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RESEARCH ARTICLE

Impact of IL-27 on hepatocyte antiviral gene expression and function [version 1; referees: 1 approved, 2 approved with reservations]

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Abstract

Background: Interleukin (IL)-27 is a member of the IL-6/IL-12 family of cytokines. It is a potent cytokine, with potential antiviral impact, and has been shown to play a role in modulating functions of diverse cell types, including Th1, Th2, and NK and B cells, demonstrating both pro- and anti-inflammatory roles. In hepatocytes, it is capable of inducing signal transducer and activator of transcription (STAT)1, STAT3 and interferon-stimulated genes.
Methods: To address its role in viral hepatitis, the antiviral activity of IL-27 against hepatitis C virus (HCV) and hepatitis B virus (HBV) was tested *in vitro* using cell-culture-derived infectious HCV (HCVcc) cell culture system and the HepaRG HBV cell culture model. To further investigate the impact of IL-27 on hepatocytes, Huh7.5 cells were treated with IL-27 to analyse the differentially expressed genes by microarray analysis. Furthermore, by quantitative PCR, we analyzed the up-regulation of chemokine *(CXCL)-10* in response to IL-27.
Results: In both HCV and HBV infection models, we observed only a modest direct antiviral effect. Microarray analysis showed that the up-regulated genes mostly belonged to antigen presentation and DNA replication pathways, and involved strong up-regulation of *CXCL-10*, a gene associated with liver inflammation. Overall, gene set enrichment analysis showed a striking correlation of these genes with those up-regulated in response to related cytokines in diverse cell populations.
Conclusion: Our data indicate that IL-27 can have a significant pro-inflammatory impact *in vitro*, although the direct antiviral effect is modest. It may have a potential impact on hepatocyte function, especially chemokine expression and antigen presentation.

Open Peer Review

Referee Status: ? ✓ ?

	Invited Referees		
	1	**2**	**3**
version 1 published 17 Nov 2016	? report	✓ report	? report

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Introduction

Interleukin (IL)-27 is a member of the IL-6/IL-12 family of cytokines that includes IL-12 and IL-23. It is secreted by antigen presenting cells and has diverse impacts on host immune responses, including inducing the formation of antiviral Th1 cells[1,2]. It also inhibits Th2 humoral immune responses[2] and inhibits Th17 cells by blocking IL-17A production, and preventing up-regulation of RORγT[3]. Recent data suggest it may also act on CD4+ T cells to negatively regulate function in tuberculosis[4].

IL-27 has also been described to possess antiviral functions. It inhibits HIV replication in CD4 cells and has been reported to control hepatitis C virus (HCV) in the cell-culture-derived infectious HCV (HCVcc) model, while in hepatitis B virus (HBV) infection, the virus activates IL-27 and interferon (IFN)λ1 and these co-ordinate to inhibit HBV replication[5,6]. In hepatoma cell lines, human and primary rat hepatocytes, the antiviral activity is thought to be induced by the induction of signal transducer and activator of transcription (*STAT)1* and *STAT3*, leading to the induction of IFN regulated proteins, such as interferon response factor (*IRF)-1, IRF-9*, guanylate binding protein 2 and myxovirus resistance A[6,7].

In this study, we used the HCVcc model and the HepaRG cell line (an HBV infection model) to address the antiviral impact of IL-27 on hepatocytes, and used a genome wide microarray to analyse the interplay of different genes that were regulated after stimulation with IL-27.

Methods

HCV infection

Huh7.5 cells (Apath) were maintained in DMEM supplemented with Glutamax with 10% fetal calf serum (FCS), 100U/ml penicillin and 100μg/ml of streptomycin (cat# 15140122), and MEM non essential amino acids (cat# 11140035) at 37°C and 5% CO_2. All reagents were GIBCO products from Thermo Fisher Scientific. IL-27 was obtained from R&D Systems Europe. Cells were stimulated with 100ng/ml IL-27 for 72 hrs at 37°C.

