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In vitro effects of Type I interferons (IFNτ and IFNα) on bovine hepatocytes cultured with or without Kupffer cells

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Abstract. In cattle, maternal recognition of early pregnancy depends on the effects of the embryonic signal interferon (IFN)-τ. IFN-stimulated genes have been upregulated in the maternal liver during early pregnancy. In this study, primary hepatocyte cell culture models were evaluated for their suitability to test Type I IFN effects *in vitro*. The expression of target genes (interferon-stimulated gene 15 (*ISG-15*), interferon-induced GTP-binding protein (*MX-1*), C-X-C motif chemokine 10 (*CXCL-10*), *CXCL-5*, insulin-like growth factor 1 (*IGF-1*), IGF binding protein 2 (*IGFBP-2*)) was measured using reverse transcription–quantitative polymerase chain reaction in hepatocytes from monoculture or in indirect coculture with Kupffer cells (HKCid) on Days 1, 2, 3 and 4 of culture ($n = 21$ donor cows). Gene expression was also measured on Day 4 after challenging the cultures with recombinant IFNτ, IFNα, progesterone (P4), IFNτ + IFNα or IFNτ + P4 for 6 h. A significant increase in the mRNA expression of target genes in hepatocytes was shown in response to stimulation with IFNτ. The Kupffer cells in coculture did not influence the effects of IFNτ in hepatocytes. In conclusion, primary bovine hepatocyte cultures are suitable for stimulation experiments with Type I IFNs and as an extrauterine model for embryo–maternal communication. The proposed endocrine action of IFNτ in the liver may affect maternal metabolism and immune function in the liver.

Keywords: cattle, cell culture, embryo, embryo-maternal communication, endocrinology, gestation, hepatocytes, IGF binding protein, insulin-like growth factor, interferone tau.

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Introduction

In sheep and cattle, there is evidence that interferon (IFN)-τ reaches the blood stream and induces expression of IFN-stimulated genes (ISG) in the endometrium and other organs. In 2008, an 'antiviral activity' was detected in the uterine vein in sheep at Day 15 of pregnancy (Oliveira *et al.* 2008). Two years later this 'antiviral activity' was confirmed to be IFNτ, and ISG expression was detected in ovarian tissue (Bott *et al.* 2010). Recently, significantly higher gene expression of *ISG-15* and MX dynamin like GTPase 1 (*MX1*) and MX dynamin like GTPase 2 (*MX2*) was noted in cervical and vaginal tissues on Days 17–18 after AI in cows (Kunii *et al.* 2018). Increased expression of ISG genes has also been detected in leucocytes between Days 15 and 32 after insemination in both sheep (Yankey *et al.* 2001) and cattle (Han *et al.* 2006). This can be assumed to be in response to IFNτ. Similarly, several studies indicated a difference in *ISG-15* expression in the liver between pregnant and non-pregnant sheep on Days 14 (Romero *et al.* 2015) and 15 (Bott *et al.* 2010). In cows, studies in Holstein Friesian and Angus heifers detected increased mRNA levels of ISG (*MX1*, *MX2*, *ISG-15* and oligoadenulate synthetase 1(*OAS-1*)) in the liver on Day 18 of pregnancy compared with

non-pregnant heifers (Meyerholz *et al.* 2015; Ruhmann *et al.* 2017). Ruhmann *et al.* (2017) also demonstrated OAS-1 protein expression in hepatocytes via immunohistochemistry. However, there is still no direct proof (e.g. immunoassays) of IFNτ in the blood circulation. Nakamura *et al.* (2016) detected IFNτ in extracellular vesicles and discussed its involvement in the regulation of ISGs.

MX1, *MX2*, *ISG-15* and *OAS-1* are classical ISGs. They are induced in response to IFNτ after binding to Type 1 IFN receptors (interferon-α/β receptor (IFNAR1/2)) via induction of the Janus tyrosine kinase (JAK) signal transducers and activators of transcription (STAT) pathway. In addition, Type I IFNs stimulate increased expression of other genes via non-classical signalling pathways. Arosh *et al.* (2004) showed that IFNτ reduced the expression of prostaglandin F synthases on Day 16 of pregnancy in cows. In contrast, increased expression of the non-classical ISG chemokine (C-X-C motif) ligand 6 (CXCL-6) in response to IFNτ was shown in endometrial cells (Teixeira *et al.* 1997).

The present study focused on two chemokines that are traditionally produced during an inflammatory reaction. The chemokine CXCL-10 is produced in Kupffer cells (KCs) and induces decreased angiogenesis, whereas CXCL-5 supports angiogenesis and activates neutrophil granulocytes (Fernandez and Lolis 2002; Dembic 2015). Metabolic genes also seem to be affected by IFNτ. For example, there were changes in the expression of insulin like growth factor 1 (*IGF-1*) and IGF binding protein 2 (*IGFBP-2*) mRNA expression in the endometrium (Robinson *et al.* 2008) and peripheral blood (Meyerholz *et al.* 2015) during early pregnancy in cows. An effect of IFNτ on the somatotropic axis can therefore be speculated, because *IGFBP-2* is mainly produced in the liver. However, it is still unclear whether non-classical ISG expression is induced by IFNτ in bovine hepatocytes and whether it is affected by local liver KCs or progesterone (P4). KCs are located in the endothelium of liver sinusoids and displace the largest population of tissue macrophages. KCs play an important role in host defence and stay in close paracrine contact to hepatocytes (Naito *et al.* 2004). It remains unknown whether all IFNτ effects are specific or whether other Type I IFNs can induce comparable expression of ISGs. IFNτ belongs to the group of Type I IFNs (IFNα, IFNβ, IFNε, IFNκ and IFNω; Roberts *et al.* 1998; Capobianchi *et al.* 2015) and shows 45–55% homology to IFNs of the IFNα group and 70.3% homology to bovine IFNα (Imakawa *et al.* 1987). All Type I IFNs bind to the same receptor (*IFNAR1/2*), but stimulate the expression of different ISGs downstream (Mogensen *et al.* 1999; Uzé *et al.* 2007).

In this study, a well-established bovine liver cell culture (Ehrhardt and Schmicke 2016; Witte *et al.* 2019) was used and a KC coculture was established to test the endocrine effects of IFNτ. The following questions were investigated: (1) is the liver cell culture suitable for testing the effects of Type I IFNs; (2) does the Type I IFN effect differ between pure hepatocytes and hepatocytes cocultured with KCs; (3) does the expression of ISGs differ between hepatocytes stimulated with IFNτ and other Type I IFNs; and (4) does simultaneous stimulation with P4 and IFNτ affect the expression of single target genes in the maternal liver?

Materials and methods

Extraction of hepatocytes and KCs

Between March 2017 and July 2018, 15 bovine livers (six obtained at an abattoir, nine that had been 'patients' of the Clinic for Cattle at the University of Veterinary Medicine Hannover; see below) were used to establish a hepatocyte coculture with KCs without dexamethasone. A further six livers from female pluriparous Holstein Friesian cows (also clinic 'patients') were used to investigate the effects of type I IFNs on bovine hepatocytes as a model for the metabolic effects of early pregnancy signals in ruminants.

All cows used in this study that had been patients of the Clinic for Cattle at the University of Veterinary Medicine Hannover were killed for medical reasons, not including any signs of metabolic disease and/or general inflammation with fever. Within 20 min (abattoir) or 5 min (clinic 'patients') of death, the caudate process of the caudate lobe of the liver was removed. It was then rinsed with 200 mL ice-cold EGTA buffer, followed by 200 mL EGTA-free perfusion buffer. Hepatocytes were isolated using a two-step collagenase perfusion as described previously (Witte *et al.* 2019), with one modification: in order to test the effects of Type I IFNs without bias, all buffers were used without addition of dexamethasone.

