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Oncostatin M Enhances the Antiviral Effects of Type I Interferon and Activates Immunostimulatory Functions in Liver Epithelial Cells[▽]

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Oncostatin M (OSM) is released together with type I interferon (IFN) by activated dendritic cells, suggesting a concerted action of these cytokines in the biological response against infection. We found that OSM increases the antiviral effect of IFN-α in Huh7 hepatoma cells infected with hepatitis A or hepatitis C virus and synergizes with IFN-α in the induction of antiviral genes. The combination of OSM and IFN-α led to upregulation of both STAT1 and STAT3 together with intense and prolonged activation of STAT1, STAT3, and Jak1. OSM with or without IFN-α also activated p38 mitogen-activated protein kinase, which is known to enhance transcription of IFN-α-inducible genes. Interestingly, OSM combined with IFN-α strongly induced immunoproteasome genes and other genes involved in antigen processing and presentation. Moreover, OSM, alone or in combination with IFN-α, upregulated relevant innate immunity molecules and increased the expression of intracellular adhesion molecule 1 and interleukin-15 receptor alpha (IL-15Rα) in liver cells. Hepatoma cells transfected with a plasmid encoding a viral antigen were able to activate effector T cells when pretreated with IFN-α plus OSM but not with each cytokine separately. Also, OSM, more than IFN-α, augmented the ability of Huh7 cells to transpresent IL-15 to responding lymphocytes and increased the immunostimulatory activity of liver epithelial cells by presenting a short viral peptide to sensitized cytotoxic T cells. In conclusion, OSM enhances the antiviral effects of type I interferon and cooperates with it in the induction of adaptive immune responses to pathogens. These findings may have therapeutic implications.

Oncostatin M (OSM) is a member of the interleukin-6 (IL-6) cytokine family, which includes IL-6, cardiotrophin-1 (CT-1), IL-11, leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (1–3). All of them share a common signal-transducing receptor component called gp130 (13, 19, 23). In humans, OSM binds to a heterodimer composed of gp130 and LIFR, which is common to OSM and LIF. OSM also binds with high affinity to a receptor formed by gp130 and the OSM receptor (OSMR), which specifically recognizes OSM (1). Binding of OSM to its receptor complex activates Janus tyrosine kinases (Jak1, Jak2, and Tyk2) as well as STAT1 and STAT3 (19).

OSM is produced by activated monocytes and macrophages (31, 52), and it is also secreted by dendritic cells in response to pathogen-associated molecular patterns (47). It has also been shown that neutrophils produce and release OSM upon stimulation with lipopolysaccharide (LPS) or granulocyte-monocyte colony-stimulating factor (4, 18, 20). OSM, as IL-6, is known to enhance the synthesis of acute-phase proteins by hepatocytes (25). Altogether, these findings indicate that OSM might be a player of innate immunity. However, its role in the defense against pathogens and in the orchestration of immune responses has not yet been defined.

Type I interferons (IFN-α/β) constitute a group of closely related molecules fulfilling essential functions in the early reaction against infectious agents. IFN-α/β are rapidly produced in response to viral infections and are also induced by bacteria (2). IFN-α/β interacts with a single receptor composed of two subunits, IFNAR1 and IFNAR2 (2). Signal transduction is mediated by Jak1 and Tyk2, which phosphorylate and activate STAT1, STAT2, and STAT3 proteins (2, 8). STAT1 and STAT2 dimerize and together with ISGF3G form the ISGF3 transcription factor complex. In addition, activated STAT1 and STAT3 can form homodimers or STAT1-STAT3 heterodimers, which also drive gene transcription (54). Binding of IFN-α/β to its receptor activates the expression of a variety of genes that interfere with viral replication and induce an antiviral state in neighboring noninfected cells. This effect, together with the enhancement of the cytotoxic activity of NK cells and macrophages (16), makes IFN-α/β a master player in innate immunity.

Type I IFNs are instrumental in linking natural and adaptive immune responses (16). In particular, IFN-α is an efficient Th1-biasing cytokine which is necessary for priming and cross-priming $CD8^+$ T cells by antigen-presenting cells (28) and for the generation and activity of cytotoxic T lymphocytes (CTL) (5). Since both OSM and IFN-α activate Jak/STAT pathways after binding to their specific receptors and the two cytokines are induced in response to infection, we hypothesized the existence of functional interactions between them. Here we show that OSM acts at the interphase of innate and adaptive immu-

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nity, enhancing the antiviral effect of IFN-α and stimulating the processes of antigen processing and presentation in liver epithelial cells. In addition, OSM activates the immunostimulatory functions of liver epithelial cells and increases their ability to transpresent IL-15 to the effector lymphocytes. These novel properties of OSM could be exploited in the clinic to enhance the antiviral and immunostimulatory effects of IFN-α-based therapies.

MATERIALS AND METHODS

DCs. Dendritic cells (DCs) were generated as described previously (43). DCs (10^5/well) were seeded in 96-well plates and stimulated with 1 μg/ml of LPS for different times (from 0 to 40 h) or 20 μg/ml of poly(I-C) for 8 and 24 h. The antiviral activity of IFN was measured in supernatants of DCs after 24 h of LPS or poly(I-C) stimulation as described previously (27). Protein levels of OSM were determined in an enzyme-linked immunosorbent assay (ELISA; R&D Systems) according to the manufacturer's instructions.

Antiviral assays. Antiviral assays were performed in Huh7 cells transfected with full-length hepatitis C virus (HCV) replicon (26, 38) and in Huh7 cells infected with hepatitis A virus (HAV) (4.6×10^3 PFU/ml). These Huh7 cells were seeded onto 24-well plates (2×10^4 cells/well) in Dulbecco's minimum essential medium (Gibco) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Twenty-four h later, cells were left untreated or treated with 20 ng/ml of IL-6, CT-1, or OSM (R&D Systems) plus different amounts of IFN-α2 (from 0 to 100 IU/ml; Sicor Biotech) for 72 h.

RNA extraction and real-time RT-PCR. Total RNA extraction was performed using a nucleic acid purification lysis solution (Applied Biosystems) and the semiautomated ABI Prism 6100 Nucleic Acid PrepStation system (Applied Biosystems). Real-time reverse transcription-PCR (RT-PCR) was performed as described previously (26) using specific primers for each gene.

Western blot assays. A total of 1.5×10^4 Huh7 or HepG2 cells were seeded onto six-well plates. After 24 h, cells were left untreated or treated with IFN-α2 (50 IU/ml), OSM (20 ng/ml), or IFN-α2 (50 IU/ml) plus OSM (20 ng/ml). At different time points, cells were washed with phosphate-buffered saline and collected in 150 μl of protein loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate, 0.006% bromophenol blue). Western blotting was performed (26) using the following antibodies: anti-phospho-STAT1^{tyr701}, anti-phospho-STAT3^{tyr705}, anti-phospho-JAK1$^{tyr1022/1023}$, anti-phospho-Tyk2$^{tyr1054/1055}$, anti-phospho-p38$^{thr180/tyr182}$ mitogen-activated protein kinase (MAPK), and anti-rabbit immunoglobulin G (IgG)–horseradish peroxidase conjugate (all from Cell Signaling Technology); anti-STAT3, anti-Tyk2, anti-STAT2, anti-phospho-STAT2^{tyr689} antibodies (Upstate Biotechnology); anti-STAT1 and anti-p38 MAPK antibodies (Santa Cruz Biotechnology), anti-Tap1, anti-ICAM-1, anti-PSMB9, anti-OSMR, and anti-B2M (Abcam); anti-actin and anti-mouse IgG–horseradish peroxidase–linked antibodies (Sigma-Aldrich); anti-HCV core (kindly provided by Martinez-Anso, CIMA, Pamplona, Spain).

Microarray analysis. Huh7 cells were seeded at 1×10^6 cells/plate in Dulbecco's minimum essential medium plus 10% fetal bovine serum. After 18 h, cells were left untreated or treated with IFN-α2 (50 IU/ml), OSM (20 ng/ml), or IFN-α2 (50 IU/ml) combined with OSM (20 ng/ml). Three days later, cells were harvested in 1 ml of TRIzol reagent (Invitrogen). The experiments were performed in quadruplicate. Samples were then processed following Affymetrix recommendations and cRNA was hybridized to the Affymetrix human U133A 2.0 array. Both background correction and normalization were done using the Robust Multichip average algorithm (21). After calculation of the expression for each probe set in all the microarrays, a filtering process was performed to eliminate low-expression-level probe sets. Applying the criterion of an expression value greater than 16 in 17% of the samples, 17,927 probe sets were selected for the statistical analysis. The program Linear Models for Microarray Data (45) was used to find which probe sets showed significant differential expression under experimental conditions. Genes affected by IFN-α2, OSM, or the combination of IFN-α2 plus OSM treatments were identified as significant based on a B statistic cutoff ($B > 0$). Genes were selected based on a change criterion of 1.2-fold in the following ratios: (IFN-α2 + OSM)/OSM and (IFN-α2 + OSM)/IFN-α2. Functional categories were studied by using Ingenuity Pathways Analysis (Ingenuity Systems; www.ingenuity.com) and Webgestalt (55).

