OSM (Fig. 3A and B).

### 3.5. IL27 stimulation does not lead to STAT3-dependent responses in HCC cells

We next investigated the expression of STAT3-dependent genes upon IL27 stimulation in HepG2 cells. IFNγ or hy-IL6 stimulation was

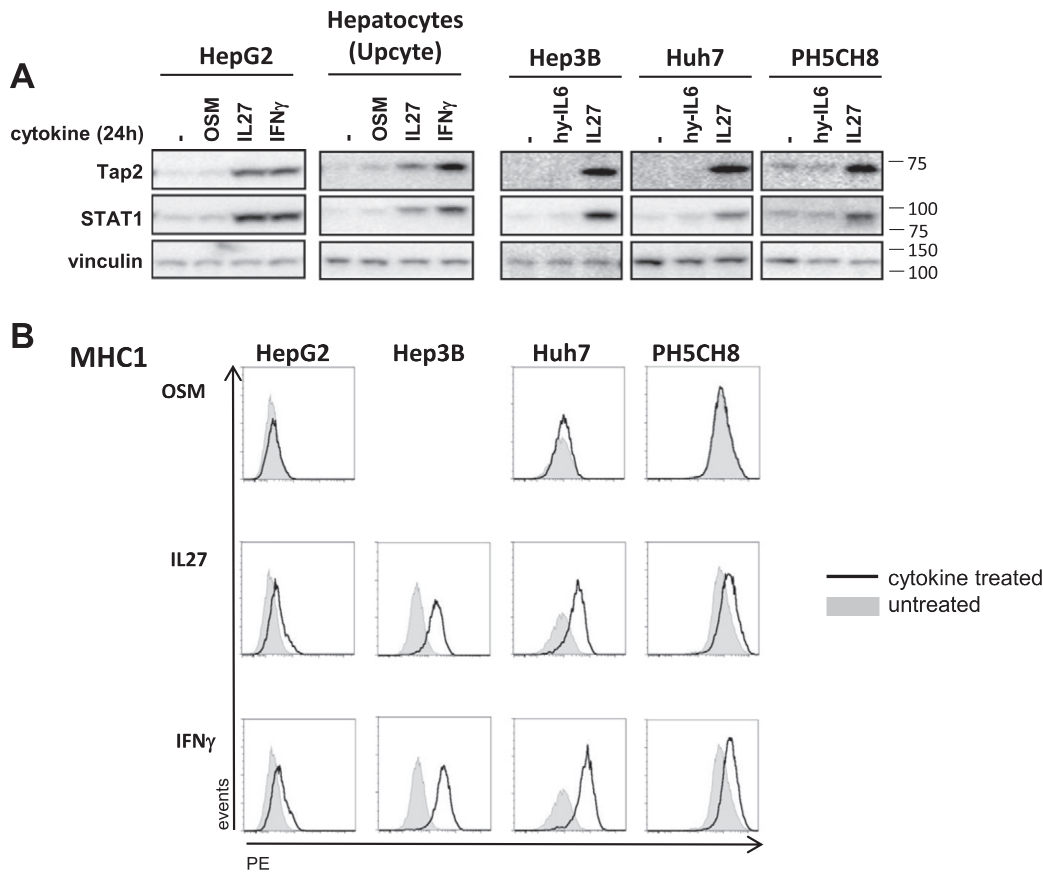


**Fig. 3.** IL27 stimulation of HCC cells and hepatocytes leads to an IFNγ-like response. A) A panel of HCC cells and hepatocytes was stimulated for 24h with OSM, IL27 or IFNγ or left untreated. Lysates were analyzed by Western blot immunodetection using antibodies against the STAT1 target genes Tap2 and STAT1. Vinculin detection was performed to show equal loading. Representative blots of three biological replicates are shown. B) Hepatic cell lines were stimulated for 24h with OSM, IL27 or IFNγ or left untreated. STAT1-dependent enhanced surface expression of MHCI was investigated by flow cytometry after incubation of the cells with a PE-labeled anti-MHCI antibody. Representative results of three biological replicates are shown.

used as negative or positive control, respectively. The STAT3-dependent genes γ-fibrinogen and JunB were upregulated at the protein level upon hy-IL6 stimulation but not upon IL27 or IFNγ stimulation (Fig. 4A). SOCS3 mRNA was only slightly and transiently upregulated upon IL27 and IFNγ stimulation, while a much stronger and prolonged upregulation became evident for the known SOCS3 regulators hy-IL6 and OSM (Fig. 4B). In addition, IL27 stimulation did not induce expression of γ-fibrinogen in HepG2, Hep3B or Huh7 cells, in contrast to hy-IL6 treatment. IL27 on the other hand induced expression STAT1, a known auto-regulated STAT1-dependent gene which was not induced by hy-IL6 (Fig. 4C).

