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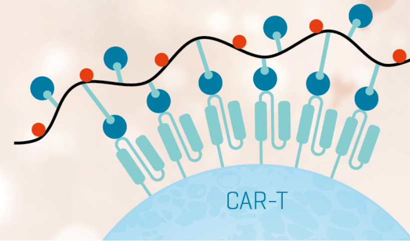

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Notch-1 Up-Regulation and Signaling following Macrophage Activation Modulates Gene Expression Patterns Known to Affect Antigen-Presenting Capacity and Cytotoxic Activity[1]

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Notch signaling has been extensively implicated in cell-fate determination along the development of the immune system. However, a role for Notch signaling in fully differentiated immune cells has not been clearly defined. We have analyzed the expression of Notch protein family members during macrophage activation. Resting macrophages express Notch-1, -2, and -4, as well as the Notch ligands Jagged-1 and -2. After treatment with LPS and/or IFN-γ, we observed a p38 MAPK-dependent increase in Notch-1 and Jagged-1 mRNA and protein levels. To study the role of Notch signaling in macrophage activation, we forced the transient expression of truncated, active intracellular Notch-1 (Notch-IC) proteins in Raw 264.7 cells and analyzed their effects on the activity of transcription factors involved in macrophage activation. Notch-IC increased STAT-1-dependent transcription. Furthermore, Raw 264.7 Notch-IC stable transfectants increased STAT1-dependent transcription in response to IFN-γ, leading to higher expression of IFN regulatory factor-1, suppressor of cytokine signaling-1, ICAM-1, and MHC class II proteins. This effect was independent from an increase of STAT1 Tyr or Ser phosphorylation. However, inducible NO synthase expression and NO production decreased under the same conditions. Our results show that Notch up-regulation and subsequent signaling following macrophage activation modulate gene expression patterns known to affect the function of mature macrophages. ***The Journal of Immunology,*** **2006, 176: 5362–5373.**

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Macrophages are cells essential for the immune response. After their precursors are produced in the bone marrow, they are transported in the blood to the different tissues in the organism, where they can differentiate to mature macrophages and be activated by pathogens and proinflammatory cytokines (1, 2).

Macrophages, and other phagocytic cells, such as neutrophils and dendritic cells, can discriminate between pathogens and self through the signals triggered by specific TLRs present at the cell membrane. TLRs recognize different pathogens' components, called pathogen-associated molecular patterns. LPS, an integral element present at the outer membrane of Gram-negative bacteria, is recognized by the TLR4, thus initiating a complex signaling pathway finally leading to the activation of several transcription factors that control macrophage activation, such as NF-κB, IFN regulatory factor (IRF)[3]-3, and AP-1, among others (3, 4).

In addition to pathogen-associated molecular patterns, macrophages can also become fully functional after the binding of IFN-γ, a cytokine released by activated T lymphocytes and NK cells, to its specific membrane receptor, IFN-γR (5–7). Following this interaction, activated IFN-γR binds and activates the JAK 1 and 2 that, in turn, trigger the phosphorylation of STAT1. Phosphorylated STAT1 dimerizes and translocates to the nucleus, where it binds to the IFN-γ activation promoter site (GAS) to initiate or suppress transcription of IFN-γ-regulated genes (5). Some of these genes, like *IRF-1*, are transcription factors able to drive regulation of a second wave of gene expression. Although biochemical and genetic studies show that STAT1 plays a critical role in IFN-γ-dependent signaling, recent work has revealed that additional signals are required for the full range of responses elicited by IFN-γ (7).

Notch proteins comprise a family of epidermal growth factor-like transmembrane receptors that control different aspects of tissue development and homeostasis (8, 9). Four distinct Notch genes have been identified in mammals, *Notch-1*, *-2*, *-3*, and *-4* (9). Notch ligands are also epidermal growth factor-like transmembrane proteins. Five different Notch ligands have been characterized in mammals, Jagged-1 and -2, and Delta-1, -3, and -4 (8). After effective ligand interaction, Notch receptors undergo at least two proteolytic cleavage steps that release the intracellular Notch (Notch-IC) receptor region from the membrane. Notch-IC then translocates to the nucleus, where it associates with the transcription factor CSL (CBF1, Suppressor of Hairless, LAG-1/RBP-Jκ). In the absence of Notch-IC, CSL binds to corepressors, thus inhibiting transcription. Notch-IC displaces the

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[3] Abbreviations used in this paper: IRF, IFN regulatory factor; GAS, IFN-γ activation promoter site; Notch-IC, intracellular Notch; iNOS, inducible NO synthase; RAM, RBP-Jκ-associated molecule; PEST, proline-glutamate-serine-threonine-rich; SOCS, suppressor of cytokine signaling.

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corepressors, recruits coactivators, and activates CSL-dependent transcription. Some target genes of this process have been characterized, including the basic helix-loop-helix transcription factors belonging to the HES family, including *Hes-1* and *Hes-5*, that are expressed by cells of the hemopoietic lineage (10). Although some CSL-independent Notch signaling can occur, its mechanism is not well characterized (11). Notch signaling is modulated by extracellular, cytoplasmic, and nuclear proteins (8). Several enzymes, like Fringe, glycosylate specific extracellular Notch sites, modifying ligand-mediated signaling (12, 13).

Notch signaling is highly involved in lymphopoiesis and, to a lesser extent, in myeloid differentiation and generation of embryonic hemopoietic stem cells (10, 14, 15). Notch signaling in myeloid cells has been associated with maintenance of undifferentiated phenotypes (16, 17), but recently it has been implicated in monocyte differentiation to dendritic cells (18).

Although Notch signaling has been extensively implicated in cell-fate selection through the development of the immune system, less attention has been paid to the function of Notch receptors in different mature immune cells expressing high levels of those proteins. In this study, we show that different members of the Notch family are expressed in macrophages, and some of them, like Notch-1 and Jagged-1, are up-regulated in the course of macrophage activation. In addition, transfection of constitutively active Notch-IC proteins in Raw 264.7 cells induced elevated responses to IFN-γ through increased STAT1-dependent transcription, leading to higher expression of ICAM-1 and class II MHC proteins. In contrast, after activation with LPS or IFN-γ, these transfectant cells displayed lower expression of inducible NO synthase (iNOS) and decreased NO production. We show in this study for the first time that Notch expression is modulated in the course of macrophage activation, and that Notch signaling can regulate specific gene expression patterns induced by different activation signals that may modulate the function of mature macrophages.

Materials and Methods

Chemicals

Reagents were purchased from Sigma-Aldrich, Boehringer Mannheim, or Merck. Abs were purchased from Santa Cruz Biotechnology or Sigma-Aldrich. LPS from *Salmonella typhimurium*, kinase inhibitors, and cytokines were purchased from Sigma-Aldrich, Calbiochem, and Roche, respectively. Serum and culture medium were acquired from BioWhittaker. Electrophoresis equipment and reagents were acquired from Bio-Rad.

Cell culture

Raw 264.7 and J774 cells were subcultured at a density of 6–8 $\times$ 10^4/cm^2 in RPMI 1640 supplemented with 2 mM glutamine, 10% FCS, and antibiotics (50 mg/ml each of penicillin, streptomycin, and gentamicin). After 2 days in culture, the medium was replaced by RPMI 1640 containing 1% FCS, and then cells were used in the next 24 h. 293T cells were cultured in DMEM supplemented with glutamine, 10% FCS, and antibiotics, as described previously. Elicited peritoneal macrophages were obtained from male mice 4 days after i.p. inoculation of 1 ml of sterile 10% (weight/volume) thioglycollate broth as described previously (19). Cells were seeded at a density of 10^5/cm^2 in RPMI 1640 supplemented with 10% heat-inactivated FCS and 50 μg/ml gentamicin, penicillin, and streptomycin.

Plasmids

pNIC-2 plasmid encodes for an intracellular active form of human Notch-1 protein (aa 1759–2556) containing the RAM (RBP-Jκ-associated molecule) domain, the six cdc10/ankyrin repeats, a homopolymer repeat of glutamine (OPA) domain, three nuclear localization signal sequences, and a proline-glutamate-serine-threonine-rich (PEST) domain, linked to a *myc* epitope fused to the C-terminal region. pNIC-1 plasmid encodes for a human Notch-1-IC protein (aa 1759–2237), lacking the OPA and PEST domains. Both plasmids were derived from the pLNCX2 retroviral vector (BD Biosciences), engineered by us to contain a *myc* tag epitope. The *myc* tag epitope was generated by hybridization of two complementary oligonucleotides (5′-TCG ACT CAA TGC AGA AGC TGA TCT CAG AGG AGG ACC TCT AAT-3′ and 5′-CGA TTA GAG GTC CTC CTC TGA GAT CAG CTT CTG CAT TGA G-3′), which were then cloned at the *Sal*I and *Cla*I restriction sites of pLNCX2. Human intracellular *Notch-1* cDNAs were obtained by RT-PCR amplification of total RNA from human HepG2 cells, by using *Pfu* DNA Polymerase (Stratagene). The insert to be cloned into pNIC-2 was amplified with the following primers: 5′-CCG GAA GCT TGC ACC ATG GCA CGC AAG CGC CGG CGG CAG-3′ and 5′-GCG ACG TCG ACC TTG AAG GCC TCC GAA T-3′; and the insert to be cloned into pNIC-1 was amplified with the following primers: 5′-CCG GAA GCT TGC CAC CAT GGC ACG CAA GCG CCG GCG GCA G-3′ and 5′-GCG ACG TCG ACG AGG GGC ACG GAC GGA GAC T-3′. Both amplified fragments were cloned at the *Hin*dIII and *Sal*I restriction sites of the pLNCX2-*myc* vector. All vectors were confirmed by restriction analysis and sequencing. CBF1-Luc, NF-κB-Luc, AP-1-Luc, and m67-Luc plasmids, used to detect Notch-, NF-κB-, AP-1-, or STAT-dependent transcription activities, respectively, have been described previously (20, 21). All plasmids were purified using EndoFree Qiagen columns.