KC were also extracted from the livers using the cell suspension that was obtained after collagenase perfusion. In addition to hepatocytes, this suspension contained all non-parenchymal cells of the liver. This total cell suspension was centrifuged at 300*g* for 5 min at 4°C. The pellet was resuspended in William's E medium (PAN-Biotech) and 10% fetal bovine serum (FBS; PAN-Biotech). According to the protocol of Smedsrød *et al.* (1985), the KCs were further purified by two-density gradient centrifugation (gradient: 5 mL of 50% Percoll (Sigma-Aldrich), 5 mL of 25% Percoll and 5 mL resuspended pellet). After centrifugation (1250*g*, 8 min, 4°C), the fraction between the two layers of Percoll contained KCs and fibroblasts. Further purification was based on selective adhesion properties between the two cell types on plastic surfaces (Smedsrød *et al.* 1985). After 20 min adhesion time at 37°C under 95% O_2 and 5% CO_2, cells were washed twice with phosphate-buffered saline (PBS) and harvested by the addition of 1 mL of 10% trypsin (Sigma-Aldrich) to the cells. Williams E Medium (PAN-Biotech) with 10% FBS (PAN-Biotech) was used to stop the enzymatic reaction, and the cells were centrifuged at 1300*g* for 1 min at 4°C. The cell pellet was resuspended in 1 mL Williams E Medium (PAN-Biotech) and cell vitality was tested by Trypan blue exclusion. The number of KCs was counted under a microscope (Fig. 1).

Establishment of hepatocyte–KC cocultures

In order to find the most suitable model, two different coculture methods were tested and compared with a hepatocyte monoculture (HMC) as a control. Hepatocytes were cocultured with KCs at a ratio of 6:1 (0.5 million hepatocytes:0.15 million KCs per well) in direct (HKCd) or indirect contact (HKCid). The three different models are shown in Fig. 2.

Basal expression of genes of interest

Six livers were used to test whether the basal expression of genes of interest is comparable between hepatocytes extracted

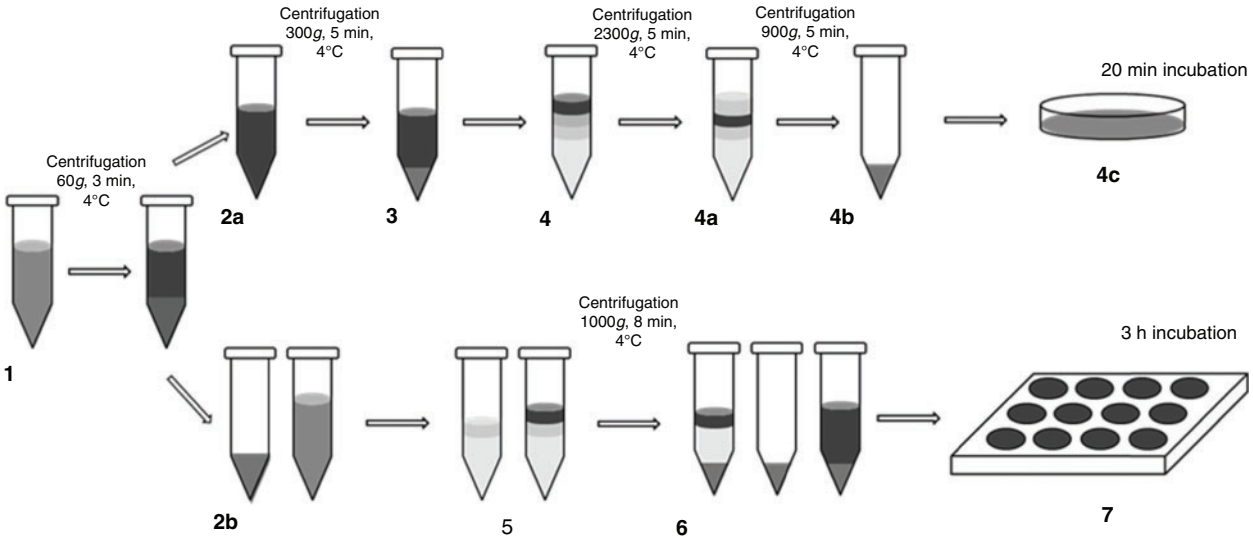

Fig. 1. After collagenase perfusion of the liver, the cell suspension (1) containing hepatocytes, Kupffer cells (KCs) and other non-parenchymal cells (NPC) was centrifuged (3 min, 60*g*) and divided into an NPC supernatant (2a) and a sediment containing hepatocytes (2b). The NPC fraction was again centrifuged (5 min, 300*g*; 3) and the resulting pellet was loaded into two density gradients (25% and 50% Percoll; 4). The next centrifugation (5 min, 2300*g*) led to accumulation of KCs and endothelial cells between the two density layers (4a). After collecting this middle layer, the cells were again centrifuged (5 min, 900*g*; 4b) and the resulting pellet was placed on a glass Petri dish (4c). In the sediment, hepatocytes were counted (2b) and a 25% Percoll gradient was loaded with 5000–6000 cells (5). After centrifugation (8 min, 1000*g*), hepatocytes in the pellet (6) were seeded onto collagen-coated plates (7).

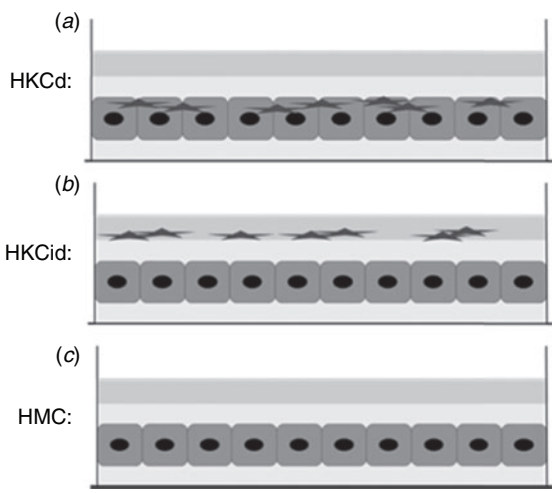

Fig. 2. Illustration of two different coculture methods for bovine hepatocytes (H) and Kupffer cells (KCs) in (*a*) direct (HKCd) or (*b*) indirect (HKCid) contact compared with (*c*) hepatocyte monoculture (HMC). H are represented by squares, whereas KC are represented by stars. The light grey line represents the collagen layers and the dark grey line represents the medium.

from different donors and on different days. To this end, hepatocytes were immediately frozen at –80°C after Percoll density centrifugation. Cell samples were also taken from HMC and HKCid on Days 1, 2, 3 and 4 of culture. The mRNA expression of *MX1*, *ISG-15*, *CXCL-10*, *CXCL-5*, *IGFBP-2*, *IGF-1* and albumin was analysed by reverse transcription–quantitative polymerase chain reaction (RT-qPCR) and compared between the six donors. Concentrations of lactate dehydrogenase (LDH) and urea in the culture supernatant were measured as indicators of vitality.

Stimulation of hepatocytes and KC coculture with IFNτ, IFNα and P4

HMC and HKCid were established from five consecutive livers and cultured for 4 days. Each stimulation condition was evaluated in 12 wells. Two wells were pooled prior to analysis. The culture medium was changed daily. On Day 4, cells were stimulated for 6 h with 0.06 or 0.6 ng well^{-1} IFNτ or IFNα. Other cells were stimulated simultaneously with 0.6 ng well^{-1} IFNτ and 0.6 ng well^{-1} IFNα or 0.06 ng well^{-1} IFNτ and 0.06 ng well^{-1} IFNα. Cells were also stimulated with 0.6 ng well^{-1} IFNτ with or without 5 or 10 ng well^{-1} P4 progesterone and were compared with cells stimulated with 5 and 10 ng well^{-1} P4 alone. Cells and supernatants were stored at –80°C until further processing.

