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ARTICLE

The PD-L1- and IL6-mediated dampening of the IL27/STAT1 anticancer responses are prevented by α-PD-L1 or α-IL6 antibodies

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Abstract

Interleukin-27 (IL27) is a type-I cytokine of the IL6/IL12 family and is predominantly secreted by activated macrophages and dendritic cells. We show that IL27 induces STAT factor phosphorylation in cancerous cell lines of different tissue origin. IL27 leads to STAT1 phosphorylation and recapitulates an IFN-γ-like response in the microarray analyses, with up-regulation of genes involved in antiviral defense, antigen presentation, and immune suppression. Like IFN-γ, IL27 leads to an up-regulation of TAP2 and MHC-I proteins, which mediate increased tumor immune clearance. However, both cytokines also upregulate proteins such as PD-L1 (CD274) and IDO-1, which are associated with immune escape of cancer. Interestingly, differential expression of these genes was observed within the different cell lines and when comparing IL27 to IFN-γ. In coculture experiments of hepatocellular carcinoma (HCC) cells with peripheral blood mononuclear cells, pre-treatment of the HCC cells with IL27 resulted in lowered IL2 production by anti-CD3/-CD28 activated T-lymphocytes. Addition of anti-PD-L1 antibody, however, restored IL2 secretion. The levels of other T_H1 cytokines were also enhanced or restored upon administration of anti-PD-L1. In addition, we show that the suppression of IL27 signaling by IL6-type cytokine pre-stimulation—mimicking a situation occurring, for example, in IL6-secreting tumors or in tumor inflammation–induced cachexia—can be antagonized by antibodies against IL6-type cytokines or their receptors. Therapeutically, the antitumor effects of IL27 (mediated, e.g., by increased antigen presentation) might thus be increased by combining IL27 with blocking antibodies against PD-L1 or/and IL6-type cytokines.

KEYWORDS
cytokine, interferon, IFN-γ, IDO-1, OSM

Abbreviations: CD, Cluster of differentiation; CD274, See PD-L1; CLF1, Cytokine-like factor 1; CTLA4, Cytotoxic T-lymphocyte associated protein 4; CXCL, C-X-C motif chemokine ligand; DC, Dendritic cell; DMEM, Dulbecco's modified Eagle medium; EBI3, Epstein-Barr virus induced gene 3; subunit of IL27; FBS, Fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HLA, Human leukocyte antigen; hy-IL6, Hyper-IL6; IFN, Interferon; IL, Interleukin; IL18BP, Interleukin 18 binding protein; LAP3, Leucine aminopeptidase 3; MCP, Monocyte chemoattractant protein; MHC, Major histocompatibility complex; NK, Natural killer cell; OSM, Oncostatin M; PBMC, Peripheral blood mononuclear cells; PD-L1, Programmed cell death 1 ligand 1; PSME, Proteasome activator subunit; rPAP, Rat pancreatitis-associated protein 1; SOCS, Suppressor of cytokine signaling; STAT, Signal transducer and activator of transcription; TAP, Transporter, ATP binding cassette subfamily B member; TNF, Tumor necrosis factor

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1 | INTRODUCTION

The heterodimeric interleukin 27 (IL27), composed of the noncovalently linked subunits p28 and EBI3 (Epstein-Barr virus induced gene 3), is related to the IL12-type-cytokines IL12, IL23, and IL35 composed of two heterodimeric subunits, which are either covalently linked (IL12, IL23) or not (IL27, IL35). IL27 signals via a receptor complex composed of the IL27-specific receptor chain IL27Rα (WSX1)[1] and the common receptor subunit of IL6-type cytokines, IL6Rβ (gp130).[2] Thus, IL27 is also related to the IL6-type cytokines, as the IL6- and IL12-type cytokines form a common superfamily.

Cytokines such as interferons, IL2, IL12, IL7, IL15, IL27, and others are tested as potential anticancer immuno-stimulatory agents and some have progressed into clinical trials.[3–5] IFN-γ is one of those cytokines long known to be involved in antitumor defense, because IFN-γR and STAT1 deficiency has been described to increase cancer susceptibility upon treatment with chemical pathogens in mice.[6,7] Interferon adjuvant therapy has unfortunately invariably been reported to have high toxicity, for example, in hepatocellular carcinoma (HCC) patients.[8] Also the antitumor effects of other cytokines such as IL12 have been attributed to expression of IFN-γ.[9,10] IL27 has been described to have pro- or anti-inflammatory functions in different systems[11–14] and it is capable of exerting antitumor effects. IL27 seems to be better tolerated than interferons[8] and IL12[15] in preclinical models, probably because less IFN-γ is released by IL27 compared to IL12. Combination of IL27 and IL2 has been shown to be strongly synergistic in a murine neuroblastoma model.[16] Treg levels were suppressed and IFN-γ levels elevated for the combination treatment compared to the single treatments. IL2 therapy in humans has been shown to have a relatively low risk of severe side effects, although it was only effective in 10–20% of the patients.[17–19] Therefore it still remains to be seen whether a combination of IL27 and IL2 will be more effective—as in the murine model—and still well tolerated.

IL27 is discussed to be a candidate cytokine for therapeutic approaches and acts on both the cancer cells themselves and on immune cells. IL27 has been shown to enhance antitumor immune responses[20–25] via, for example, $CD8^+$ T cells[23] or NK cells.[26,27] IL27 is involved in T_H1 commitment by suppressing GATA3 (GATA binding protein 3), inducing T-bet, resulting in IL-12Rβ2 expression in naïve T cells, thereby supporting the IL12-dependent IFN-γ secretion.[1,28–30] It activates $CD8^+$ cells, NK cells, induces T_H1 responses and acts on endothelial cells to secrete anti-angiogenic factors, as does IFN-γ.[13] IL27 also induced antibody-dependent cell-mediated cytotoxicity (ADCC).[31] IL27 has also been described to limit IL2 production by T-cells during T_H1 differentiation.[32] The promotion of $CD8^+$ T-, NK or T_H1-cells leads to the production of IFN-γ and other T_H1 cytokines that promote differentiation of M1 macrophages. The M1 macrophages secrete cytokines such as IFN-γ, IL12, TNF-α, and IL1, which promote cancer clearance by T_H1-, NK-cells, and CTLs (reviewed in[33–36]). IL12 also induces differentiation of mature dendritic cells (DCs) that present cancer antigens on their surface. Recently, IL27 has emerged as an important immune-regulatory cytokine, antagonizing the development of T_H17 cell responses,[34–38] which have cancer promoting effects (e.g., angiogenesis promoting functions).[39] Immunosuppressive inducible $Foxp3^+$ and $Foxp3^-$ regulatory T cells (iTreg) are also inhibited by IL27.[40–45] Thus the effects of IL27 on the immune system show a profile that might be compatible with its proposed function as an anticancer cytokine (see also Supporting Information Fig. 5).

IL27 does, however, not only act on immune cells but can also elicit antitumor responses in cancer cells directly, such as antiproliferative, pro-apoptotic, anti-angiogenic, and anti-metastatic functions, which have recently been reviewed extensively.[13] Briefly, IL27 showed antiproliferative effects on various melanoma cell lines[46] and, like IFN-γ, inhibition of angiogenesis.[20,47] The growth of poorly immunogenic B16/F10 melanoma tumors in syngeneic mice was reduced when the cells had been transfected with scIL27.[15] We have previously reported IFN-γ-like responses and antiviral activity of IL27 in hepatoma cells, primary hepatocytes, and hepatic stellate cells,[48,49] as has also been described for other cell types.[13,24,50]

We have described before that IL27 signaling is susceptible to inhibition by SOCS3, which is induced, for example, upon phosphorylation of STAT3 by IL6-type cytokines.[50] Many tumors have been shown to secrete IL6-type cytokines and/or to have constitutive STAT3 activation (and thus elevated SOCS3 levels).[51–53] In addition, IL6 has been shown to be crucial for colorectal cancer and HCC development.[54–56] In cancer cachexia, IL6 and other pro-inflammatory cytokines are produced due to a tumor-induced systemic inflammation.[57,58] IL6 and TNF-α blockade have been used successfully to suppress cancer cachexia.[59,60]

Here we describe that IL27 can stimulate cancer cells of different lineages and further characterize IL27 signaling in these cell lines. Microarray analysis showed that IL27 induced an IFN-γ-like, STAT1-dependent transcriptional response, mediating, for example, up-regulation of genes associated with increased tumor immune clearance and also mediating gene expression profiles associated with cancer immune escape, for example, PD-L1 and IDO-1 up-regulation. We show that IL27-induced PD-L1 can suppress IL2 production by peripheral blood mononuclear cells (PBMCs) and that this effect can be rescued by anti-PD-L1 antibody administration. Additionally, we used an IL6-type cytokine pre-stimulation approach to mimic tumor inflammation–mediated STAT3- and SOCS3-dependent IL27 inhibition and we show that this inhibition can be prevented by blocking anti-IL6, -IL6R, or -OSM antibodies.