Cell transfection

Subconfluent Raw 264.7 cells were seeded in 24-well or 6-cm plates and transfected on the following day with 600 ng or 2.5 μg of plasmid DNAs by using Fugene 6 reagent (Roche), according to the manufacturer's recommendations (20). HEK 293T cells were transfected with Superfect reagent (Qiagen), following the manufacturer's recommendations. The amount of transfected DNA was kept constant by addition of appropriated amounts of the parental empty vectors. β-galactosidase or GFP expression vectors were used as internal controls for transfection efficiency. Luciferase and β-galactosidase activities were measured by using a commercial kit (Promega). Raw 264.7 stable transfectants were obtained by transfection of pLNCX2, or pLNCX2-Notch-IC plasmids with Fugene 6 reagent. Twenty-four hours later, cells were released by trypsin treatment and diluted 1/10 with fresh medium. After the cells were attached to the plates, transfected cells were selected by resistance to neomycin (350 μg/ml G-418 (Sigma-Aldrich) in complete culture medium). Cells resistant to the drug were pooled and screened for Neo and human Notch-IC expression. Selected cells were always maintained with medium containing G-418.

Analysis of gene expression by RT-PCR

Total RNA (2–4 $\times$ 10^6 cells) was extracted following the TRIzol method (Sigma-Aldrich) and treated with DNase to eliminate any potential DNA contamination. For RT-PCR, cDNA was generated from 2 μg of total RNA by using the cDNA synthesis kit RevertAid HMinus First Strand (Fermentas). Semiquantitative RT-PCR was performed as described previously (22). PCR assays were run for 25–40 cycles, depending on the gene to be studied, to obtain a PCR yield proportional to the amount of initial cDNA template. Amplification of the housekeeping gene P0 was used as a quality and loading control (23). The forward and reverse primers, respectively, used for PCR amplification are as follows: Notch-1 (5′-CTG TGT GGA TGA GGG AGA TAA-3′ and 5′-GGC ATA GAC AGC GGT AGA AA-3′); Notch-2 (5′-CAG CCG GTC TCC GTG TAA AAA CAA AG-3′ and 5′-GCG AAG AGT GGA GGT GCA GTT G-3′); Notch-3 (5′-ACA CTG GGA GTT CTC TGT-3′ and 5′-GTC TGC TGG CAT GGG ATA-3′); Notch-4 (5′-CCT CCA GCC TCC AGC CAG TG-3′ and 5′-TGT TTG TCC AGT TCG GGT GTT TTG-3′); Jagged-1 (5′-CCT CCA GCC TCC AGC CAG TG-3′ and 5′-TGT TTG TCC AGT TCG GGT GTT TTG-3′); Jagged-2 (5′-AAG GAC ATA CTC TAC CAG TGC-3′ and 5′-ACG TCC TGG TAC TTC TGA CG-3′); Hes-1 (5′-CGA AAA TGC CAG CTG ATA TAA TGG-3′ and 5′-GCA GTG GCC TGA GGC TCT CAG TTC-3′); Hes-5 (5′-GAG AAA AAC CGA CTG CGG AAG-3′ and 5′-TGT AGT CCT GGT GCA GGC TCT T-3′); Delta-1 (5′-CCA CGG AAG CTT AGC GGT ACC ATG GGC CGT CGG AGC-3′ and 5′-GCC GCG TCG ACA TCT TAC ACC TCA CTC GCT ATA ACA-3′); Presenilin-1 (5′-TGG TGT GGT CGG GAT GAT TGC C-3′ and 5′-GTC TCC TCC TCG GGC TTG CTC T-3′); Kuzbanian (5′-AGG AGC CCG GGC ACA TCC AGA G-3′ and 5′-AGG AGC CCG GGC ACA TCC AGA G-3′); Lunatic Fringe (5′-GGA TCC ACC GCC CGG GGT CGC T-3′ and 5′-GAG GGG TAC ACC CAG CAG AGC C-3′); Manic Fringe (5′-GGC CGC CCA GCT TCC GGA GCA GG-3′ and 5′-GGC CAG CTG AGC AGC GCC AGG A-3′); Radical Fringe (5′-CAG CAG AGC GCG TTC GGC TGC C-3′ and 5′-GGA CCT GTG GCT GGG CTG GGA A-3′); and P0 (5′-GCA CTT TCG CTT TCT GGA GGG TGT C-3′ and 5′-TGA CTT GGT TGC TTT GGC GGG ATT AG-3′).

Quantitative PCR was performed with SYBR Green PCR mix from Applied Biosystems. Quantification of gene expression was performed using P0 expression as a control. The forward and reverse primers used, respectively, are as follows: P0 (5′-AAG CGC GTC CTG GCA TTG TCT-3′ and 5′-CCG CAG GGG CAG CAG TGG T-3′); Notch-1 (5′-GCT

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GAG CAT GTA CCC GAG C-3′ and 5′-ATG ACG CTT GAA GAC CAC GTT-3′); and Jagged-1 (5′-AGA AGT CAG AGT TCA GAG GCG TCC-3′ and 5′-AGT AGA AGG CTG TCA CCA AGC AAC-3′).

Preparation of cell extracts and Western blot analysis

Adherent macrophages (1–3 × 10^6 cells) were washed twice with ice-cold PBS, scraped off the dishes, and collected by centrifugation. Cell pellets were homogenized with 200 μl of lysis buffer (20 mM Tris-HCl (pH 7.8), 0.4 M NaCl, 5 mM $MgCl_2$, 10 mM KCl, 0.5 mM EGTA, 1 mM PMSF, 0.5 mM DTT, 10 μM leupeptin, 10 mM sodium fluoride, and 1 mM sodium vanadate). After 10 min at 4°C, Nonidet P-40 was added to reach a 0.5% final concentration. The tubes were gently vortexed for 15 s. Nuclei were collected by centrifugation at 8,000 × *g* for 15 min, and the supernatants were stored at −80°C (cytosolic extracts). To obtain nuclear protein extracts, the pellets were resuspended in 50 μl of lysis buffer supplemented with 20% glycerol and 0.4 M KCl, and gently shaken for 30 min at 4°C, followed by centrifugation at 13,000 × *g* for 15 min. To obtain total extracts, cell pellets were homogenized with 200 μl of lysis buffer supplemented with 1% Nonidet P-40, and then gently shaken for 30 min at 4°C. Protein concentration of the extracts was determined by using the Bio-Rad detergent-compatible protein reagent. Depending on the experiment, a volume containing from 80 to 120 μg of total cell protein extract from each sample was boiled in denaturing buffer (250 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 2% 2-ME) and subjected to 10% polyacrylamide SDS-PAGE. The gels were transferred to Hybond-C extra (Amersham Biosciences) nitrocellulose membranes, which were then processed according to the recommendations provided by the Ab suppliers. Several Abs were used for the Western blot assays: anti-Notch-1 (C-20) intracellular region; anti-Notch 1 (H-131) extracellular region; anti-Notch 2 (M-20); anti-Jagged 1 (H-66); anti-Jagged 2 (H-143); anti-IRF-1 (M-20); anti-suppressor of cytokine signaling (SOCS)-1 (H-93); anti-NOS 2 (H-174); anti-Stat1 p84/p91 (C-136); anti-phospho-Tyr^{701}-Stat-1; anti-Stat-3 (F-2); anti-phospho-Tyr-Stat 3 (B-7); anti-Myc (9E10); anti-β-actin; anti-GFP; and anti-Hes1 (H-20) were all purchased from Santa Cruz Biotechnology. Anti-phospho-Ser^{727}-STAT-1 was acquired from Sigma-Aldrich. Proteins were detected by the ECL technique (Amersham Pharmacia Biotech). In each assay, several film exposure times were used to avoid film saturation.

EMSAs

EMSAs were performed as described previously (19). Briefly, binding reactions were prepared with 5–20 μg of nuclear extracts and 50,000 cpm ^{32}P-labeled probe in the presence of 2 μg of poly(dI-dC) in a final volume of 15 μl of 1× binding buffer (10 mM HEPES, (pH 7.9), 100 mM KCl, 5 mM MgCl, 0.12 mM EDTA, and 1 mM DTT). After 15 min of incubation at 4°C, the DNA-protein complexes were separated on native 6% polyacrylamide gels in 0.5% Tris-borate-EDTA buffer. A double-stranded oligonucleotide probe corresponding to the high-affinity SIE m67 site (5′-GTC GAC ATT TCC CGT AAA TCG-3′) was 5′-end labeled using T4 polynucleotide kinase (Fermentas) and used as described previously (21).

Determination of NO synthesis

NO release was spectrophotometrically determined by measuring the accumulation of nitrite in the medium 24 h after cell activation, as described previously (24). Nitrite concentrations were calculated from a standard curve derived from the reaction of $NaNO_2$ in the assay. Results were expressed as the amount of nitrite released per milligram of cell protein.

Confocal microscopy

Raw 264.7 cells were grown on coverslips and activated with both LPS and IFN-γ for 24 h. After washing the covers twice with ice-cold PBS, cells were fixed for 2 min with methanol at −20°C, blocked with 3% BSA for 30 min at room temperature, and incubated for 1 h with specific primary Abs. Biotin anti-mouse CD54 (ICAM-1) and biotin anti-mouse I-A^k were purchased from BD Pharmingen. Anti-Notch 1 extracellular region (H-131), anti-Jagged-1, and anti-NOS 2 (H-174) were obtained as indicated above. Ab dilutions used ranged from 1/500 to 1/1000. After washing the coverslips twice with PBS, the cells were incubated for 1 h with secondary Abs (diluted 1/1000) marked with Alexa 488 (BD Pharmingen). The cells were visualized using a MRC-1024 confocal microscope (Bio-Rad).