Huh7.5 cells were infected with genotype 2a chimeric HCV (MOI 0.05; J6/JFH1) (kindly provided by Apath)[8]. Infected hepatocytes were treated with IL-27 at 100ng/ml and controls cultured with PBS as a control for up to 20 days. Cells were stimulated with IFNa (R&D systems Europe) at 1000u/ml as a positive control for antiviral activity. Immuno-fluorescence assays (IFA) were performed over this timecourse; cells were probed with 9E10 mouse monoclonal anti-non-structural protein 5 (NS5A) antibody (a gift from Dr. Charles Rice, Rockefeller University, USA) conjugated to Alexa 488 for the presence of HCV by fluorescence microscopy. Visualisation was performed using a Nikon Eclipse TE2000-U inverted microscope at x20 magnification using a Nikon DXM1200F camera. The software used for taking pictures was ACT-1 v2.63 and Adobe photoshop CS4 was used to count the infected cells.

HBV infection

HepaRG cells (a gift from Dr. Nicole Zitzmann, University of Oxford) were cultured in Williams medium containing 10% FCS,

5μg/ml insulin and 50mM hydrocortisone at 37°C and 5% CO_2. Cell differentiation was induced two weeks prior to infection by adding 2% v/v DMSO and epidermal growth factor (5ng/ml). Infectious HBV particles, for infection, were obtained from a culture of HepG2.2.15 cells (a gift from Dr. Nicole Zitzmann, University of Oxford). Briefly culture supernatants from HepG2.2.15 cells that were stably transfected with HBV plasmid were shaken in an orbital shaker overnight and following the addition of PEG8000 at a final concentration of 8%. The inoculum was pelleted by centrifugation at 13000xg for 30 mins, and reconstituted with sterile PBS.

To obtain viral load genome equivalents, HBV DNA was isolated from the inoculum using Invitrogen PureLink Viral DNA Mini kit (cat# 12280050), following the manufacturer's instructions. The viral DNA was then quantified using forward: 5'-GGT CTC TTT CGG AGT GTG GA-3'; reverse: 5'-ATA GGG GCA TTT GGT GGT CT-3' primers. qPCR was performed on a Roche 480 light cycler machine and using the Roche LC480 SYBR green master mix (cat# 04707516001) as below.

HepaRG cells were infected with the above inoculum with a genome equivalent of 20GE/cell overnight at 5% CO_2 at 37°C. The infected HepaRG cells were treated with IL-27 (100ng/ml) or IFN-α (1000IU/ml) (R&D systems Europe).

HBV ELISA

At day 7 post infection hepatitis B surface (HBs) antigen levels were assayed using MONOLISA HBs Ag Ultra Kit (Bio-Rad), according to the manufacturer's instructions.

RNA extraction and relative reverse transcription quantitative (q)PCR analysis

Total RNA was prepared using RNeasy kits (Qiagen). In-column DNAse treatment was performed. The quality of RNA was checked using Agilent Technologies 2100 Bioanalyser.

Two-step reverse transcription was performed using Superscript III Reverse Transcriptase (Invitrogen) and qPCR was performed using Roche Light Cycler 480 to detect CXCL10, low molecular mass peptide 7 (*LMP7*) and transporter associated with antigen processing 1 (*TAP1*). Primers were designed using the Roche Universal Probe Library system as follows: *CXCL10*, forward: 5'-GAA AGC AGT TAG CAA GGA AAG GT-3' and reverse: 5'- GAC ATA TAC TCC ATG TAG GGA AGT GA-3'; *LMP7*, forward: 5'-CAA GTT CCA GCA TGG AGT GA-3' and reverse: 5'-TCA CCC GTA AGG CAC TAA TGT-3'; *TAP1*, forward: 5'-GCA AGA AAT AAA GAC ACT CAA CCA-3' and reverse: 5'-CCC ACT TTC AGC AGC ATA CC-3'; *GAPDH* forward: 5'-AGC CAC ATC GCT CAG ACA C-3' and reverse: 5'-GCC CAA TAC GAC CAA ATC C-3'.

Relative gene expression was calculated using the comparative cycle threshold method, as described previously[9].

Gene expression and statistical analysis

Gene expression profiles were obtained by hybridising the samples to GeneChip Human Gene 1 STU Arrays (HuGene-1_0-st-v1; Affymetrix), according to the manufacturer's instructions.

Statistical testing was performed using Linear Models for Microarray Analysis (LIMMA) package (http://bioconductor.org/packages/release/bioc/html/limma.html)[10]. Raw p-values were corrected using the false discovery rate controlling procedure of Benjamini and Hochberg[11]. Following this correction, adjusted p-values <0.01 were considered significant. Gene annotation was added to the final probe list from the NetAffx™ Analysis Center (https://www.affymetrix.com/analysis/index.affx).