2 | MATERIALS AND METHODS

2.1 | Materials and cell culture

Different cancer and noncancer cell lines were used: HCC cell lines (HepG2, Hep3B, Huh7), a normal hepatocyte cell line (PH5CH8), melanoma cell lines (A375, MeWo, IGR39, IGR37, FM55P, FM55M1), normal human epidermal melanocytes (NHEM), colon carcinoma cell lines (HCT116 and HT29), normal colon mucosa epithelial cells (NCM460), epidermoid carcinoma cells (A431), epithelial cervix carcinoma (HELA), breast adenocarcinoma cells (MCF7), and fibrosarcoma cells (2C4).

All cells were grown in a water-saturated atmosphere at 5% CO2 and at 37°C. HepG2, Hep3B, Huh7, PH5CH8, A431, MCF7, HELA, and 2C4 cells were maintained in DMEM medium (Lonza, Switzerland) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 ug/L streptomycin and 60 mg/L penicillin (Lonza) and 25 mM HEPES (Lonza). A375, MeWo, IGR39, IGR37, FM55P, FM55M1 were maintained in RPMI (Lonza), whereas HCT116 and HT29 were maintained in DMEM/F12 (Lonza), both media being supplemented with 10% FBS, 100 mg/L streptomycin, 60 mg/L penicillin. NHEM were maintained in melanocyte growth medium M2 (PromoCell, Germany). NCM460 were maintained in M3:BaseA (Incell, USA) supplemented with 10% fetal bovine serum.

Cells were stimulated according to the molecular masses of the different cytokines, with 20 ng/mL human recombinant OSM, IL6, and IFN-γ (Peprotech, USA), 50 ng/mL IL27 (R&D Systems, USA), or 40 ng/mL hyper-IL6 (hy-IL6) (provided by Prof. Dr. Stefan Rose-John, University of Kiel, Germany) unless stated otherwise in the figure legends.

The blocking antibodies used to antagonize IL6-type cytokine signaling were anti-human IL6 (R & D Systems Cat# MAB206, RRID: AB_2127617), anti-human IL6Rα (R & D Systems Cat# MAB227, RRID: AB_2127908) and anti-human OSM (R & D Systems Cat# MAB295, RRID: AB_2298931).

2.2 | Cytokine expression analysis in HCC/PBMC cocultures

The experiment was performed as described[61] with minor changes. The cryopreserved PBMCs were from Zenbio. Huh7 and Hep3B (7×10^4 per well) were pre-incubated in 48-well plates (in RPMI medium) with or without 100 ng/ml of IL27 for 24h. They were then washed 3 times with RPMI and incubated for 3 days with PBMC (3×10^5 per well) in the presence of Immunocult human CD3/CD28 T Cell activator (8 μl per well, Stemcell technologies) and a neutralizing anti-PD-L1 antibody (eBioscience, Cat# 16-5983-82, RRID:AB_469180; 10 μg/ml). The cell culture supernatants were collected and applied to a Q-Plex human cytokine array IR (Quansys biosciences), according to the manufacturers protocol. The fluorescent signals were detected on an Odyssey Infrared Imaging System (Li-COR Biosciences) and quantitated using the Q-view software (Quansys biosciences) according to the manufacturer's protocol. Detections of single cytokines by ELISA were performed using the IL2 (#88-7025-88) and IL6 ELISA kits (#88-7066-88) from Invitrogen according to the manufacturer's protocol.

2.3 | Cell lysis and Western blot analysis

Cell lysis was performed as described before.[62] 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), phospho-STAT1 (pSTAT1) (BD Biosciences Cat# 612233, RRID:AB_399556), STAT1 (BD Biosciences Cat# 610116, RRID:AB_397522), tubulin (Santa Cruz Biotechnology Cat# sc-32293, RRID:AB_628412), phospho-STAT3 (pSTAT3) (Cell Signaling Technology Cat# 9145S, RRID:AB_561305), TAP2 (Cell Signaling Technology Cat# 12259 RRID:AB_2619687), Vinculin (Abcam Cat# ab18058, RRID:AB_444215), phospho-STAT5 (pSTAT5) (BD Biosciences Cat# 611965, RRID:AB_399386), phospho-STAT4 (pSTAT4) (Cell Signaling Technology Cat# 4134S, RRID:AB_11179071), IDO-1 (Cell Signaling Technology Cat# 86630, RRID: AB_2636818). The 4 SOCS3 antibodies tested here were i) Santa Cruz Biotechnology Cat# sc-7009, RRID:AB_2193311, ii) Santa Cruz Biotechnology Cat# sc-73045, RRID:AB_1129585 iii) Cell Signaling Technology Cat# 2932, RRID:AB_2286460 and iv) IBL-America Cat# JP18395, RRID:AB_1643379. The 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 pH8.8 and 0.2 mM para-coumaric acid[63] and a CCD camera system (BioRad) or an Odyssey Infrared Imaging System (Li-COR Biosciences). The fluorescently labelled secondary antibodies (IRDye 680RD and IRDye 800CW) were from Li-COR Biosciences. Quantitation of the fluorescent signals was performed on single channels with the analysis software Image Studio Lite V4.0 provided by LI-COR Biosciences (Lincoln, NE, USA) as described before.[50]

2.4 | Quantitative PCR

RNA extraction and qPCR was performed as described before.[50] Primers were purchased from Eurogentech and the sequences were as follows: IL27Rα-F (GCATCCTATTCTTGTGGGG), IL27Rα-R (CACTTTGTGCCTTAGGTGGT), IL6Rβ-F (TGAAACTGCTGTGAATGTGG), IL6Rβ-R (CATCCTTCCCACCTTCATCT), SOCS3-F (ATGAGAACTGCCAGGGAATC), SOCS3-R (CCCAGGCTCCACAA CCA).

2.5 | 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 2000 (Life Technologies) transfection reagent or PromoFectin-Hepatocyte (for Hep3B, PromoCell). Twenty four hours after transfection, cells were stimulated for 24 h as described in the figure legends. Lysis and luciferase assay were carried out as described before.[64] Experiments were performed at least in biological triplicate, each with 3 technical replicates. Figures show a representative biological replicate with mean and standard deviation calculated from 3 technical replicates. The STAT3 specific luciferase reporter, pXP2d2-rat pancreatitis-associated protein 1 (rPAP1), was described earlier.[65]

2.6 | 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, Germany, Cat# 21159034, RRID: AB_2629494), anti-IL6Rβ-PE (BD Biosciences, USA, Cat#555757, RRID: AB_396098), anti- IL27Rα-PE (R & D Systems, USA, Cat# FAB14791P, RRID:AB_10718687), and anti-CD274-PE (PD-L1, eBioscience, USA, Cat# 12-5983-42, RRID:AB_11042286),

or corresponding IgG control antibodies for 1 h at 4°C. Cells were then washed with PBS/azide. Cells analyzed on a FACSCanto II flow cytometer using FACSDiva software (BD Biosciences). Overlays were created using the FlowJo software.

2.7 | Whole genome microarray analysis

The cell lines (PH5CH8, Hep3B, Huh7, NHEM, A375, MelJuso, NCM460, HCT116 and HT29) were left untreated or were stimulated with hyper-IL6 (hy-IL6)[66] (20 ng/ml), OSM (20 ng/ml), IFN-γ (50 ng/ml), or IL27 (50 ng/ml) for 24 h. Total RNA was extracted using Quick-RNA (Zymo Research, USA). For each treatment, two independent biological replicates were analyzed. RNA integrity was verified with a Bioanalyzer 2100 (Agilent, USA) and only RNA with a RIN $>$ 8 was used. 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). A total of 100 ng of RNA was used as starting amount for microarray experiments. Microarray data were normalized and summarized to gene level by using the RMA[67] algorithm with GC correction in the 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[68] and using linear models in an empirical Bayesian approach implemented in the R/Bioconductor package *limma*.[69] A global linear model accounting for cell lines and treatments was fit, and then *P*-values were estimated for various contrasts. *P*-values were adjusted for multiple comparison by Benjamin-Hochberg's FDR method[70] and genes with FDR $<$ 0.05 were selected.

2.8 | Principal component analysis

The microarrays of the cell lines responding to the 4 cytokine treatments (PH5CH8, Hep3B, Huh7, NHEM, A375, NCM460 and HT29) were subjected to principle component analysis (PCA). Computation and plotting were performed using R (v3.4.0)[68] and Rstudio (v1.1).[71] The PCA was computed using the R package *FactoMineR* (v1.36)[72] and plotted by the R package *factoextra* (v1.0.4).[73] Other plots were generated using the R package *ggplot2* (v2.1)[74] and the collection of packages *tidyverse* (v1.1).[75] All experiments were scaled to the same unit, as the IFN-γ stimulation for the NHEM cells possessed a larger variance compared to the other experiments.

3 | RESULTS

3.1 | IL27 triggers phosphorylation of STAT1 and STAT3 transcription factors in many different cancer cell lines

IL27 has been described to use the ubiquitously expressed common signal transducing receptor of the IL6-type cytokines, IL6Rβ, in combination with IL27Rα. We stimulated a number of cancer and noncancer cell lines from various tissues (see materials and methods) with IL27 to test their responsiveness (Fig. 1A). Phosphorylation of the STAT factors was investigated by Western blot analysis after one hour of stimulation. The cell lines all reacted to IL27 with a robust phosphorylation of STAT1 (pSTAT1) and a phosphorylation of STAT3 (pSTAT3), the latter being only slightly over background for some of the cell lines. STAT5 and STAT4 were not phosphorylated by IL27 in any of the cell lines (Data no shown).