Flow cytometric analysis

Raw 264.7 cells were stimulated for 24 h with LPS and/or IFN-γ, washed twice with PBS, collected, incubated with biotin anti-mouse-CD54 (ICAM-1, 1/500 dilution) or biotin anti-mouse I-A^k (1/200 dilution) for 1 h at 4°C, washed twice with PBS, and stained with streptavidin-PE for 30 min. Following incubations with the Abs, cells were washed and analyzed with a FACSVantage (BD Biosciences). Data were processed using the CellQuest program (BD Biosciences). Abs were purchased from BD Pharmingen.

Data analysis

The number of experiments performed and analyzed is indicated in the corresponding figure legends. Statistical differences ($p < 0.05$) between mean values were determined by one-way analysis of the variance, followed by Student's *t* test. In experiments using x-ray films (Hyperfilm), different exposure times were used to avoid signal saturation.

Results

Macrophages express different molecules involved in the Notch signaling pathway

We analyzed by RT-PCR the expression patterns of the Notch receptor and ligand mRNAs in macrophages. We found that mouse peritoneal macrophages and two monocyte cell lines, Raw 264.7 and J774 cells, express *Notch-1*, *-2*, and *-4*, as well as the Notch ligands *Jagged-1* and *-2* (Fig. 1*A*). We could not evidence expression of members belonging to the Delta family of Notch-ligand proteins (data not shown). We observed only small differences among the patterns of expression of peritoneal macrophages and the cell lines. In general, *Notch-2* and *Notch-4* were more intensely expressed than *Notch-1* in macrophages, which is in agreement with what has been observed previously (22, 25, 26). Expression of *Jagged-2* was slightly higher than that of *Jagged-1* in cell lines and cultured macrophages, but the contrary was observed in recently isolated peritoneal macrophages (data not shown), an observation also in agreement with previously reported data (22).

We analyzed also the expression of genes implicated in Notch receptor processing, such as Kuzbanian, which has been related with the generation of heterodimeric mature receptor forms (8, 11), and Presenilin, one of the best candidate enzymes to catalyze the internal membrane cleavage essential for Notch signaling (27, 28). As shown in Fig. 1*B*, both genes were expressed at similar levels to those found in mouse brain or 3T3-L1 fibroblasts.

We also studied the expression in macrophages of genes implicated in posttranslational modification of Notch receptors, such as *Radical*, *Manic*, and *Lunatic Fringe* glycosyltransferases (29, 30). The three genes were expressed in these cells, with *Lunatic Fringe*

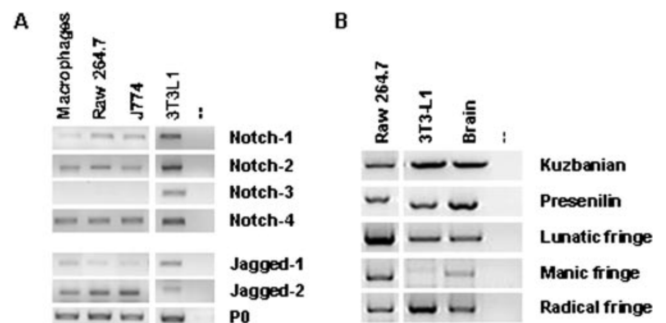

FIGURE 1. Expression analysis by RT-PCR of different Notch family members and other molecules implicated in Notch processing in macrophages. *A*, Expression pattern of Notch receptors and ligands in macrophages and monocyte cell lines Raw 264.7 and J774. *B*, Expression of molecules implicated in Notch processing (Kuzbanian and Presenilin-1) and glycosylation (Radical, Manic, and Lunatic Fringe). Total RNA was extracted from each cell type, and cDNA was prepared and diluted. The amount of template cDNA was standardized with primers specific for the riboprotein P0. PCR products were analyzed by agarose gel electrophoresis, followed by ethidium bromide staining. 3T3-L1 cells and brain were used as positive expression controls. Results are representative of three individual experiments.

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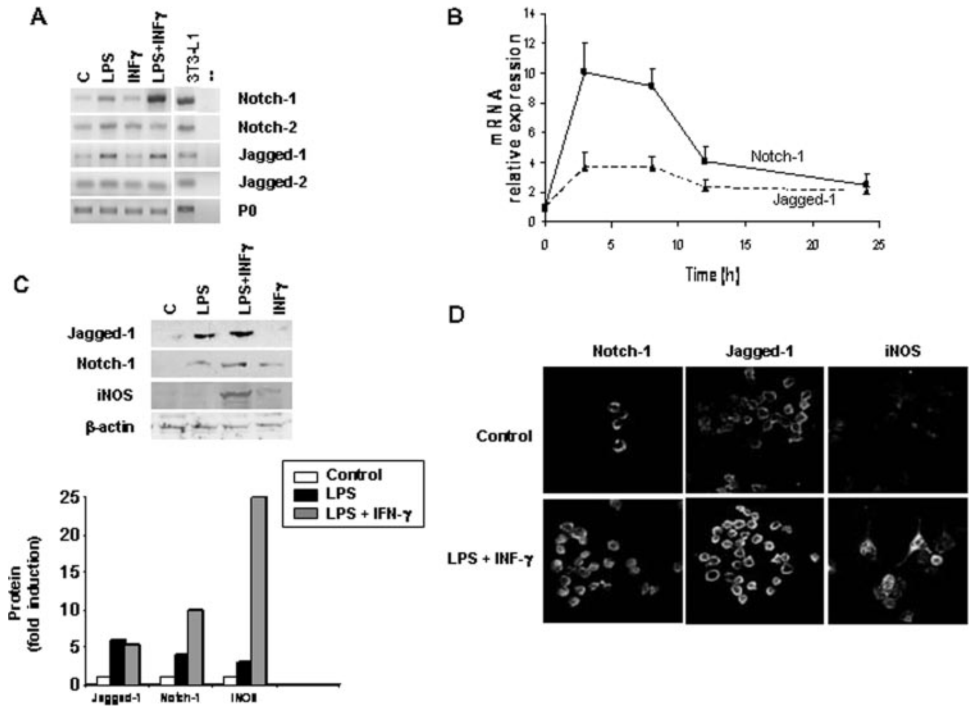

FIGURE 2. Different members of the Notch family are up-regulated after macrophage activation with LPS and IFN-γ. Raw 264.7 cells were triggered with LPS (50 ng/ml) and/or IFN-γ (20 U/ml) for different times. *A*, mRNA expression was evaluated at 6 h by semiquantitative RT-PCR analysis. *B*, Time-course analyses for *Notch-1* and *Jagged-1* expression were also performed using real-time quantitative RT-PCR (mean + SD; $n = 4$). *C*, The amount of Notch-1, Jagged-1, and iNOS proteins was evaluated by Western blot in 120 μg of total cell protein extracts 18 h after macrophage activation. Results show the mean of three independent experiments and a representative blot. *D*, Immunolocalization of Notch-1, Jagged-1, and iNOS proteins in macrophages cultured on glass slides (see *Materials and Methods*) activated for 18 h. Cells were fixed and the protein detected by binding of specific Abs that were revealed with Alexa 488-labeled anti-rabbit or anti-goat Ig.

being the most represented (Fig. 1*B*). These results indicate that different genes, implicated in Notch processing and modification, are expressed in macrophages.

LPS and IFN-γ up-regulate Notch-1 *and* Jagged-1 *expression in macrophages*

As shown in Fig. 2, *A* and *B*, the treatment of macrophages with bacterial LPS (100 ng/ml) increased the level of *Notch-1* (~3-fold increase) and *Jagged-1* (~4-fold increase) mRNAs, but not those of *Notch-2*, *Jagged-2*, or *Delta* mRNAs (data not shown). Simultaneous treatment of macrophages with LPS and IFN-γ (10 U/ml) induced an increase of ~10-fold in *Notch-1* expression. However, *Jagged-1* induction remained similar to that obtained with LPS treatment alone. As a control for macrophage activation by LPS and IFN-γ, we determined NOS expression and NO production (data not shown).

Notch-1 and *Jagged-1* up-regulation was a rapid process, which reached maximal mRNA expression levels ~2 h after macrophage stimulation (Fig. 2*B*). This expression pattern is similar to that shown by other genes implicated in macrophage activation, such as NOS or cyclooxygenase-2 (31). Notch-1 and Jagged-1 protein up-regulation after macrophage activation could be evidenced also by Western blot or by immunocytochemistry assays (Fig. 2, *C* and *D*). Confocal microscopy studies showed that Notch-1 and Jagged-1 proteins were mainly present at the plasma membrane, although some protein was intracellular, probably located at the endoplasmic reticulum. NOS, used as a positive control for macrophage activation, was mainly detected in the cytoplasm (Fig. 2*D*).

To study the effects of proinflammatory cytokines in *Notch-1* and *Jagged-1* gene expression, cultured peritoneal macrophages or Raw 264.7 cells were stimulated with TNF-α, IL-1, or IL-6, and Notch-1 and Jagged-1 protein levels were determined by Western blot. Maximal Notch-1 induction was obtained after stimulation with both LPS and IFN-γ. Much smaller induction was observed after treatment with IL-1 or IL-6, alone, or in combination with IFN-γ (data not shown). Treatment with TNF-α did not induce Notch-1 expression, although it was able to induce NOS or IκB-α expression (data not shown). Maximal expression of Jagged-1 (~5-fold increase) was obtained by treatment with LPS or IL-6 alone. As observed for LPS, IFN-γ did

Table I. *Effect of different protein kinase inhibitors in the up-regulation of Notch-1 and Jagged-1 mRNA and protein after macrophage activation by LPS and IFN-γ*[a]

	Jagged-1		Notch-1	
	mRNA	Protein	mRNA	Protein
	100	100	100	100
PD98059 (20 μM)	80 + 15	102 + 12	65 + 15	93 + 11
SB203580 (10 μM)	20 + 7*	55 + 8*	5 + 3*	50 + 7*
Wortmanin (10 μM)	110 + 20	114 + 17	97 + 16	120 + 13

LPS + INFγ
\- \- + SB203580
Notch-1
Jagged-1
β-actin

[a] Cells were pretreated with the kinase inhibitors and after 1 h, stimulated with 50 ng/ml LPS and 20 U/ml IFNγ. mRNA and protein levels were evaluated after 6 and 18 h, respectively. The image shows a representative Western blot. The table shows the mean + SD of four experiments.