Live/dead staining

To evaluate the vitality of the hepatocytes, cells were stained using a LIVE/DEAD Viability/Cytotoxicity Kit (ThermoFisher Scientific) according to the manufacturer's instructions and evaluated under an inverted fluorescence microscope with phase contrast (Axiovert 200M) using AxioVision 4.8.2.0 software. The proportion of live and dead cells was determined by counting 100 hepatocytes in different visual fields (various fields were used until 100 cells were counted).

Staining of hepatocytes with liver-specific markers

For staining, hepatocytes were cultured on collagen-coated coverslips. Cells were collected on Days 1 and 5. A cryosection

Table 1. Genes of interest and primers used in RT-qPCR

MX1, Myxovirus-resistance protein 1; *ISG-15*, interferon-stimulated gene 15 kDa; *CXCL-5*, chemokine (C-X-C motif) ligand 5; *CXCL-10*, chemokine (C-X-C motif) ligand 10; *IGF-1*, insulin-like growth factor-1; *IGFBP-2*, insulin-like growth factor-binding protein 2

Gene	Primer sequence (5′–3′)	Amplicon size (bp)	Accession no.
Albumin	Forward: GGGGTGTGTTTCGTCGAGAT Reverse: CTCACAGCAGTCAGCCATGT	293	NM_180992.2
18S rRNA	Forward: ACCCATTCGAACGTCTGCCCTATT Reverse: TCCTTGGATGTGGTAGCCGTTTCT	1873	NR_036642.1
MX1	Forward: AATCCCAGGTCTCAGAGCCC Reverse: CGGAGCACGAAGAACTGGA	2434	NM_173940.2
ISG-15	Forward: GACCTGACGGTGAAGATGCT Reverse: TGATCTTCTGGGCGATGAAC	591	NM_174366.1
CXCL-5	Forward: GCCACCTTGAAGAATGGAAG Reverse: TTGGAGAAAATGAGCCCTTG	1489	NM_174300.2
CXCL-10	Forward: GGTGGCATGTCATTCTTCACT Reverse: TCCACGGACAATTAGGGCTT	1194	NM_001046551.2
IGF-1	Forward: ATGCCCAAGGCTCAGAAG Reverse: GGTGGCATGTCATTCTTCACT	841	NM_001077828.1
IGFBP-2	Forward: CACATCCCCAACTGTGACAA Reverse: GATCAGCTTCCCGGTGTTAG	954	NM_174555.1

of bovine liver was used as a control. Cells were fixed using methanol and paraformaldehyde (PFA). Methanol (at –20°C) was added to the cultures for 10 min. Next, the cells were rehydrated in Tris-buffered saline (TBS) for 5 min, followed by treatment with 10 mL of 10% PFA. The cells were then washed three times with TBS and non-specific interactions were blocked using 5% skimmed milk with 20% Tween. After 45 min, the blocking solution was removed and primary antibodies were added to the cells (1 : 1000 dilutions; mouse anti-CK18 monoclonal antibody (C8541; Sigma-Aldrich) and rabbit anti-panCK monoclonal antibody (17916; Sigma-Aldrich). Cells were incubated overnight at 4°C, then washed with TBS for 5 min and then incubated with secondary fluorescence antibodies for 3 h at room temperature (goat anti-rabbit IgG (green; Alexa Fluor 488) and goat anti-mouse IgG (red; Alexa Fluor 546); dilution of 1:2000). Cells were incubated with 500 µL staining solution for 45 min, then washed with Tris-buffered saline Tween-20 (TBST) for 5 min, followed by 4 min incubation with Hoechst dye nucleus stain (1:1000 in TBS; 33342; ThermoFisher Scientific). After a final washing step, cover slides were gently removed from the cell culture plate and mounted on a microscope slide with Prolong Gold anti-fade reagent (P36930; Invitrogen). Cells were examined under an inverted fluorescence microscope (Zeiss Axiovert 25 with phase contrast).

Detection of functional KCs with latex beads

To assess the vitality of the gathered KCs in the culture model, specific latex beads (2.0 µm; L2778; Sigma-Aldrich) were used as vitality markers. Vital KCs phagocytose fluorescent microspheric polystyrol particles, which can be detected microscopically. To avoid non-specific binding, latex beads were opsonised by incubation for 1 h at 37°C with 1:1 PBS/FBS. HMC and HKCid were incubated with latex beads at a ratio of 1:40 (KCs:latex beads) for 24 h. Then, plates were washed twice with PBS to remove any non-phagocytosed beads. The phagocytosed particles were counted using a fluorescence microscope with phase contrast equipment (Zeiss Axiovert 25) in four fields of view per well.

Urea concentrations and LDH activity in the cell culture medium

Urea concentrations were measured using a Urea Assay Kit (ab83362; Abcam) and LDH activity was measured using a Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich), as described previously (Witte *et al.* 2019). Both assays were performed according to the manufacturers' instructions (https://www.sigmaaldrich.com/catalogue/product/sigma/mak066?lang=deandregion=DE). The intra-assay CVs were 6.1% for urea and 5.2% for LDH.

Isolation of mRNA and RT-qPCR

The expression of *S18*, *MX1*, *ISG-15*, *CXCL-10*, *CXCL-5*, *IGFBP-2*, *IGF-1* and albumin mRNA in cell samples was determined using RT-qPCR. To this end, total RNA was extracted using TRIzol reagent (Sigma-Aldrich), as described previously (Ehrhardt and Schmicke 2016). The extracted RNA was quantified using a NanoDrop One spectrophotometer (ThermoFisher Scientific). RNA quality was checked in HKCid samples using the 2100 Bioanalyzer System (Agilent 6000 Nano Kit). All samples had RNA integrity numbers (RIN) >8.5. A CFX96 C1000 Touch thermocycler (Real-Time PCR Detection System; Bio-Rad) was used to reverse transcribe mRNA into cDNA, as described previously (Witte *et al.* 2019). Each PCR contained 2.5 ng μL^{-1} cDNA, 4 µL HOT FIREPol EvaGreen qPCR Supermix (5×; Solis BioDyne) and 0.4 µM forward and reverse primers (Eurofins MWG Operon). The primer sequences and Accession numbers are listed in Table 1. The PCR cycler was programmed as follows: activation of the DNA polymerase at 95°C for 10 min, followed by 40 cycles of RNA denaturation at 95°C for 15 s, annealing at 60°C for 30 s and elongation at 72°C

Table 2. Results of viability tests and the quantity of hepatocytes and Kupffer cells obtained from of 21 perfused livers

Livers were obtained from an abattoir (Long warm ischaemia interval) or from cows that had been 'patients' of the Clinic for Cattle at the University of Veterinary Medicine Hannover and were killed for the medical reasons listed (short warm ischaemia interval). Asterisks indicate that viability was only estimated on the basis of the test procedure, but was always above 95%.