To further investigate whether IL27 can induce transcriptionally active STAT3 homo-dimeric complexes we used two reporter gene constructs. Both show an efficient induction upon STAT3 homodimer binding and can be used as STAT3 sensitive probes. HepG2 and Huh7 cells were transfected with reporter plasmids containing the STAT3-dependent promotors of rPAP1 or hepcidin and were then stimulated with cytokines. Luciferase activity was strongly induced by OSM and hy-IL6, while IFNγ and IL27 did not significantly induce the reporter genes (Fig. 4D). Thus, we conclude that although IL27 leads to phosphorylation of both STAT1 and STAT3, the protein expression of known STAT3 target genes was not observed and STAT3-specific reporter genes were not induced.

### *3.6. Revealing the hidden IL6-like character of IL27: STAT1 availability orchestrates the IL27 signaling output*

We then investigated the effects of a suppression of STAT1 or STAT3 expression on the inducibility of their target genes. STAT1 siRNA knock down had different repercussions on the actions of IL27, IFNγ and hy-IL6 (Fig. 5). For IFNγ (which does not induce phosphorylation of STAT3) knock-down of STAT1 only leads to a marginal upregulation of γ-fibrinogen even in untreated cells, while a STAT3 knock-down has no effects on STAT1 gene expression. For IL27, on the other hand, STAT1 knock-down led to a markedly increased STAT3 phosphorylation and an IL27-mediated expression of γ-fibrinogen. Knock-down of STAT3 had little repercussion on STAT1 protein levels upon IL27 stimulation. Thus, knock-down of STAT1 is sufficient to transform the IFNγ-type response of IL27 into an IL6-type response, while STAT3 knock down has little effects on IL27-induced STAT1 upregulation. In contrast, for hy-IL6, STAT3 knock-down led to a markedly increased STAT1 phosphorylation and an hy-IL6-dependent expression of STAT1, while knock-down of STAT1 had little repercussion on the γ-fibrinogen expression. Thus, knock-down of STAT3 is sufficient to transform the hy-IL6 response into an IFNγ-type response, as was observed before by others [42].

Considering the aforementioned effects we performed microarray analysis in stably transfected HepG2 cells expressing either a scrambled shRNA (HepG2-shScr) or a STAT3 shRNA (HepG2-shSTAT3) [43]. In the cells stably expressing the shRNA against STAT3, STAT3 protein is efficiently down-regulated (Supplemental Fig. 6A). Upon IL6-type cytokine stimulation only a residual STAT3 phosphorylation is observed while STAT1 phosphorylation is prolonged, which also leads to a STAT1 protein upregulation (Supplemental Fig. 6A). Thus those stable shRNA transfectants show the same effects upon cytokine stimulation than observed upon STAT3 down-regulation by siRNA as shown in Fig. 5.

Analysis of the microarray data of those cells revealed that STAT3 down-regulation did not change the expression pattern of the IL27-