We used the online Database for Annotation, Visualization and Integrated Discovery (DAVID v6.8) bioinformatics database (https://david.ncifcrf.gov/) to analyze pathways[12]. Gene set enrichment analysis (GSEA) from the Broad Institute (http://software.broadinstitute.org/gsea/msigdb/) was used to assess significant enrichment of immunological signature gene sets in IL-27 up-regulated genes.

Results
Antiviral studies
In the HCVcc model, it was previously demonstrated[6] that IL-27 had antiviral properties. Using the same model, we have demonstrated that there is no significant reduction in viral infectivity at 10 days post treatment with IL-27 at 100ng/ml, as observed by an immuno-fluorescence assay (IFA) of infected hepatocytes (Figure 1A). However, at 16 days a modest impact of IL-27 on infection was observed with 73.8% of cells infected compared to controls (normalized to 100%; p=0.0116 t test; Figure1B). Analysis of viral RNA at day 10 revealed a small difference between treated and untreated cells (Figure 1C); however, the effect is overall limited compared to the IFNγ positive control (Figure 1B) and was not significantly different when measured at a later time point (day 13).

Similarly, at day 7 post-infection, HepaRG cells infected with HBV showed modest reductions in HBsAg levels after treatment with IL-27 (Figure 1D).

Gene expression studies
To further understand the impact of IL-27 on hepatocytes, we next addressed the gene expression changes occurring during IL-27 treatment. Microarray analysis showed that IL-27 significantly induced (>two-fold) a total of 446 genes, while 129 genes
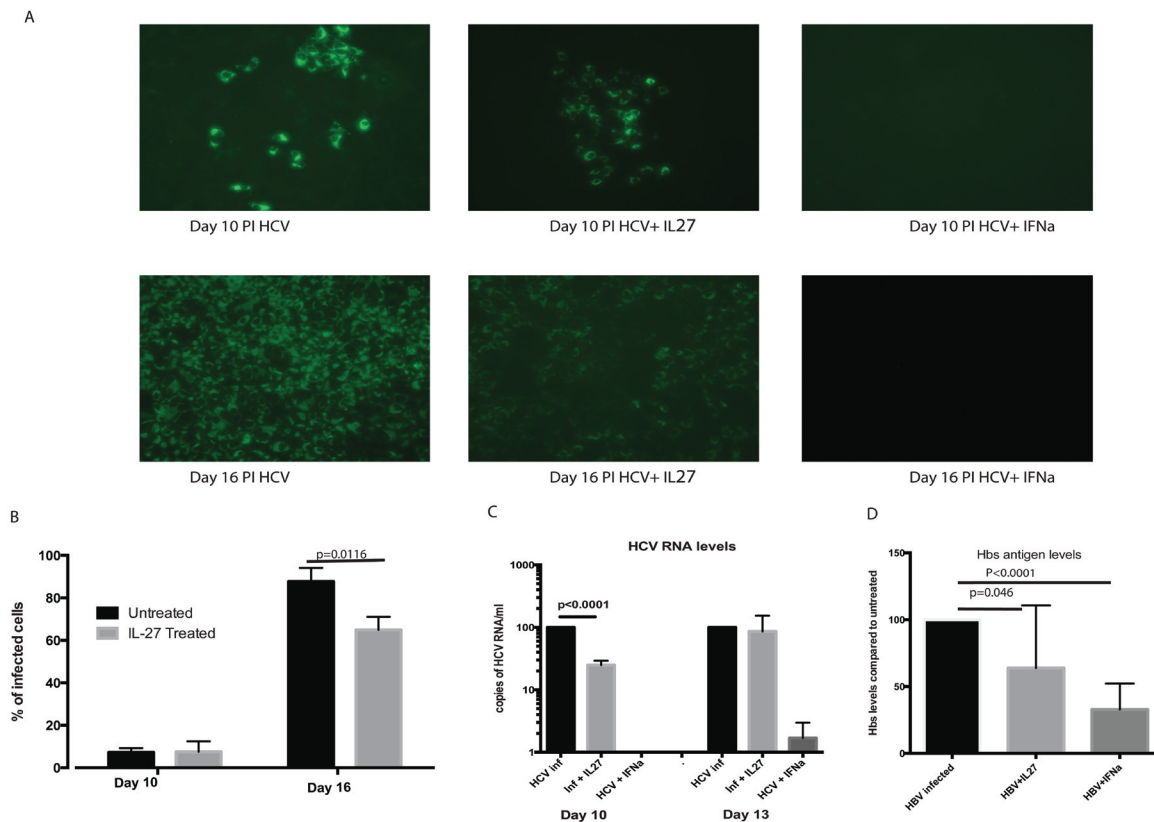