	Hepatocytes (cells g^{-1})	Viability before Percoll (%)	Viability after Percoll (%)	Estimated no. adhesions after 3 h	Kupffer cells	Viability (%)
Long warm ischaemia interval (~25 min) after death						
Liver 1	9 567 669	70.50	71.00	40	286 036	60.10
Liver 2	9 253 246	52.40	71.40	50	732 142	75.40
Liver 3	7 050 556	53.60	86.80	50	250 000	68.20
Liver 4	11 312 790	61.50	81.30	55	612 209	80.50
Liver 5	8 534 211	50.60	81.10	45	680 701	83.50
Liver 6	431 250	51.50	53.50	20	240 885	74.90
Mean ± s.d.	6 641 620 ± 4 776 137	56.68 ± 7.82	74.18 ± 11.86	43.33 ± 12.52	467 050 ± 231 474	73.77 ± 8.51
Short warm ischaemia interval (~5 min) after death						
Hip luxation	4 787 879	57.80	84.00	99	884 848	94.20
Hip luxation	2 807 992	82.40	93.70	90	431 250	89.70
Mastitis	43 712 121	93.90	–[B]	80	284 091	96.20
Claw defect	23 881 579	70.70	88.40	90	331 579	98.40
Hip luxation	32 375 000	89. 0	95.90	85	418 421	97.40
Arthritis	10 356 164	64.50	94.70	99	164 384	99.00
Claw defect	32 571 429	88.10	87.10	85	222 857	98.60
Muscular disease	15 408 163	67.60	87.20	70	367 346	99.00
Cataract	117 441 860	98.20	92.50	99	920 930	97.00
Arthritis[A]	18 675 000	94.40	93.50	95	100 000	*
Claw defect[A]	10 050 000	81.20	87.00	90	125 000	*
Claw defect[A]	14 200 000	92.80	90.60	85	280 000	*
Fracture[A]	8 300 000	91.70	85.90	90	700 000	*
Tendovaginitis[A]	11 025 000	90.90	84.50	85	345 000	*
Arthritis[A]	11 825 000	92.70	89.80	95	490 000	*
Mean ± s.d.	23 827 812 ± 28 313 775	83.73 ± 12.63	89.63 ± 3.91	89.13 ± 7.74	387 340 ± 269 546	96.61 ± 3.02

[A]Cells from these livers were used for mono- and coculture experiments after stimulation with Type I IFNs and P4.
[B]Due to the high initial viability, Percoll centrifugation was not used.

for 30 s. A melting curve analysis was performed between 55 and 95°C in 0.5°C increments to confirm the identity of the amplicons. Gene expression levels were determined by relative quantification (ΔΔCt method) using *S18* as a reference gene. Determinations were performed in duplicate.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 8. All data were tested for normal distribution using the Shapiro–Wilk normality test. Normally distributed data were evaluated using Student's *t*-test. Data that were not normally distributed were evaluated using the Mann–Whitney *U*-test. The mRNA data are presented as ΔΔCt relative to a calibrator with respect to the different efficiencies of the primers. Data are presented as the mean ± s.d. if data was normally distributed, or mean ± 95% CI if data was not normally distributed. Differences were considered significant at two-tailed $P < 0.01$.

Results

Establishment of KC cocultures

On average, 23 827 812 hepatocytes were isolated per gram liver tissue, with 84% viability, and 387 340 KCs were isolated (Table 2). The hepatocytes showed typical cell morphology and vitality. Initially, KCs were morphologically round with a diameter of 10 µm (Fig. S1, Fig. S2). After Day 1, the cells developed typical stellate cell extensions (Fig. S1). Liver-specific markers were detected by immunostaining on Days 1 and 5 with CK18 and panCK (Fig. 3). On Day 2, 41.3% of KCs contained phagocytosed fluorescent latex beads. This decreased to 25.1% on Day 3 and to 13.6% on Day 4 (Fig. 4).

Suitability of cell culture conditions during stimulation experiments

Mean viability after Percoll density centrifugation was 91.4 ± 1.9% in the six livers used for the experiments with IFNτ. The mean urea concentration in each experiment was comparable, with concentrations of 12.43 ± 0.94, 12.26 ± 1.25 and 12.62 ± 1.55 nmol $well^{-1}$ in ES1 (HKCd), ES2 (HKCid) and ES3 (HMC) respectively. LDH concentrations were also comparable in each experiment: 3.32 ± 0.51, 1.79 ± 0.84 and 4.97 ± 2.89 mU $well^{-1}$ in ES1, ES2 and ES3 respectively. There were also no significant differences in albumin expression (9.62 ± 1.85, 10.77 ± 2.21, 11.60 ± 1.91 and 12.09 ± 1.92 for basal albumin expression and expression in ES1, ES2 and ES3 respectively).

Basal expression of genes of interest

The intra-assay CVs for basal expression were 7.5% for *MX1*, 11.6% for *ISG-15*, 5.9% for *CXCL-5*, 7.4% for *CXCL-10*, 12.5% for *IGF-1* and 7.9% for *IGFBP-2* (Table 3). The relative mRNA expression of the target genes did not differ significantly between the six livers analysed in the stimulation experiments.

Stimulation of hepatocytes and KC coculture with IFNτ, IFNα and P4

The mRNA expression of ISGs (*MX1*, *ISG-15*, *CXCL-5* and *CXCL-10*) increased in mono- and cocultures during the first days of culture until Day 3, but decreased sharply when morphological restructuring of hepatocytes was finished on Day 4 (Fig. 5). On Day 4, the expression of the inflammatory-/IFN-related genes *MX1*, *ISG-15*, *CXCL-5* and *CXCL-10* was back to baseline levels in HMC and HKCid. In contrast, mRNA levels of the metabolic genes (*IGF-1* and *IGFBP-2*) increased towards Day 3 and remained higher on Day 4 than on Day 1. The overall expression profiles were comparable between mono- and cocultures (Fig. 5).

The addition of 0.6 ng mL^{-1} IFNτ upregulated *ISG-15* and *MX1* mRNA levels in HMC and HKCid (Fig. 6*a*, *b*). *CXCL-10* mRNA levels increased sharply in HMC stimulated with 0.6 ng mL^{-1} IFNτ or IFNα, but not in HKCid after stimulation with 0.6 ng mL^{-1} IFNτ. In HKCid, the addition of 0.06 ng mL^{-1} IFNα resulted in significant upregulation of *CXCL-10* mRNA expression ($P = 0.0041$; Fig. 6*c*). Stimulation of HMC with 0.06 ng mL^{-1} IFNτ or IFNα resulted in higher *CXCL-5* mRNA expression than without stimulation (Fig. 6*d*). The addition of IFNα increased *IGFBP-2* mRNA expression exclusively in HKCid, whereas the addition of IFNτ reduced the expression of *IGFBP-2* (Fig. 6*f*).

The addition of P4 alone had no effect on *MX1* and *CXCL-10* mRNA expression in HMC or HKCid (Fig. 7*a*, *c*). However, *CXCL-5* mRNA levels increased significantly after stimulation with 10 ng mL^{-1} P4 ($P = 0.0055$; Fig. 7*d*). The addition of IFNτ increased *MX1*, *ISG-15*, *CXCL-10* and *CXCL-5* mRNA expression, whereas additional stimulation with 5 ng mL^{-1} P4 reduced the expression of these four genes. This reduction was more pronounced when the P4 concentration was increased to 10 ng mL^{-1} (Fig. 7*a–d*). In HKCd, *IGF-1* and *IGFBP-2* mRNA expression was comparable in the presence and absence of P4, but decreased after stimulation with 0.6 ng mL^{-1} IFNτ alone. However, this decrease was reversed by the addition of 5 ng mL^{-1} P4 to the HKCd (Fig. 7*e*, *f*).

Discussion

Establishment of KC cocultures

The first aim of the present study was to establish a hepatocyte coculture with KCs to test whether hepatocytes respond to IFN directly or whether KCs transduce the signal instead. The hepatocyte sandwich culture had been established previously (Witte *et al.* 2019). The present work confirmed that, in a sandwich culture system, the shape of hepatocyte cells changed to cuboidal and polygonal, and hepatocytes formed cords reminiscent of the hepatic trabeculae with bile canaliculi, which can be detected as bright elucidations between hepatocytes. This has already been reported by Witte *et al.* (2019).