Using flow cytometry we analyzed the surface expression of the IL27 signaling receptors, IL6Rβ, and IL27Rα, on a selection of cell lines (Fig. 1B) and found that both receptors were expressed on the surface of all investigated cell lines.

3.2 | In contrast to IFN-γ, IL27 induces a stronger STAT3 phosphorylation, which is insufficient to activate STAT3-mediated reporter gene transcription

We compared the STAT1/3 phosphorylation pattern obtained for IL27 signaling to efficient activators of each type of STAT (OSM for STAT3 and IFN-γ for STAT1) by Western blot immuno-detection analyses in a number of cell lines (Fig. 2A). IL27-mediated STAT1 phosphorylation was robust, as for IFN-γ, and STAT1 protein expression was induced. As expected, OSM did not induce STAT1 protein up-regulation. Phosphorylation of STAT3 by IL27 was generally weaker than that induced by the strong STAT3 activator OSM. Thus, IL27 stimulates many cell types resulting in robust phosphorylation of STAT1 and a less efficient STAT3 phosphorylation (compared to IFN-γ and OSM, respectively).

To test for transcriptional activity of STAT3 downstream of IL27 we used a STAT3-specific reporter gene assay containing the rPAP1 promotor,[65] which is very sensitive and highly specific for STAT3 (Fig. 2B).[50] IL27, as IFN-γ (which in contrast to IL27 only leads to STAT1 phosphorylation), did not activate the STAT3-specific reporter gene construct, whereas hy-IL6- and OSM-induced transcription of the reporter gene. Of note, Hep3B cells do not express the oncostatin M receptor (OSMR) and thus do not activate reporter gene expression upon OSM.[50] Thus, although IL27 led to detectable STAT3 phosphorylation, it did not induce STAT3-specific promotor construct transcription (similarly to IFN-γ, which does not trigger STAT3 phosphorylation in our system).

3.3 | IL27 and IFN-γ stimulations lead to similar transcriptome expression profiles

The fact that we did not detect STAT3-dependent reporter gene activity led us to compare the IL27, IFN-γ, IL6, and OSM responses in a set of microarrays of cancer and noncancer cell lines of different tissue origin. We performed a PCA of the dataset to compare the IL27 response to those of the STAT1 (IFN-γ) or STAT3 (hy-IL6, OSM) activating cytokines. The percentile variance of the dataset contributed by the different dimensions of the PCA was 40% for the first dimension, whereas the succeeding dimensions account for 13.7 and 9.8% (data not shown). The first two dimensions of the PCA represent 53.7% of the total variance of the dataset, and contain most of the information on the variance related to cytokine stimulation. This value is significant

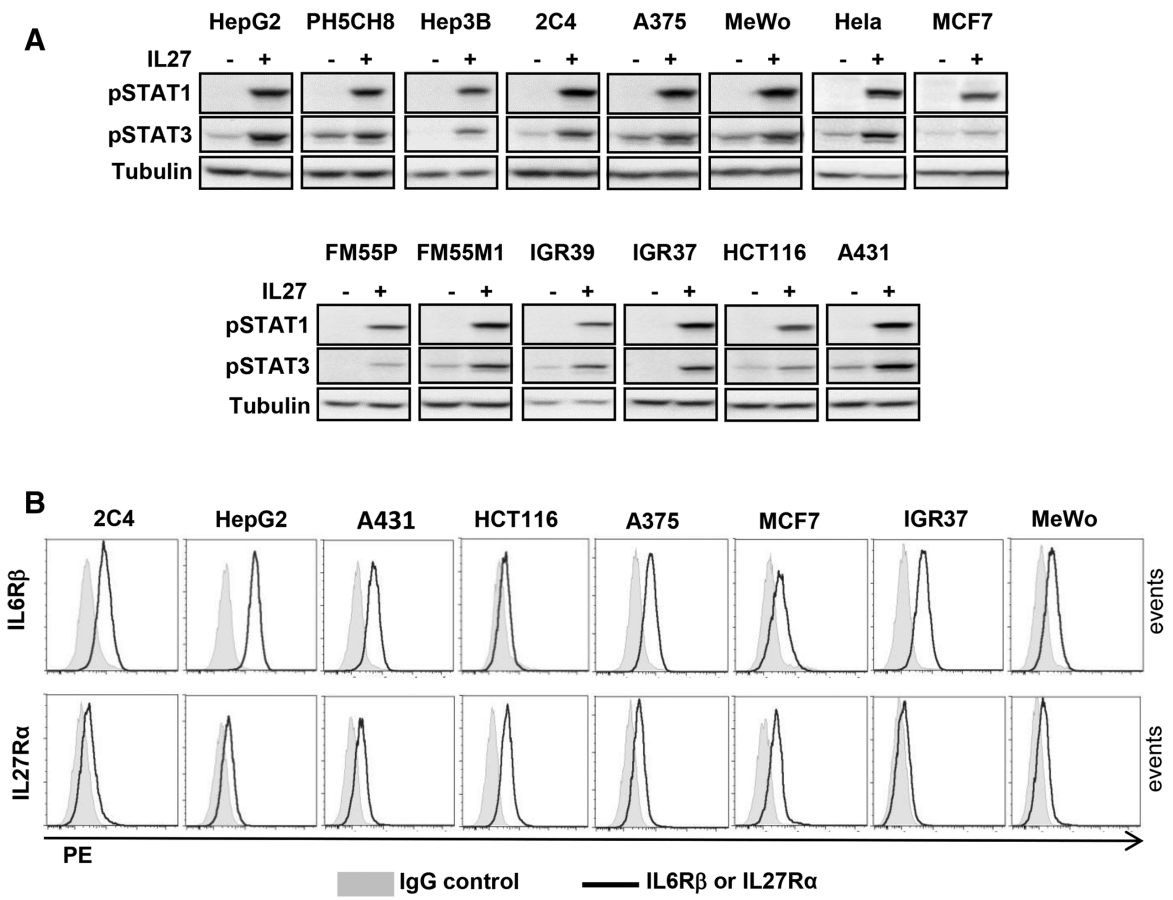

FIGURE 1 **Cancer cell lines of different origin are sensitive to IL27 stimulation**. (A) A panel of cancer and noncancer cell lines of different tissue origin was stimulated for 1 h with IL27 or was left untreated. Lysates were analyzed by Western blot immuno-detection, using phospho-specific antibodies against pSTAT1, pSTAT3. Anti-tubulin immuno-detection was performed as loading control. (B) Different cell lines were incubated with antibodies against IL6Rβ or IL27Rα and analyzed by flow-cytometry as described in materials and methods

because it is greater than the reference value of 10.2% (obtained by the R package *FactoInvestigate* v1.0[76]), which is the 0.95-quantile of the variable percentage distribution obtained by stimulating 616 data tables of equivalent size on the basis of a normal distribution. Analysis of the first two dimensions of the PCA shows that for most cell lines the projection of the "combined effect" for the different cytokine treatments (represented by the direction of the arrows) show orthogonal orientations when comparing the hy-IL6 or OSM effect to the ones of IL27 or IFN-γ (Fig. 3A and Supporting Information Fig. 1A and1B). Orthogonal orientation between these "combined effects," representing, for example, the hy-IL6- and IL27-responses, indicates that overall those genes, which are regulated by one of the treatments, are not regulated by the other. IL27 and IFN-γ showed an almost identical orientation, which is indicative of very similar responses. The same effect was observed when comparing the hy-IL6 to the OSM responses. In summary, the IL27 and IFN-γ transcriptomic responses were very similar throughout all cell lines and cell types (Fig. 3, Supporting Information Fig. 1A and B), and they did not regulate genes characteristic of an IL6-type cytokine response. The NHEM cells did not show such a clear distinction between the IL6 and OSM responses compared to the IL27 and IFN-γ responses. Especially the IFN-γ response was shifted toward an intermediate direction between IL6-type cytokines and the interferon-like responses. Closer examination of this phenomenon showed that the different behavior could be attributed to the fact that the IFN-γ stimulation for this cell line possessed a larger variance compared to the other cell lines (data not shown). Because the IFN-γ stimulation serves as a control stimulus of a STAT1-activating cytokine, this interferes with the interpretation of the results for this particular cell line.

A closer inspection of the differentially regulated genes showed that IFN-γ and IL27 efficiently upregulate genes involved in antigen presentation, which are important for a potential "anticancer" cytokine. IFN-γ and IL27 treatment upregulated the expression of proteasomal subunits, HLA subunits, TAP transporters and other molecules involved in tumor antigen presentation. In addition, chemokines associated with a T_H1 response and the suppression of angiogenesis were also upregulated (CXCL9, CXCL11) (Fig. 3B).

Interestingly, the microarray analyses also revealed that IFN-γ and IL27 regulate a set of genes associated with immune evasion of cancer (Fig. 3C). In a potential cancer treatment involving IL27, however, immune-inhibitory proteins such as CD274 (hence forward referred to as PD-L1), IDO-1, IL18BP, or LAP3 might prevent an efficient immune clearance and instead induce immune tolerance. These genes were described to limit inflammation and tissue destruction upon IFN-γ and IL27 stimulation. In chronic cancer inflammation these genes are associated with a bad prognosis.