Antigen processing and presentation assays. Peripheral blood mononuclear cells obtained from an HLA-A2$^+$ healthy donor were pulsed with 1 μg/ml of HLA-A2-restricted influenza A virus matrix 58–66 peptide (GILGFVFTL) for 2 h at 37°C, washed, and cultured on 24-well plates at a density of 3×10^6 cells/well. Three days later, IL-2 (10 U/ml) was added and cells were cultured for an additional 5 days. On day 8, recovered cells (10^5/well) were cocultured in 96-well round-bottom plates with 5×10^4/well of the following stimulator hepatoma cells: (i) HepG2 cells untreated or previously treated for 4 days with IFN-α2 (50 IU/ml), OSM (20 ng/ml), or the combination IFN-α2 (50 IU/ml) plus OSM (20 ng/ml), in the presence or absence of 1 μg/ml of GILGFVFTL peptide; (ii) Huh7 cells untreated or previously treated for 3 days with IFN-α2 (50 IU/ml), OSM (20 ng/ml), or the combination and cotransfected 24 h after cytokine addition with plasmid pLNCX encoding HLA-A2 (kindly provided by N. Aptsiauri, Hospital Universitario Virgen de las Nieves, Granada, Spain) and plasmid pSV982 encoding influenza matrix protein (a gift from J. Ortín, CNB, Madrid, Spain). Transfection (1.5×10^5 cells/well) was carried out using 10 mM polyethylenimine (18 μl; high molecular weight, pH 7; Aldrich) and plasmids (3 μg of each plasmid). Cotransfected cells treated with both cytokines and the proteasome inhibitor Z-LLF-CHO (Sigma) at 1 μM were also employed. In all cases, after 24 h of coculture the supernatants were collected to measure IFN-γ production by ELISA (BD Biosciences).

IL-15Rα activity assay. Huh7 cells were seeded and treated with IFN-α2 (50 IU/ml), OSM (20 ng/ml), or the combination. Three days later, they were harvested and incubated for 1 hour with or without 50 ng/ml of exogenous IL-15, washed three times, and irradiated at 15,000 cGy in a Gammacell 3000 Elan apparatus. Then, 3×10^4 irradiated Huh7 cells were cocultured with 1×10^4 CTLL-2 cells in 96-well plates. On day 2, cells were pulsed with 0.5 μCi/well of tritiated thymidine for 8 h and harvested, and thymidine incorporation was measured in a scintillation counter (Topcount; Packard).

Statistical analysis. Statistical methods used were as described previously (26). Data are means ± standard deviations (SD); a P value of <0.05 was considered significant. To study the type of interaction between IFN-α2 and the members of the IL-6 cytokine family (IL-6, OSM, and CT-1), we performed multivariant analyses following the method previously described (7). The type of interaction between two molecules was fixed by the interaction index, which was calculated as follows: $I = d1/D1 + d2/D2$ (d1 and d2 are the inhibitor concentrations in the combination, and D1 and D2 are the concentrations of the inhibitors 1 and 2 that separately exert the same inhibition as the combination). Therefore, if I is equal to 1 this indicates that there is no interaction and that the effect is additive. If I is lower than 1, the combination exerts synergism, and if I is higher than 1 the combination is antagonistic.

Microarray data accession number. The microarray data for Huh7 cells untreated or treated with IFN-α2, OSM, or IFN-α2 plus OSM have been deposited in the GEO database under accession number GSE13046.

RESULTS

OSM is released by activated DCs and synergizes with IFN-α in the inhibition of HCV and HAV replication in hepatic Huh7 cells. It has been already shown that DCs release OSM upon Toll-like receptor (TLR) ligation (47). We observed that incubation of DCs with LPS (a TLR4 agonist) caused rapid upregulation of OSM mRNA, with two peaks at 1 h and 8 h and returning to basal values by 16 h. This was accompanied by secretion of the cytokine to the extracellular space starting at 8 h and reaching maximum levels at 24 h (Fig. 1A and B). TLR3 ligation also induced OSM and promoted its release to the extracellular milieu, although the levels were lower than those observed following TLR4 activation (Fig. 1C and D). At 24 h after TLR stimulation the secretion of OSM was accompanied by the release of type I IFN to the medium (Fig. 1E). The simultaneous secretion of type I IFN and OSM led us to hypothesize that these two cytokines might act in concert in the defense against pathogens.

The induction of OSM in DCs upon TLR activation was not accompanied by any modification in the expression of *OSMR* or *LIFR* mRNAs. These two transcripts were maintained at extremely low levels in DCs (Fig. 1F and G). Western blot analysis showed that while OSMR was abundantly expressed in cells of hepatocellular lineage, Huh7 and HepG2, this receptor

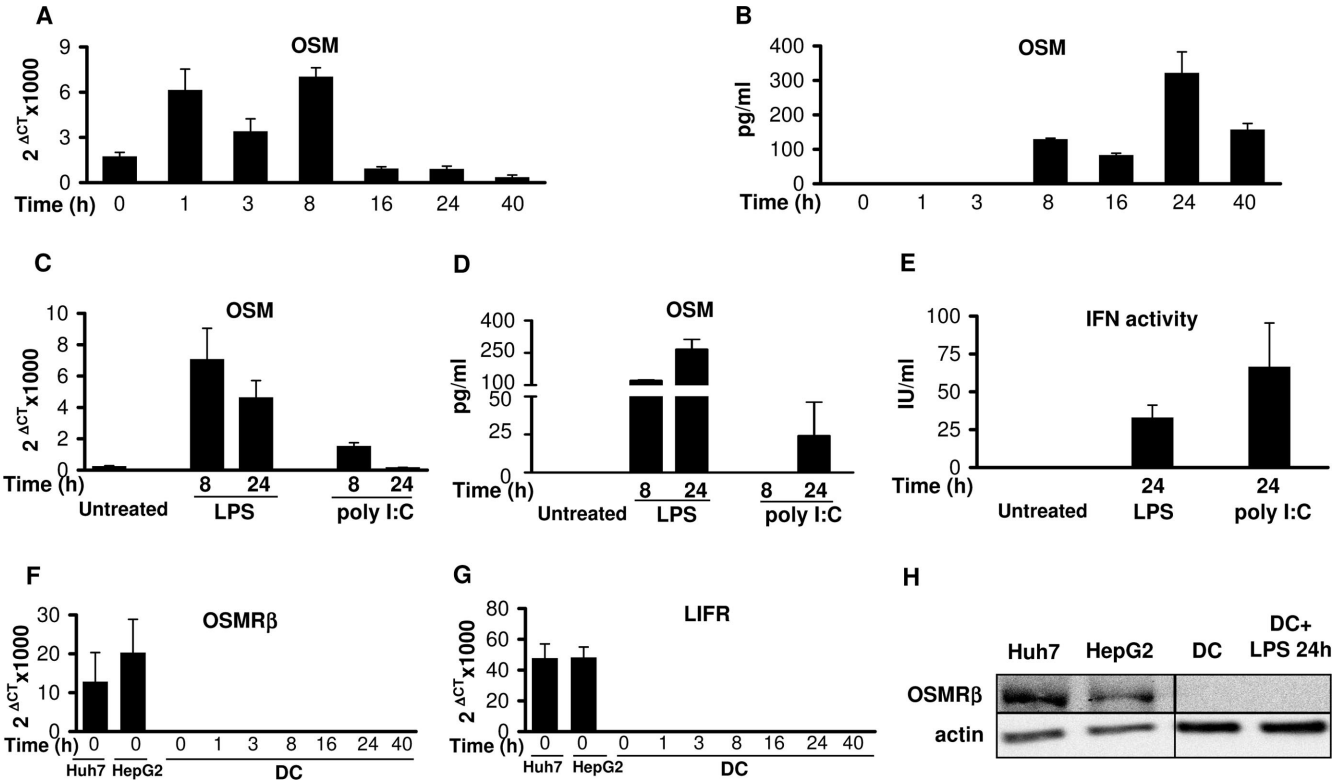


FIG. 1. DCs release OSM and type I IFN upon TLR ligation but lack OSM receptors, as opposed to liver epithelial Huh7 and HepG2 cells. The transcriptional expression of *OSM* (A), *OSMR*β (F), and *LIFR* (G) was analyzed by quantitative RT-PCR in monocyte-derived DCs treated with LPS (TLR4 ligand) for the indicated periods of time, and the release of OSM to the medium in the same time periods was determined by ELISA (B). A Western blot assay for OSMRβ was performed in Huh7 and HepG2 cells and in monocyte-derived DCs stimulated or not with LPS for 24 h (H). (C to E) Comparison of the ability of TLR4 (LPS) and TLR3 [poly(I-C)] ligands to stimulate OSM expression (C) and release to the medium (D) and type I IFN secretion (E). Values are means ± SD of three experiments performed in quintuplicate.

was undetectable in resting and LPS-activated DCs (Fig. 1H), suggesting that DC-derived OSM targets epithelial cells rather than DCs themselves. Indeed, we found that neither the addition of OSM nor its blockade with anti-OSM antibodies modified *CD80* expression nor the synthesis of IL-12 or IL-10 in LPS-stimulated DCs (data not shown).