Figure 1. Impact of IL-27 in HCV replication *in vitro*. Huh7.5 cells were infected with HCV and treated with IL-27 (100ng/ml). (**A**) Immuno-fluorescence assays were performed during the experimental period to assess for antiviral activity. (**B**) Infected cells were counted and the percentage of infected cells compared to the control untreated well. (**C**) Supernatants from cells infected with HCV and control untreated wells were analysed by real time RT-PCR and represented as copies of HCV/ml of supernatant. (**D**) HBs antigen levels as assessed by ELISA. Results from three independent experiments are shown (p-value assessed using t-test). IL, interleukin; PI, post-infection; HCV, hepatitis C virus; IFN, interferon; HBs, hepatitis B surface antigen; HBV, hepatitis B virus; Inf, infection.

were down-regulated in Huh 7.5 cells, 72 hours post-stimulation (Supplementary Table 1). Table 1 shows the top 20 differentially regulated genes obtained.

Amongst the top 20 hits in our data were a number of signaling pathway genes notably Rab-like protein 2A (*RABL2a*) (a GTPase that mediates signal transduction), neurotensin (*NTS*) and signal peptidase complex subunit 2 homolog (*SPCS2*). Cell cycle proteins, e.g. the calmodulin binding *ASPM* gene, *CDC6*, which regulates DNA replication, along with DNA replication checkpoint gene,

CDC45L, were also up-regulated. Other relevant up-regulated genes were those involved in inflammation, including *CXCL-10*, which is known to be IFN responsive, orosomucoid (*ORM*)-*1/2* and lysozyme (*LYZ*) (Table 1).

The DAVID program was used to analyse the data set to identify pathways that are differentially regulated in response to stimulation by IL-27. Kegg functional annotation analysis within this program (Supplementary Table 2A) of the up-regulated genes, showed high fold-enrichment and significance in genes involved

Table 1. Table showing top 20 genes differentially regulated in Huh7.5 cells in response to 100ng/ml of IL-27 at 72 hrs post stimulation.

Up-regulated genes		Down-regulated genes	
Gene symbol	**Gene title**	**Gene symbol**	**Gene title**
SLC6A14	solute carrier family 6 (amino acid transporter),	USP17L6P	ubiquitin specific peptidase 17-like 6
LOC100289612	arsenic transactivated protein 1	UIMC1	ubiquitin interaction motif containing 1
RABL2A	RAB, member of RAS oncogene family-like 2A	FOS	FBJ murine osteosarcoma viral oncogene homolog
SPCS2	signal peptidase complex subunit 2 homolog	EGR1	early growth response 1
UBD	ubiquitin D	JUN	jun oncogene
DKK1	dickkopf homolog 1 (Xenopus laevis)	RPPH1	ribonuclease P RNA component H1
CXCL10	chemokine (C-X-C motif) ligand 10	TMEM191A	transmembrane protein 191A
ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	PIK3IP1	phosphoinositide-3-kinase interacting protein 1
NTS	neurotensin	RNU22	RNA, U2 small nuclear 2
NME1	non-metastatic cells 1, protein (NM23A	GADD45B	growth arrest and DNA-damage-inducible, beta
MCM10	minichromosome maintenance complex component 10	SMA4	glucuronidase, beta pseudogene
ORM2	orosomucoid 2	FMO1	flavin containing monooxygenase 1
ESCO2	establishment of cohesion 1 homolog 2 (S. cerevisiae)	LOC642838	similar to hCG1742442
CDC6	cell division cycle 6 homolog (S. cerevisiae)	BTG2	BTG family, member 2
CDC45L	CDC45 cell division cycle 45-like (S. cerevisiae)	GDF15	growth differentiation factor 15
LYZ	lysozyme (renal amyloidosis)	RFC1	replication factor C (activator 1) 1, 145kDa
CDC25A	cell division cycle 25 homolog A (S. pombe)	PLK2	polo-like kinase 2 (Drosophila)
MCM4	minichromosome maintenance complex component 4	FOSB	FBJ murine osteosarcoma viral oncogene homolog B
LCORL	ligand dependent nuclear receptor corepressor-like	DKFZP564O0823	prostatic androgen-repressed message-1
DTL	denticleless homolog (Drosophila)	LOC100128868	testin-related protein TRG
GINS1	GINS complex subunit 1 (Psf1 homolog)	JUNB	jun B proto-oncogene
HIST1H2AB	histone cluster 1, H2ab	DIO1	deiodinase, iodothyronine, type I
DHFR	dihydrofolate reductase	DUSP1	dual specificity phosphatase 1