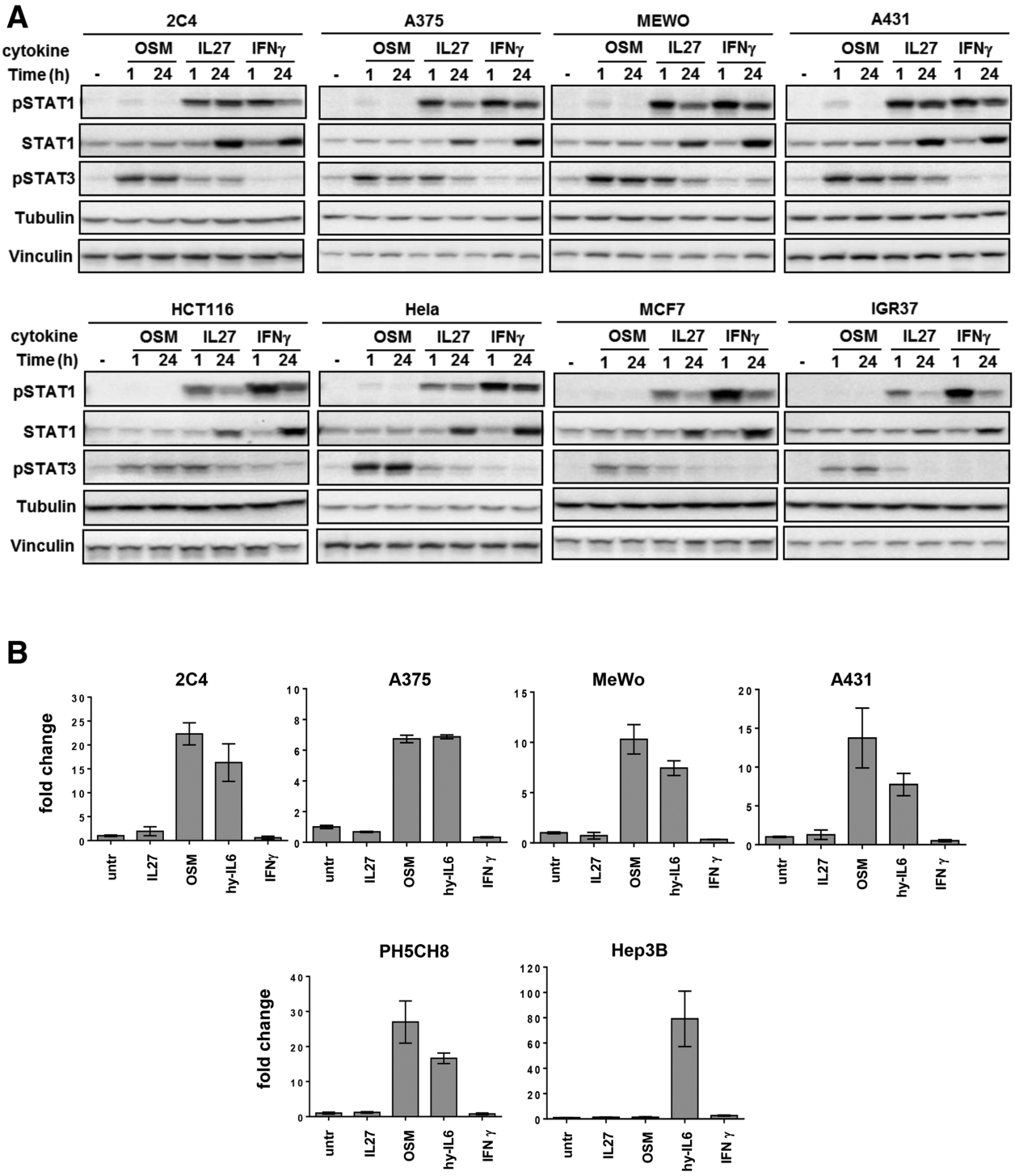

FIGURE 2 **IL27 stimulation induces a robust STAT1 phosphorylation**. (A) Different cell lines were stimulated for 1 or 24 h with OSM, IL27, or IFN-γ or were left untreated. Lysates were analyzed by Western blot. Anti-tubulin and -vinculin were used to show equal loading. (B) Different cell lines were transfected with the STAT3-specific reporter gene plasmid (under the control of the rPAP promotor). 24 h after transfection the cells were stimulated for 24 h with IL27, OSM, hyperIL6, or IFN-γ or were left untreated before lysates were prepared and luciferase activity was measured

3.4 | IL27 and IFN-γ differentially regulate proteins involved in antigen presentation and immune escape in HCC cells and nontransformed liver cells

Validation of some interesting genes at the protein level was performed by flow cytometry (MHC-I, PD-L1) or by Western blot analysis (STAT1, TAP2, IDO-1) in different cell types (Fig. 4 and Supporting Information Fig. 2). Because HCC is our main focus,[50] we also performed quantitative Western blot analysis for three HCC cell lines and the PH5CH8 cells for several proteins (Fig. 4D). In general, proteins associated with increased immune clearance of cancer such as Tap2 and MHC-I were upregulated upon IL27, IFN-γ and IFN-α treatment,

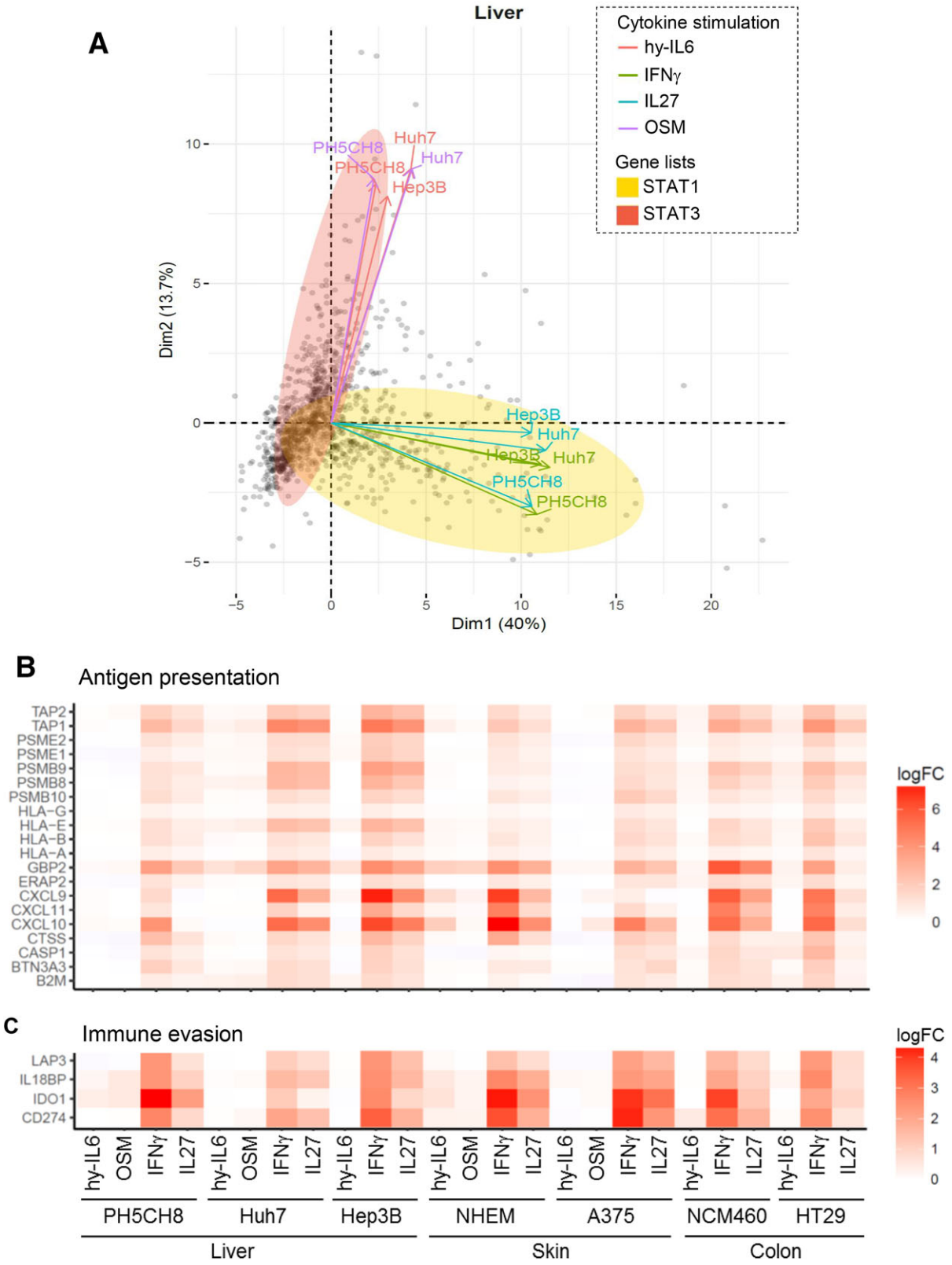

FIGURE 3 **IL27 recapitulates an IFN-γ-like transcriptomic response.** (A) Representation of dimensions 1 and 2 of a PCA of microarray data of cells stimulated as indicated. Arrows (one for each treatment in each cell line) represent the projection of the total contribution of all differentially regulated genes of this treatment. For visualization reasons this figure shows only the cell lines of hepatic origin (for colon and skin cells see Supporting Information Figure 1A and 1B). The colored ellipses show the 0.95% quantile distribution of validated STAT1- (yellow) or STAT3-regulated genes (rose). Individual genes are represented as gray spots. For detailed lists see Supporting Information Figures 1C and D. (B) Heatmap representing the regulation of genes involved in antigen presentation or induction of T_H1 responses. (C) Heatmap representing the regulation of genes involved in immune escape. Heatmaps were drawn using the R package *ggplot2*[74]

