Interferon- γ regulates nucleoside transport systems in macrophages through signal transduction and activator of transduction factor 1 (STAT1)-dependent and -independent signalling pathways

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The expressions of CNT and ENT (concentrative and equilibrative nucleoside transporters) in macrophages are differentially regulated by IFN- γ (interferon- γ). This cytokine controls gene expression through STAT1-dependent and/or -independent pathways (where STAT1 stands for signal transduction and activator of transcription 1). In the present study, the role of STAT1 in the response of nucleoside transporters to IFN- γ was studied using macrophages from STAT1 knockout mice. IFN- γ triggered an inhibition of ENT1-related nucleoside transport activity through STAT1-dependent mechanisms. Such inhibition of macrophage growth and ENT1 activity by IFN- γ is required for DNA synthesis. Interestingly, IFN- γ led to an induction of the CNT1- and CNT2-related nucleoside transport activities independent of STAT1, thus ensuring the supply of extracellular nucleosides for the STAT1-independent RNA synthesis. IFN- γ up-regulated CNT2 mRNA and CNT1 protein levels and down-regulated ENT1

mRNA in both wild-type and STAT1 knockout macrophages. This is consistent with a STAT1-independent, long-term-mediated, probably transcription-dependent, regulation of nucleoside transporter genes. Moreover, STAT1-dependent post-transcriptional mechanisms are implicated in the regulation of ENT1 activity. Although nitric oxide is involved in the regulation of ENT1 activity in B-cells at a post-transcriptional level, our results show that STAT1-dependent induction of nitric oxide by IFN- γ is not implicated in the regulation of ENT1 activity in macrophages. Our results indicate that both STAT1-dependent and -independent pathways are involved in the regulation of nucleoside transporters by IFN- γ in macrophages.

Key words: interferon- γ , macrophage, nucleoside transporter, signal transduction.

INTRODUCTION

Nucleoside transport into cells is a key step in the salvage pathway for nucleotide synthesis, thus avoiding the high energetics cost of nucleoside biosynthesis *de novo*. Moreover, cells that lack this ability, such as haematopoietic cells, rely exclusively on extracellular nucleosides to fulfil these requirements [1–4]. Thus a major function of nucleoside transporters is to supply substrates for nucleotide and nucleic acid syntheses. Among other functions, nucleoside transporters also control extracellular nucleoside concentration, regulating purine receptor signalling and, therefore, processes such as neurotransmission and cardiovascular activity [1–4]. Furthermore, nucleoside transporters mediate the uptake of many nucleoside analogue-derived drugs used in antiviral or anticancer therapies [5,6].

Several nucleoside transport systems that differ in their energetics, substrate specificity and inhibitor sensitivity have been described in mammalian cells [1–4]. The Na⁺-independent ENTs (equilibrative nucleoside transporters) show broad selectivity for both purine and pyrimidine nucleosides. To date, two plasma membrane ENTs have been identified in mammalian cells: ENT1, which is potently inhibited by NBTI (nitrobenzylthioinosine)

[7,8], and ENT2, which is insensitive to this compound [9]. In addition, three Na⁺-dependent CNTs (concentrative nucleoside transporters) have been characterized at the molecular level: CNT1 [10–12], CNT2 [13] and CNT3 [14]. CNT1 prefers pyrimidine and CNT2 prefers purine, whereas CNT3 shows broad specificity [10–14].

The expression pattern of nucleoside transporters differs depending on cell type and is regulated by several stimuli. Thus ENT1 is the major nucleoside transporter in hepatoma cell lines and is more abundant in the highly proliferating foetal hepatocytes than in neonatal or adult hepatocytes [15–17]. Similarly, ENT1-related activity is higher in T lymphoblastic leukaemia cell lines than in resting peripheral T lymphocytes [18]. In contrast, B- and T-cell-induced activation, which triggers proliferation, leads to up-regulation of specific concentrative nucleoside transport systems [18,19]. Furthermore, differentiation of the promyelocytic leukaemic HL60 cells to macrophages/monocytes by PMA induces CNT3 and represses ENT1 activity [14]. Moreover, nucleotide metabolism may modulate the expression of CNTs in an adaptive manner [20].