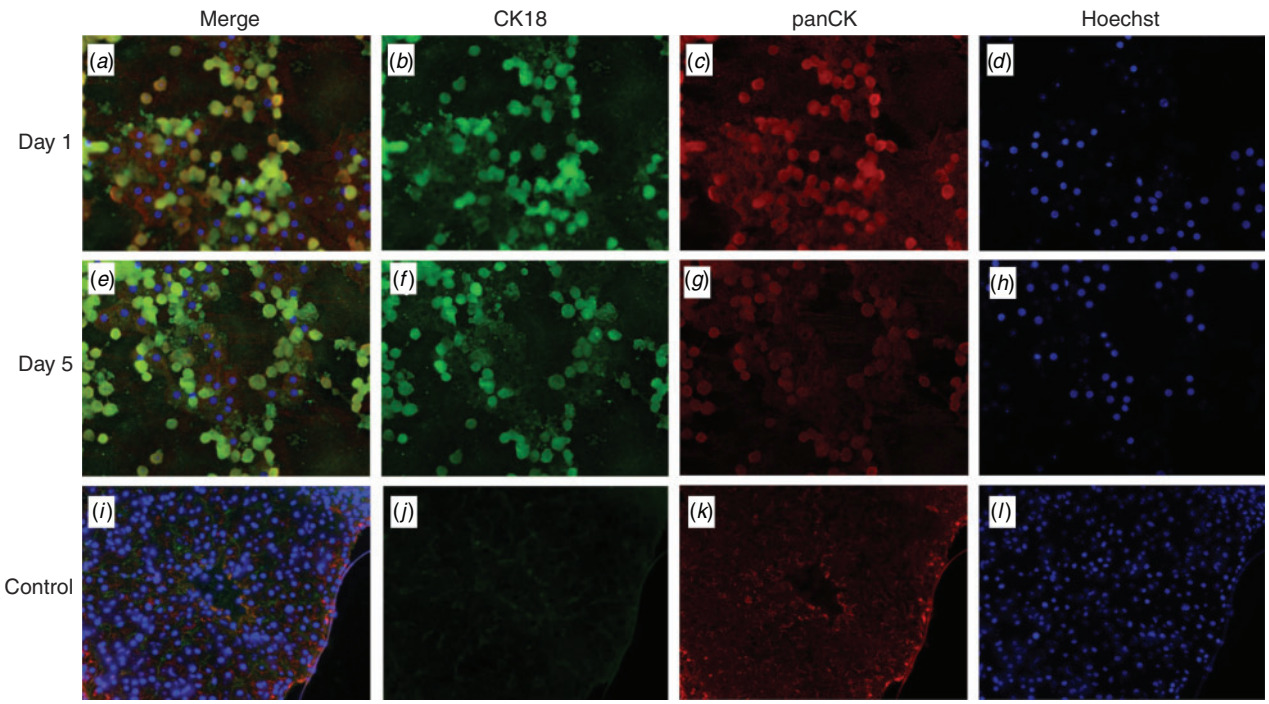

Fig. 3. Hepatocytes on Day 1 (*a–d*) and Day 5 (*e–h*) in sandwich monoculture stained with CK18 (red) and panCK (green) to confirm liver-specific cell surface markers in culture. (*i–l*) A cryosection used as a control. Intact nuclei were stained blue using a Hoechst stain.

The separation of sessile KCs from bovine livers was established using a previously established protocol (Smedsrød *et al.* 1985). KCs in culture were viable, as indicated by the phagocytosis of fluorescent latex beads. In HKCid cocultures, KC could be detected based on phagocytosed latex beads on Days 2, 3 and 4, suggesting immunologically active cells. KCs are located in the endothelium of liver sinusoids and are responsible for the phagocytosis of pathogens before they enter the liver, as well as the elimination of apoptotic endothelial cells. KCs are normally in an inactive state, but can be activated by inflammatory signals (Duque and Descoteaux 2014). The activation of sessile KCs can be differentiated into classical activation (e.g. by IFNγ or toll-like receptors (TLRs)/interleukin (IL)-1 receptors). This results in the production of proinflammatory cytokines, such as IL-1, IL-6, tumour necrosis factor-α, transforming growth factor-β, IFNγ, epidermal growth factor and leukaemia inhibitory factor (Gordon and Martinez 2010). In addition to the classical activation, IL-4 and/or IL-13 can also activate M2 macrophages (Stein *et al.* 1992; Wynn *et al.* 2013) to exhibit anti-inflammatory functions. The third group of activated KCs are regulatory KCs, which need two activation signals: a priming-signal from apoptotic cells, IL-10, prostaglandins or glucocorticoids and a second signal via their TLRs (Mosser and Edwards 2008). It would be interesting to determine whether the sessile KCs in the hepatocyte culture are inactivated or activated, but unfortunately mRNA expression analyses of factors differentiating between the two states were not performed in the present study.

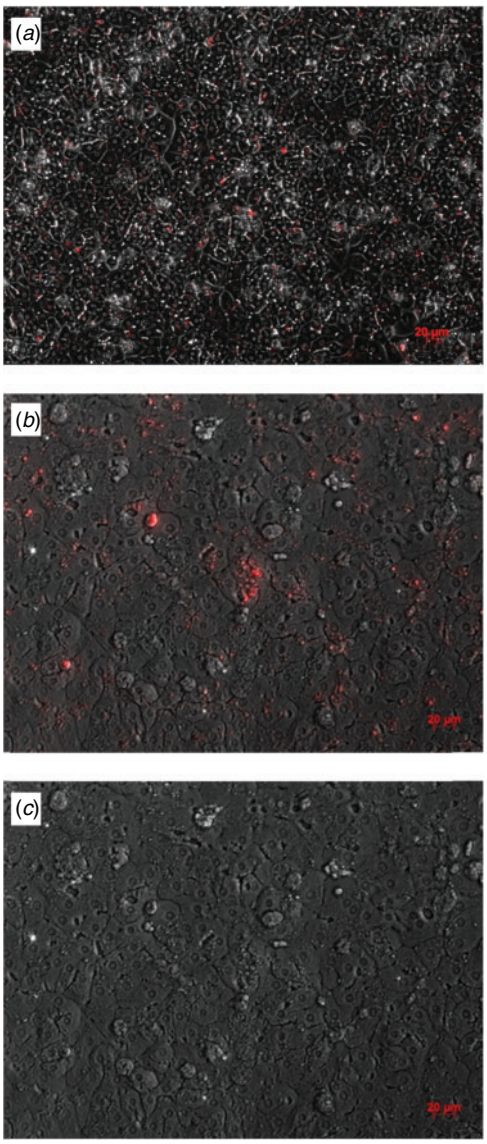

Fig. 4. Inverse phase contrast images of hepatocytes in indirect coculture with Kupffer cells (HKCid), with (*a*, *b*) and without (*c*) fluorescence. Viable Kupffer cells can be recognised by phagocytosed red fluorescent beads.

The basal expression of the genes of interest varied less than 13% between the six livers from different donors. This leads to the assumption, that regardless of the donor animal, mono- and cocultures of hepatocytes lead to comparable results. During the 4 days of incubation, the expression profiles of *MX1*, *ISG-15*, *CXCL-5* and *CXCL-10* were also comparable. The expression of inflammatory genes was initially increased, which paralleled the morphological changes noticeable in the cultures and is suggestive of a proinflammatory state. Separation of hepatocytes leads to an increase in nuclear factor (NF)-κB expression (Li and Verma 2002; Vinken *et al.* 2014). NF-κB is an important regulator of inflammation (Li and Verma 2002) and may indirectly induce ISG via IFNβ (Sen and Sarkar 2007; Oeckinghaus and Ghosh 2009). Although not further examined in the present study, this may be a possible pathway for the upregulation of the chemokines investigated in this study.