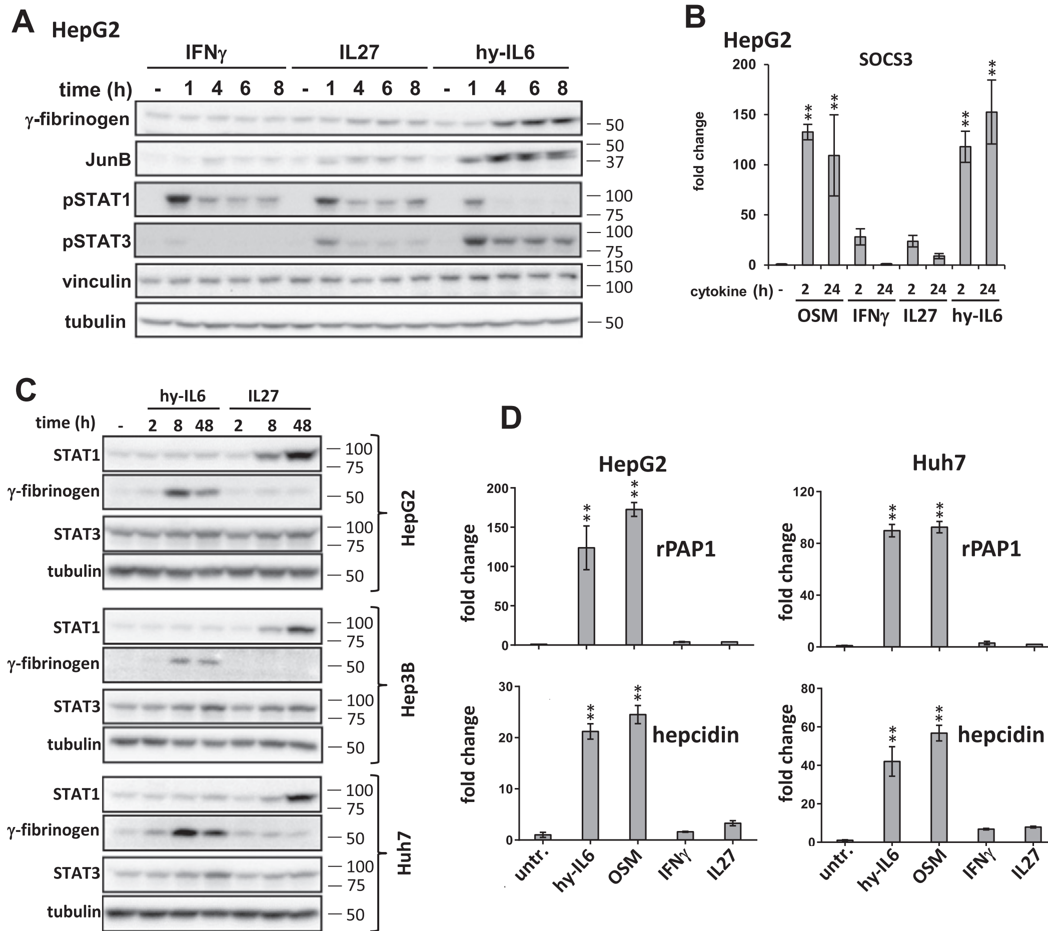


**Fig. 4.** IL27 stimulation does not induce an IL6-like response in hepatocellular carcinoma cells. A) HepG2 cells were treated for 1, 4, 6, or 8h with IFNγ, IL27 or hyper-IL6. Western blots were probed with antibodies against pSTAT1, pSTAT3, γ-fibrinogen, JunB, tubulin or vinculin. Representative blots of three biological replicates are shown. B) HepG2 cells were treated for 2 or 24h with cytokines as indicated. RNA was extracted and SOCS3 levels were analyzed by quantitative PCR. Standard deviations were calculated from 3 biological replicates. Statistical analysis was performed as decribed in materials and methods. **: $P < 0.01$ C) HepG2, Hep3B and Huh7 cells were treated for 2, 8, or 48h with IL27 or hyper-IL6. Western blots were probed with antibodies against STAT1, γ-fibrinogen, STAT3 or tubulin. Representative blots of three biological replicates are shown. D) HepG2 or Huh7 cells were transfected with the rPAP1 or hepcidin luciferase reporter plasmid. 24h after transfection, cells were stimulated with hyper-IL6, OSM, IFNγ or IL27 for 24h before lysates were prepared and luciferase activity was measured. Representative results of 3 biological replicates – each performed in technical triplicate - are shown. Standard deviations are calculated from 3 technical replicates. Statistical analysis was performed as decribed in materials and methods. **: $P < 0.01$.

regulated genes in comparison to IFNγ. However, hy-IL6 showed a profound upregulation of STAT1-dependent genes in these cells; its response was largely identical to the IFNγ and IL27 responses. Only a few genes regulated by IL6 in parental cells were still regulated in the shSTAT3 cells (Supplemental Fig. 5 and Supplemental tables 5 and 6). Thus, interestingly, the residual STAT3 phosphorylation observed upon hy-IL6 treatment in HepG2-shSTAT3 cells (corresponding to 9–11% of the hy-IL6 signal observed in HepG2-shScr) still leads to upregulation of a few IL6 regulated genes (in spite of mostly upregulating Interferon genes) while the STAT3 phosphorylation observed upon IL27 in HepG2-shScr (corresponding to 19–35% of the hy-IL6 signal observed in HepG2-shScr) does apparently not lead to “IL6-type” transcriptional changes (Supplemental Figs. 5A and 6B). This might suggest that the IL27-induced STAT3 responses are actively suppressed by a yet unknown mechanism.