Therefore cell-selective regulation of nucleoside transporters occurs during cell activation, proliferation and differentiation to

Abbreviations used: c/EBP β , CCAAT-enhancer-binding protein β ; CNT, concentrative nucleoside transporter; DMEM, Dulbecco's modified Eagle's medium; ENT, equilibrative nucleoside transporter; IFN, interferon; NOS, nitric oxide synthase; iNOS, inducible NOS; ISGF, IFN-stimulated response element; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; NBTI, nitrobenzylthioinosine; RT, reverse transcriptase; STAT1, signal transduction and activator of transcription 1; TNF- α , tumour necrosis factor- α .

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fulfil specific nucleoside requirements. In this context, we have recently demonstrated that macrophages require a number of nucleoside transporters for proliferation and activation. Thus M-CSF-induced macrophage proliferation (where M-CSF stands for macrophage colony-stimulating factor) leads to up-regulation of ENT1, whereas macrophage activation by IFN (γ) or LPS (lipopolysaccharide), which triggers growth arrest, results in the induction of CNT1 and CNT2 and in the down-regulation of ENT1s [21,22]. Since an inhibition of ENT1 function blocks the DNA synthesis induced by M-CSF, but does not block RNA synthesis induced by M-CSF, IFN- γ or LPS, the differential regulation of nucleoside transporters by these stimuli seems to respond to specific nucleoside requirements for DNA and RNA synthesis [21]. These results indicate that CNT-related nucleoside uptake is involved in macrophage activation, whereas ENT1-mediated nucleoside transport is mainly associated with cell proliferation [21,22].

IFN- γ , a cytokine that triggers diverse cellular responses ranging from regulation of immune response to control of cell growth, controls the expression of nearly 500 genes [23,24]. Regulation of this expression by IFN- γ , either positively or negatively, is mostly mediated by STAT1 (signal transduction and activator of transcription 1), a latent cytosolic transcription factor that also acts as a signal-transduction molecule. Binding of IFN- γ to its receptor triggers the activation of the receptor-associated Janus kinases 1 and 2 and further phosphorylation and dimerization of STAT1. The dimers translocate into the nucleus and regulate gene expression by binding to γ -activated sequence elements [23–26]. Nevertheless, a large number of genes are regulated by STAT1-independent pathways [27–29]. Indeed, there is increasing evidence confirming that signalling by IFN- γ is the result of a crosstalk between STAT1-dependent and -independent pathways.

In the present study, we analysed the role of STAT1 in the differential regulation of the nucleoside transporters present in macrophages by IFN- γ . We show that the IFN- γ inhibition of ENT1-related activity is STAT1-dependent, whereas the up-regulation of the CNT1- and CNT2-related activities is not mediated by STAT1 signalling. The STAT1-independent regulation of CNT1 and CNT2 genes seems to occur at the transcriptional level. On the other hand, STAT1-dependent mechanisms are involved in the regulation of ENT1 at the post-transcriptional level.

EXPERIMENTAL

Reagents

Recombinant mouse IFN- γ and TNF- α (tumour necrosis factor- α) were obtained from Preprotech (London, U.K.) and IFN- α/β and LPS from Sigma (St. Louis, MO, U.S.A.). [^3H]Uridine and [α - ^{32}P]dCTP were obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.). DMEM (Dulbecco's modified Eagle's medium) and other culture-medium reagents were obtained from BioWittaker (Verviers, Belgium). All other chemical reagents were of the highest quality and were purchased from Sigma, Merck (Darmstadt, Germany) or Fluka Chemie (Buchs, Switzerland).

Rabbit polyclonal antibodies against the rat CNT1 protein were raised and characterized as described previously [30,31] and have been shown to cross-react specifically with the mouse orthologue [21,22]. Rabbit polyclonal antibodies raised against mouse iNOS (inducible nitric oxide synthase) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Mouse anti-mouse β -actin antibody was purchased from Sigma and the

goat peroxidase-conjugated anti-rabbit and anti-mouse IgGs from Cappel (Durham, NC, U.S.A.).