Because OSMR is highly expressed in cells of hepatocellular lineage, we centered our study on the role of OSM in the defense of liver cells against infection. We found that OSM reduced viral load in Huh7 cells supporting HCV or HAV replication. This antiviral activity was significantly higher than that exerted by other members of the IL-6 superfamily, namely, CT-1 and IL-6 (Fig. 2A and B). Importantly, the combination of IFN-α2 plus each one of these cytokines enhanced the antiviral potency of IFN-α2, and the mixture IFN-α plus OSM was the most effective in reducing replication of both HCV and HAV (Fig. 2A and B). The calculation of the interaction index (I) of IFN-α2 with OSM, CT-1, or IL-6 showed synergism in all cases ($I < 1$), but it was stronger with the combination IFN-α2 plus OSM (0.11 and 0.17 for HCV and HAV replication, respectively). We also analyzed the levels of HCV core protein in HCV replicon cells after incubation for 3 and 4 days with IFN-α, OSM, or the combination. As shown in Fig. 2C, OSM decreased core protein only modestly and IFN-α2 caused a marked reduction of this viral antigen, while the combination of OSM plus IFN-α2 completely abrogated HCV core expression at day 4 of incubation.

In line with these findings we observed that OSM (and substantially less IL-6 and CT-1) synergized with IFN-α2 in the induction of the interferon-sensitive genes *OAS*, *ISG20*, and *GBP1* in HCV- or HAV-infected Huh7 cells (Fig. 3A to F). Notably, OSM alone upregulated some interferon-inducible genes, such as *ISG20* and *GBP1*. The synergisms of OSM (and that of IL-6 and CT-1) with IFN-α2 on antiviral activity and induction of antiviral genes were observed not only with IFN-α2 but also with other IFN-α subtypes, such as IFN-α5 (data not shown), which is the IFN-α subtype most abundantly expressed in the liver (3).

Jak/STAT signaling in Huh7 cells treated with IFN-α and/or OSM. To analyze cell signaling mechanisms activated by the combined effect of OSM and IFN-α, we performed immunoblotting analysis of Jak/STAT proteins in Huh7 cells treated for 1, 3, 24, 48, and 72 h with IFN-α2, OSM, or both. As shown in Fig. 4, STAT2 was only activated by IFN-α2 or by its combination with OSM being transient and not detectable by 24 h. Similarly, STAT1 was strongly phosphorylated by IFN-α2 at 1 and 3 h but its activation was no longer present at 24 h. However, IFN-α2 caused an increase of total STAT1 protein which was apparent from 24 h onwards. OSM activated STAT1 at 1 h, and the signal was faint during the following time points

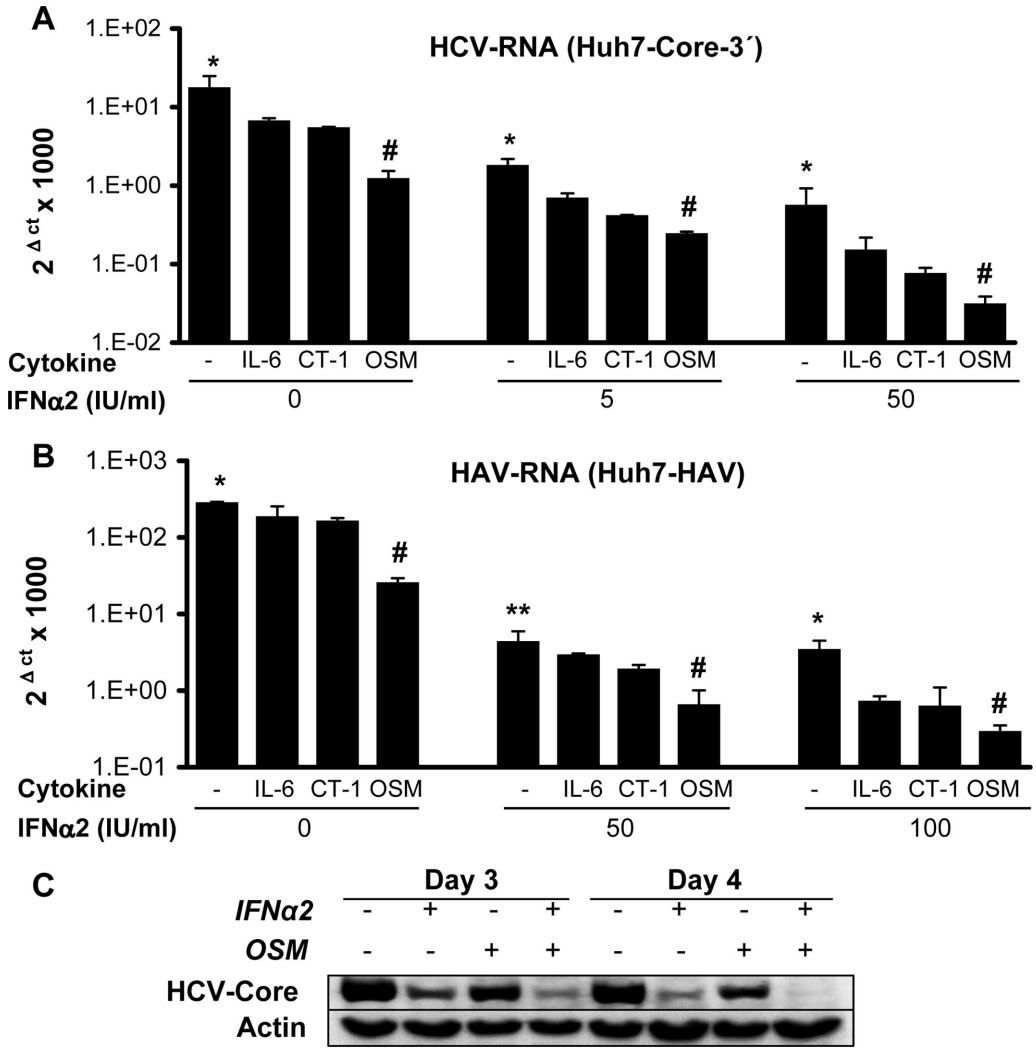


FIG. 2. Antiviral effect of cytokines of the IL-6 family (IL-6, CT-1, and OSM) used alone or in combination with different doses of IFN-α2 in cells transfected with full-length HCV replicon or HAV infected. Cells were treated with the cytokines (or were left untreated) for 72 h. HCV-RNA and HAV-RNA were determined by quantitative RT-PCR (A and B). HCV core protein was estimated by Western blotting (C). Values are means ± SD of three experiments performed in triplicate. *, $P < 0.05$ versus IL-6, CT-1, and OSM; **, $P < 0.05$ versus CT-1 and OSM; #, $P < 0.05$ versus IL-6 and CT-1.

but lasted 72 h. OSM also increased, albeit moderately, the levels of total STAT1 protein. When IFN-α2 was combined with OSM we observed an additive effect of the two cytokines, resulting in increased levels of total STAT1 and prolonged activation of this molecule, leading to a strong activation signal of STAT1 lasting up to 72 h. Relating STAT3, IFN-α2 caused only a mild and transient activation of the molecule which was no longer detectable after 1 h. In contrast, OSM alone and the combination OSM plus IFN-α2 induced a rapid and very robust activation of STAT3 that persisted at 72 h. This was accompanied by increased levels of STAT3 protein from 24 h onwards. Moreover, OSM, alone or in combination with IFN-α2, caused stronger and more prolonged activation of Jak1 than when using IFN-α2 alone (Fig. 4A). It seems possible that the longer and stronger activation of Jak1, STAT1, and STAT3 caused by OSM plus IFN-α2 might facilitate durable formation of STAT1 and STAT3 homodimers and heterodimers and enhanced expression of IFN-α-responsive antiviral genes.

Since activation of p38 MAPK has been shown to facilitate IFN-α-driven gene expression through ISRE and GAS elements (36), we also analyzed the effect of both cytokines in the activation of this signaling molecule. We found that in Huh7 cells IFN-α failed to induce p38 phosphorylation while OSM with or without IFN-α caused marked p38 activation for at least 72 h (Fig. 4B). This effect on p38 might contribute to enhance the expression of IFN-α-sensitive genes when both cytokines are used in combination.

Microarray analysis of genes induced by IFN-α and/or OSM. To gain insight into the transcriptional program activated by the joint action of IFN-α2 plus OSM, we studied the transcriptome of Huh7 cells incubated for 72 h in basal medium or in the presence of IFN-α2 (50 IU/ml), OSM (20