Within the first 3 days of incubation, hepatocytes developed the specific polygonal cell shape, cell–cell contacts and canaliculi (Ehrhardt and Schmicke 2016; Witte *et al.* 2019). In parallel with an end to the morphological changes, the expression of inflammatory genes decreases. In agreement with Ehrhardt and Schmicke (2016) and Witte *et al.* (2019), this led us to conclude that Day 4 of culture is most suitable for experiments in primary bovine hepatocytes. Moreover, the continuously increasing

Table 3. Basal expression of target genes in six livers (for two samples per liver)

MX1, Myxovirus-resistance protein 1; ISG-15, interferon-stimulated gene 15 kDa; *CXCL-5*, chemokine (C-X-C motif) ligand 5; *CXCL-10*, chemokine (C-X-C motif) ligand 10; *IGF-1*, insulin-like growth factor-1; *IGFBP-2*, insulin-like growth factor-binding protein 2

	MX1	*ISG-15*	*CXCL-5*	*CXCL-10*	*IGF-1*	*IGFBP-2*
Mean (±s.d.) expression	19.6 ± 1.5	16.3 ± 1.9	22.6 ± 1.3	20.5 ± 1.5	18.2 ± 2.3	11.8 ± 0.9
CV (%)	7.5	11.6	5.8	7.4	12.5	8.0

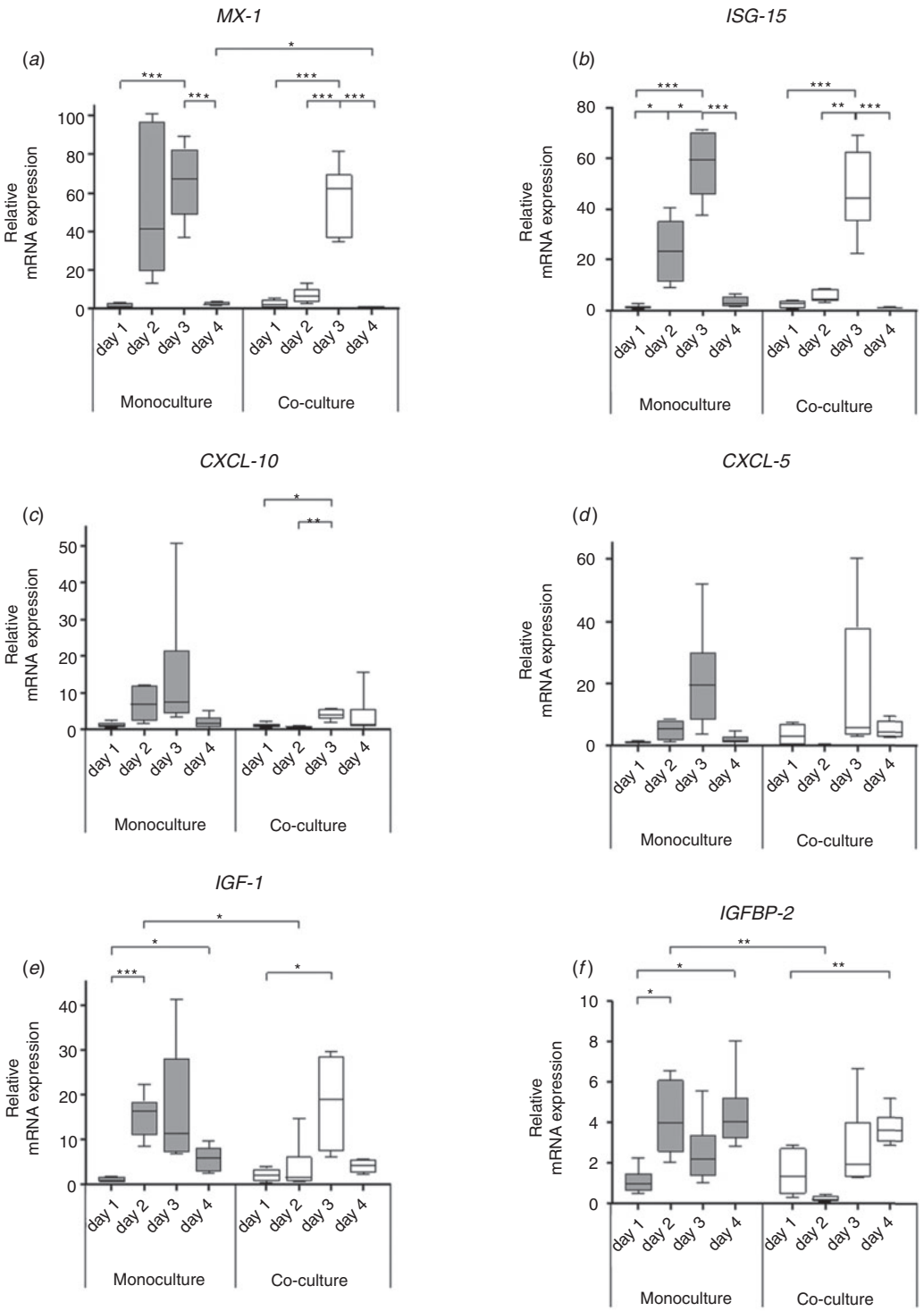

Fig. 5. Relative mRNA expression of (*a*) Myxovirus-resistance protein-1 (*MX1*), (*b*) interferon-stimulated gene 15 kDa (*ISG-15*), (*c*) chemokine (C-X-C motif) ligand 10 (*CXCL-10*), (*d*) chemokine (C-X-C motif) ligand 5 (*CXCL-5*), (*e*) insulin-like growth factor 1 (*IGF-1*) and (*f*) insulin-like growth factor-binding protein 2 (*IGFBP-2*) in hepatocyte monoculture (HMC) and indirect coculture of hepatocytes with Kupffer cells (HKCid) on Days 1, 2, 3 and 4. The mRNA data are presented as ΔΔCt relative to a calibrator. The boxes show the interquartile range, with the median value indicated by the horizontal line; whiskers show the range ($n = 6$). *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$.