Bone-marrow-derived macrophages from mice and their culture conditions

Studies were performed in bone-marrow-derived macrophages from wild-type mice, STAT1 knockout mice (strain 129/Sv/Ev) [32] kindly provided by Robert D. Schreiber (Washington University, St. Louis, MO, U.S.A.) and iNOS knockout mice (strain C57BL/6; The Jackson Laboratory, Bar Harbor, ME, U.S.A.). Cells were isolated and cultured as described previously [33]. Briefly, the ends of femurs were cut off and the marrow tissue was flushed by irrigation with DMEM. Once dispersed by passing through a 25 gauge needle, the cells were cultured on plastic dishes (150 mm) with DMEM containing 20 % (v/v) foetal bovine serum and 30 % (v/v) L-cell conditioned media as a source of M-CSF, and kept at 37 °C in a humidified 5 % CO₂ atmosphere. Macrophages were obtained as a homogeneous population of adherent cells after culturing for 7 days. For experiments, cells were cultured in fresh media either in the presence or absence of IFN- γ (300 units/ml), IFN- α/β (1000 units/ml), LPS (100 ng/ml) or TNF- α (100 ng/ml).

Transport measurements

For transport experiments, macrophages were seeded on 35 mm tissue culture dishes. Uptake was measured as described previously [21,22], using [^3H]uridine as a substrate in either 137 mM NaCl or 137 mM choline chloride medium, containing 5.4 mM KCl, 1.8 mM CaCl₂, 1.2 mM Mg₂SO₄ and 10 mM Hepes (pH 7.4). After 3 min, monolayer cells were washed three times in 2 ml of an ice-cold buffer containing 137 mM NaCl and 10 mM Hepes (pH 7.4). Cells were lysed in 0.5 ml of 0.5 % Triton X-100 and, subsequently, 0.4 ml of cellular extracts was used for radioactivity counting and the rest for protein determination by the Bradford technique using the Bio-Rad protein reagent (Bio-Rad Laboratories, Madrid, Spain). Concentrative (Na⁺-dependent) uptake was calculated as the difference between transport rates in the presence of Na⁺ and in the absence of Na⁺ (choline medium). The relative contribution of the CNT2 transport system to Na⁺-dependent transport was assessed by inhibiting uridine uptake using saturating concentrations of formycin B, a CNT1 substrate. The formycin B-resistant Na⁺-dependent transport corresponds to the CNT1-related transport uptake. The equilibrative (Na⁺-independent) transport inhibited by NBTI (1 μM) accounts for the ENT1-related nucleoside transport activity, whereas the NBTI- and uridine-resistant transport includes a residual ENT2-related uptake plus diffusion and binding components.

Nitric oxide (NO) measurement

NO was measured as nitrite using the Griess reagent, as described previously [34]. Cell culture supernatant was mixed with 100 μl of 1 % sulphanilamide, 0.1 % *N*-(1-naphthyl)ethylenediamine dihydrochloride and 2.5 % (v/v) H₃PO₄. Absorbance was measured in a microplate reader (Molecular Devices, Ismaning, Germany).

Arginase activity

Arginase activity was measured as described previously [34]. Briefly, cells were lysed with 100 μl of 0.1 % Triton X-100.

After 30 min on a shaker, 100 μ l of 25 mM Tris/HCl was added. The enzyme was activated by heating for 10 min at 56 °C after the addition of 10 μ l of 10 mM MnCl₂ to 100 μ l of this lysate. Arginine hydrolysis was performed by incubating the lysate with 100 μ l of 0.5 M L-arginine (pH 9.7) at 37 °C for 15–120 min. The reaction was stopped with 900 μ l of H₂SO₄ (96 %)/H₃PO₄ (85 %)/H₂O (1:3:7, by vol.). The urea concentration was measured at 540 nm after the addition of 40 μ l of α -isonitrosopropiophenone, followed by heating at 95 °C for 30 min. One unit of enzyme is defined as the amount of enzyme that catalyses the formation of 1 μ mol of urea/min.