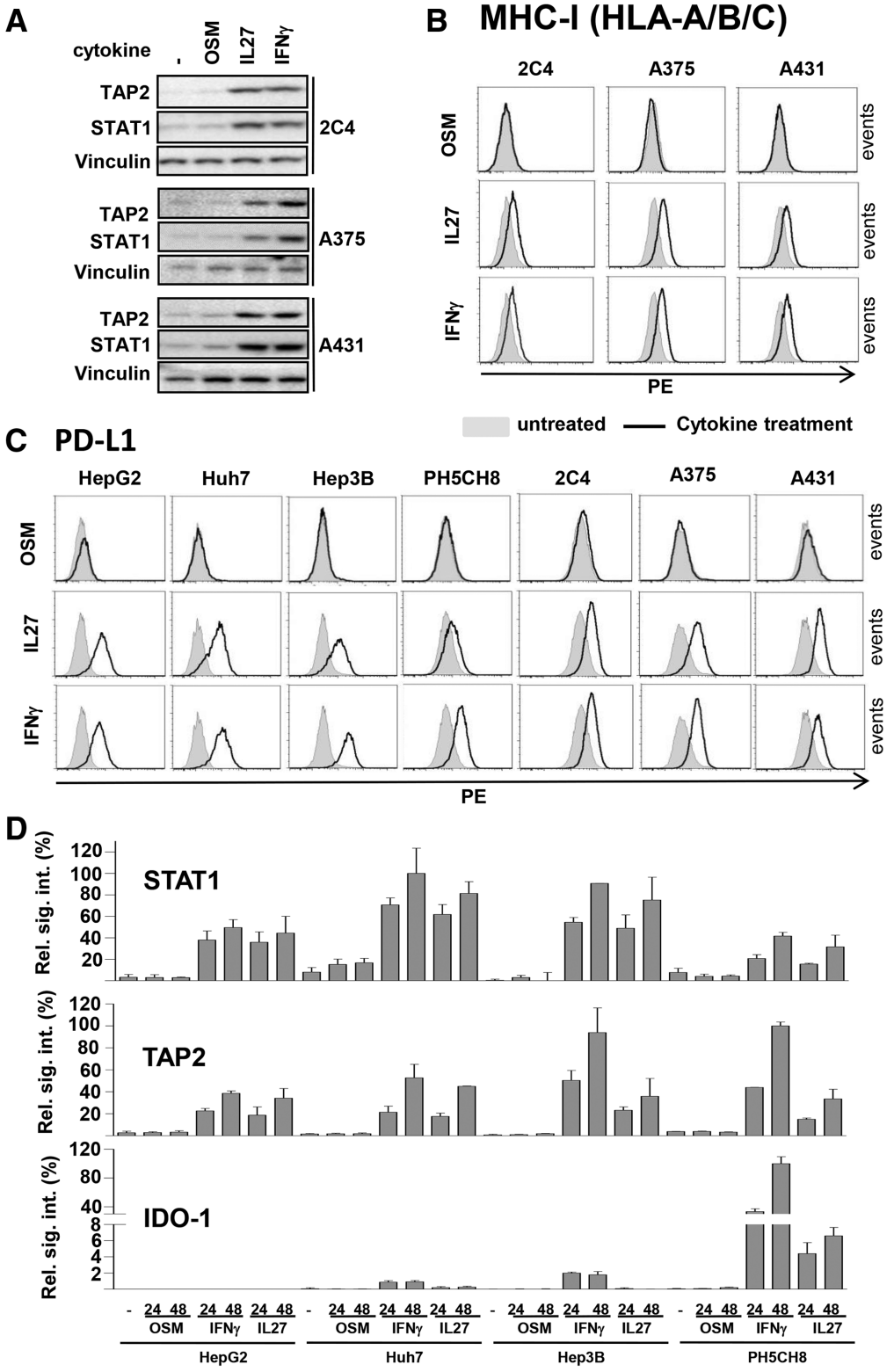

FIGURE 4 **Validation of IL27 gene regulation on protein level.** (A) 2C4, A375, and A431 cells were stimulated for 24 h with IL27, OSM, or IFN-γ or were left untreated. Western blots of the lysates were immuno-detected using antibodies against TAP2, STAT1, and vinculin (the latter to show equal loading). (B) Expression of MHC1 was studied by flow cytometry in cells stimulated with OSM, IL27, or IFN-γ for 24 h. A representative from 2 biological replicates is shown. (C) Expression of PD-L1 was studied by flow cytometry in a panel of cancer cell lines. Cells were left untreated or stimulated with OSM, IL27, or IFN-γ for 24 h. A representative of 3 biological replicates is shown. (D) HepG2, Hep3B, Huh7, and PH5CH8 cell lines were stimulated for different time points with IL27, OSM, or IFN-γ or were left untreated. Western blots of the lysates were then immuno-detected using antibodies against IDO-1, TAP2, and STAT1 and quantitated as described in materials and methods and represented as relative signal intensity compared to the strongest signal on the blots (mean and standard deviation of 3 biological replicates are shown). Tubulin served as control detection (see also Supporting Information Figure 2C)

but not after OSM or hy-IL6 treatment in different cell types (Fig. 4A and B and Supporting Information Fig. 2A and C) (for HCC cells see also[50]). Thus, IL27-treated cells are likely to present more antigen on the cell surface than untreated cells. FACS analysis showed that IL27, IFN-γ, and IFN-α led to an efficient PD-L1 protein up-regulation in different cancer cell lines, and again, OSM or hy-IL6 did not (Fig. 4C and Supporting Information Fig. 2B). Interestingly, quantitative Western blot analysis revealed that IDO-1 was inefficiently induced by IL27 in contrast to STAT1 or TAP2, whereas IFN-γ induced IDO-1 more efficiently (Fig. 4D and Supporting Information Fig. 2C). In addition, IDO-1 protein induction was especially strong in the IFN-γ-stimulated nontransformed cell line PH5CH8.

In summary, IL27, like IFN-γ, induces protein expression associated with increased antigen presentation (TAP2, MHC-I) and tumor immune escape (PD-L1).

3.5 | In cocultures of HCC and PBMC, HCC cell-expressed PD-L1 can suppress IL2 production in anti-CD3/-CD28 activated T lymphocytes

To analyze if IL27-mediated PD-L1 expression in HCC cells can have an immune-suppressive effect, we performed coculture experiments in which we pre-stimulated HCC cells with IL27 for 24 hours, washed IL27 away and incubated the PD-L1-expressing HCC cells with anti-CD3/CD28-activated PBMCs for 3 days (Fig. 5A). Pre-treatment of the HCC cells with IL27 resulted in lower IL2 production in the coculture, which shows that PD-L1 expressed at the surface of the HCC cells can influence T-cell functions (Fig. 5B). Addition of a blocking PD-L1 antibody (concomitantly with the PBMCs), raised the IL2 secretion at least to the level observed before (or higher) (Fig. 5B and Supporting Information Fig. 3A). The levels of other pro-inflammatory cytokines (e.g., IL1β, TNF-α, and MCP1) were either restored or upregulated by anti-PD-L1 administration (Fig. 5C). However, IL6 levels, a cytokine described to mediate "pro-tumor" effects, were also decreased by IL27 treatment and efficiently increased upon anti-PD-L1 administration (Fig. 5D and Supporting Information Fig. 3B).

3.6 | The inhibition of IL27 responses by IL6-type-cytokines is prevented by administration of blocking antibodies against these cytokines or their receptors

IL27 signaling is susceptible to inhibition by SOCS3, which is induced, for example, upon phosphorylation of STAT3 by IL6-type cytokines.[50] IL6-type cytokine and non-IL6-type cytokine-mediated STAT3 phosphorylation is often observed in a cancer-associated inflammatory context and is thought to have "pro-tumor" effects[77] (see also Supporting Information Fig. 5). In addition, as shown in Figure 5, IL6 levels are also upregulated upon anti-PD-L1 administration.