### *3.7. IL27 responses are suppressed by IL6-type cytokine pre-stimulation*

IL27 is using its canonical receptor system composed of gp130 and WSX1 for signalling in hepatic cell lines, while it does not upregulate SOCS3 efficiently, compared to hy-IL6 or OSM (Fig. 4B). Since SOCS3 is described to bind gp130 on phosphorylated tyrosine 759 and to negatively regulate kinase activity of Jak1 [44], we hypothesised that IL6 or OSM pre-stimulation could lead to an attenuation of IL27-induced signalling events. HCC cell lines were pre-stimulated with OSM, hy-IL6 or IL6 for 4 h before IL27, IFNα or IFNγ stimulation. After 28 (OSM, hy-IL6 or IL6) respectively 24 (IL27, IFNγ, IFNα) hours of stimulation, STAT1 protein upregulation was analysed. IL27-dependent STAT1 protein expression was reduced efficiently if OSM, hy-IL6 or IL6 pre-stimulation had occurred (Fig. 6A). The down-regulation observed upon IL6 pre-stimulation was less pronounced but in agreement with the reduced signal strength observed for IL6 compared to OSM and hy-IL6 in the respective cell lines (Fig. 6A and Supplemental Fig. 1B). The effect was not at all observed in Hep3B cells upon OSM stimulation since they do not express the OSMR. IFNγ- or IFNα-induced STAT1 expression was not efficiently inhibited by IL6-type cytokine pre-stimulation.

To show that the IL6-type cytokine-mediated down-regulation can be mediated by SOCS3, we co-transfected a SOCS3 expression vector together with a vector containing a STAT1 responsive (GAS or ISRE) luciferase reporter gene, and stimulated the transfected cells with IL27 to see if SOCS3 expression is sufficient to suppress the IL27-mediated STAT1 transcriptional activity. In cells transfected with the SOCS3 plasmid, the GAS element-dependent luciferase activity was efficiently down-regulated in comparison to cells which were transfected with empty vector control (Fig. 6B). For ISRE sequence-containing reporter gene plasmids comparable results were observed.