*, $p < 0.05$ relative to the control conditions in the absence of inhibitors.

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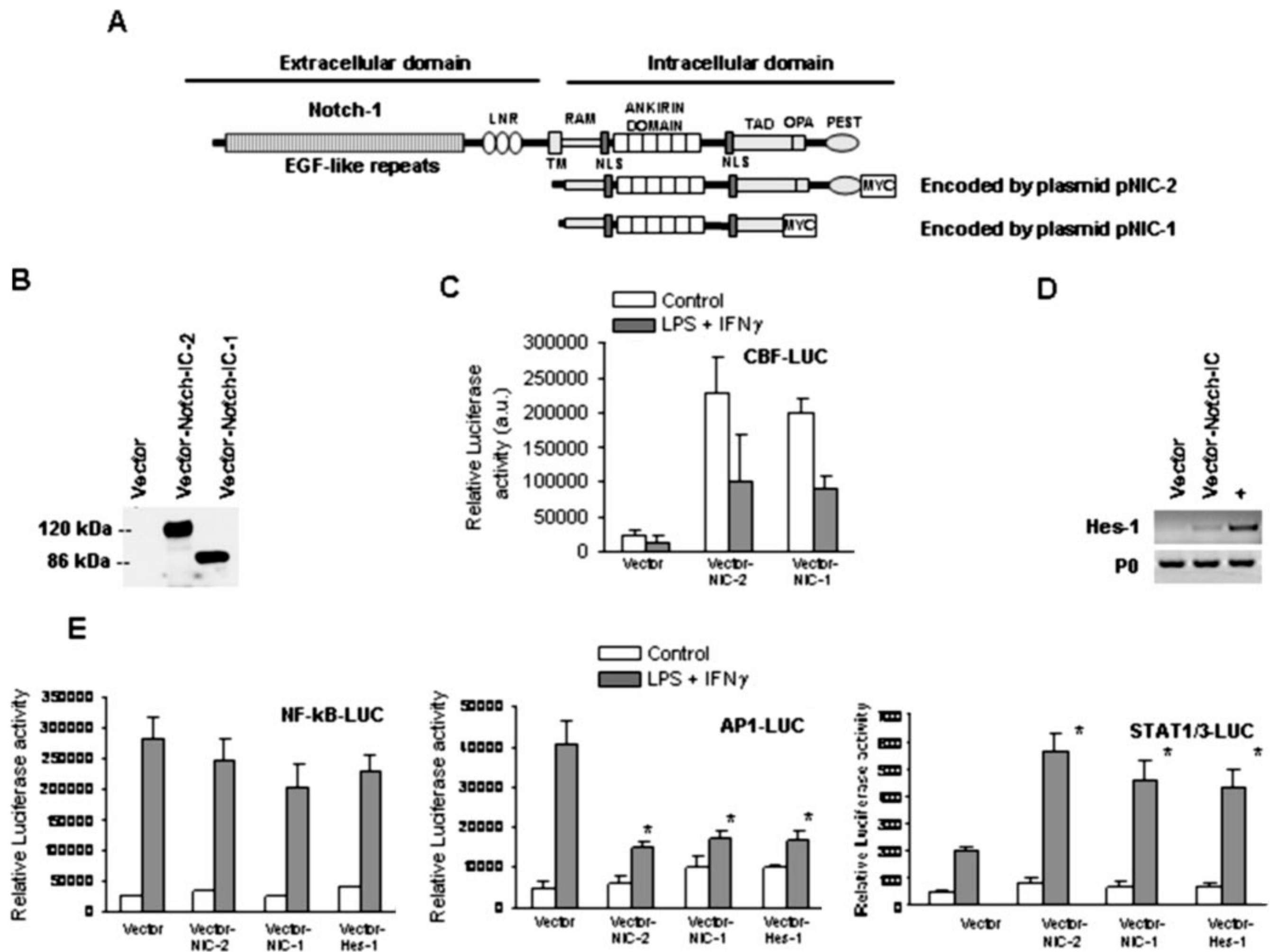

FIGURE 3. Constitutively active Notch-IC modifies the activity of AP-1 and STAT1/3 reporter genes in activated macrophages. Two expression vectors encoding for active forms of human Notch-1 were constructed. One of them (pNIC-2, aa 1759–2556) contains the RAM domain, the ankyrin repeats, the glutamine-rich region, and the PEST domain linked to a *myc* epitope. The second one (pNIC-1, aa 1759–2232) lacks the glutamine-rich region and the PEST domain. *A*, The structure of the entire Notch-1 receptor and the two truncated IC proteins used in this work. *B*, Expression of active Notch-IC proteins was assessed in HEK 293T cells by Western blot after transient transfection. *C*, Activity of truncated proteins was checked by transient transfection with a CBF1-dependent reporter gene (CBF-Luc) in Raw 264.7 cells stimulated (▨) or not (□) with LPS (50 ng/ml) and IFN-γ (50 U/ml). *D*, Vectors encoding for the intracellular active Notch-1 proteins were transiently transfected with a GFP expression vector into Raw 264.7 cells. GFP^+ cells were separated in a sorter flow cytometer, and the level of *Hes-1* mRNAs was analyzed by RT-PCR. 3T3-L1 cells were used as a positive control for *Hes-1* expression. *E*, Intracellularly active *Notch-1* expression vectors and a *Hes-1* expression vector were transiently cotransfected in Raw 264.7 cells with NF-κB-Luc, AP-1-Luc, or m67-Luc plasmids, and a β-galactosidase expression vector. Luciferase activity was measured 24 h after activation with LPS and IFN-γ, and results were normalized by using the data from β-galactosidase activity. Results represent the mean + SD of four experiments. The * indicates a statistically significant difference in reporter gene activity ($p < 0.05$), relative to that of the respective control in the absence of Notch-1 proteins.

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not increase further Jagged-1 induction by IL-6 (data not shown). These results indicate that the intracellular signals implicated in Notch-1 and Jagged-1 induction are not identical, although some of them are probably shared.

We did not observe changes in the expression of the different genes implicated in Notch processing, such as *Kuzbanian* and *Presenilin*, or those implicated in Notch glycosylation, such as *Manic*, *Lunatic*, or *Radical Fringe*, in macrophages activated by proinflammatory signals.

p38 MAPK is implicated in Notch-1 *and* Jagged-1 *induction by proinflammatory signals*

We used different protein kinase inhibitors to characterize the signaling pathways implicated in the induction of *Notch-1* and *Jagged-1* expression following macrophage activation. As shown in Table I, only SB203580, a p38 MAPK inhibitor, but neither PD98059, a MEK-1 inhibitor upstream of p42/44 MAPKs, nor Wortmanin, a phosphoinositide 3-OH kinase inhibitor, blocked *Notch-1* and *Jagged-1* induction. In addition, inhibition of prostaglandin synthesis by the cyclooxygenase inhibitor 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)phenyl-2(5*H*)-furan one did not modify *Notch-1* and *Jagged-1* expression, revealing that prostaglandins are not implicated in the regulation of these genes after macrophage activation (data not shown).

Constitutively active Notch-IC proteins modify the activity of AP-1- and STAT1/3-dependent reporter genes in activated macrophages

What is the biological meaning of *Notch-1* up-regulation in the process of macrophage activation? In the last years, some controversial data have been published describing interactions between the Notch-IC proteins and different transcription factors, some of

which are involved in macrophage activation, such as NF-κB and AP-1 (3). To study whether these interactions could be happening in macrophages, we decided to analyze the effects of Notch signaling in NF-κB, AP-1, and STAT-1 activation after macrophage triggering with LPS and IFN-γ. Because Notch activation implies its transmembrane proteolysis and the release and translocation to the nucleus of its intracellular active region to activate expression of target genes, we studied the relationship between typical inflammatory signaling pathways and Notch-1 activation in Raw 264.7 cells, using the expression vectors pNIC-1 and pNIC-2 (see *Materials and Methods*). pNIC-1 and pNIC-2 encode for active forms of human Notch-1 intracellular protein, from aas 1759 to 2237 and 1759 to 2556, respectively. Unlike pNIC-2, pNIC-1 lacks the glutamine-rich region and the PEST domain (Fig. 3*A*). Expression of these active Notch-1 polypeptides was assessed by Western blot after transient transfection of HEK 293T cells (Fig. 3*B*).

Expression of both intracellular forms of human Notch-1 proteins in Raw 264.7 cells induced a strong activation of a CBF1-dependent reporter gene (CBF1-Luc) in control or activated macrophages, demonstrating that these Notch-1-truncated proteins were active, and that the glutamine-rich region and the PEST domain were not essential for Notch-1-induced CBF1 activation in these cells (Fig. 3*C*). CBF1 activation was greater in control than in activated macrophages. This could be the result of a competition for coactivators between Notch and NF-κB, as it has been described in 32D cells activated with TNF-α (32, 33). Interaction of Notch-IC with the CBF1 complex in the nucleus leads to dissociation of corepressors from the CBF1 protein enabling it to up-regulate downstream Notch-1 target genes, such as *Hes-1* (10–12). As shown in Fig. 3*D*, transient expression of the active pNIC-1 protein induced *Hes-1* expression in activated macrophages. Fig. 3*E* shows that, after macrophage triggering with LPS and IFN-γ, expression of both Notch-IC proteins did not modify significantly the activity of a NF-κB-dependent reporter gene (NF-κB-Luc). However, both proteins drastically inhibited the activity of an AP-1-dependent reporter gene (AP-1-Luc) and strongly increased the activity of a STAT1/3-dependent reporter gene (m67-Luc). It is noteworthy that despite Notch-IC activity being lower in activated macrophages than in control cells, it is nonetheless sufficient to modify the activity of AP-1 and STAT1/3 proteins.