in DNA replication, the cell cycle and homologous recombination. Gene functional classification classified genes into 14 clusters, the largest, with a high enrichment score, included genes involved in the cell cycle, cell division, DNA replication, the spliceosome, and nucleic acid metabolism, and a strong signal from the proteasomal signaling pathway (Supplementary Table 2B).

Analysing the down-regulated genes, gene functional classification showed only one gene cluster with a significant score, which included transcriptional regulators v-maf musculoaponeurotic fibrosarcoma oncogene homolog *MAF*, early growth response 1 (*EGR1*) and Jun- D proto-oncogene (*JUND*). *NR1D1*, a nuclear receptor and transcription repressor involved in circadian regulation, which is normally highly expressed in the liver, was also reduced in response to IL-27. Target genes of *NR1D1* are ApoA1 and ApoCIII and anti-fibrinolytic factor PAI-1, while *NR1D2* is involved in the control of lipid and energy homeostasis in skeletal muscle[13].

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was used to understand the significance of IL-27 induced gene expression, in comparison with other published experimental datasets. Genes differentially regulated in response to IL-27 were compared against a list of immunological signatures from the Broad Institute GSEA database. We found linked gene expression sets in experiments using related cytokines on distinct cell types. Figure 2 shows representative enrichment plots of relevant gene signatures from

datasets with significant correlation to our experiments on Huh7.5 cells (CD8+ T cells and NK cells following treatment with IL-15, a related cytokine *in vitro*). Supplementary Table 3 give a more comprehensive list (top 20 most significant enriched gene sets) of experiments that have similar differentially up (S-2A) or down (S-2B) regulated genes, respectively. Overall, this shows clear overlapping gene sets between genes induced in our experiments and cellular activation by lymphocytes in response to related cytokines.

Quantitation of CXCL-10, TAP-1 and LMP7

One of the genes that was up-regulated by IL-27 was *CXCL-10* - an IFNγ-responsive gene. Therefore, as a test of principle and to reconfirm the results obtained by microarray analysis, we tested the level of gene expression of *CXCL10* in hepatocyte lines in response to IL-27. qPCR analysis showed that the levels of *CXCL-10* increased with time, reaching a peak at about 24 hrs post-stimulation (p=0.0001; Figure 3A).

Since proteasomal signaling emerged as an enriched pathway, *TAP1* and *LMP7*, important proteasomal genes involved in MHC class I presentation, were also examined by qPCR[14]. We addressed this by analyzing the amount of *TAP1* in cells stimulated with IL-27 over time. qPCR analysis showed that the expression levels of *TAP1* increased from 6 hrs post stimulation (p=0.0001) and *LMP7* significantly increased increased from 24 hours (p=0.0132) post-stimulation with IL-27 (Figure 3B).
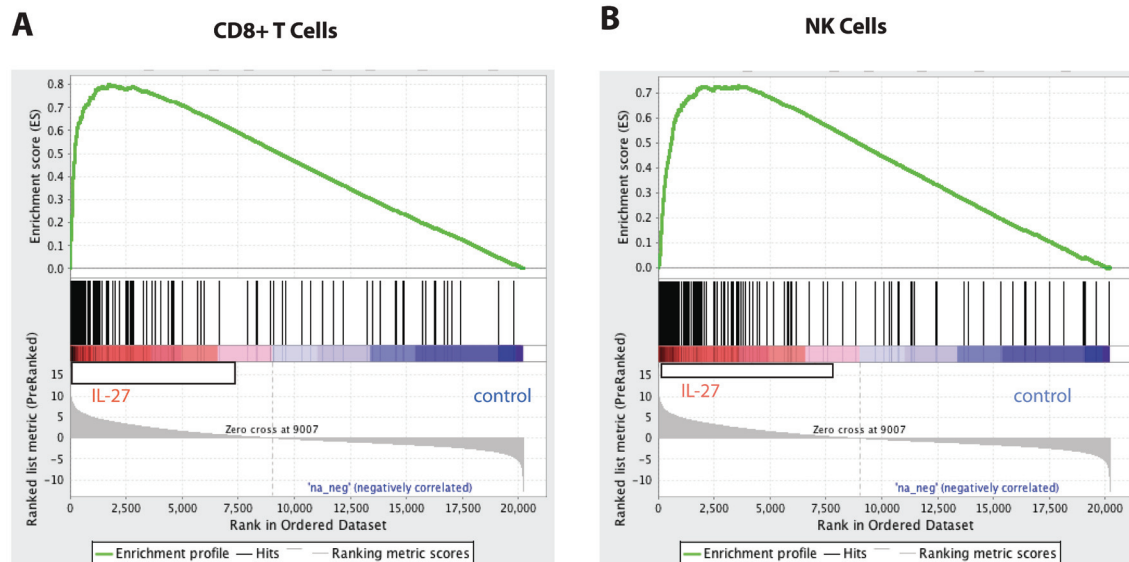