Northern-blot analysis

Total RNA was isolated using the TRIzol[®] reagent (Gibco BRL, Life Technologies, Gaithersburg, MD, U.S.A.) according to the manufacturer's instructions. Northern-blot analysis was performed as described previously [21,22]. The RNA was size-separated on 1 % agarose-formaldehyde gels, transferred overnight to an Immobilon filter (Amersham Biosciences) and cross-linked to the filter by irradiation with UV light. Filters were analysed with [α -³²P] dCTP-labelled random-primed cDNA probes for CNT2, ENT1, *c-myc* and 18 S RNA. Expression levels were quantified using a Molecular Imaging System (Bio-Rad, Richmond, CA, U.S.A.).

Quantitative RT (reverse transcriptase)-PCR analysis

RNA was treated with DNase (Ambion, Austin, TX, U.S.A.) to eliminate contaminating DNA. For cDNA synthesis, 1 μ g of RNA and TaqMan reverse transcription reagents (including Multiscribe RT and random hexamers) were used according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, U.S.A.). The primers and probes used to amplify mouse CNT2 (Mm00445488) and ENT1 (Mm00425176) cDNAs by real-time PCR were obtained from Applied Biosystems. Real-time monitoring of PCR amplification of cDNAs was done using the TaqMan Universal master mix (Applied Biosystems) in the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Relative quantification of gene expression was performed as described in the TaqMan User's Manual using human 18 S (18 S Hs99999901; Applied Biosystems) as an internal control. The threshold cycle (C_T) is defined as the cycle number at which the fluorescence corresponding to the amplified PCR product is detected. The PCR arbitrary unit of each transporter was defined as the mRNA levels of these genes normalized to the 18 S expression level in each sample. RNA from control samples (wild-type and knockout in the absence of stimuli) were used as the reference control to normalize nucleoside transporter/18 S amplification rates.

Western-blot analysis

Western-blot analyses were performed basically as described in [21,22]. To obtain cell extracts, cells were washed twice in cold PBS, then solubilized in lysis buffer [1 % Nonidet P40/10 % (v/v) glycerol/50 mM Hepes (pH 7.5)/150 mM NaCl], 1 mM Na₃VO₄, 10 μ g/ml aprotinin and leupeptin, 86 μ g/ml iodoacetamide and 1 mM PMSF for 15 min at 4 °C, and centrifuged at 10 000 *g* for 10 min. Protein concentration was measured as indicated above. Samples (100 μ g) were heated at 95 °C in a Laemmli buffer, subjected to SDS/PAGE (10 % gel) and transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences). The membranes were blocked and then probed

Figure 1 Increase in CNT1 and CNT2 activities independent of STAT1 and suppression of STAT1-dependent ENT1 activity by IFN- γ

Macrophages from wild-type (upper panel) and STAT1 knockout (lower panel) mice were incubated in the absence (open bars) or presence (black bars) of IFN- γ for 24 h. Concentrative uridine transport activity associated with CNT1 and CNT2, equilibrative uridine transport activity associated with ENT1 and ENT2 plus diffusion (D) and binding (B) were measured as described in the Experimental section. Results are the means $\pm$ S.E.M. from three independent experiments.

with antibodies raised against CNT1, iNOS and β -actin. Subsequently, the membranes were incubated with the corresponding peroxidase-conjugated secondary antibodies. Detection was performed using enhanced chemiluminescence detection reagents from Amersham Biosciences. The bands were quantified using densitometric analysis by Phoretix software (Phoretix, Newcastle upon Tyne, U.K.).

RESULTS AND DISCUSSION

Role of STAT1 in IFN- γ -mediated regulation of nucleoside transporters in macrophages

Nucleoside transporters are among the many genes regulated by IFN- γ [21]. They constitute a first key regulatory step for nucleic acid synthesis in cells like macrophages that lack synthesis of nucleotides *de novo* [1,2]. Signalling through STAT1 is critical and specific for most of the biological functions of IFN- γ , including immune responses and control of cell growth, as demonstrated by disruption of the STAT1 gene [25,29,32,35]. Although IFN- γ regulates gene expression mainly by STAT1-dependent mechanisms, several genes are either induced or repressed by IFN- γ , independent of STAT1 signalling [27–29] or through both STAT1-dependent and -independent signalling [36]. To determine the role of STAT1 in IFN- γ -mediated regulation of nucleoside transporters, we used bone-marrow macrophages from STAT1 knockout mice. Nucleoside uptake in bone-marrow macrophages was mainly mediated by CNT1, CNT2 and ENT1 [21,22]. IFN- γ increased CNT1 and CNT2 activities similarly (approx. 2.5-fold induction) in wild-type and STAT1 knockout macrophages (Figure 1). In contrast, ENT1 activity was repressed by IFN- γ in wild-type macrophages (approx. 50 %), but not in STAT1 knockout macrophages. No changes in the non-concentrative NBTI- and uridine-resistant uptake [ENT2