Because Notch-1 functional requirements for CBF1-, AP-1-, and STAT-dependent transcription seem similar, and transcription of *Hes-1* is dependent upon CBF1 activation, we examined whether the protein Hes-1 was also able to modulate AP-1- and STAT-dependent reporter gene activity. As shown in Fig. 3*E*, the expression of *Hes-1* did not modify NF-κB-reporter gene activity, but inhibited AP1 activity and increased STAT-reporter gene expression, similarly to that observed with Notch-IC proteins. These results suggest that the protein Hes-1 mediates the cross-talk between Notch and AP-1/STAT signaling pathways after macrophage triggering with LPS and IFN-γ.

The specific sequences used in the STAT-reporter construct (m67) have been previously characterized as binding mainly STAT1 homodimers (34). However, binding of STAT3 homodimers or STAT1-STAT3 heterodimers cannot be completely ruled out. Related to this data, Kamakura et al. (35) have recently published that STAT3 mediates the cross-talk between Notch and JAK-STAT signaling in neuroepithelial cells. Macrophage activation triggered by IFN-γ induces STAT1 activation, which is the predominant and essential transcription factor in the IFN-γ transduction pathway; however, STAT3 is also weakly activated (36). For that reason, we decided to study the effects of Notch-IC and Hes-1 expression in Raw 264.7 cells overexpressing STAT1 or STAT3 proteins. As shown in Fig. 4, overexpression of both STAT proteins increased m67 reporter activity in the

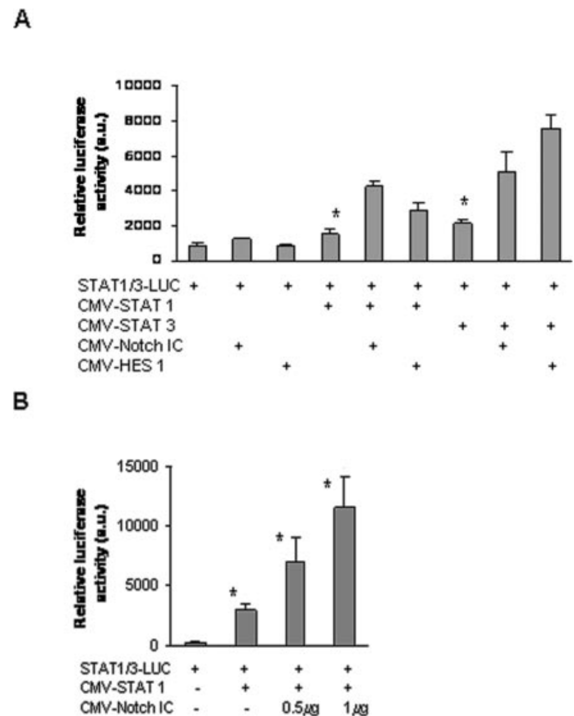

FIGURE 4. Intracellular active Notch-1 and Hes-1 proteins increase STAT1/3-dependent transcription activity. *A*, Raw 264.7 cells were co-transfected with 1 μg of m67-Luc STAT1/3 reporter gene vector and 500 ng of expression vectors encoding for intracellular active Notch-1 (CMV-Notch), Hes-1 (CMV-Hes-1), STAT1 (CMV-STAT1), or STAT3 (CMV-STAT3) expression vectors, as indicated by +. After 24 h, cells were harvested, and luciferase activity was determined and normalized to β-galactosidase activity. Data correspond to the mean + SD of four determinations. *B*, Relative luciferase activity from Raw 264.7 cells transfected with m67-Luc reporter gene vector in the presence or absence of STAT1 expression vector and increasing amounts of CMV-Notch-IC. Results are expressed as the mean + SD of three independent experiments. The * indicates a statistically significant difference in reporter gene activity in presence of STAT1 or STAT3 expression vectors ($p < 0.05$), relative to that of the respective control in the absence of these expression vectors.

absence of macrophage activation, and either Notch-IC or Hes-1 expression further augmented this activity. Notch-IC was stronger than Hes-1 in potentiating STAT1 activity. Nevertheless, Hes-1 potentiation of m67-LUC reporter activity was stronger in the presence of STAT3 than in presence of STAT1. In addition, Hes-1 potentiation of STAT3 activity was stronger than the potentiation caused by Notch-IC. These results suggest that Hes-1 activation may account for all Notch-IC effects on STAT3 activity but that other mediators besides Hes-1 are involved in Notch-IC potentiation of m67-Luc activity in the presence of STAT1.

Raw 264.7 stable transfectants constitutively expressing Notch-IC show increased responses to IFN-γ

To confirm the results previously obtained, we generated Raw 264.7 stable transfectants constitutively expressing the Notch-1 intracellular protein encoded by pNIC-1 (Raw-Notch-IC cells). Unfortunately, we were unsuccessful in obtaining stable transfectants with the larger protein encoded by pNIC-2. As a control, we used Raw 264.7 cells stably transfected with the empty cloning vector (Raw-Vector cells). As shown in Fig. 5, *A* and *B*, pNIC-1 transfectants expressed human Notch-IC mRNA and protein, although the level of expression was low as compared with that obtained by transient transfection (Fig. 3*B*). We also detected increased *Hes-1* expression in Raw-Notch-IC transfectants, as compared with the controls (Fig. 5*C*).

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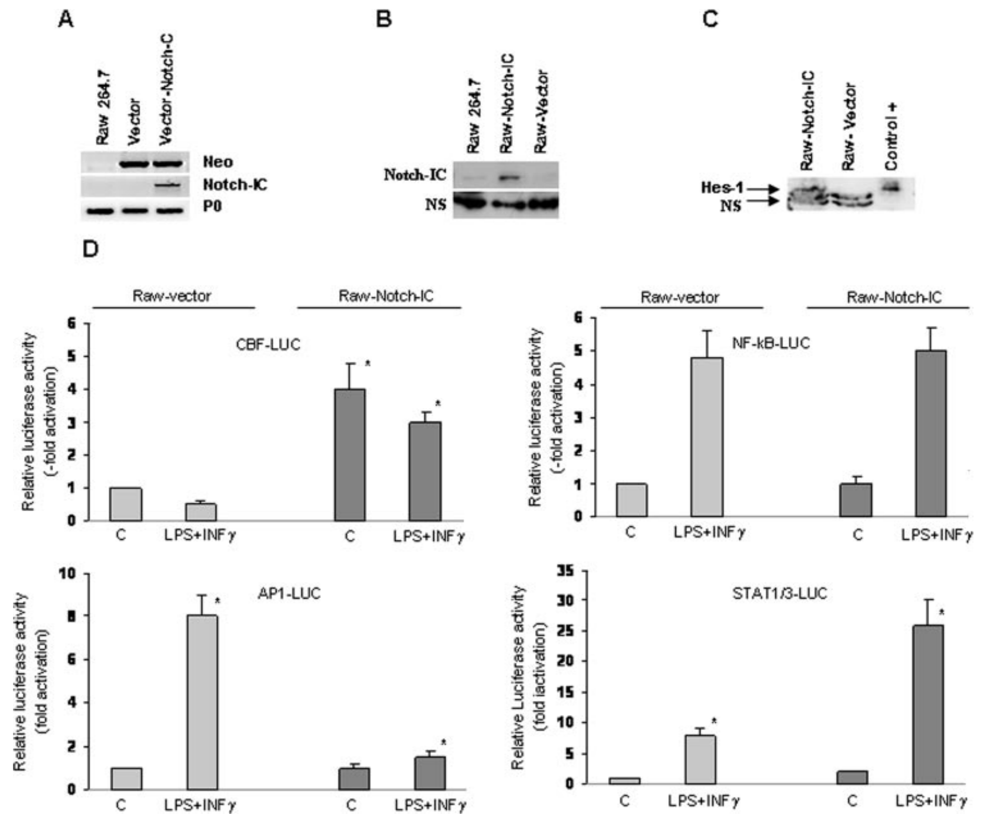

FIGURE 5. Raw 264.7 stable transfectants constitutively expressing Notch-IC present increased CBF1 activity and modified AP-1- and STAT1/3-dependent reporter gene expression after activation with LPS and IFN-γ. Raw 264.7 pNIC1 stable transfectants express human Notch mRNA (*A*) and protein (*B*) and show increased expression of the protein Hes-1 (*C*). *D*, Stable Raw 264.7-transfected cells, Raw-vector (empty vector), or Raw-Notch-IC (pNIC1 expression vector) were transiently transfected with CBF-Luc, NF-κB-Luc, AP-1-Luc, or m67-Luc plasmids, and a β-galactosidase expression vector. Luciferase activity in control and LPS plus IFN-γ-activated cells was measured after 24 h and normalized with β-galactosidase activity. Results are expressed as the mean + SD of three individual experiments. The * indicates a statistically significant difference in reporter gene activity ($p < 0.05$), relative to that of the respective control in the absence of Notch-1 proteins.

CBF1-, NF-κB-, AP-1-, or STAT-dependent reporter genes were transiently transfected in Raw-Vector or Raw-Notch-IC cells. As expected, CBF1-dependent reporter activity increased (~4- to 6-fold) in pNIC-1 stable transfectants (Fig. 5*D*). In contrast, NF-κB-dependent reporter activity was not significantly modified by Notch-IC expression. However, AP1- and STAT-dependent reporter activities were significantly affected. Whereas AP1 activity decreased, STAT activity increased in cells expressing the Notch-1 intracellular domain. These results confirm the results obtained previously, and further suggest that endogenous activation of the Notch signaling pathway can modulate gene expression triggered by macrophage activation.