To mimic cancer-associated constitutive activation of STAT3, we pre-stimulated the cells with OSM, hy-IL6 or left them untreated for 4 h before a 24 h stimulation with IL27. To monitor IL27-induced responses, we measured PD-L1 surface expression by flow cytometry and detected STAT1, TAP2, and IDO-1 by Western blot analysis in HCC cell lines. OSM or hy-IL6 pre-stimulation (Fig. 6A, solid line) reduced the IL27-dependent PD-L1 up-regulation (Fig. 6A, red peak) almost to the level of the non-IL27-stimulated sample (Fig. 6A, gray peak). Treatment with anti-cytokine or anti-cytokine receptor antibodies efficiently restored IL27-induced PD-L1 expression (Fig. 6A, dotted line). The identical observations were made for STAT1, TAP2, and IDO-1 as investigated by Western blot analysis (Fig. 6B). Thus, an anti-IL6-type cytokine approach might have beneficial effects in IL27 antitumor treatment, if constitutive STAT3 phosphorylation is observed. We have described before that IL27 can be inhibited by SOCS3 (which is a STAT3 target gene). We show that phosphorylation of STAT3 (Fig. 6B, lower panels) and up-regulation of SOCS3 mRNA (Fig. 6C) and protein (see Supporting Information Fig. 4) are suppressed by anti-IL6 treatment.

4 | DISCUSSION

We show and discuss in the following that (i) IL27 induces STAT factor phosphorylation in cancerous cells lines of different tissue origin. (ii) IL27 leads to STAT1 phosphorylation and recapitulates an IFN-γ-like response in the microarray analyses, with up-regulation of many genes involved in antiviral defense, antigen presentation and an up-regulation of some genes involved in immune escape. (iii) However, the STAT3 response to IL27 remains elusive and a sensitive STAT3 reporter gene construct did not respond to the IL27 stimulus. (iv) Like IFN-γ, IL27 leads to an up-regulation of TAP2 and MHC-I protein, which are associated with an increased tumor immune clearance. However, both cytokines also upregulate proteins such as PD-L1 (CD274) and IDO-1, which are associated with an immune escape of cancer. (v) PD-L1 up-regulation upon IL27 treatment reduced IL2 secretion in coculture experiments of HCC cells and PBMCs, but blocking antibodies targeting PD-L1 overcompensated the effect and led to significantly enhanced secretion of IL2. (vi) Finally, the attenuation of IL27 responses by pre-stimulation with IL6-type cytokines can be prevented by use of anti-cytokine antibodies. Thus, this might represent a possible co-treatment option with IL27 in IL6-type cytokine-expressing tumors or in case of tumor-induced systemic inflammation.

We investigated a number of cell lines from various tissue origins concerning their responsiveness to IL27. IL27 induced a robust phosphorylation of STAT1 and a phosphorylation of STAT3, the latter being only slightly over background for some of the different cell lines (Fig. 1A). The investigated cells also expressed the IL6Rβ and IL27Rα, the canonical receptor subunits for IL27 signaling (Fig. 1B).[50] Since IL27 is a noncovalent dimer of the EBI3 and p28 subunits, a dissociation of IL27 in the presence of other competing cytokine subunits (p35, CLF1) might occur. p28 alone or in complex with CLF1 (cytokine-like factor 1) has been described to signal through IL6Rα/ IL6Rβ (although only at high cytokine concentrations) to activate STAT3,[78,79] which (i) is described to be pro-tumorigenic[80] and (ii) can inhibit IL27 signaling (by SOCS3 induction).[50] p28 (also called IL30) expression correlates with advanced grade of prostate cancer and promotes breast cancer growth.[81,82] In addition, local

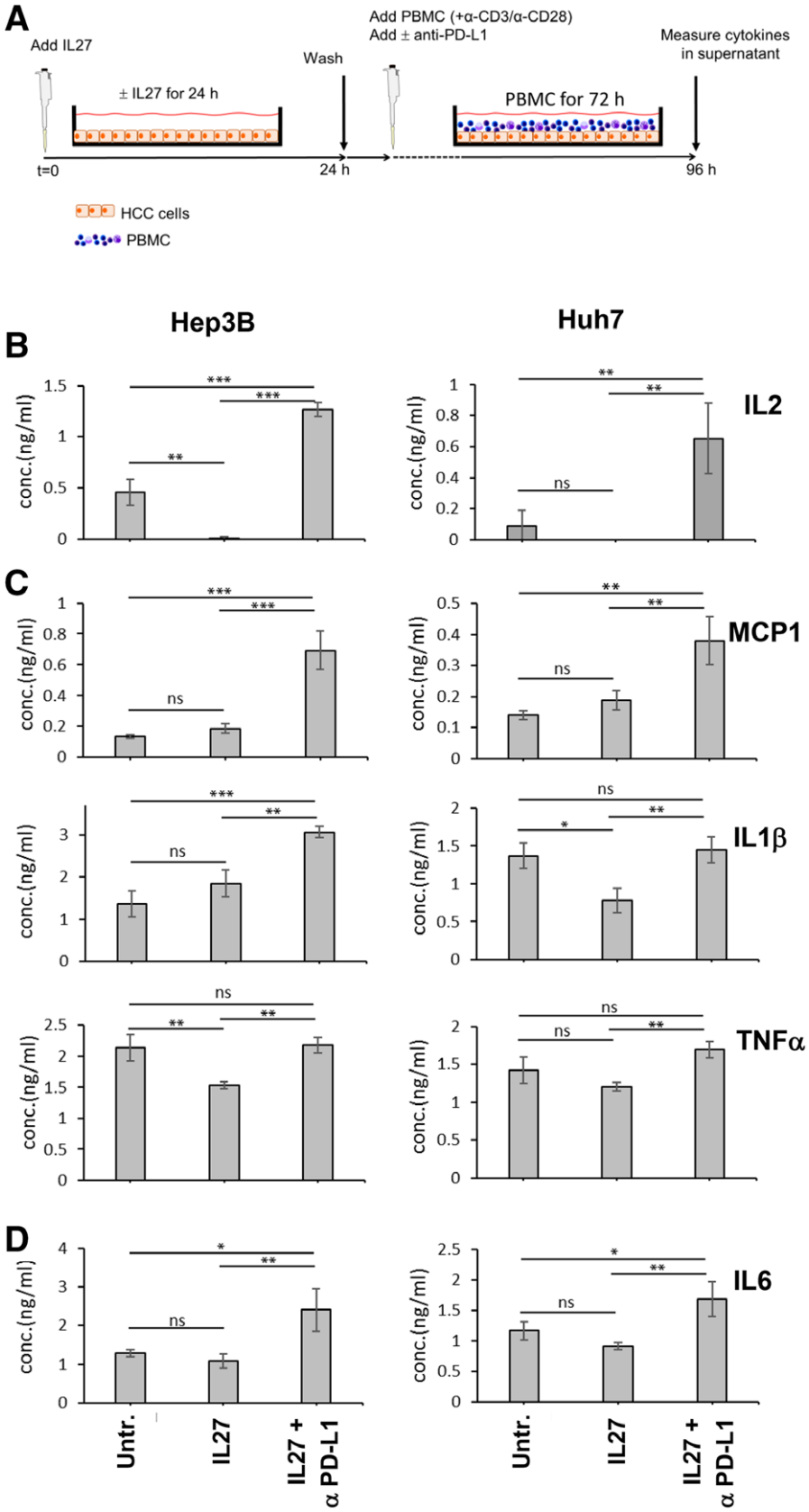

FIGURE 5 **Cytokine expression from cocultures of PBMCs with IL27-pre-stimulated HCC cells in presence or absence of a blocking anti-PD-L1 antibody**. (A) Scheme of the experimental layout. (B to D) Cytokine levels were analyzed by cytokine arrays. IL2 (B), MCP1, IL1β, TNF-α (C) and IL6 (D) levels are expressed as the mean ± standard deviation of 3 biological replicates. One-way ANOVA with Bonferroni posttest was performed using Graphpad InSTAT version 3.10 (www.graphpad.com). For statistical analysis error probabilities <0.05 were considered to be significant, ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$