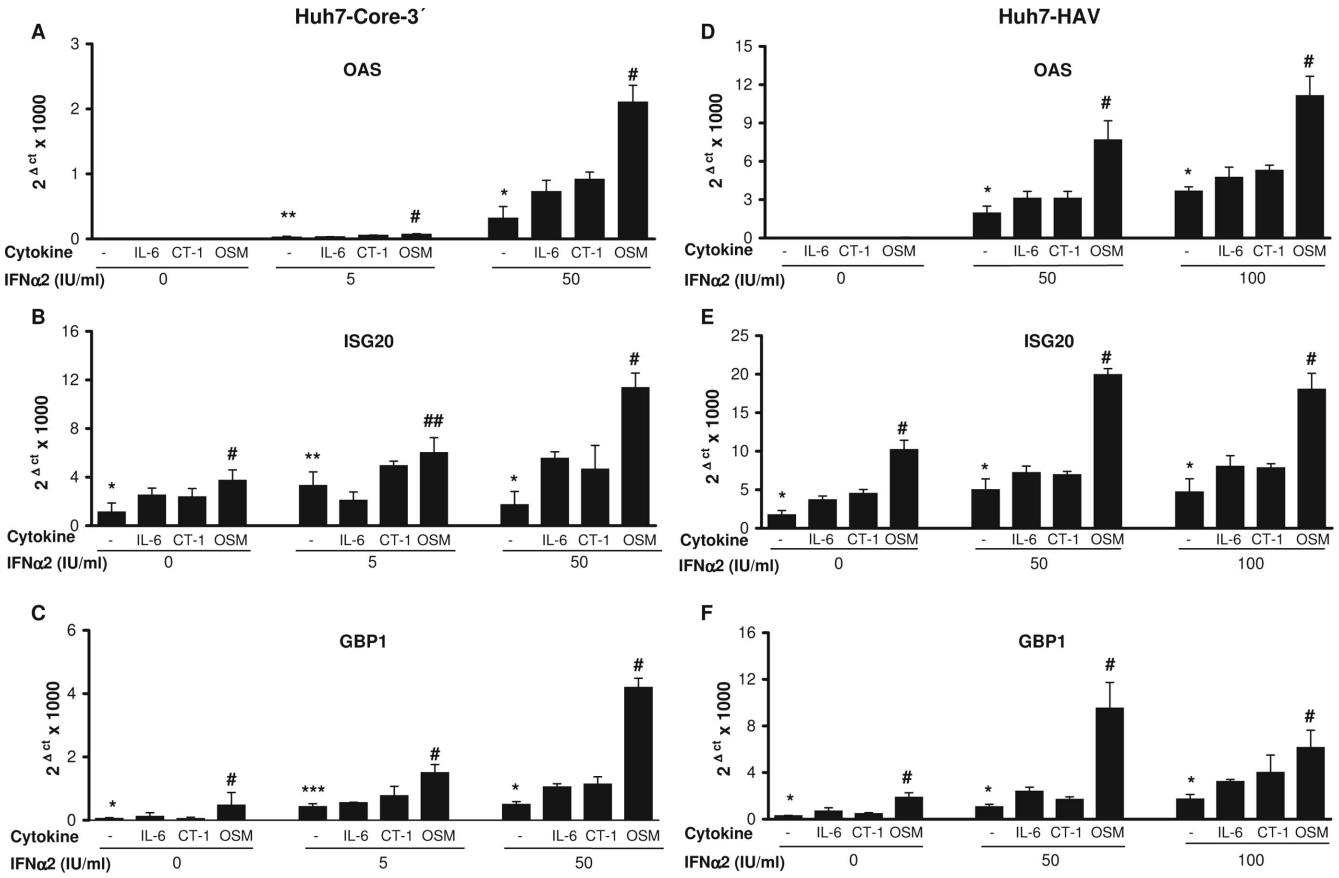


FIG. 3. Effect of cytokines of the IL-6 superfamily (IL-6, CT-1, and OSM) used alone or in combination with different doses of IFN-α2 on the expression of antiviral genes in cells transfected with full-length HCV replicon or HAV infected. Cells transfected with full-length HCV replicon or HAV infected were treated with the cytokines (or were left untreated) for 72 h. Gene expression levels of *OAS* (A and D), *ISG20* (B and E), and *GBP1* (C and F) were determined by quantitative RT-PCR. Values are means ± SD of three experiments performed in triplicate. *, $P < 0.05$ versus IL-6, CT-1, and OSM; **, $P < 0.05$ versus CT-1 and OSM; ***, $P < 0.05$ versus OSM; #, $P < 0.05$ versus IL-6 and CT-1; ##, $P < 0.05$ versus IL-6.

ng/ml), or both. After functional analysis studies with the genes differently expressed, we found an enrichment of biological categories that included antiviral genes, genes involved in antigen presentation, and genes encoding key immunoregulatory factors (Fig. 5). Validation of these genes was performed by quantitative RT-PCR after RNA extraction from Huh7 cells treated with IFN-α2, OSM, or both for 24, 48, and 72 h. Validated genes could be grouped into two clusters: (i) genes sensitive or not to IFN-α which showed little or no change with OSM alone but manifested vigorous upregulation with the combination treatment (Fig. 5); (ii) genes that were induced by OSM as well as by the combination of the two cytokines (Fig. 5). The positive interaction of OSM with type I IFN in the induction of antiviral genes and other immunoregulatory molecules was observed not only with IFN-α but also with IFN-β (data not shown).

Cluster A comprised mainly antiviral genes and genes implicated in antigen processing and presentation. Antiviral genes in this cluster include *ZC3HAV1*, *TRIM22*, *Mx1*, *IFI35*, *TLR3*, and *ISGF3G*, in addition to *GBP1*, *ISG20*, and *OAS*, as mentioned above (Fig. 3). Mx proteins bind viral ribonucleoprotein structures and block replication of viral RNA (9). TRIM22 and ZC3HAV1 have been implicated in the defense against retroviruses and alphaviruses (30, 56). TLR3 is localized in endosomes acting as a sensor of virus-derived double-stranded RNA that mediates type I IFN induction (22). ISGF3G contributes to efficient transcription of IFN-α/β-sensitive genes (51).

Cluster B included genes encoding molecules relevant to innate immunity and genes implicated in lymphocyte activation and expansion, as well as specific antiviral genes and genes involved in antigen presentation.

OSM induces key players of innate immunity. OSM was able to directly induce a variety of molecules that are essential in the natural defense against infection, including *MYD88*, *S100A9*, *ULBP2*, *IL-32*, *IRF1*, and *GBP2* and the chemokine genes *CXCL1*, *CXCL2*, and *CXCL3* (several of these genes were more intensely induced with OSM plus IFN-α than with OSM alone) (Fig. 6). MYD88 is the adapter protein for TLR2, -4, -5, -7, -8, and -9, and S100A9 contributes to MYD88 translocation to the TLR4-MD2 complex, thus enhancing TLR4 signaling (53). IL-32 is a proinflammatory cytokine that activates monocytes/macrophages (11). ULBP2 is a stress-induced molecule and a ligand for NKG2D that activates NK cells and provides costimulation for T cells by acting as a danger signal to alert the immune system of the presence of DNA damage or

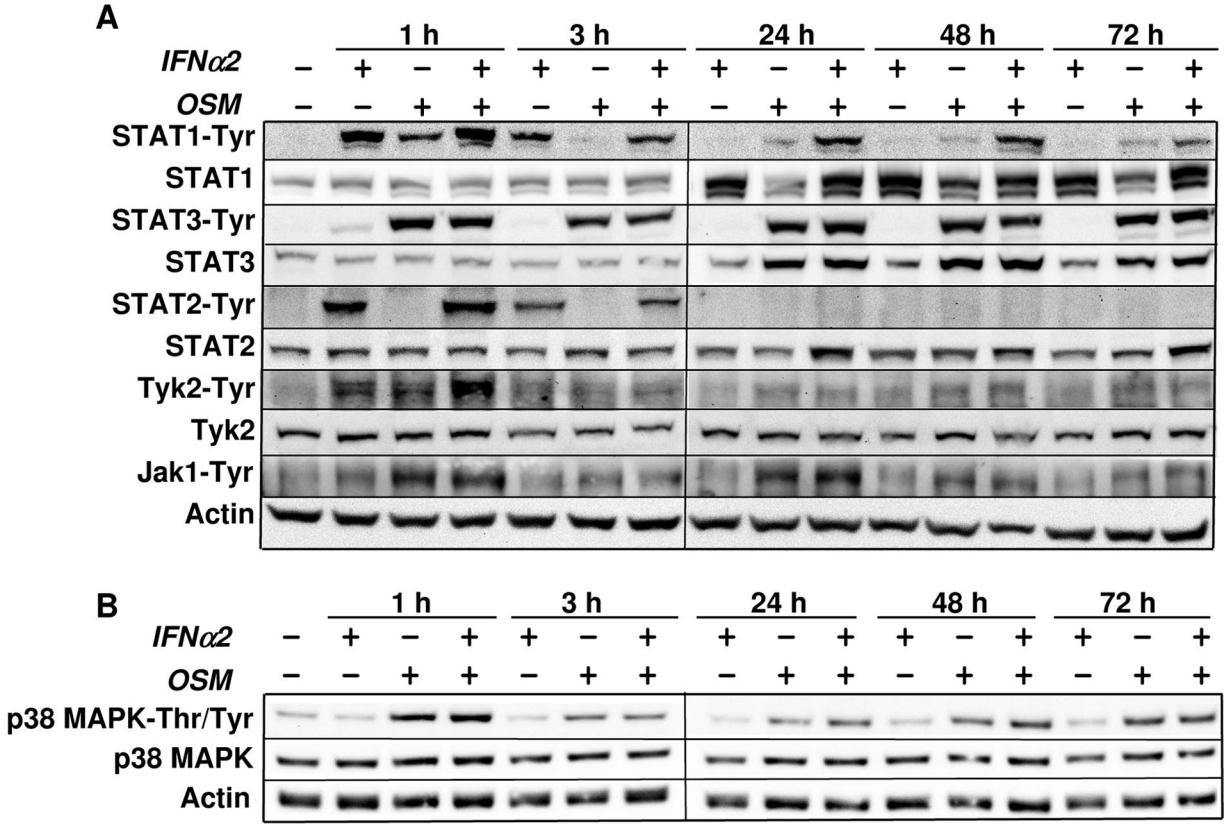


FIG. 4. Jak/STAT signaling pathway and p38 MAPK activation in Huh7 cells treated with IFN-α2, OSM, or the combination. (A) Representative Western blot analysis of phosphorylated and total protein levels of STAT1, STAT2, STAT3, Tyk2, and Jak1 in Huh7 cells cultured in medium alone or in the presence of IFN-α2, OSM, or IFN-α2 plus OSM during 1, 3, 24, 48, and 72 h of incubation. (B) Representative Western blot analysis of phosphorylated and total protein of p38 MAPK levels in Huh7 cells untreated or treated with IFN-α2, OSM, or IFN-α2 plus OSM during 1, 3, 24, 48, and 72 h. Actin levels are shown as a loading control.

intracellular infection (15). IRF1 is a factor known to enhance type I IFN production upon TLR ligation (34).