Because STAT activation is a key event in the IFN-γ signaling pathway, we analyzed the expression of well-characterized IFN target genes in our stable transfectants. We studied the expression of both IRF-1, a transcription factor involved in type I and II IFNs signal cascades, and SOCS-1, a protein involved in the feedback inhibition of IFNs signaling pathways. Both genes present GAS elements in their promoter regions, and studies with $STAT1^{-/-}$ mice showed that their expression is mediated by STAT1 and not by STAT3 (6, 7). As shown in Fig. 6*A*, both proteins were more intensely induced by treatment with LPS and IFN-γ in Raw-Notch-IC cells than in control cells. We also analyzed two surface proteins involved in macrophage function that are up-regulated after IFN-γ treatment: the adhesion protein ICAM-1 and class II MHC molecules, implicated in Ag presentation (5). Fig. 6, *B* and *C*, show the mean of three experiments in which surface expression of these proteins was evaluated by flow cytometric analysis. Induction of both ICAM-1 and class II MHC molecules was significantly higher in transfectants expressing the Notch-IC protein than in control cells.

Increased STAT1 phosphorylation, nuclear translocation, or DNA binding are not responsible for the elevated IFN-γ response in Raw-Notch-IC stable transfectants

Biochemical and genetic studies have shown that STAT1 plays a critical role in IFN-γ-dependent signaling. STAT1 and, to a lesser extent, STAT3 are rapidly Tyr-phosphorylated and activated after IFN-γ triggering. Recently, it has been described that the Notch effectors Hes-1 and Hes-5 facilitate STAT3 phosphorylation and activation (35). We analyzed STAT1 and STAT3 phosphorylation in our Raw 264.7 stable transfectants after their activation with LPS and IFN-γ. As shown in Fig. 7*A*, we found no differences in STAT1, and only a small increase in STAT3 Tyr phosphorylation in these transfectants; however, an increase in the levels of IRF-1 was detected. We analyzed also STAT1 nuclear translocation using cytosolic and nuclear extracts; no differences in STAT1 translocation were detected (data not shown). Binding of STAT1 to high-affinity SIE m67 site was similar in extracts from control cells or from Notch-IC Raw 264.7 stable transfectants (Fig. 7*B*).

Because the levels of Notch-IC and Hes-1 proteins were lower in stable than in transient transfectants, we decided to study the Notch-1 and Hes-1 implication in STAT phosphorylation by using transient transfectants of HEK 293T cells, which generally show abundant mRNA and protein expression levels of the transfected genes. HEK 293T cells were cotransfected with GFP, STAT1, Notch-IC, or Hes-1, or cotransfected with GFP, STAT3, Notch-IC, or Hes-1 expression vectors, and the efficiency of transfection was determined by examining GFP expression by fluorescence microscopy. As shown in Fig. 7*C* (*upper panel*), we observed increased STAT3 phosphorylation in *Hes-1* and, to a lesser extent, in Notch-IC HEK 293T STAT3 cotransfectants (probably due to the much lesser expression of *Hes-1*, undetectable in our experiments), but STAT1 phosphorylation levels remained the same in STAT1 cotransfectants. Nevertheless, overexpression of STAT1 and Notch-IC or Hes-1 increased *IRF-1* expression to levels similar to those obtained after treatment with IFN-γ (Fig. 7*C*, *lower panel*). STAT1 overexpression increased the basal level of STAT1 Tyr phosphorylation (Fig. 7*C*, *right lower panel*), probably due to the basal activity of JAKs (21). This activation resulted in a weak increase of *IRF-1* expression that was further promoted by Notch-IC and *Hes-1*. In contrast, STAT3 overexpression did not

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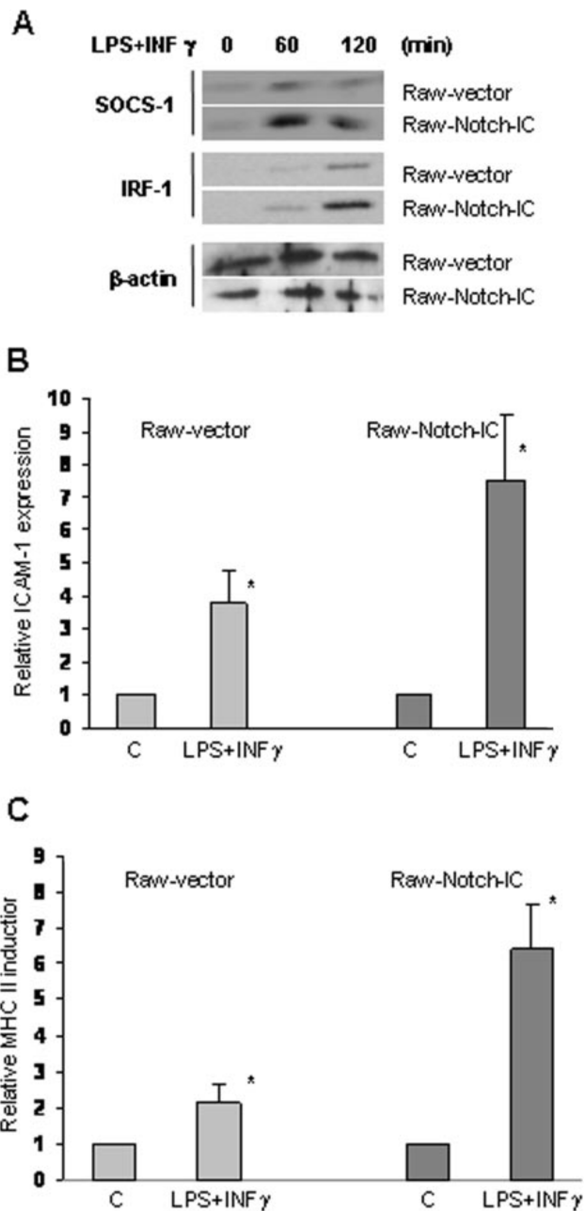

FIGURE 6. Raw 264.7 stable Notch-IC transfectants present increased response to IFN-γ. *A*, Raw 264.7 stable Notch-IC transfectants were stimulated with LPS (50 ng/ml) and IFN-γ (50 U/ml) for the indicated times. Total cell extracts were obtained after 1 or 2 h of incubation, and 80 μg of proteins were analyzed by Western blot to determine SOCS-1 and IRF-1 expression. Expression of β-actin was used as a loading control. The blot shown is representative of three individual experiments. ICAM-1 (*B*) and type II MHC protein expression (*C*) was evaluated by flow cytometry 24 h after macrophage activation. Cells were first labeled with biotin anti-mouse CD54 (ICAM-1) or biotin anti-mouse I-A^k, followed by streptavidin-PE incubation. Results are expressed as the mean + SD of three experiments. The * indicates a statistically significant difference in protein expression ($p < 0.05$), relative to that of the respective control.

show the same pattern of *IRF-1* induction (data not shown). Multiple studies in the last years have shown that STAT1 phosphorylation at serine 727 is essential for maximal transcription of target genes (5, 37). For that reason, we studied whether Ser^{727} phosphorylation could be responsible for the increased transactivation capacity of STAT1 induced by Notch-IC in Raw 264.7 stable transfectants. We could not observe differences in STAT1 Ser^{727} phosphorylation between control transfectants and cells expressing Notch-IC (Fig. 7*D*).

All of these results suggest that Notch-IC and Hes-1 proteins potentiate the transactivation capacity of STAT1 without increasing its level of Tyr phosphorylation, nuclear translocation, or DNA binding. Our results also confirm that Notch-IC and Hes-1 increase STAT3 phosphorylation in transiently transfected HEK 293T cells and in the Raw 264.7 stable transfectants.

iNOS expression is inhibited in Raw-Notch-IC stable transfectants

We monitored *iNOS* expression and NO production in stable transfectants expressing or not expressing active Notch-IC proteins. Because STAT1 and IRF-1, together with NF-κB, are the major transcription factors implicated in *iNOS* gene expression (38), we expected to find an increase in *iNOS* expression. Nevertheless, we were surprised to find weaker *iNOS* expression and NO production in Raw-Notch-IC transfectants than in control Raw-Vector transfectants (Fig. 8, *A–C*). This lower *iNOS* expression resulted from diminished transcriptional activity of the *iNOS* promoter (Fig. 8*D*). Further studies are needed to dissect the mechanism by which Notch-IC appears to down-regulate *iNOS* expression, but our results suggest that macrophage activation, gene expression, and production of macrophage cytotoxic substances are modified by Notch-1 activation.

Discussion

In the last years, multiple lines of evidence have implicated Notch signaling in lymphoid (39, 40) and myeloid differentiation (16). Nevertheless, less attention has been focused on the role of Notch proteins in mature, fully differentiated cells, despite Notch receptors and ligands being often highly expressed by these cells (10). In this study, we show that macrophages express *Notch-1*, *-2*, and *-4*, as well as the ligands *Jagged-1* and *-2*. These results agree with those from different authors who have reported the expression of these genes and proteins by monocytes and macrophages (25, 26, 41). Our results indicate that macrophages also express different proteins implicated in Notch posttranslational modification, processing and signaling, such as Kuzbanian, Presenilin, and Fringe. We also show in this study that macrophage activation by LPS specifically increases the expression of *Notch-1* and *Jagged-1* (Fig. 2). Moreover, IFN-γ, the most potent activator of macrophages, synergizes with LPS to up-regulate the expression of *Notch-1*, but not that of *Jagged-1*, *Jagged-2*, or *Notch-2*. In addition, the expression patterns of *Notch-1*, *Jagged-1*, and *iNOS* genes during macrophage activation are similar (Fig. 2).

Bacterial lipopolysaccharides are recognized by the macrophage's Toll-4 membrane receptors. Signal transduction through Toll-4 receptors implicates the activation of multiple pathways, including NF-κB activation, p42/44 MAPKs, stress-activated protein kinases, JNK and p38 kinase, and phosphoinositide 3-OH kinase (3). Our results implicate the p38 signaling pathway in the induction of *Notch-1* and *Jagged-1* expression by LPS. Previous work has implicated NF-κB proteins in *Jagged-1* induction (42). Recently, Amsen et al. (43) have shown that LPS induces *Jagged-1* expression via a Myd88-independent pathway. Li et al. (44) found that *Notch-1* expression is dependent on NF-κB activity in pre-B cell lines. Very recently, Adler et al. (45) have shown that, in primary CD4$^+$ T cells, *Notch-1* expression is induced after specific peptide-Ag stimulation, for which NF-κB is also a key element (46, 47). *Notch-1* induction and activation have also been described after oncogenic Ras signaling through a p38-mediated pathway (48). Our results, together with those mentioned above, demonstrate that Notch proteins can be up-regulated in different immune cells, including macrophages, and that activation of p38 MAPK and NF-κB seems to be an essential element in this induction.