Figure 2. Enrichment of immune signatures in IL-27 treated hepatocytes. (**A**) Representative analysis of our genes when compared by gene enrichment analysis against the immunological signals from the Broad Institute gene enrichment database showed genes up-regulated with IL-27 were also those that were found up-regulated in CD8+ T cells (GSE 15750; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?targ =self&form=html&view=brief&acc=GSE15750) and (**B**) NK cells in response to IL-15 (GSE 22886; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?targ=self&form=html&view=brief&acc=GSE25616). The steep upslope of the curve (leading edge) indicates strong, statistically robust enrichment of relevant genes. IL, interleukin.
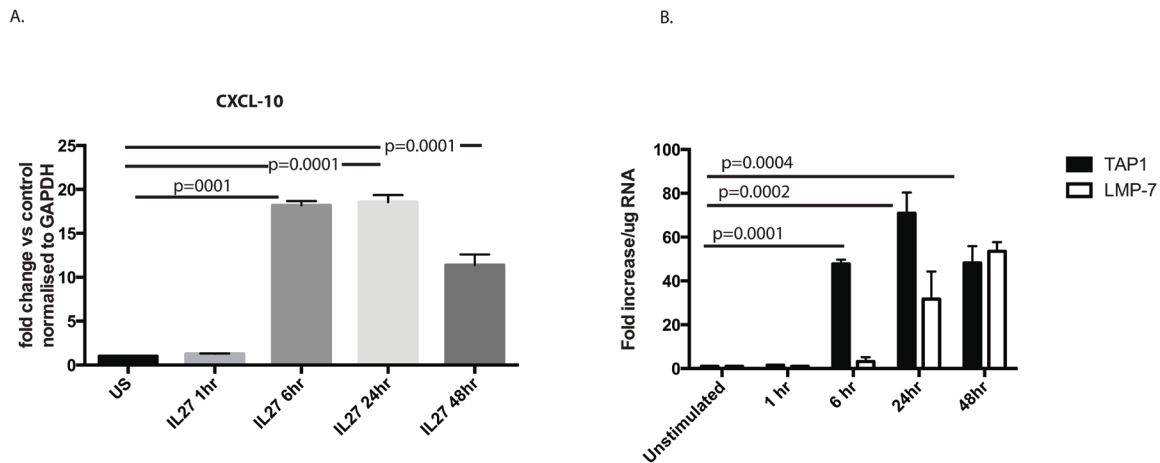
A.

B.

Figure 3. qPCR for CXCL-10, TAP1 and LMP7. (**A**) IL-27 strongly induces CXCL-10 (IP-10) in hepatocytes. Huh7 cells were treated with IL-27 and quantitative PCR for CXCL-10 was performed over time (p value assessed using t test). (**B**) mRNA expression of TAP1 and LMP7 in Huh7.5 cells treated with IL-27. Results are from three individual experiments. US, unstimulated; IL, interleukin; CXCL-10, chemokine 10; TAP1, transporter associated with antigen processing 1; LMP-7, low molecular mass polypeptide 7. Significant difference between values of unstimulated (US) and TAP1 were observed only after 6 hrs p=0.0001, and for US Vs LMP7 expression significant values were observed after 24 hrs of stimulation p=0.0132 and significance further increased at 48 hrs to p<0.0001.