Upregulation of molecules involved in antigen processing and presentation by the combined effect of OSM and IFN-α or by OSM alone. As previously indicated, a group of genes encoding molecules with essential functions in antigen processing and presentation were strongly upregulated in Huh7 cells treated with OSM plus IFN-α2. These genes include the following: (i) members of the ubiquitin-immunoproteasome system, *UBE2L6*, *PSMB8*, and *PSMB9*, which are implicated in the generation of peptides from cytosolic proteins; (ii) transporters of peptides into the endoplasmic reticulum for association with major histocompatibility complex class I molecules, namely, *TAP1* and *TAP2*; (iii) HLA class I genes, particularly *HLA-B* and *HLA-C*; and (iv) *B2M*, which encodes β_2-microglobulin, an essential molecule for stable expression of class I molecules on cell surfaces (41) (Fig. 7A to H). *HLA-A*, which shows high basal expression, was also upregulated by the combination treatment but to a lesser extent than *HLA-B* and *HLA-C* (data not shown). OSM per se was also able to induce other genes which are critical for antigen presentation, such as *TAPBP* (Fig. 7I), whose gene product mediates the interaction between TAP1 and HLA class I (41).

Western blot analysis of PSMB9 and TAP1 in Huh7 cells demonstrated that treatment with IFN-α2 plus OSM induced the expression of these molecules at day 3 of incubation with persisting strong overexpression at day 4, while each cytokine alone caused only a mild elevation of the same proteins (Fig. 7J). In addition, B2M protein was upregulated by IFN-α2, and to a lesser extent by OSM, at day 3 but required the combined action of IFN-α2 plus OSM to be expressed at high levels on day 4 (Fig. 7J).

These results indicate that the combination of IFN-α2 and OSM strongly stimulates in liver epithelial cells the functional chain responsible for the generation and presentation of antigenic peptides to the executors of the adaptive immune response. This effect may be relevant for immune clearance of virus-infected cells.

OSM increases the immunostimulatory function of Huh7 cells and their ability to transpresent IL-15. We also found that OSM induces in Huh7 cells genes that encode molecules favoring activation and expansion of lymphocytes, namely, *ICAM-1*, *IL-15R*α, and *IL-7* (Fig. 8A to C). Western blot analysis indicated that OSM alone or in combination with IFN-α2 upregulated ICAM-1 with a pattern of multiple bands consistent with hyperglycosylation (Fig. 8D), a modification that has been reported to be associated with higher immunostimulatory activity of the protein (10).

Another relevant molecule conferring immunostimulatory properties to epithelial cells is IL-15Rα, which is essential for

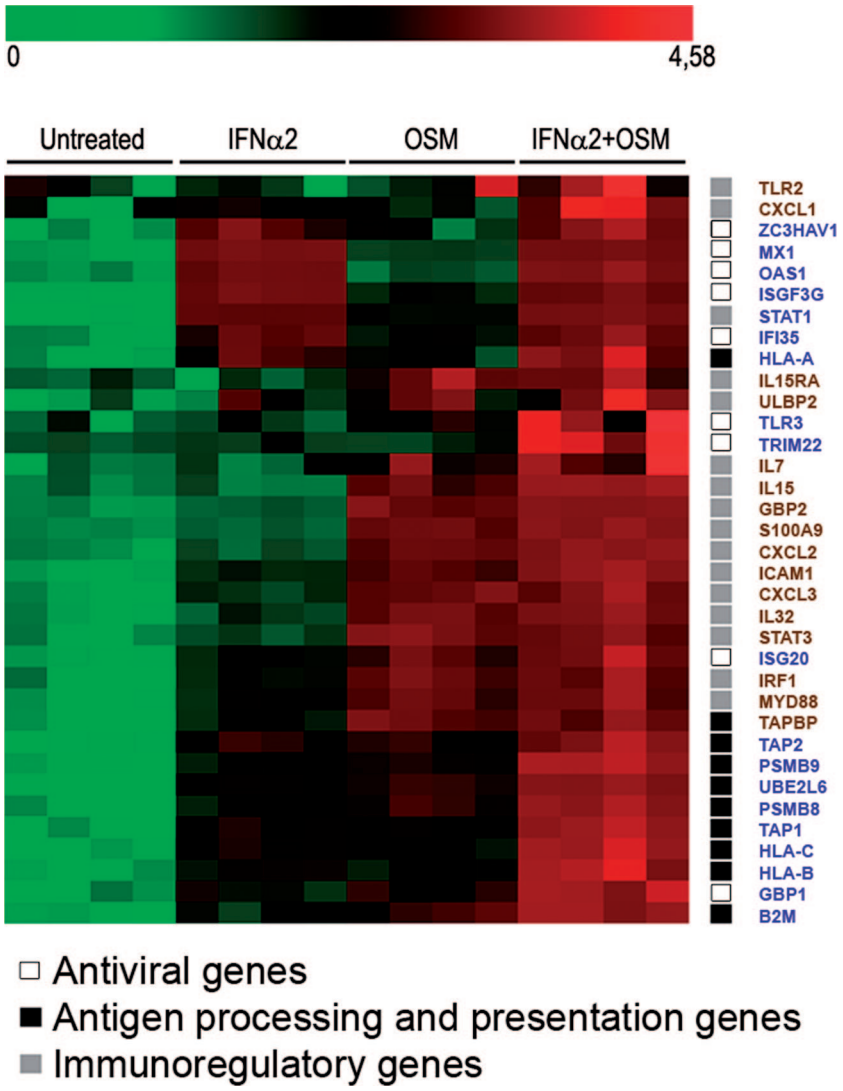


FIG. 5. Affymetrix microarray of Huh7 cells that received no treatment or were treated for 72 h with IFN-α2, OSM, or the combination. Control and treatment groups were analyzed in quadruplicate. Columns represent the specific treatments and rows represent individual genes. Genes upregulated by the combination therapy at levels higher than that by each cytokine in isolation are represented in blue (cluster A), and genes upregulated by OSM alone (or plus IFN-α) are represented in brown (cluster B). All genes in the figure have been validated by quantitative RT-PCR. Next to the array a color code indicates the gene function category.

efficient transpresentation of IL-15 to $CD8^+$ T cells. To ascertain the role of OSM in boosting the expression of functional IL-15Rα we studied the effect of OSM, IFN-α2, or OSM plus IFN-α2 on the ability of IL-15-pulsed Huh7 cells to sustain the proliferation of CTLL-2 cells (an IL-15-responsive cell line). As depicted in Fig. 8E, OSM alone or in combination with IFN-α2 caused significant stimulation of CTLL-2 proliferation, while cell growth was similar with all forms of treatment in the absence of IL-15. Importantly, OSM was more potent than IFN-α in enhancing IL-15 transpresentation by the epithelial cells to the responding lymphocytes (Fig. 8E).

We further investigated whether OSM alone or in combination with IFN-α2 could increase the immunostimulatory activity of liver epithelial cells. In two different sets of experiments we used hepatoma cells either pulsed with the short peptide GILGFVFTL or transfected with a plasmid encoding influenza A virus matrix to stimulate lymphocytes specific for GILGFV FTL, which is an HLA-A2-restricted epitope from the influenza A virus matrix. In these experiments hepatoma cells had been previously treated with OSM, IFN-α2, or the combination or had not received any previous treatment. In the first experiment HepG2 cells were employed, as they are HLA-$A2^+$, and were shown to respond to OSM with upregulation of genes involved in antigen presentation (such as *B2M*) and immunostimulation (such as *ICAM-1* and *IL-15R*α) in the same manner as Huh7 cells (data not shown). We found that pretreatment with OSM or the combination OSM plus IFN-α2 enhanced the ability of peptide-pulsed HepG2 cells to stimulate the production of IFN-γ by CTL more efficiently than when using IFN-α2 alone (Fig. 8F). In the second experiment, we used Huh7 cells transfected with two plasmids, one encoding the influenza A virus matrix protein and the other HLA-