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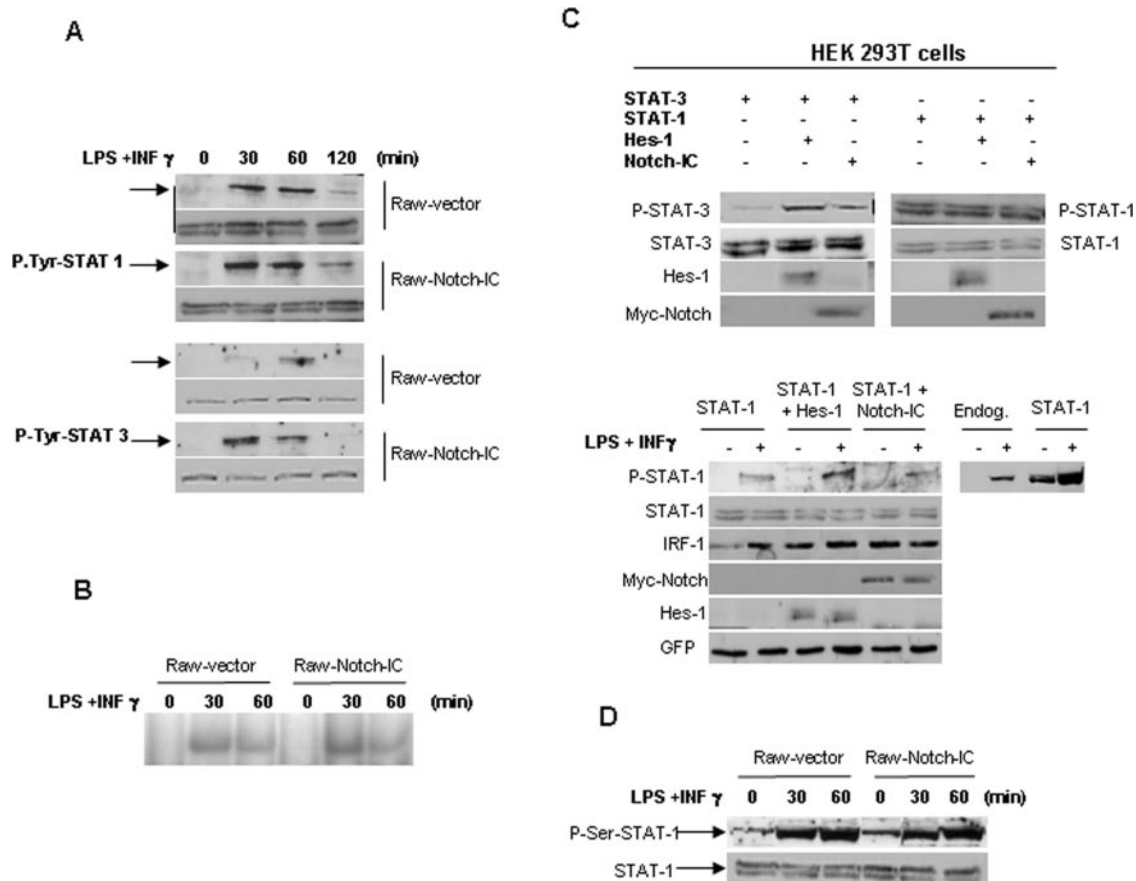

FIGURE 7. Increased STAT1 phosphorylation is not responsible for the elevated IFN-γ response in Raw-Notch-IC. *A*, Raw 264.7 stable transfectants were stimulated with LPS (50 ng/ml) and IFN-γ (50 U/ml) for the indicated times. After stimulation, total extracts were obtained, and 80 μg of proteins were analyzed by Western blot to determine STAT1/3 Tyr phosphorylation. Blots were also revealed with anti-STAT1 or anti-STAT3 Abs to evaluate gel loading. *B*, EMSA with the high-affinity SIE m67 probe and nuclear extracts prepared at different times after triggering macrophage activation. Results show a representative experiment of three. *C* (*upper panel*), Hes-1 and Notch-IC overexpression induces STAT3, but not STAT1 phosphorylation, in HEK 293T cells transiently transfected with CMV-STAT1 or CMV-STAT3. Transfection efficiency was assessed by determining GFP fluorescence 24 h after transfection, and total protein extracts were subjected to immunoblot analysis with Abs to phospho-Tyr^{701}-STAT1, phospho-Tyr^{705}-STAT-3, STAT1, STAT3, Hes-1, and *myc* (Notch-IC). *Lower panel*, Overexpression of STAT1 and Hes-1 or STAT1 and Notch-IC induced IRF-1 expression in HEK 293T cells. Cells were transiently transfected, and 24 h later, cells were stimulated or not with 50 U/ml human IFN-γ for 2 h. Then, total extracts were obtained and analyzed by Western blot. Endogenous human Hes-1 protein is not detected by this Ab, but its expression was assessed by RT-PCR (data not shown). On the *right panel*, an overexposed blot shows that overexpression of STAT1 is sufficient to increase basal STAT1 Tyr phosphorylation levels. *D*, STAT1 Ser phosphorylation was also evaluated in Raw 264.7 stable transfectants by using a specific anti-phospho-Ser^{727}-STAT1 Ab. In all of the cases, blots correspond to representative experiments of a minimum of three individual assays.

Given that high levels of Notch-1 receptors are present in activated macrophages after triggering with LPS and IFN-γ, we have investigated the potential role of Notch-1 signaling in macrophage biology. Truncated Notch molecules lacking the transmembrane and extracellular regions (Notch-IC) behave as constitutively active Notch receptors and have been used extensively to study the function of Notch in multiple systems (49–51). Using this strategy, we have shown that Notch-IC interferes with the activity of different transcription factors implicated in macrophage activation, such as AP-1 or STAT1/3, without significantly affecting others, such as NF-κB (Figs. 3*E* and 5). Different authors have explored the potential interaction between Notch and NF-κB signaling pathways. Our results agree with those reported by Bresnick and coworkers (52), who did not find any interaction between Notch and NF-κB signaling pathways. Nevertheless, Guan and coworkers (53, 54) have reported NF-κB inhibition by interaction of its p50 subunit with Notch-IC. In addition, Oakley et al. (55) have shown that the basal expression level of the NF-κB inhibitor, IκBα, is controlled by the mammalian transcriptional repressor RBP-J (CBF1) and its activator Notch1.

In any case, because Notch signaling may be required for basal synthesis of NF-κB components (51, 56), interplay between Notch and NF-κB seems to be a complex process, probably depending on several factors, including cell type. An inhibitory cross-talk between Notch and NF-κB pathways has also been described by Espinosa et al. (32, 57), who observed that these proteins competed for nuclear corepressors, such as N-CoR. When searching for the reasons that could explain the discrepancies among all these works, we observed that NF-κB inhibition appears to occur when the Notch-IC-truncated proteins lack part of the RAM domain, but not when they correspond to the entire Notch region normally liberated after ligand activation. In support of these data, we found NF-κB inhibition when using a vector expressing an Notch-IC protein starting at aa 1771, but not starting at aa 1759 (data not shown). Notch-IC proteins starting at aa 1771 are indeed poorly active when a CBF1-reporter gene is used (57). Although we cannot completely rule out an inhibition of NF-κB by Notch signaling, according to our data, if it existed at all, it would be small.

If NF-κB activity is not strongly affected by Notch signaling, the same does not hold true for other transcription factors involved in

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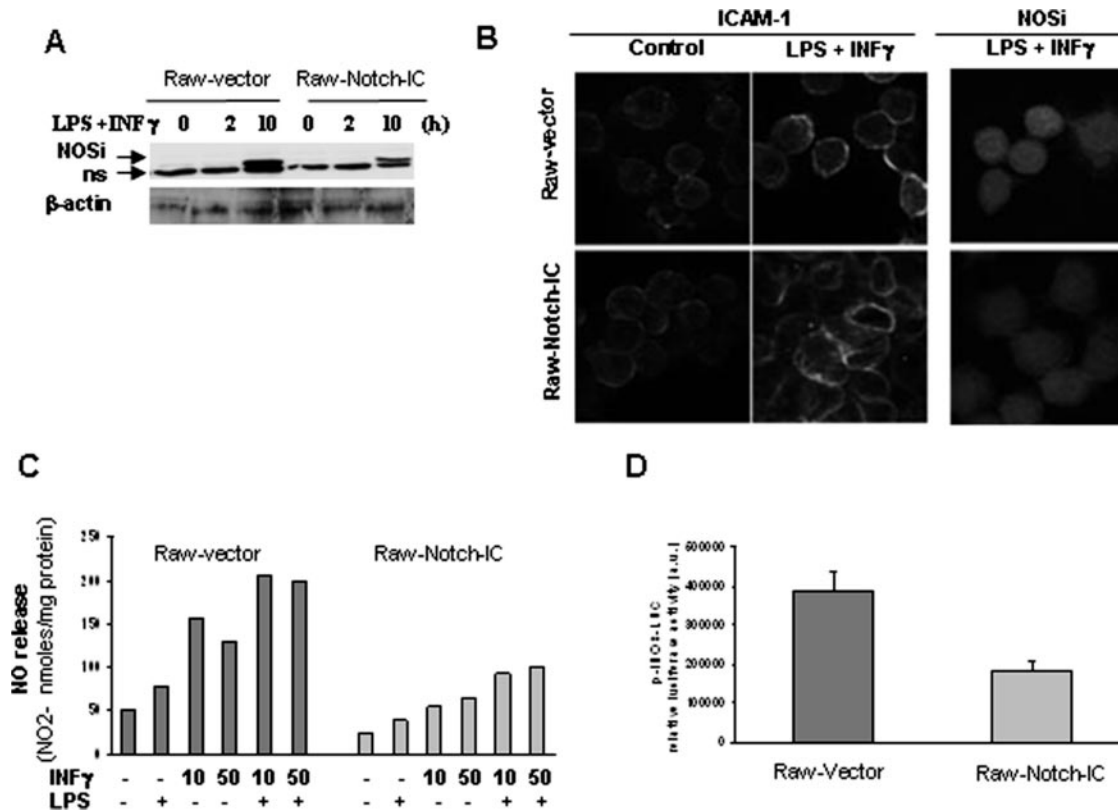