Discussion

IL-27 functions to activate diverse intracellular pathways in hepatocytes, but only a modest impact on viral replication was demonstrated in this study with HCVcc, and similarly for HBV. Consistent with this result, the gene expression dataset did not show a marked up-regulation of classical antiviral genes. This is different to previous findings using different cell models[7], where IL-27 has been shown to post-translationally modulate phosphorylation of *STAT1/3* in HepG2 cells. Other studies have also proposed that IL-27 might possess antiviral functions similar to IFNα[5,7,15]. The fact that we did not observe a similar up-regulation of gene expression in our study could be due to differences in the cell lines used in the two studies. Consistent with this, in the above report another cell line within the same study did not show similar up-regulation of STAT phosphorylation[7]. However, as suggested by others, IL-27 may synergize with other antiviral treatments, such as IFNα, for therapy for HBV[16]. One further possible difference between studies was the time-point assessed – at 72hrs we may have missed some early antiviral gene expression, although overall the data is consistent with the lack of substantial impact of IL-27 in virus culture experiments over 1–2 weeks. We chose 72 hrs based on experience with other antiviral gene expression studies responses (e.g. IFN-lambda and alpha), and RNAseq studies in the HCVcc system, where we have used time-points up to day 10[17].

Although we did not observe a clear antiviral gene set activated, we did observe some specific responses within the hepatocytes, such as up-regulation of *CXCL10, TAP1* and *LMP7*. These responses were confirmed as relevant by comparison with other datasets using GSEA, which revealed consistent patterns of response in diverse cell types, in response to related cytokines. *CXCL-10* is associated with the expression of IFNγ, but can be induced by other cytokines, and it has been shown that IL-27 is able to induce *CXCL-10* in bronchial epithelial cells[18]. In skin tissue, antagonism of IL-27 attenuated the up-regulation of IFNγ, *CXCL-9, CXCL-10,*

CXCL-11 and tumor necrosis factor α mRNA[19]. Our observation, stimulation with IL-27 up-regulated *CXCL-10*, is consistent with previous *in vitro* studies of hepatocytes[7].

The overall impact of IL-27 *in vivo* is still unclear. IL-27 has been observed to increase in patients with chronic HBV[20], and has also been shown to be able to modulate immune responses to prevent hepatic injury[21]. Our *in vitro* data indicate it may have a potential impact on hepatocyte chemokine secretion and MHC class I antigen presentation, and thus *in vivo* studies of the role of IL-27 in modulating hepatocyte interaction with host CD8+ T cell responses may be of value in future.

Author contributions

NR and PK planned the study and wrote the manuscript. NR and SB carried out the experimental protocols. NR and RA performed qPCR in Figure 3. DB performed the microarray, and NS and HL analysed the initial bioinformatics from the microarray data. EM helped with the final analysis of the bioinformatics data and generated the GSEA data.

Competing interests

No competing interest were disclosed.

Supplementary files

Supplementary Table 1: List of genes differentially regulated in response to 100ng/ml IL-27 at 72 hrs post stimulation.
Click here to access the data.

Supplementary Table 2: (A) Kegg functional annotation of genes up-regulated in response to IL-27 in Huh7.5 cells by DAVID software analysis. (B) Gene functional annotation of genes up-regulated in response to IL-27 in Huh7.5 cells by DAVID software analysis.
Click here to access the data.

Supplementary Table 3: (A) Comparative gene sets on immunological signatures from the MSigDB on genes up-regulated genes in response to IL-27. (B) Comparative Gene sets on immunological signatures from the MSigDB on genes down-regulated genes in response to IL-27.
Click here to access the data.

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Current Referee Status: ❓ ✔ ❓

Version 1

Referee Report 19 December 2016

❓ **Ruth Broering**

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Duisburg-Essen, Essen, Germany

Ramamurthy et al. aimed to investigate the antiviral effect of IL-27 on hepatotrophic viruses (HCV, HBV)
using HuH7.5 and HepaRG cell culture models. They observed a pro-inflammatory response but only
modest antiviral effects. Although the findings are interesting there are several limitations regarding the
experimental design.