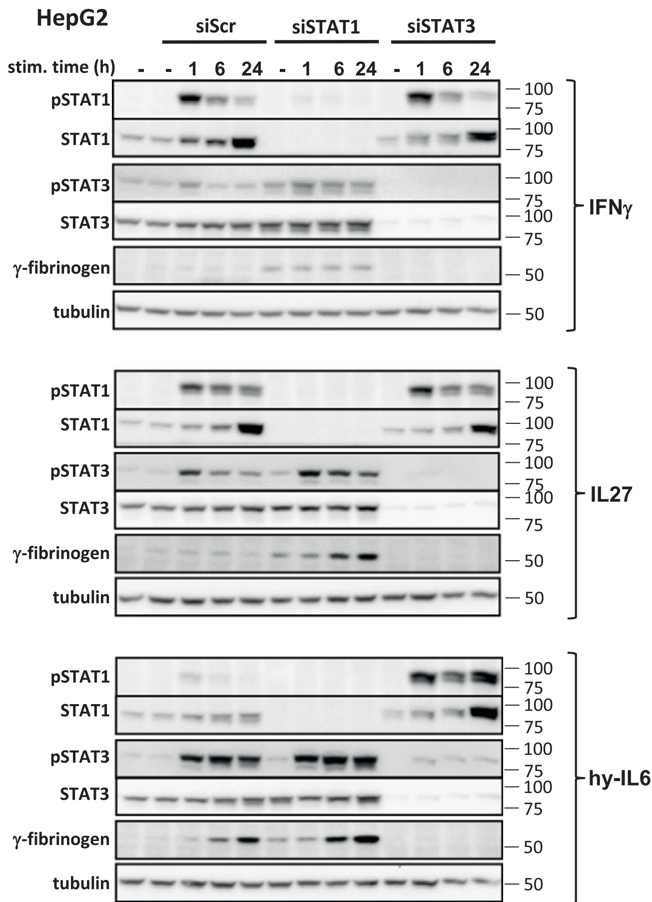


Fig. 5. In the absence of STAT1 expression IL27, in contrast to IFNγ, induces STAT3-dependent gene expression. HepG2 cells were transfected with siRNA against STAT1, STAT3 or with a scrambled control as described in materials and methods. The cells were stimulated with IFNγ, IL27 or hy-IL6 as indicated. Western blots were probed with antibodies against pSTAT1, pSTAT3, STAT1, STAT3, γ-fibrinogen or tubulin. Representative blots of three biological replicates are shown.

Thus, we conclude that IL27 is eliciting an IFNγ-type response, but that, in contrast to IFNγ, IL27 responses are susceptible to SOCS3 inhibition, and thus to cross-inhibition by pro-inflammatory IL6-type cytokine signalling (Fig. 7).

## 4. Discussion

In the present study we have compared the signal transduction of IL-27 in liver cells to the one of interferons and IL6-type cytokines. We show that (i) IL27 elicits a STAT1-dominated response and shares the majority of target genes with IFNγ. STAT3 becomes phosphorylated, albeit to a weaker extent than in response to IL-6-type cytokines, but this does not lead to a transcriptional response. (ii) IL-27 can induce STAT3 target genes only when STAT1 expression is lowered. (iii) IL-27 signaling, in contrast to IFN signaling, can be attenuated by pre-stimulation of liver cells with IL-6-type cytokines.

Using flow cytometry and siRNA approaches we showed that IL27 uses its canonical receptor complex (gp130/WSX1) [45] for signaling in hepatic cells (Fig. 1C and D). HepG2, Hep3B, Huh7, HLE, HLF, PH5CH8, THLE2 and hepatocytes (Upcyte) all reacted to IL27 with a robust phosphorylation of STAT1 (comparable to IFNγ and IFNα responses) and a phosphorylation of STAT3, the latter being weaker compared to the OSM, hy-IL6 or even IFNα responses in the same cells (Fig. 1 and Supplemental Fig. 1). It is intriguing that the STAT1 SH2 domain recognition motif (YEKH) in WSX1, which closely resembles the IFNγR1 Y440 (YDKP) motif, mediates STAT1 activation by IL27 so efficiently [46], while the 4 STAT3 recruiting tyrosines [47–50] in gp130 lead to a less efficient activation of STAT3. Nevertheless, the STAT3 phosphorylation is sustained over time and, thus, STAT3-dependent transcription might occur upon IL27 stimulation. Indeed, studies in immune cells have discussed a role of STAT3 in transcriptional responses through IL27 [46,51,52].

To better understand the STAT3 contribution to IL27 responses in HCC we first performed whole genome microarray analysis in 3 HCC cell lines (HepG2, Hep3B and Huh7). We compared the transcriptional responses of IL27 to the ones of IFNγ, hyper-L6 and OSM (OSM was not studied in Hep3B since the OSMR is not expressed [35]) after 24 h of stimulation. IFNγ, hyper-IL6 and OSM were used because they are *bona fide* activators of STAT1 (IFNγ) [53–56] and STAT3 (hyper-IL6, OSM) [39,40,57,58] responses in HCC cells. Evaluation of the genome microarray analysis of IL27 responses showed that there was little overlap with responses through IL6-type cytokines but that the IL27 response closely resembled an IFNγ response.

Validation experiments (Figs. 3 and 4) on mRNA level (SOCS3) as well as on the protein level (STAT1, Tap2, MHCI, γ-fibrinogen, JunB) and using specific STAT3-responsive promotor constructs (rPAP, hepcidin; as a STAT3 homodimer-sensitive probe), led us to conclude that although IL27 leads to sustained phosphorylation of STAT3, neither the tested STAT3 target proteins nor STAT3-dependent reporter genes were found to be induced. The reasons for this could be multiple. IFNα-induced STAT3 phosphorylation e.g. failed to induce transcriptional responses due to co-repressor complex SIN3 transcription regulator homologue A (SIN3A) binding to STAT3 and suppressing STAT3-mediated transcription [59]. In other studies the inefficient STAT1 response observed after IL-6 and OSM stimulation [60,61] has been attributed to the transient STAT1 phosphorylation [61–65] and additionally, most of the phosphorylated STAT1 seemed to be present in STAT1/STAT3 heterodimers, which translocated less efficiently into the nucleus [60]. Such a mechanism might also occur for IL27. In addition, STAT1/3 heterodimers might not be able to activate STAT3-responsive genes in an effective way.