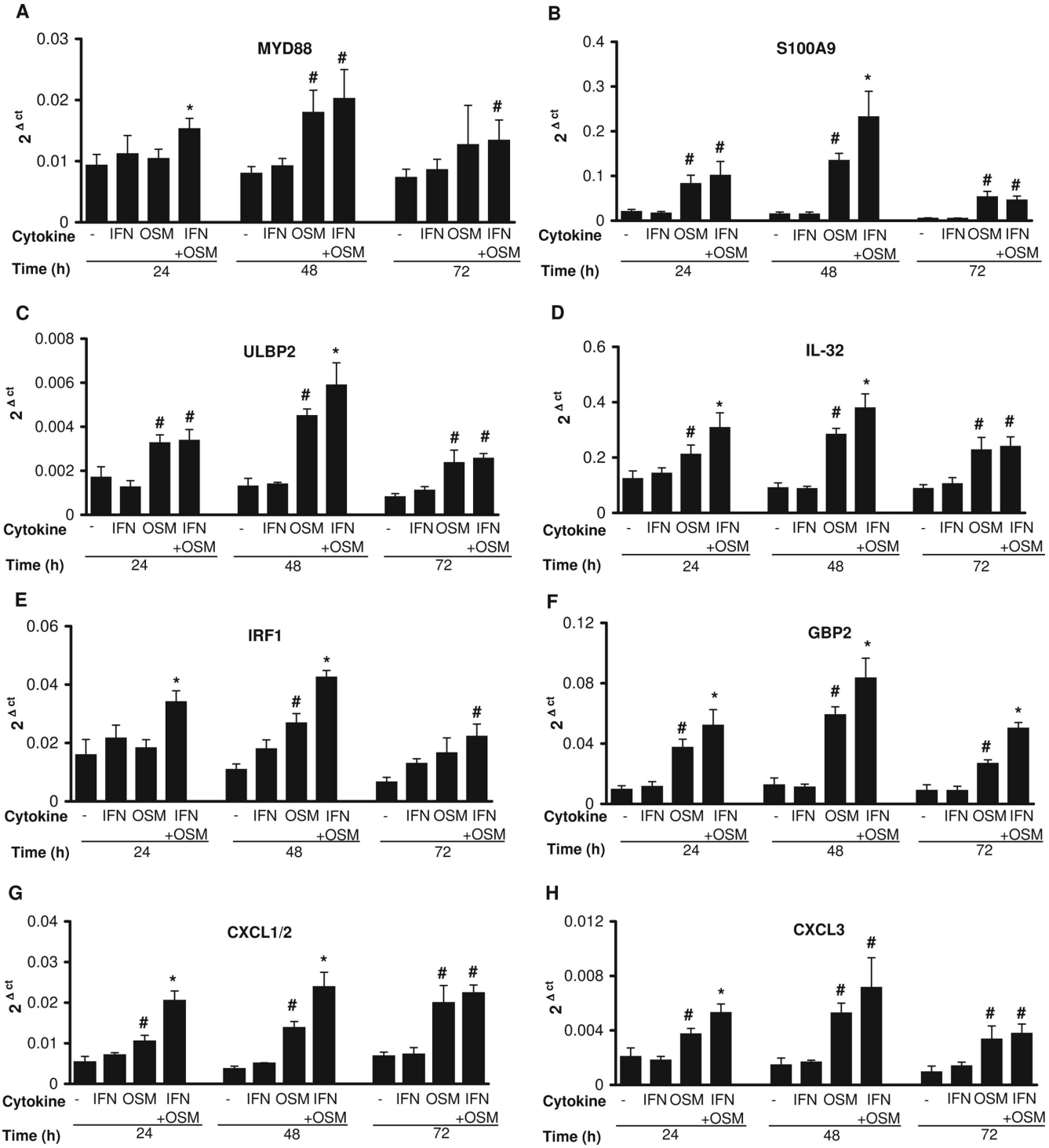
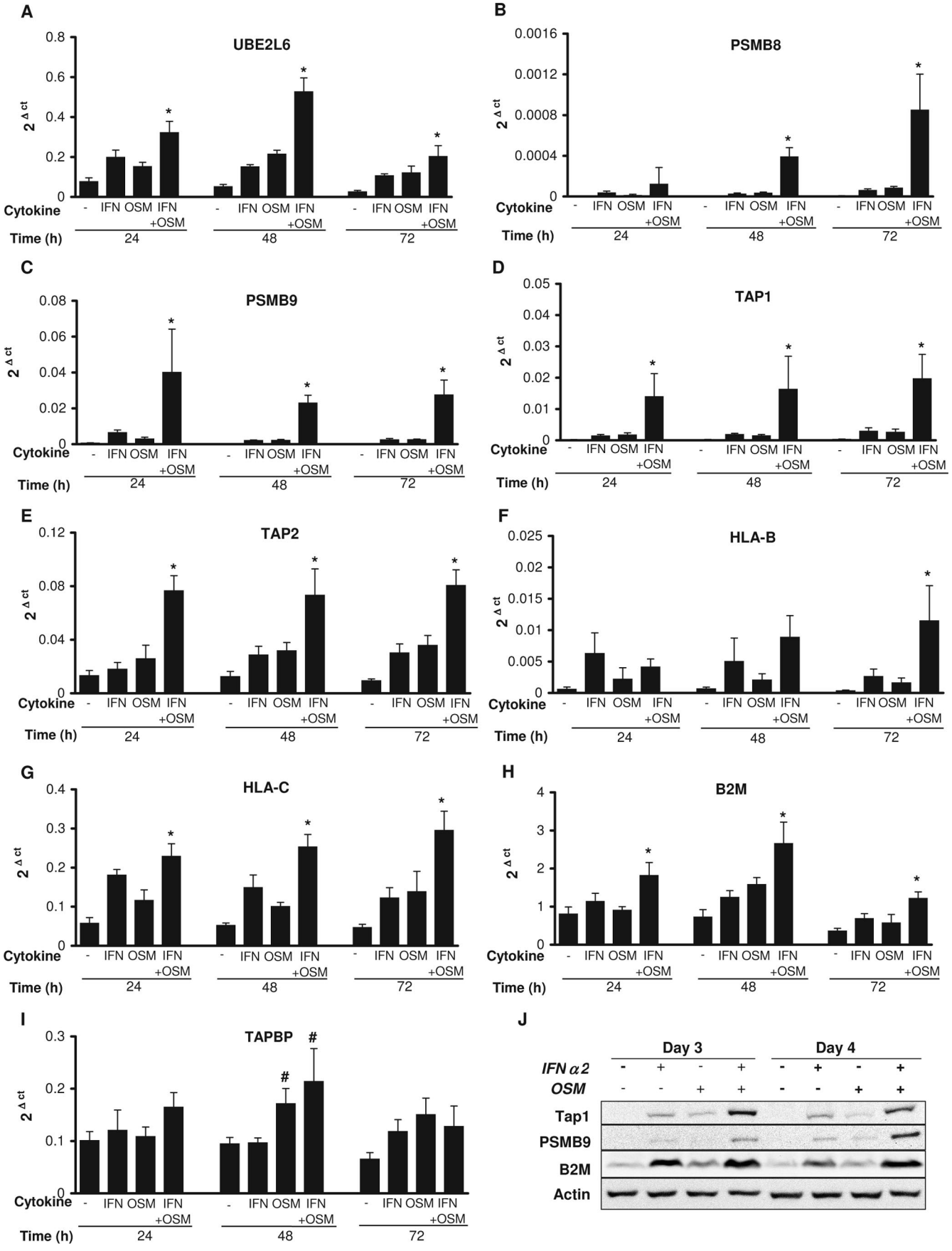


FIG. 6. OSM upregulates genes which participate in the natural defense against infection. The transcriptional expression levels of *MYD88* (TLR adapter molecule) (A), *S100A9* (modulator of TLR function) (B), *ULBP2* (ligand for NKG2D that activates NK cells) (C), *IL-32* (proinflammatory molecule that activates macrophages) (D), *IRF1* (factor that induces type I interferon production) (E), *GBP2* (molecule with antiviral activity) (F), and the chemokine genes *CXCL1/2* and *CXCL3* (G and H) were determined by quantitative RT-PCR in untreated Huh7 cells or cells treated for the indicated periods of time with IFN-α2, OSM, or the combination. Values are means ± SD of three experiments performed in quintuplicate. ⋆, $P < 0.05$ versus untreated, IFN-α2, and OSM; #, $P < 0.05$ versus untreated and IFN-α2.



A2. Higher IFN-γ production by influenza virus-specific effector lymphocytes was observed when target cells had been previously treated with OSM plus IFN-α2 than when using untreated cells or cells treated with IFN-α2 or OSM alone (Fig. 8G). The enhancement of lymphocyte response by treating the target cells with IFN-α2 plus OSM was abolished by a proteasome inhibitor (Fig. 8G). These findings are in keeping with our previous data showing activation of antigen processing by the concerted action of the two cytokines (Fig. 7).

DISCUSSION

Our findings have characterized OSM as a new cytokine involved in the defense of the liver against infection. This idea is based on the following facts: (i) in liver epithelial cells OSM increases the antiviral properties of type I IFN and induces key players of innate immunity; (ii) in these cells OSM synergizes with IFN-α to enhance antigen processing and presentation; and (iii) OSM augments the immunostimulatory properties of cells of hepatocellular lineage. Taken together these data suggest an important role for this cytokine in the activation of both innate and adaptive immunity and in linking together these two biological responses to pathogens.

As mentioned above, OSM is released by DCs and neutrophils upon stimulation (18, 20, 47). We found that TLR4 activation, and to a lesser extent TLR3 stimulation, induced OSM secretion. Although these data might indicate that bacterial products are more efficient than viruses in triggering OSM release, it should be considered that TLR4 signaling may take place in viral infections through recognition of virion surface proteins (37) or through interaction with molecules such as HMGB1, released by activated macrophages or dying cells (29). Our finding that OSM and type I interferon are secreted simultaneously upon TLR activation suggested to us a concerted action of the two cytokines at the earlier phases of pathogen recognition. The notion of a functional connection between OSM and type I IFN is also consistent with the fact that TLR4 activation (which stimulates OSM release) couples with the induction of type I IFN via the TRIF pathway (48).