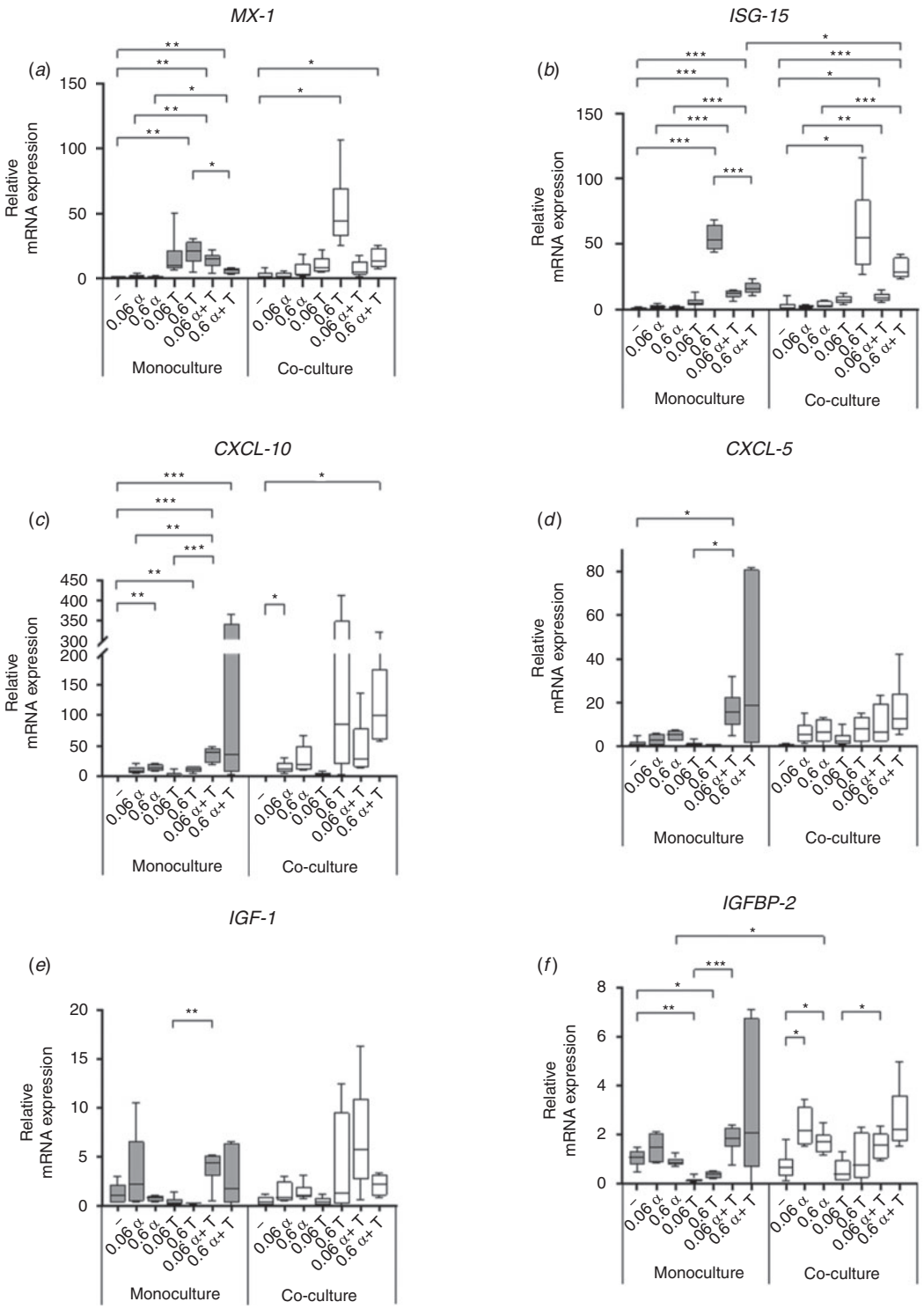

Fig. 6. Relative mRNA expression of (*a*) Myxovirus-resistance protein-1 (*MX1*), (*b*) interferon (IFN)-stimulated gene 15 kDa (*ISG-15*), (*c*) chemokine (C-X-C motif) ligand 10 (*CXCL-10*), (*d*) chemokine (C-X-C motif) ligand 5 (*CXCL-5*), (*e*) insulin-like growth factor 1 (*IGF-1*) and (*f*) insulin-like growth factor-binding protein 2 (*IGFBP-2*) in hepatocyte monoculture (HMC) and indirect coculture of hepatocytes with Kupffer cells (HKCid) stimulated with 0.6 or 0.06 ng mL^{-1} IFNα (α) or bovine recombinant IFNτ (τ) for 6 h on Day 4 of culture. The mRNA data are presented as ΔΔCt relative to a calibrator. The boxes show the interquartile range, with the median value indicated by the horizontal line; whiskers show the range ($n = 6$). $^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

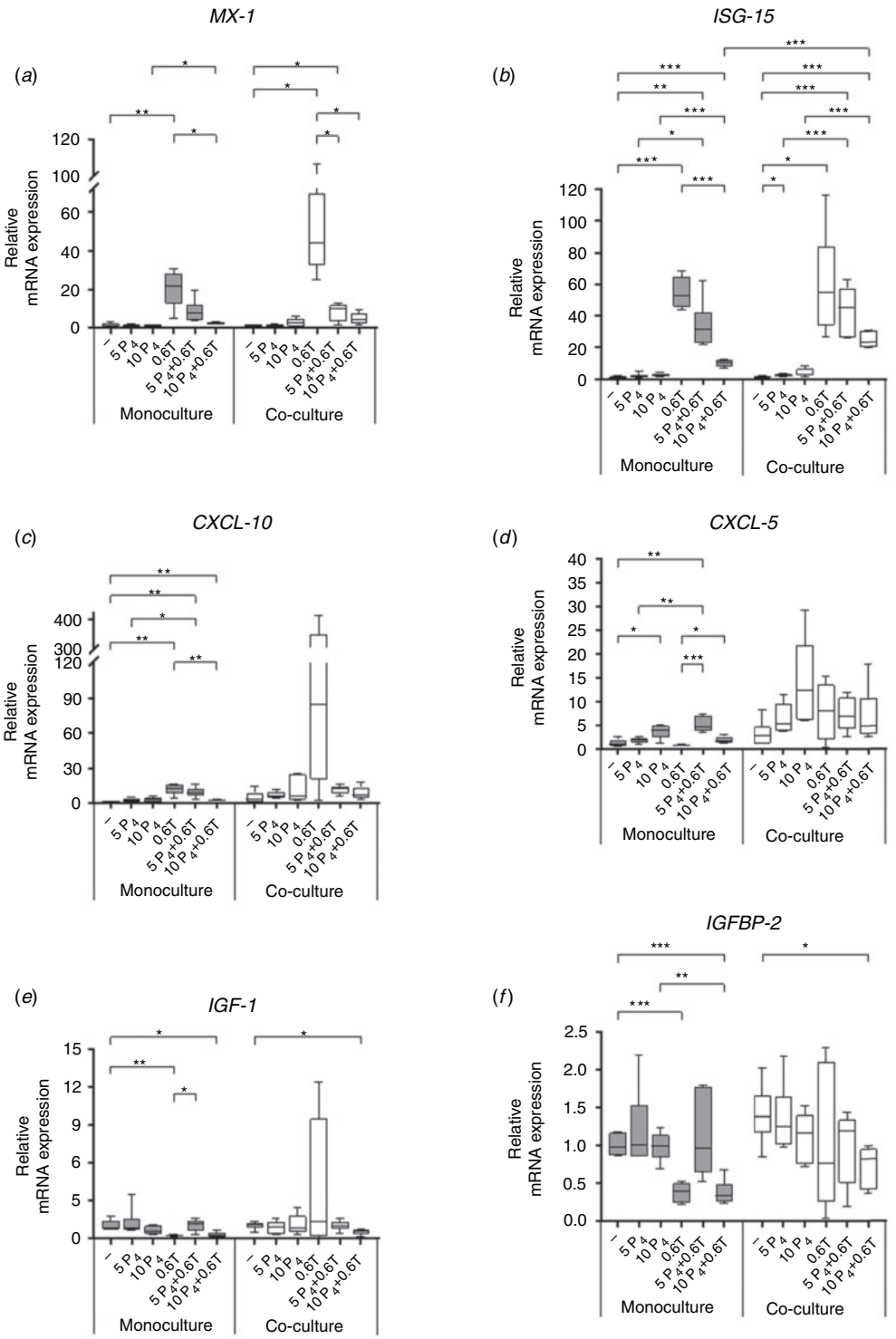

Fig. 7. Relative mRNA expression of (*a*) Myxovirus-resistance protein-1 (*MX1*), (*b*) interferon (IFN)-stimulated gene 15 kDa (*ISG-15*), (*c*) chemokine (C-X-C motif) ligand 10 (*CXCL-10*), (*d*) chemokine (C-X-C motif) ligand 5 (*CXCL-5*), (*e*) insulin-like growth factor 1 (*IGF-1*) and (*f*) insulin-like growth factor-binding protein 2 (*IGFBP-2*) in hepatocyte monoculture (HMC) and indirect coculture of hepatocytes with Kupffer cells (HKCid) stimulated with 5 or with 10 ng mL^{-1} progesterone (P4) and 0.6 ng mL^{-1} bovine recombinant IFNτ (τ) for 6 h on Day 4 of culture. The mRNA data are presented as ΔΔCt relative to a calibrator. The boxes show the interquartile range, with the median value indicated by the horizontal line; whiskers show the range ($n = 6$). *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$.