- The choice of IL-27 dosing as well as the treatment duration is unclear; rationales need to be given
  here.

- The immunofluorescent staining in Figure 1 needs a DAPI signal to indicate HCV negative cells.

- There are discrepancies between the text and the Figure. Page 4: "… however, the effect is overall
  limited compared to the IFNγ positive control (Figure 1B) and was not significantly different when
  measured at a later time point (day 13)." There is no IFNγ mentioned in Figure 1.

- The HBV data given in Figure 1D are insufficient to draw any conclusions on IL-27 treatment.
  These data are of minor quality and need to be complemented (HBeAg, HBV DNA) or discarded.

- The number of replicates and repetitions within diverse experiments is not clear, especially for the
  gene array. Furthermore, the validation was performed with a single gene (CXCL-10) at a diverse
  time point.

- The links in the legend of Figure 2 seemed to be wrong. No IL-15 treatment is mentioned there.

- Drawing conclusions on antiviral responses of hepatocytes, while using Huh7 hepatoma cell line, is
  false. Here only suggestions can be made.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that
it is of an acceptable scientific standard, however I have significant reservations, as outlined
above.**

Competing Interests: No competing interests were disclosed.

Referee Report 05 December 2016

✔ **Andrew B. Lloyd**

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This in vitro study by Ramamurthy et al. evaluated the in vitro effects of IL-27 in Huh 7.5 HCVcc and HepaRG HBV cell culture models. Limited antiviral effect was demonstrated. The scope of the experiments was modest: a single concentration of IL-27 was utilised; (presumably) simultaneous inoculation of virus and administration of IL-27 only was utilised (as opposed to pre-treatment with IL-27); limited timepoints were analysed with 72 hours being the earliest. The read-outs in virus production were appropriate. The gene expression studies by microarray appear robust (including limited validation by qPCR for CXCL10) but provided unsurprising results.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

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Ramamurthy et al. have evaluated the in vitro effects of IL-27, a novel cytokine, belonging to the IL-12 family using in vitro infectious HCV and HBV models. The results demonstrated a pro inflammatory rather than antiviral effect of IL-27.

1. The in vitro culture systems are excellent in evaluating direct antiviral effects of biologics and drugs. However, it does not allow us to quantitate the indirect effects (on T cells and other immune cells). Hence, based on these results, we cannot assume IL-27 has no antiviral effect mediated via immune cell potentiation, as it is expected to.

2. Please explain the rationale for selecting 100ng dose for IL-27. It would have been preferable to perform a dose ranging studies to better inform of antiviral effect.

3. Why was IFN-gamma used as positive control? Why not, IFN-alpha? There are more validated data about IFN-alpha mediated suppression of HCV in vitro than IFN-gamma.

4. IL-27 receptor expression on target cells was not evaluated in the study. This could be an important factor in determining what cell types respond to IL-27. Ideally, the measuring the density of the receptor on the cells would be beneficial to rule out cell line specific factors for discrepancy in the results.

5. The results shown here for HCV are quite different from previous published reports, some of which have shown significant effect on HCV replication. This study has evaluated HCV replication by

PCR and IF and demonstrates no effect on HCV replication. The reasons for such discrepancy are not clear. Cell lines could have acquired or lost characteristics over time and could behave differently. Do we know the genotype of this cell line? Is it IL28B CC or TT?

6. Gene expression results shown in Figure 2 shows overlapping patterns with IL-15 induced gene expression in CD8 and NK cells. I am not sure what this means since gene expression in different cell types are likely to be controlled by cell specific transcriptional patterns and not comparable to effects of different cytokine on completely different cell types. This would benefit form addition of a description that will explain the results better.

7. Also, what was the rationale to choose 72 hours post treatment for gene expression analysis? Earlier studies have shown immediate induction of ISGs with Interferons and other antiviral cytokines.

8. Cell type specific expression of ISGs have been associated with favorable responses to interferon-alpha in patients. For example, higher expression of ISGs in hepatocyte is associated with a poor response to IFN-alpha, while a higher expression of ISGs in monocytes is associated with SVR. Hence, a lack of ISG expression in this hepatocyte cell line may be associated with an overall favorable host ISG response. Effects of IL-27 on other cell types may provide clues on the net effect of this cytokine on host innate response to HCV.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.