Noticeably, OSMR is scarcely expressed by either DCs (Fig. 1) or peripheral blood lymphocytes (32), while it is abundant in cells of hepatocellular lineage. It is thus reasonable to think that OSM exerts its effects on epithelial cells rather than on professional antigen-presenting cells. A key observation in this paper was the synergism of OSM and IFN-α in reducing viral replication in liver cells transfected with full-length HCV replicon or infected with HAV. We have also shown that this effect is associated with enhanced expression of several antiviral genes when both cytokines are used in combination.

The differential regulation of gene expression when using OSM plus IFN-α compared with either of them alone might be due to interactions between the respective signaling pathways or to changes in the levels of signaling molecules and transcription factors, caused by one of them, that influence the transcriptional response to the other. Our data show that combination of IFN-α and OSM leads to more intense and more prolonged activation of both STAT1 and STAT3 in association with higher intracellular levels of the two proteins. While elevation of STAT1 protein is caused by IFN-α, the augmentation of STAT3 is due to OSM. We also found that OSM and its combination with IFN-α resulted in increased and lasting activation of Jak1 which might contribute to maintain STAT phosphorylation when IFN-α acts together with OSM. As a result the joint action of OSM and IFN-α could favor the formation of STAT1/STAT3 heterodimers and STAT3/STAT3 homodimers for longer times, allowing enhanced and more durable expression of IFN-α-sensitive antiviral genes (40). On the other hand, OSM alone or combined with IFN-α caused marked and sustained p38 MAPK phosphorylation. Since p38 activation has been shown to enhance transcription of IFN-α-inducible genes from both ISRE and GAS elements (36), the effect of OSM on this signaling molecule offers an additional explanation for the observed synergism between OSM and IFN-α.

OSM might also be implicated in natural defenses against infection because of its stimulatory effect on the expression of relevant components of innate immunity, such as MYD88, S100A9, IL-32, ULBP2, IRF1, and GBP2, and by its ability to induce the expression of the chemokines CXCL1, CXCL2, and CXCL3, which recruit inflammatory cells to the site of infection (39).

A crucial aspect in the defense against viral infections is the ability of the infected cells to display viral peptides on the cell membrane in the context of HLA class I molecules for presentation to primed $CD8^+$ cells. Prior to antigen presentation by major histocompatibility complex class I molecules, cytosolic antigens must be polyubiquitinated and processed to CTL epitopes by the proteasome. It has been shown that stimulation of the infected epithelial cell with IFN-γ induces a change in the composition of the 20S catalytic core of the proteasome by substituting β1, β2, and β5 subunits of the inner heptameric rings by β1i (PSMB9), β2i (MELC-1), and β5i (PSMB8), leading to the formation of the immunoproteasome, which exhibits differences in its proteolytic activity compared to the constitutive proteasome (46, 49). In fact, mice lacking PSMB8 or PSMB9 fail to process and present specific epitopes to $CD8^+$ T cells (14, 50). It has been shown recently that not only IFN-γ but also IFN-α can induce the expression of immunoproteasome subunits (44). In the present work we have demonstrated that OSM strongly enhances the ability of IFN-α to stimulate the production of both PSMB8 and PSMB9. The synergism

FIG. 7. OSM synergizes with IFN-α in the induction of genes involved in antigen processing and presentation in Huh7 cells. mRNA levels were determined by quantitative RT-PCR for ubiquitin-conjugating enzyme UBE2L6 (A), immunoproteasome subunits PSMB8 (B), and PSMB9 (C), transporters associated with antigen processing, TAP1 (D) and TAP2 (E), HLA class I B and C (F and G), β2-microglobulin (H), the TAP binding protein TAPBP (I) in Huh7 cells treated for the indicated periods of time with IFN-α2, OSM, or both, or left untreated. Values are means ± SD of three experiments performed in quintuplicate. A Western blot assay determined TAP1, PSMB9, and β2-microglobulin levels in Huh7 cells treated for 3 and 4 days with IFN-α2, OSM, or the combination. Actin levels are shown as a loading control (J). *, $P < 0.05$ versus untreated, IFN-α2, and OSM; #, $P < 0.05$ versus untreated and IFN-α2.

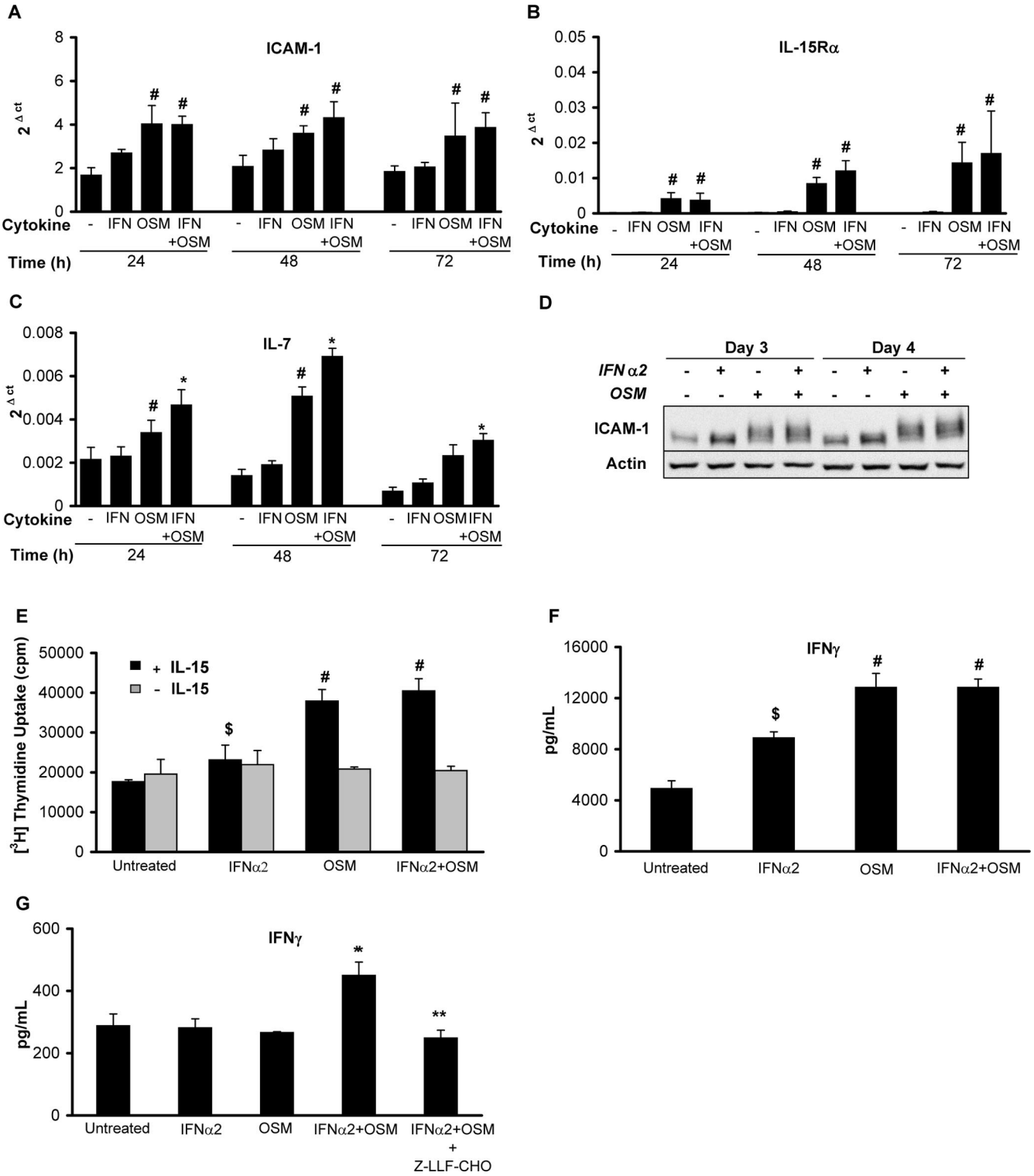


FIG. 8. OSM upregulates genes that stimulate adaptive immunity and enhances IL-15 transpresentation and the immunostimulatory activity of liver epithelial cells. (A to C) Transcriptional expression of *ICAM-1* (A), *IL-15Rα* (B) and *IL-7* (C) in untreated Huh7 cells or cell treated with IFN-α2, OSM, or both. (D) Western blot assay for ICAM-1 in Huh7 cells treated with IFN-α2, OSM, or both. (E) Proliferative activity of CTLL-2 cells (a $CD8^+$ T-cell line with IL-15 growth dependence) incubated with Huh7 cells treated for 3 days with IFN-α2, OSM, or both or left untreated. (F and G) Production of IFN-γ by CTL sensitized to the influenza virus peptide GILGFVFTL cocultured with HepG2 cells previously treated for 4 days with IFN-α2, OSM, both, or left untreated and pulsed with the synthetic peptide (F) or with Huh7 cells treated for 72 h with IFN-α, OSM, both, or left untreated and cotransfected with a plasmid encoding HLA-A2 and a plasmid encoding influenza A virus matrix protein (G). An additional group was treated with the cytokine combination plus the proteasome inhibitor Z-LLF-CHO. Values are means ± SD of three experiments performed in quintuplicate. *, $P < 0.05$ versus untreated, IFN-α2, and OSM; #, $P < 0.05$ versus untreated and IFN-α2; $, $P < 0.05$ versus untreated; **, $P < 0.05$ versus IFN-α2 plus OSM.