When abrogating STAT1 or STAT3 expression by siRNA interference we could show that IFNγ-induced STAT1 protein upregulation is unchanged if STAT3 is knocked-down while the response is absent when STAT1 is knocked down (Fig. 5). Thus, since IFNγ shows almost no detectable STAT3 phosphorylation, the IFNγR cannot efficiently recruit STAT3 in the case of a STAT1 knock-down. However, the case is very different for IL27. Since IL27 uses gp130 in its receptor complex, STAT1 knock-down transforms IL27 into an IL6-type cytokine, which leads to recruitment and increased phosphorylation of STAT3 and an upregulation of γ-fibrinogen. Thus, knockdown of STAT1 is sufficient to transform the IFNγ-type response of IL27 into an IL6-type response while STAT3 knock-down has little effects on IL27-induced STAT1 upregulation. In contrast to this and as described before [42], abrogation of STAT3 expression is sufficient to transform the hy-IL6 response into an interferon-type response. Thus, for IL6-type cytokines and IL27, absence of either STAT3 or STAT1, respectively, leads to an opportunity for “other” STATs, “normally” activated to a minor degree, to bind efficiently to the receptor complexes and to elicit transcription of their specific target genes. Thus, knocking-down STATs to show their implication in signalling pathways can potentially be misleading. Depending on the affinities of different types of STATs for receptor phospho-tyrosine residues, signalling patterns can be changed drastically.

Since we were intrigued by the fact that IL27 almost totally mirrored IFNγ responses in our microarray analysis, we also performed a microarray analysis in HepG2 cells in which STAT3 had been stably silenced by shRNA to see more clearly what set of genes would not be induced anymore. In these HepG2-shSTAT3 cells hy-IL6 stimulation elicited an IFNγ-type response (Supplemental Fig. 5) in contrast to HepG2-shScr control cells. Nevertheless, hy-IL6 still led to a significant upregulation of 13 typical IL6-dependent genes although the STAT3 phosphorylation was reduced to 9–11% in HepG2-shSTAT3 compared to HepG2-shScr