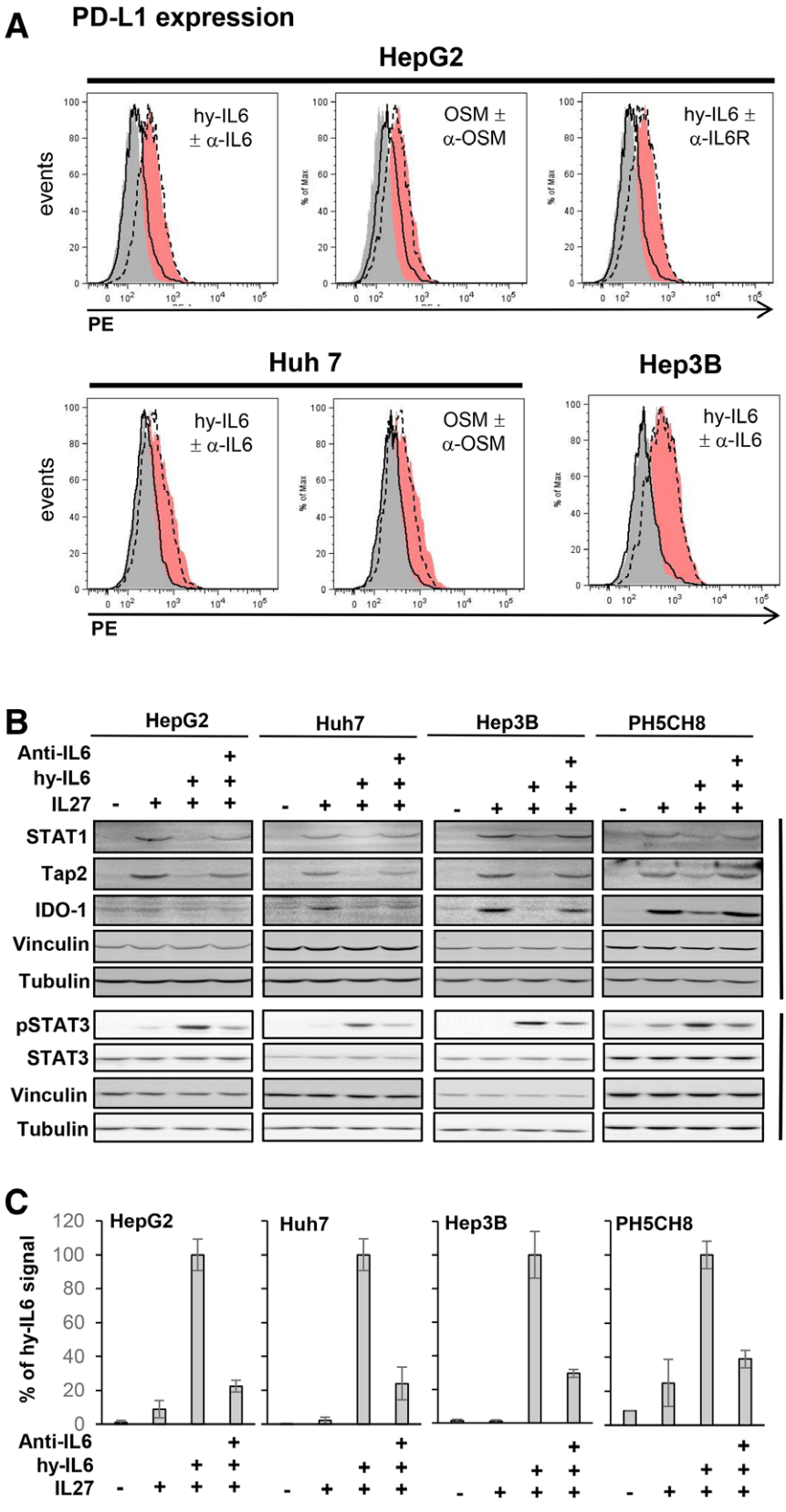

FIGURE 6 Blocking antibodies against IL6-type cytokine or receptors prevent down-regulation of IL27 signaling. (A) Expression of PD-L1 on the HCC cell lines HepG2, Huh7, and Hep3B was studied by flow-cytometry. Cells were left untreated (gray fill) or were stimulated with IL27 alone (red fill) or treated for 4 h with hy-IL6 or OSM prior to the IL27 treatment for 24 h (solid line). The dotted line indicates the situation in which blocking antibodies against IL6, IL6R, or OSM were added together with the IL6 or OSM pre-treatment. (B) HepG2, Hep3B, Huh7, and PH5CH8 cells were stimulated as described in (A) but using only hy-IL6 and anti-IL6. Western blots of the lysates were immuno-detected for the STAT1 target genes IDO-1, TAP2, STAT1. Vinculin and tubulin detections were used as loading controls. Phosphorylation of STAT3 was investigated on a separate blot using STAT3, vinculin and tubulin as controls. (C) HepG2, Hep3B, Huh7, and PH5CH8 cells were stimulated as described in (B). The cells were lysed after 6 h of treatment. SOCS3 mRNA expression was investigated by qPCR. Results show the mean of 3 biological replicates as a percentage of the highest SOCS3 induction with standard deviation

expression of p35 (a subunit of IL12), could lead to competitive displacement of p28 from EBI3, to form IL35 (composed of EBI3 and p35), a cytokine that exerts its immunosuppressive functions by promoting iTreg differentiation and activity.[83,84] Thus, IL27 administration as a potential anticancer treatment should rather be performed with a covalently linked dimer of p28 and EBI3 to avoid the aforementioned pro-tumorigenic effects.

With respect to STAT factor activation, almost all tested cells reacted to IL27 with a robust phosphorylation of STAT1 (comparable to the IFN-γ response) and a phosphorylation of STAT3, the latter

being weaker compared to the OSM-induced STAT3 phosphorylation (Fig. 2A). Because studies in immune cells have discussed a role of STAT3 in transcriptional responses through IL27,[85–87] we tested for the presence of transcriptionally active STAT3 by using the STAT3-specific and very sensitive rPAP reporter gene construct (Fig. 2B). Only OSM, but not IL27 or IFN-γ, induced STAT3-dependent reporter gene transcription in the tested cells of different tissue types. The possible reasons for such an inefficient STAT3 response have been discussed in detail before.[50]

Whole genome microarray analyses of 7 cell lines (3 different tissue types) stimulated with IL27, IFN-γ, hy-IL6, or OSM showed that the IL27 response closely recapitulates an IFN-γ response (Fig. 3 and Supporting Information Fig. 1A and B). IL27, like IFN-γ, regulates mRNAs involved in immune regulation and antigen presentation but is also related to other canonical functions of IFN-γ, for example, the antiviral defense (data not shown). In the antigen presentation pathway, genes encoding MHC-I subunits (HLA-A, -B, -E, -G, B2M), the immunoproteasome (PSMB-8 to -10), the S11 proteasome (PSME-1 and -2), and TAP transporters (TAP1 and 2) were upregulated (Fig. 3B). The up-regulation of the antigen presentation pathway is associated with increased immunogenicity of tumors. In addition, CXCL9 and 11 were upregulated, which promote anti-angiogenesis and recruit immune cells associated with TH1 responses. Interestingly, a subset of genes involved in immune escape of cancer (LAP3, IL18BP, IDO-1, and PD-L1 (CD274)) were also upregulated by IL27 and IFN-γ[88] (Fig. 3C). These genes can promote immune evasion of cancer if overexpressed, as part of a dysregulated interferon response.[89] Very recently, a number of the proteins mentioned earlier was found to be upregulated by IL27 in ovarian cancer, neuroblastoma, and lung adenocarcinoma cells.[88,90] IL27 upregulates PD-L1 in leukocytes[37,38,91,92] and other cell types including cancer cells.[61,90,93,94]

In the subsequent validations we showed that IL27, like IFN-γ, also upregulates the protein levels of a number of genes involved in increased cancer immune clearance (STAT1, TAP2, MHC-I) and proteins involved in immune escape of cancer (PD-L1, IDO-1) (Fig. 4 and Supporting Information Fig. 2). Interestingly, PD-L1 is upregulated following IL27 and IFN-γ stimulation, but not upon OSM in all tested cell lines.

Because HCC was our main focus here and represents a tumor with still limited treatment options, we tested the induction of the aforementioned genes in more detail in these cells and in the non-transformed PH5CH8 liver cell line (Fig. 4D). The liver is characterized by an intrinsic immune suppressive microenvironment, which has been described to impede the effectiveness of anticancer vaccines.[95] Moreover, HCC is known to lack the antigen processing proteins necessary to present tumor antigens, which allows escape from $CD8^+$ lymphocyte killing.[31] However, reactivation of different types of immune cells, such as cytokine-induced killer cells, NK cells or DCs pulsed with tumor lysate have been shown to be effective in clinical and preclinical studies (reviewed in[95]). Therefore, we reason that HCC antigen presentation and a subsequent immune reactivation might be achievable by using IL27. Whereas MHC-I, STAT1, and TAP2 proteins were expressed in IL27- and IFN-γ-treated cells, IDO-1 was less efficiently induced in IL27-treated cells. Interestingly, the induction of IDO-1 and, to a much lesser extent, TAP2 vary when comparing the IFN-γ and IL27 responses. This effect was not observed at the level of STAT1 protein induction, possibly, due to a different sensitivity of the different genes toward phosphorylated STAT1. Because IL27 generally shows a slightly weaker induction of STAT1 phosphorylation in HCC cells[50] than IFN-γ, it might generally induce less IDO-1. On the other hand, PD-L1 expression was equally efficiently mediated by IL27 and IFN-γ.

Thus, IL27 and IFN-γ can have immune-promoting functions but could also lead to tumor immune escape, a characteristic that might interfere with tumor clearance upon a potential IL27 therapy. Because the PD-L1 gene is efficiently induced by IL27, IL27 immune therapy might profit from anti-PD-L1 antibody co-treatment. Targeting PD-L1/PD-1 has shown impressive antitumor effects in solid tumors,[96–99] including HCC.[100] Clinical trials using immune checkpoint inhibitors, for example, anti-CTLA4 and anti-PD-1, demonstrated that the treatment was well tolerated.[100] In coculture experiments of HCC cell lines with PBMCs we could show that an IL27-dependent PD-L1 expression on HCC cells can decrease IL2 production by the PBMCs. Interestingly, a blocking anti-PD-L1 antibody could again increase IL2 secretion. (Fig. 5B).[61] The levels of other pro-inflammatory cytokines were also restored or upregulated by anti-PD-L1 administration (Fig. 5C).