IGFBP-2 mRNA expression levels throughout the 4 days of incubation indicate that the highest rate of metabolic synthesis is achieved on Day 4.

Effects of IFNτ versus IFNα on target gene expression in the HMC and HKCid models

Once established, the HMCid model was used to investigate the following questions: (1) is liver cell culture suitable for testing the effects of Type I IFNs; (2) does the effect of Type I IFNs differ between pure hepatocytes and hepatocytes cocultured with KCs; (3) does the expression of ISGs differ between hepatocytes stimulated with IFNτ and other Type I IFNs; and 4) does simultaneous stimulation with P4 and IFNτ affect the expression levels of individual target genes in the maternal liver?

As suspected, and previously shown in hepatocytes, stimulation with IFNτ induced classical ISGs (*MX1* and *ISG-15*; Ruhmann *et al.* 2017). The stimulation was dose dependent and did not depend on the presence of sessile KCs because the expression levels were comparable between the HMC and HKCid models. Others have previously reported an increase in *CXCL-10* expression in response to IFNα in human thyrocytes (Antonelli *et al.* 2010) and in response to IFNτ in sheep endometrial cells (Imakawa *et al.* 2006). In line with these findings, *CXCL-10* mRNA expression was increased in response to the higher concentration of 0.6 ng mL^{-1} IFNτ in hepatocytes in HMC in the present study. *CXCL-10* expression has previously been identified in KCs and in the uterus of sheep during the peri-implantation period (Fernandez and Lolis 2002; Imakawa *et al.* 2006; Dembic 2015). It has previously been shown that the endometrial IGF system changes in early pregnancy, and IFNτ has been speculated as a possible factor for changes in the endometrial gene expression (Robinson *et al.* 2008). Following on from this, Meyerholz *et al.* (2015) reported a decrease in *IGFBP-2* during early pregnancy (towards Day 18 of gestation) in dairy heifers. *IGFBP-2* isolated from blood samples is mainly produced in the liver. Therefore, the hypothesis that IFNτ directly affects hepatic *IGFBP-2* production was confirmed in the present study. The addition of a low concentration (0.06 ng mL^{-1}) of IFNτ to the HMC for 6 h led to a reduction in *IGFBP-2* mRNA. Furthermore, the present study examined differences in responses to IFNτ compared with other Type I IFNs. For this comparison, IFNα was chosen as an alternative Type I IFN because it is normally produced by nearly all nucleated cells, especially leucocytes (Ohmann *et al.* 1987).

IFNα was shown to induce *CXCL-5* and *CXCL-10* mRNA expression but not the expression of *MX1* and *ISG-15*. This appears to be a specific IFNτ pattern despite the fact that all Type I IFNs bind to the same receptor, namely *IFNAR1/2* (Li and Roberts 1994; Mogensen *et al.* 1999; Uzé *et al.* 2007). Future studies will need to show why *ISG-15* and *MX1* mRNA expression is exclusively induced by IFNτ in primary hepatocytes and whether comparable effects can be observed *in vivo*. However, should be noted that in the present study only mRNA expression was evaluated, and not protein expression. This aspect should also be further investigated in future studies.

Effects of P4 versus IFNτ on target gene expression in HMC and HKCid

During early pregnancy, ISG expression is dependent on steroid hormones like P4 (Spencer *et al.* 2016). In heifers supplemented with P4 on Day 16 of pregnancy, *ISG-15* and *CXCL-10* mRNA expression was higher in uterine epithelial cells than in heifers with lower endogenous P4 concentrations (Forde *et al.* 2009). In the present study, based on physiological P4 concentrations on Day 16 of pregnancy (5–10 ng mL^{-1}; Matsuyama *et al.* 2012), concentrations of 5 and 10 ng mL^{-1} P4 were used in the HKCid cultures to detect potential effects not only in reproductive tissues, but also in the liver. In liver cells, only *ISG-15* mRNA expression was slightly increased in response to P4. There was no change in *MX1* and *CXCL-10* mRNA expression after stimulation with P4. After the addition of 5 ng mL^{-1} P4 and 0.6 ng mL^{-1} IFNτ to cultures, *ISG-15* expression was higher than after the addition of 10 ng mL^{-1} P4 and 0.6 ng mL^{-1} IFNτ. This is in contrast to the results in endometrial tissue reported by Forde *et al.* (2009), who detected higher *ISG-15* and *CXCL-10* mRNA expression in samples stimulated with higher concentrations of P4. However, the P4 concentrations used by Forde *et al.* (2009) were higher *in vivo* than the concentrations used in the present *in vitro* study. Moreover, different cell types were used and the reaction to P4 may differ between the endometrium and liver. Further studies should also focus on respective hormone receptor expression and intracellular signal transduction pathways, which may differ in different tissues. Interestingly, *CXCL-5* mRNA expression was only stimulated by P4 and the additional effect of IFNτ was lower compared with that on the other genes. Increased *CXCL-5* expression in response to P4 was previously reported in vaginal tissue of mice after ovariectomy (Gillgrass *et al.* 2005). This led to speculation about the presence of a protection mechanism for pregnant animals against infectious particles during pregnancy. This is imaginable for the liver as well, because *CXCL-5* attracts and activates neutrophils and induces an immune response (Dembic 2015). However, P4 receptors were not analysed in the present study, and therefore the comparison with reproductive tissue is debatable. After stimulation of the HKCid with IFNτ and P4, *IGF-1* mRNA expression decreased, which is in line with previous studies. McCarthy *et al.* (2012) showed that *IGF-1* expression on Day 16 after ovulation decreased significantly in pregnant but not cycling cows compared with expression on Day 5. Several other studies also detected lower *IGF-1* expression during the luteal phase in endometrial (Meikle *et al.* 2001) and serum (Mense *et al.* 2015) samples.

The expression of *IGFBP-2* was lower in HMC and HKCid after stimulation with P4 and IFNτ. As mentioned above, Meyerholz *et al.* (2015) also detected a decrease in blood *IGFBP-2* in early pregnant cows (Day 14). However, McCarthy *et al.* (2012) detected an increase in *IGFBP-2* mRNA expression in the endometrium from Day 7 towards Day 13. Because the liver is the main source of *IGFBP-2*, it can be hypothesised that lower peripheral *IGFBP-2* may lead to higher free *IGF-1* concentrations, whereas higher local *IGFBP-2* may bind the additional *IGF-1*, which is essential for fetal growth (Lassarre *et al.* 1991).

In conclusion, primary bovine hepatocyte cultures were proven to be a suitable model for stimulation experiments with Type I IFNs and investigation of extrauterine embryo–maternal communication. The results of the present study show that there is a significant increase in *MX1*, *ISG-15*, *CXCL-5*, *CXCL-10*, *IGF-1*, *IGFBP-2* mRNA expression in hepatocytes in response to stimulation with IFNτ. Thus, IFNτ has been confirmed to have a direct effect on the mRNA expression of target genes in hepatocytes. The KCs in cocultures seemed to make no major contribution to the effect of IFNτ on hepatocytes. In contrast, hepatocytes seem to respond differently when P4 was added to the cultures. Although *MX1* und *ISG-15* mRNA expression was stimulated by IFNτ in both hepatocytes and endometrial cells, the downregulation of *IGFBP-2* mRNA expression was only detected in hepatocytes and not in the endometrium. The proposed endocrine action of IFNτ in the maternal liver may affect maternal metabolism, as well as maternal immune function in the liver. Further investigations are needed to elucidate the underlying mechanisms, which may be relevant for pregnancy establishment, and further research is required to examine potential systemic and endocrine effects on other organs during the early pregnancy in ruminants.

Conflicts of interest

The authors declare no conflicts of interest.

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