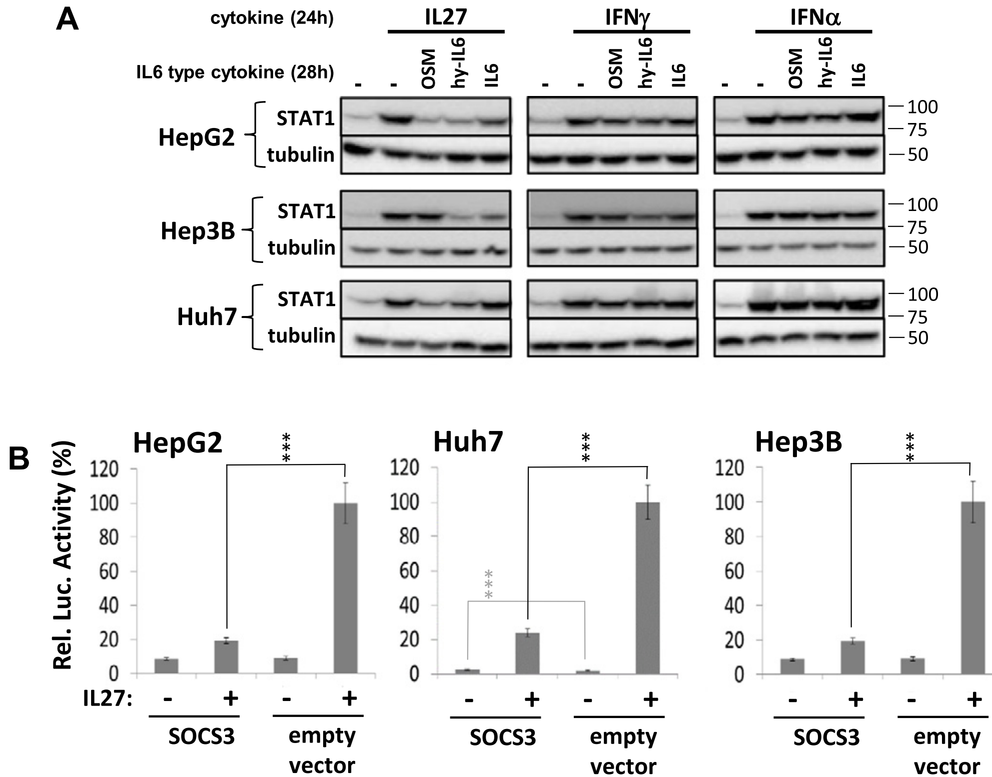


**Fig. 6.** IL6-type cytokine pre-stimulation inhibits a later IL27 response. A) HepG2, Hep3B and Huh7 cells were stimulated with OSM, hy-IL6, IL6, IFNγ, IFNα or IL27 as indicated. Western blots were probed with antibodies against STAT1 and tubulin. Representative blots of three biological replicates are shown. B) HepG2, Hep3B and Huh7 cells were co-transfected with either pcDNA5-SOCS3 and pGAS-TA-luc or pcDNA5 (empty vector) and pGAS-TA-luc. 24h after transfection, cells were stimulated with IL27 for 24h before lysates were prepared and luciferase activity was measured. Luciferase activity is represented as % of IL27-stimulated signal for the control condition (pcDNA5 + pGAS-TA-luc). One representative experiment of 3 biological replicates – each performed in 9 technical replicates - is shown. Standard deviations are calculated from 9 technical replicates. Statistical analysis was performed as decribed in materials and methods. ***: $P < 0.001$.

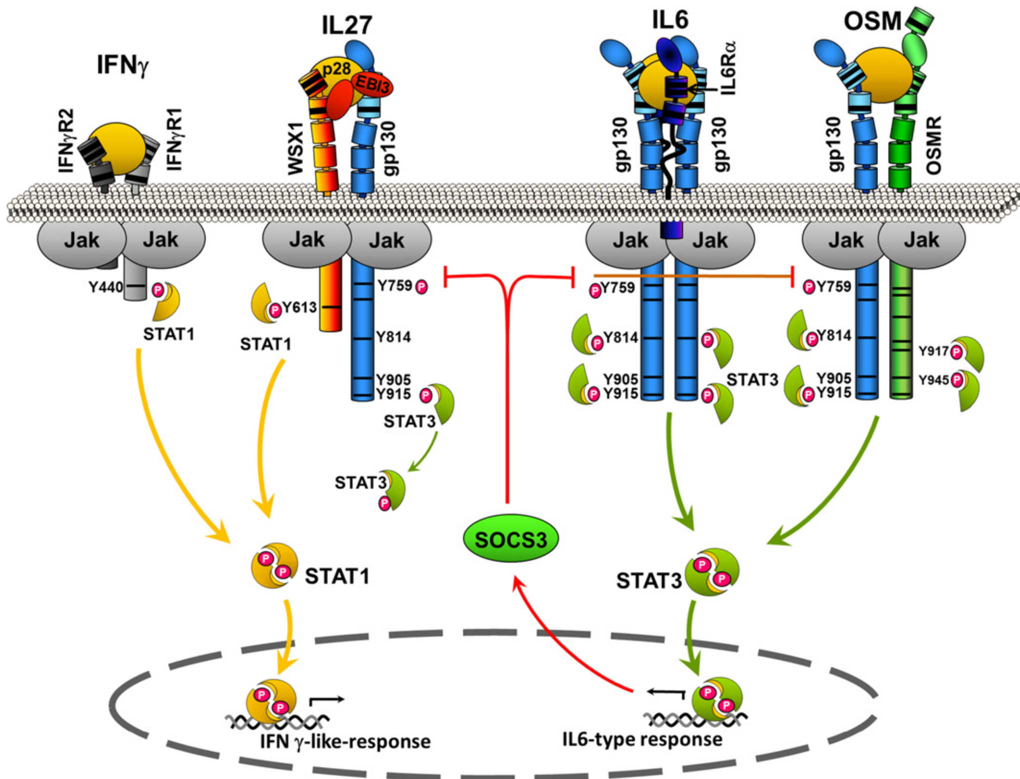


Fig. 7.

control cells (Supplemental Fig. 6B). In comparison to this, IL27 showed a STAT3 phosphorylation of 19–35% of the hy-IL6 response in HepG2-shScr. Most interestingly this much stronger response did not lead to a significant regulation of a single IL6-dependent gene. This might indicate that STAT3 transcriptional activity in HepG2 upon stimulation with IL27 could actively be prevented. A possible mechanism might include STAT1 dependent expression of co-repressors complexes in analogy to what was described for IFNα [59], but many other mechanisms are conceivable.