IL6, a "pro-tumor" cytokine, was also secreted into coculture supernatants upon anti-PD-L1 administration (Fig. 5D). IL6 and OSM were shown to be present in inflammatory tumors. Inflammation has been established to be involved in cancer development,[101] and constitutive activation of STAT3 found in many cancers is mediated by different mechanisms.[57,58,80,102–104] In addition, IL6-type cytokines have been shown to be essential players in the development of at least colon and liver cancers.[55,77,105,106] We have shown before that IL6-type cytokine pre-stimulation, which induces SOCS3 expression, can suppress a subsequent IL27-response in a SOCS3-dependent manner.[50,107] Due to this cross-inhibition of "pro-tumor" IL6-type cytokines, IL27 responses and thus its antitumor efficacy might be considerably reduced in "inflammatory" tumors. We now show that anti-IL6-type cytokine antibodies can reconstitute efficient IL27 responses (Fig. 5).

In addition, IL6 can have other immunosuppressive effects in cancer. Tumor-induced systemic IL6 can induce systemic metabolic changes. It induces lipolysis of white adipose tissue, which can cause hyperlipidemia and results in insulin resistance of skeletal muscle (reviewed in[108]). In the liver, IL6 inhibits PPARα, which reduces ketone generation under low energy/insulin resistance. The resulting hypoketonemia stimulates the adrenal gland to induce glucocorticoids, which suppress intratumoral immunity and even prevent anticancer immunotherapy with anti-PD-L1 antibodies.[109] In addition, IL6 can act locally in the tumor on immune cells to suppress anticancer immunity (reviewed in[33]). IL6 favors the development of M2 macrophages, MSDCs and T_H17 cells, which is associated with secretion of immunosuppressive factors such as IL10 and IL4 (which again inhibit T_H1 cells, CD8 positive CTLs and NK cells). Cancer-associated fibroblasts and regulatory B cells have also been described to secrete IL6. Thus, blocking IL6 in patients with a prominent tumor-induced IL6-type cytokine-dependent inflammation might have more anticancer effects

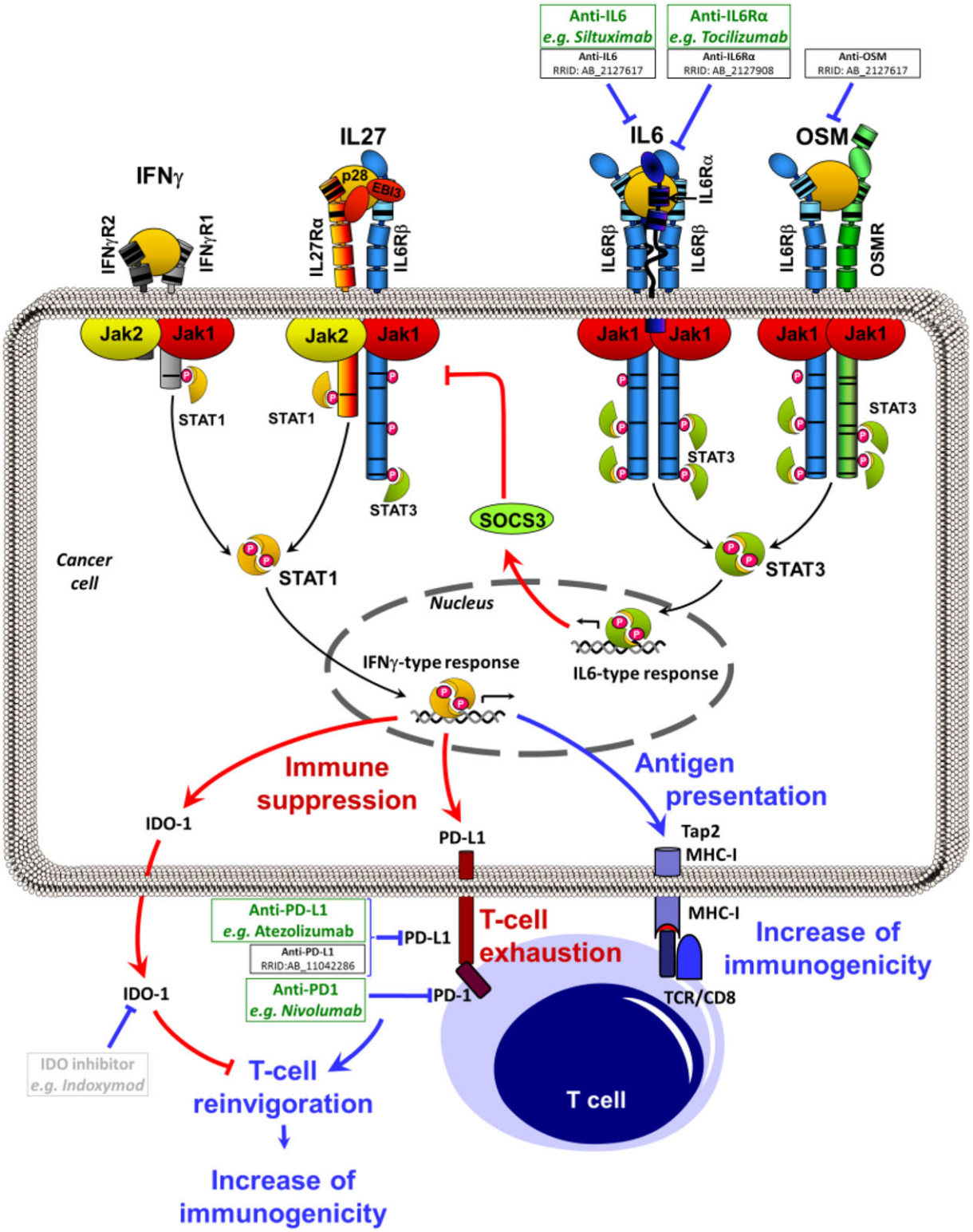

FIGURE 7 **Major IL27-mediated signaling pathways in cancer cells and possible treatment options to improve immune clearance of cancer cells.** Pathways and treatments that promote antitumor actions are indicated by blue arrows. Pathways that promote tumor immune escape are indicated using red arrows. Black boxes indicate those blocking antibodies that have been used in this study. Already FDA-approved blocking antibodies inhibiting the same targets mentioned above are shown in dark green boxes. An IDO-1 inhibitor that entered clinical trials but is not yet FDA-approved is indicated in a grey box. In tumors with high IL6-type cytokine levels, anti-cytokine-receptor- or anti-cytokine-antibodies could be used in addition to IL27, to relieve the SOCS3-mediated cross-inhibition of IL6-type cytokines on IL27 signaling. PD-L1 is expressed in all investigated cancer cell types in response to IL27 and thus anti-PD-L1 antibodies could be envisaged as standard co-treatment with IL27 (or Interferon-) therapies to prevent immune cell deactivation (e.g., a $CD8^+$T-cell as shown in the scheme) by cancer cell-expressed PD-L1. IDO-1 is not induced by IL27 in all cells and thus IDO-1 inhibitor treatment could only be applicable to a subset of tumors

than just preventing the inhibition of a potential IL27 treatment (see also Supporting Information Fig. 5).

In summary, we hypothesize that HCC (characterized by an intrinsic immune suppressive microenvironment) could benefit from IL27 treatment, which upregulates proteins involved in antigen presentation (Fig. 7). In addition, a co-treatment with anti-PD-L1 or anti-PD-1 antibodies might prevent immune suppressive effects also mediated by prolonged IL27 or interferon stimulation. A number of studies even suggested that blockade of the PD-1/PD-L1 pathway is a prerequisite for the necessary stimulatory functions of interferons in the antitumor response.[110–114] Due to the suppression of IL27 signaling, mediated by IL6-type cytokine-induced SOCS3 protein, patients with high circulating IL6 levels (e.g., by cancer inflammation-associated cachexia or IL6 producing tumors) will very likely only be responsive to IL27 treatment if an anti-IL6-type cytokine or anti-IL6-type cytokine receptor antibody is used in combination. Last but not least, IL27 responses are not restricted to the cancer cell itself. IL27-induced T_H1 responses in immune cells are known to be enhanced by blocking PD-L1 and IDO-1 or other immune-suppressive stimuli. Concomitant blocking of CTLA4 and PD-L1 has shown significant clinical responses and has been described to improve antitumor immune responses by mechanisms relying on IFN-γ and IL7 signaling.[113] Cytokine induced cancer-cell (IL27) and immune-cell (IL27, IL7, IL15, IL2, IL12) stimulation combined with blockade of crucial immune checkpoints (αPD-L1, αCTLA4) might be what is needed to elicit more effective anticancer responses in an immuno-suppressive microenvironment.

AUTHORSHIP

All authors approve to the publication of this paper. C.R. performed and planned experiments and wrote the paper. A.D.Z., C.M., D.P., M.K., F.S., S.H., and N.N. performed and planed experiments. A.G. and P.V.N. performed bioinformatic analysis. S.K., H.M.H., S.H., I.B., and C.H. planned experiments and wrote the paper. Datasets supporting the conclusions of this article are included within the article and its additional file. Microarray raw data were deposited in the ArrayExpress public repository with the reference number E-MTAB-6080.

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DISCLOSURE

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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