Figure 2 IFN- γ differentially regulates *c-myc* expression in wild-type and STAT1 knockout macrophages

Macrophages from wild-type and STAT1 knockout mice were incubated in the absence or presence of IFN- γ for the indicated times. The expressions of *c-myc* and 18 S RNA were studied by Northern-blot analysis. A representative Northern blot from two independent experiments is shown (left panel). Gene product expression was quantified as described in the Experimental section and results are expressed in arbitrary units, considering the value 1 as basal condition (absence of IFN- γ) for wild-type (right panel, black bars) and STAT1 knockout (open bars) macrophages.

activity + diffusion + binding] were observed in either wild-type or STAT1 knockout cells treated with IFN- γ . Therefore IFN- γ increased concentrative nucleoside uptake independent of STAT1, whereas it inhibited equilibrative nucleoside transport through STAT1-dependent mechanisms. In macrophages, ENT1 is essential for DNA synthesis and proliferation, but not for RNA synthesis, whereas up-regulation of the concentrative nucleoside uptake is related to an increase in nucleoside needs for transcriptional activity [21]. Interestingly, IFN- γ requires STAT1 to either repress ENT1 activity (Figure 1) or to block cell proliferation [27,36]. The STAT1-independent increase in concentrative uptake ensures supply of extracellular nucleosides for the STAT1-independent RNA synthesis induced by IFN- γ . These results reinforce the hypothesis of a selective regulation of nucleoside transporters, which may respond, at least in macrophages, to the need to fulfil specific requirements for DNA and RNA synthesis [21] or for other specialized functions which are yet to be determined.

We also monitored the expression of *c-myc*, since it is a transcription factor that participates in the regulation of cell proliferation [37] and is STAT1-dependently repressed and STAT1-independently up-regulated by IFN- γ in fibroblasts [36]. IFN- γ also led to the down-regulation of *c-myc* expression in wild-type macrophages and triggered an induction of expression in STAT1 knockout macrophages (Figure 2). Thus repression of *c-myc* expression [36–38], induction of the cyclin-dependent kinase inhibitors p21^{waf} and p27^{kip} [38,39] and decrease in nucleoside uptake for DNA synthesis through ENT1 [21] are some of the diverse STAT1-dependent responses that may be involved in IFN- γ -mediated growth arrest of macrophages.

Next, to determine whether the STAT1-independent and -dependent mechanisms, by which IFN- γ regulated the CNT and ENT transport system activities respectively, were consistent with changes in transporter expression, we studied the effects of IFN- γ on CNT2 and ENT1 mRNA and on the amount of CNT1 protein in wild-type and STAT1 knockout macrophages (Figure 3). Regulation of the expressions of CNT2 and ENT1 mRNAs was analysed by Northern blotting (Figure 3A) and also by quantitative RT-PCR (Figure 3B) to assess accurately the mRNA levels. Results obtained from Northern-blot analysis correlated well with quantitative RT-PCR analysis. In agreement with the transport activity shown in Figure 1, IFN- γ increased CNT2 mRNA and CNT1 protein levels in wild-type and STAT1

Figure 3 IFN- γ regulates CNT1, CNT2 and ENT1 expressions independent of STAT1

Macrophages from wild-type and STAT1 knockout mice were incubated in the absence or presence of IFN- γ for the indicated times. The expressions of CNT2, ENT1 and 18 S RNA were studied by Northern-blot analysis (A) and quantitative RT-PCR (B), whereas the expressions of CNT1 and β -actin (β -act) protein levels were measured by Western-blot analysis (A). The amounts of 18 S RNA and β -actin protein were used as a loading control. Representative Western and Northern blots from three independent experiments are shown and gene product expression was quantified as described in the Experimental section. Results are expressed in arbitrary units, considering the value 1 as basal condition (absence of IFN- γ) for the wild-type mice (A, black bars; B, $\bullet$, $\blacksquare$) and the STAT1 knockout mice (A, open bars; B, $\circ$, $\square$). Basal CNT2 mRNA levels from STAT1 knockout macrophages were 50 % lower when compared with wild-type cells obtained by either Northern-blot or quantitative RT-PCR analysis.