FIGURE 8. iNOS expression is diminished in activated Raw-Notch-IC cells. *A*, Raw 264.7 stable transfectants were stimulated with LPS (50 ng/ml) and IFN-γ (50 U/ml) for the indicated times. Total extracts were obtained, and 80 μg of protein were analyzed by Western blot to determine iNOS expression. Blots were also revealed with anti-β-actin to evaluate gel loading. The blot shown here corresponds to a representative experiment of three different assays. *B*, Immunolocalization of ICAM-1 and iNOS in Raw 264.7 Notch-IC stable transfectants stimulated with LPS and IFN-γ as described above for 18 h. *C*, Nitrite accumulation in the culture medium 18 h after macrophage activation. *D*, Stable transfectant cells, Raw-vector or Raw-Notch-IC were transiently transfected with p-iNOS-Luc and a β-galactosidase expression vector and stimulated with LPS and IFN-γ. Luciferase activity was measured 24 h later and normalized by β-galactosidase activity. Results represent the mean + SD of three independent experiments.

macrophage activation. We have observed an intense inhibition of AP-1-dependent reporter activity in the presence of Notch-IC (Fig. 5). AP-1 is a transcription factor involved in macrophage activation that modulates the expression of cytokines and enzymes controlling matrix remodeling (58). Although its role appears to be less important than that of NF-κB, AP-1 inhibition could have repercussions on macrophage gene expression. Inhibition of AP-1 activity by Notch-IC has been recently described by other investigators, and it has been assigned to the Notch-IC RAM domain and its ability to bind CBF1 (52, 59). The mechanism responsible for the inhibition of AP-1-dependent transcription is still unknown, but it does not involve AP-1 binding changes or c-Jun amino-terminal modifications (59). Indeed, we report in this study that, at least in part, Notch inhibition of AP-1 activity could be explained by the action of Hes-1 (Fig. 3*E*). Further work is needed to fully understand the molecular mechanism of this inhibition, but two models have been proposed to explain it: either a disruption of AP-1 complex assembly, or an impaired coactivator use by the AP-1 nucleoprotein complex (52, 59). Our data support the idea that either AP-1 complex disruption or impaired AP-1 coactivator recruitment are mediated by Hes-1, but not by Notch-IC directly.

Interestingly, contrary to what was seen with AP-1, we observed that Notch-IC increases the activity of a STAT1 reporter gene previously used to evaluate STAT1 activity (21). Although biochemical and genetic studies show that STAT1 plays a critical role in IFN-γ-dependent signaling (5), recent work reveals that additional signals are required for the full range of responses caused by IFN-γ (6, 7). STAT3 activation has been observed also after macrophage activation (36). Recently, cross-talking between STAT3 and Notch has been described in neuroepithelial cells. In these cells, the Notch effector proteins Hes-1 and Hes-5 associate with JAK2 and STAT3 to promote STAT3 phosphorylation and activation (35). We have not observed differences in STAT1 phosphorylation in the presence or absence of Notch-IC. In addition, we have not detected differences between control and Notch-IC stable transfectants in STAT1 nuclear translocation or DNA binding. Moreover, phosphorylation of STAT1 at Serine 727, shown to be essential to reach its maximal transactivation potential (5), was not affected by the expression of Notch-IC. However, an increase in STAT3 phosphorylation was detected in Raw 264.7 cells overexpressing Notch-IC. Our studies in HEK 293T cells confirmed that Hes-1 and, to a lesser extent, Notch-IC expression increased STAT3 phosphorylation levels, as described previously (35), but left STAT1 phosphorylation levels unaffected (Fig. 7). Although a Notch-IC-dependent increase of STAT1 phosphorylation, translocation or binding did not seem to occur in Raw 264.7 Notch-IC stable transfectants, following activation of these cells with LPS and IFN-γ we observed a clear increase in transcription of STAT1-dependent genes, such as IRF-1, SOCS-1, ICAM-1, and class II MHC proteins (Fig. 6), typically induced by STAT1 activity. Expression of IRF-1, SOCS-1, and ICAM-1 appears to be strictly dependent on STAT1 activity, because macrophages from STAT1$^{-/-}$ mice triggered with IFN-γ, despite showing increased phosphorylation and activation of STAT3 (36), cannot express these genes (6, 7). For that reason, it appears that increased activation of STAT3 by Notch/Hes-1 cannot compensate for the lack

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of STAT1 activity. This result suggests that the increased STAT1 transcription activity observed in Raw 264.7 cells stably transfected with Notch-IC expression constructs and activated by LPS and IFN-γ is not caused by the increase in STAT3 phosphorylation that we observe. Thus, this led us to consider three potential explanations, which were not mutually exclusive, for these data. First, it is still possible that small undetectable changes in STAT1 phosphorylation, which could explain the increased STAT1 transcription activity observed, may occur in the presence of Notch-IC. Indeed, after triggering HEK 293T cells with IFN-γ, we observed a discrete increase in STAT-1 phosphorylation in the presence of Hes-1, but not in the presence of Notch-IC (Fig. 7*C*), suggesting that intense Notch activity through Hes-1 may lead to an increase in STAT1 phosphorylation. Second, it is equally possible that, in the presence of Notch-IC, STAT3 could potentiate STAT1 transcriptional activity independently of changes in STAT1 phosphorylation levels. However, transient transfection of HEK 293T cells with STAT3 and Hes-1 or Notch-IC expression vectors does not result in changes in IRF-1 expression, making this second possibility unlikely. Finally, Hes-1, Notch, or some other Notch pathway downstream proteins could affect STAT1 activity by providing coactivators, or by competing for and displacing nuclear corepressors, as has been described previously (8, 9). Further work is needed to fully elucidate the molecular mechanism of this potentiation. However, our work adds further evidence to the increasing number of works showing the existence of different mechanisms able to modify, promote, or inhibit IFN-γ/STAT signals (60). Notch signaling can be one of them.

We evaluated *iNOS* expression and NO production as a control to check for adequate macrophage activation. We expected to find a similar increase of *iNOS* expression after IFN-γ stimulation of transfected or control cells, because *iNOS* is a gene up-regulated by IFN-γ (1). Surprisingly, Raw 264.7 Notch-IC transfectants showed a lower capacity to increase *iNOS* expression and NO production than control cells (Fig. 8). *iNOS* transcription depends on the activation of multiple transcription factors. Its promoter region contains binding sites for at least two NF-κB complexes, AP-1, two GAS elements binding STAT1 homodimers, and a complex IFN-stimulated regulatory element binding IRF-1 (61). Although specific mutation of the different binding elements will permit to dissect what sites are involved, we can already conclude that transcriptional inhibition of *iNOS* must not be directly due to STAT1 or IRF-1 proteins, because expression of other STAT1-dependent genes, including IRF-1, are increased in macrophages or Raw 264.7 Notch-IC transfectants. It is possible that AP-1 inhibition could account, at least partially, for the lower *iNOS* expression observed. Moreover, two studies have shown inhibition of NF-κB-dependent transcription after STAT3 activation. Yu and Kone (62) reported *iNOS* inhibition by direct interaction of STAT3 with p50 and p65, without affecting NF-κB binding to DNA. More recently, Hoentjen et al. (63) have shown that STAT3 inhibits cRel recruitment to the IL-12p40 promoter. Indeed, genetic deletion has shown that STAT3 has a pivotal role in controlling innate immune responses, and it is a key element in signal transduction by inhibitory cytokines, like IL-10 (64). Considering these data, it is reasonable to speculate that Notch-dependent increase of STAT3 activation and AP-1 inhibition could be responsible for the lower increase of *iNOS* expression in Notch-IC-transfected cells.

What is the role that Notch-1 plays in macrophage immune function? In view of our results, the Notch-1 receptor signaling pathway can cross-talk with different routes induced during macrophage activation, modulating specific gene expression patterns that could affect macrophage function. In this context, Notch activation seems to drive macrophages toward a more prominent role as APCs by increasing ICAM-1 and MHC type II protein expression, and by limiting their cytotoxic capacity through diminishing NOS expression. Notch activation has been found to modulate the function and fate of other immune cells. For instance, Notch activation appears to play a role in dendritic cell differentiation and maturation (65, 66). Recent reports have implicated Notch signaling in the interaction between APCs and T cells. It has been reported that Notch signaling augments T cell responsiveness by increasing the expression of the high-affinity IL-2R (45), and that it is required for TCR-mediated proliferation and production of IFN-γ in peripheral T cells (67). In addition, T cell stimulation by APC engineered to overexpress *Jagged-1* can induce Ag-specific regulatory T cells (68, 69). Finally, expression of different Notch ligands on APC can instruct CD4 T cells to differentiate into the Th1 or Th2 lineages (43).

The results presented in this study invite us to consider the possibility that Notch signaling could also be involved in the modulation of macrophage function depending on the immunological environment in which these cells may work. For example, macrophages fighting in an infectious focus would not find the appropriated ligands to activate Notch, a situation that would favor their cytotoxic activity. On the contrary, macrophages that have migrated to lymphoid organs to activate T cells could receive appropriate Notch-activating signals potentiating their Ag-presenting capability and limiting their cytotoxic effects. We are currently working to confirm this hypothesis.

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Disclosures

The authors have no financial conflict of interest.

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