Our observations and the ones of others show that expression levels of the various STATs in different cell lines could account for cell type-specific differences that are observed concerning the different STAT factor contributions [46,51,52]. In addition, dynamic stimuli-dependent changes in expression levels of the different STATs can alter the STAT activation pattern of a given cytokine in one and the same cell type. E.g. in NK cells IFNα was described to activate STAT4, which these cells express at high levels, and which in turn enhances the STAT1 levels, so that the canonical STAT1/STAT2 signaling pattern emerges upon enhanced STAT1 expression [66].

Concerning IL27 signalling, STAT3 was shown to be important for regulating transcriptional output in activated $CD4^+$ T cells, and to up-regulate significant numbers of STAT3-specific genes [46,51,52]. In contrast to what we find, IL27 induced a STAT3 phosphorylation which was comparable in strength and duration to the IL6 response, and the STAT3 phosphorylation was not enhanced in $STAT1^{-/-}$ cells. Thus, in T cells STAT3 activation upon IL27 seems to be intrinsically more efficient concerning the intensity and duration of STAT3 phosphorylation. This could be dependent on STAT factor expression levels but also on those of negative regulators, such as SOCS proteins or phosphatases. In T cells STAT3 homodimers are also found in EMSA, which we could not see in hepatic cells [18,51]. Our results in hepatocytes, however, show that the IL27 responses in HCC lines and hepatocytes are mainly STAT1-mediated and no significant contribution of STAT3 to the transcriptional response was detected.

Compared to hy-IL6 and OSM, IL27 and IFNγ only weakly and transiently induce SOCS3 (Fig. 4B). Since IL27 uses gp130 as signalling receptor (which binds SOCS3 on the phosphorylated tyrosine 759 [44]), we hypothesized that SOCS3 should be able to suppress IL27 responses via binding to gp130. SOCS3 was shown to be upregulated STAT3-dependently and to down-regulate IL6-type cytokine signalling but not IFNγ signalling in vivo [67,68]. Interestingly, IL6-type cytokine pre-stimulation (4 h), which induces SOCS3 expression, can suppress a subsequent IL27-dependent STAT1 protein upregulation (Fig. 6A). Heterologous expression of SOCS3 was sufficient to mimic the IL6-pre-stimulation effect on STAT1-dependent reporter gene constructs upon IL27 stimulation (Fig. 6B). Thus, IL27 responses are susceptible to cross-inhibition by e.g. pro-inflammatory IL6-type cytokines, which is very likely mediated by STAT3-driven SOCS3 expression (Fig. 7). IFNγ and IFNα seem less susceptible to this type of cross-inhibition. Thus, although IL27 responses in HCC are almost identical to those observed upon IFNγ stimulation, IL27 is still different from IFNγ by the fact that its IFNγ-like activity can be dampened in the inflammatory context of IL6-type cytokine stimulation. Since IL27 is capable of exerting anti-tumour effects and is discussed as a candidate cytokine for enhancing anti-tumour immune responses [69–76], one has to keep in mind that a considerable reduction of its efficacy might occur in tumours with high levels of STAT3 activating cytokines.

Taken together, we have shown that IL27 behaves like a IFNγ-like cytokine in liver cells. However, under certain conditions, IL27 may reveal its hidden characteristics of a typical IL6-type cytokine family member: it can induce STAT3 targets (if STAT1 expression is very low), and its signaling is susceptible to inhibition by SOCS3 (if expressed, e. g. upon pre-stimulation with other members of the IL-6 family). It will be interesting to further elucidate how this flexibility in responses contributes to the various effects which have been attributed to this cytokine. Moreover, a detailed knowledge of the spectrum of its responses needs to be taken into account when considering to use IL27 in therapy as discussed [69–76].

## Transparency document

The transparency document associated with this article can be found, in online version.

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Microarray raw data were deposited in the ArrayExpress public repository with the reference number E-MTAB-4570.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2016.12.006.

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