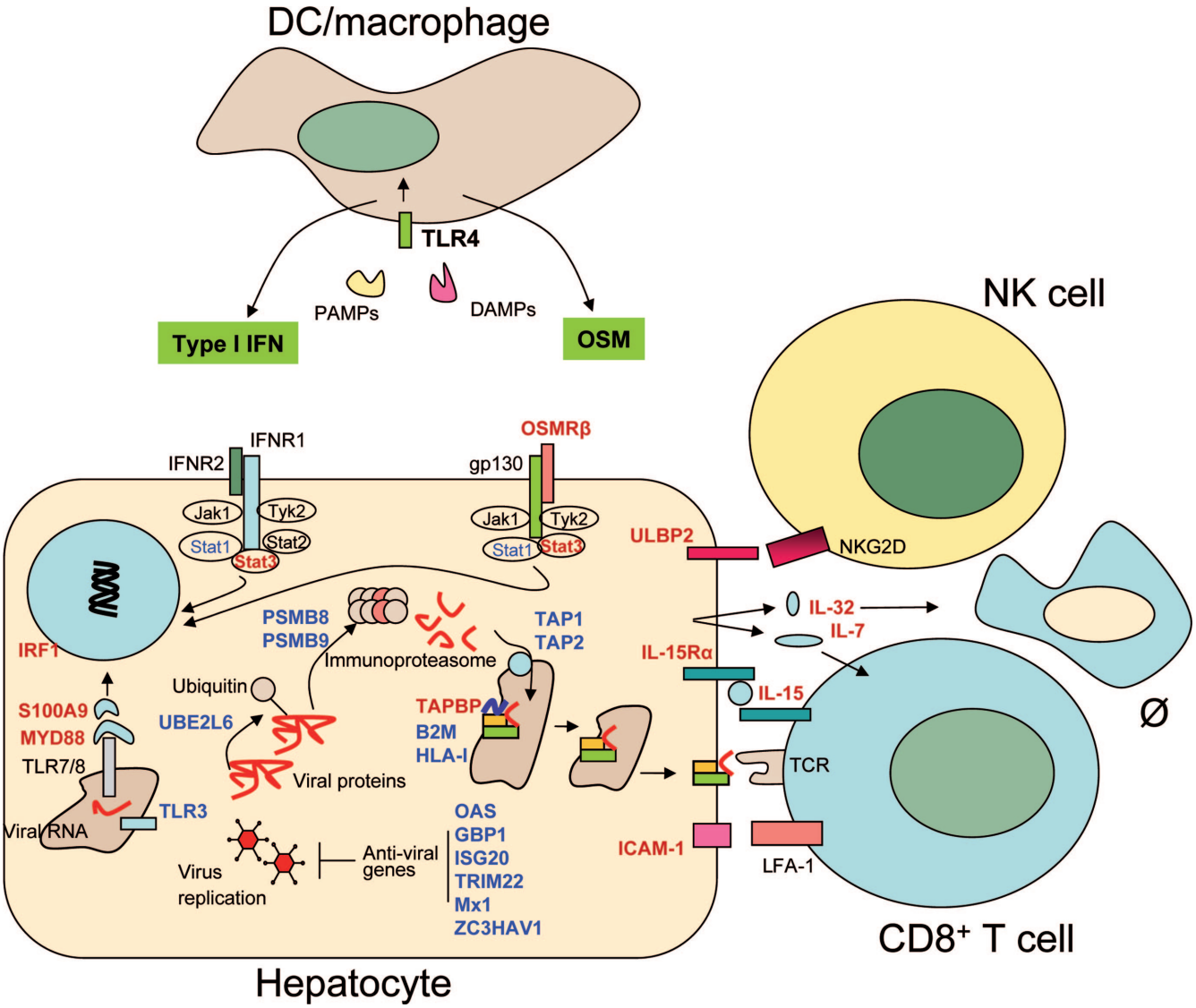


FIG. 9. Representative scheme illustrating the molecules highly upregulated by the combination of OSM with type I IFN (in blue) or OSM alone (in brown) in liver epithelial cells. OSM per se or in association with type I IFN induces a chain of antiviral defense genes, including viral sensors (TLR3), antiviral genes (OAS1, Mx1, GBP1, ZC3HAV1, and TRIM22), antigen processing genes (UBE2L6, PSMB8, PSMB9), genes involved in antigen presentation (TAP1, TAP2, TAPBP, HLA class I, and β2-microglobulin), genes implicated in activation of innate immunity (IRF1, STAT1, STAT3, MYD88, S100A9, IL-32, and ULBP2), and genes triggering adaptive immunity (ICAM-1, IL-15Rα, and IL-7). Ø indicates a macrophage. OSMR is also upregulated by OSM (data not shown). PAMPs, pathogen-associated molecular patterns; DAMPs, danger-associated molecular patterns.

OSM and IFN-α also extends to the synthesis of TAP1 and TAP2, two proteins that are critical for loading the antigenic peptides onto HLA class I. In addition TAP1 has been shown to participate in host resistance to infection by stimulating IFN-γ-producing NK cells (17). Interestingly, the immunoproteasome genes *PSMB8*, *PSMB9* map between *TAP1* and *TAP2* on 6p21.3, and *PSMB9* and *TAP1* share a common promoter (33), suggesting coordinated regulation of these functionally related genes. It has been recently reported that *PSMB9* expression is stimulated by a heterodimer formed by unphosphorylated STAT1 and IRF1 (6). The regulation of *PSMB9* by these two factors explains the synergism IFN-α and OSM in the induction of this gene, since OSM upregulates IRF1 while IFN-α elevates STAT1 levels.

Consistent with the concept that OSM operates at the interface between natural and adaptive immunity, we observed that this cytokine increases mRNA and protein levels of ICAM-1 in epithelial cells. Moreover, in OSM-treated cells Western blot studies showed a pattern of multiple bands compatible with ICAM-1 hyperglycosylation, which is a posttranslational modification that accrues the immunostimulatory activity of this costimulatory protein (10). As it has been shown that the ICAM-1–LFA-1 interaction boosts central memory $CD8^+$ T cells (35), our findings suggest a role of OSM-activated epithe-

lial cells in the expansion of this cell subset which is critical for long-term protection against infection (35). The fact that OSM upregulates IL-7 expression (a cytokine that promotes both CD4 and CD8 expansion [42]) and IL-15Rα is consistent with the idea that OSM may be important in the stimulation of CD8 responses in viral infections. In this context the effect on IL-15Rα is of considerable relevance since this receptor interacts with high affinity with IL-15, forming stable complexes on the cell surface for transpresentation of the cytokine to neighboring target cells, mainly $CD8^+$ memory T cells and NK cells (12). Due to endosomal recycling, IL-15Rα/IL-15 complexes may persist for long periods on the cell membrane (12), and it has been shown that transpresented IL-15 is much more efficient than soluble IL-15 in the stimulation and expansion of antigen-experienced $CD8^+$ T cells (24). In agreement with the observed *IL-15R*α upregulation induced by OSM, we found that liver epithelial cells stimulated with this cytokine, with or without IFN-α, were able to transpresent IL-15 to $CD8^+$ T cells more efficiently than control cells or cells treated with IFN-α alone. Although IFN-α was able to increase the ability of liver cells to transpresent IL-15 to $CD8^+$ lymphocytes, the effect of OSM was significantly higher. The stimulation of IL-15 transpresentation is a novel contribution of OSM to antiviral defense of the liver since it will enhance the capacity of hepatic parenchymal cells to activate and expand cytotoxic $CD8^+$ T lymphocytes specific for viral epitopes. The role of OSM in boosting the immunostimulatory properties of liver cells was confirmed by our results showing that HepG2 cells incubated with a viral peptide were able to stimulate the production of IFN-γ at higher levels when pretreated with OSM or the combination OSM plus IFN-α than when using IFN-α alone. This higher immunostimulatory ability of liver cells treated with OSM plus IFN-α was found not only when using peptide-pulsed HepG2 cells but also when using Huh7 cells transfected with a plasmid encoding a viral protein. This effect was abolished by proteasome inhibitors, in agreement with previous data showing a higher induction of genes involved in antigen processing by the combination IFN-α and OSM. Thus, our findings show that the concerted action of IFN-α and OSM activates in liver cells the whole functional chain leading to efficient presentation of antigenic peptides to lymphocytes by inducing (i) UBE2L6 expression, (ii) formation of the immunoproteasome, (iii) upregulation of TAP1, TAP2, and TAPBP, and (iv) enhanced expression of HLA class I molecules and β2-microglobulin and upregulation of immunostimulatory molecules ICAM-1, IL-7, IL-15Rα. A scheme depicting the functions of genes implicated in natural and adaptive immunity modulated by OSM and IFN-α in liver cells is presented in Fig. 9.

In conclusion, this paper describes a novel role of OSM in the orchestration of the defense of the liver against infection. OSM activates natural immunity and reinforces the antiviral effects of IFN-α. On the other hand, OSM may behave as a trigger of adaptive immune responses to hepatotropic viruses by stimulating antigen processing and presentation and by boosting the immunostimulatory properties of hepatic epithelial cells. These findings open new avenues for more efficient antiviral therapies.

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