knockout macrophages. Although basal levels of CNT2 mRNA were lower in STAT1 knockout macrophages than in wild-type macrophages, the time course of induction of CNT2 mRNA was similar, beginning to increase as early as 2 h after IFN- γ treatment and reaching a peak after 6 h. Lower expression of CNT2 mRNA in macrophages lacking STAT1 indicates that this transcription factor could participate in the constitutive expression of CNT2, as described for other genes [40,41]. The kinetics of CNT1 protein induction by IFN- γ was also similar in STAT1 knockout and wild-type macrophages, as shown by an increase after 6 h and the highest levels after 24 h cytokine treatment. Surprisingly, IFN- γ repressed ENT1 mRNA expression at the same level and followed similar kinetics in macrophages with and without STAT1 expression (Figure 3A), contrasting with the

Figure 4 IFN- α/β , TNF- α and LPS regulate the expressions of CNT1, CNT2 and ENT1 independent of STAT1

Macrophages from wild-type and STAT1 knockout mice were incubated in the absence or presence of IFN- α/β , TNF- α or LPS for 24 h. The expressions of CNT2, ENT1 and 18 S RNA were studied by Northern-blot analysis, whereas the expressions of CNT1 and β -actin protein levels were measured by Western-blot analysis. The amounts of 18 S RNA and β -actin protein were used as a loading control. Representative Western and Northern blots from two independent experiments are shown (left panel). Gene product expression was quantified as described in the Experimental section and results are expressed in arbitrary units, considering the value 1 as basal condition (absence of stimuli) for the wild-type (right panel, black bars) and the STAT1 knockout (open bars) macrophages.

STAT1-dependent inhibition of ENT1 activity (Figure 1). This apparent discrepancy could be explained by a STAT1-dependent post-transcriptional regulation of ENT1 activity. On the other hand, STAT1-independent regulation of concentrative nucleoside uptake by IFN- γ seems to take place only at the transcriptional level, since the increase in concentrative nucleoside uptake correlates with the up-regulation of CNT1 and CNT2 both in wild-type and STAT1 knockout cells.

To determine further the role of STAT1 in the regulation of nucleoside transporters, we studied their regulation by several cytokines or stimuli that can signal through STAT1, such as IFN- α/β [23–25], LPS [24,42] or TNF- α [40,43,44] (Figure 4). Similar to the effects of IFN- γ , IFN- α/β , LPS and TNF- α , macrophage activation triggered an up-regulation of CNT2 mRNA and CNT1 protein levels and led to a down-regulation of the amount of ENT-1 mRNA in wild-type and STAT1 knockout macrophages. Thus the regulation of CNT1, CNT2 and ENT1 expressions by these agents is also mediated by STAT1-independent mechanisms.

Therefore IFN- γ triggered the induction of CNT1 and CNT2 and the repression of ENT1 expression, independent of STAT1, indicating that regulatory sequence elements and transcription factors other than γ -activated sequence and STAT1 drive the IFN- γ effects on the expression of nucleoside transporters. Functional analysis of the ENT1 promoter indicates that the transcription factors Sp1 (zinc-finger specificity protein-1) and MAZ (myc-associated zinc protein) are positive regulators of ENT1 gene transcription [45]. Interestingly, IFN- γ suppresses the transcription of the lipoprotein lipase gene in macrophages by decreasing the DNA-binding activity of Sp1 [46]. Furthermore, a closer promoter analysis using the 'motif search' web program (www.motif

genome.ad.jp), revealed that the mouse ENT1 promoter contains putative response elements for transcription factors that are induced by IFN- γ independent of STAT1, such as c/EBP β (CC-AAT-enhancer-binding protein β) and EGR1 (early growth response gene product 1) [28], whereas putative STAT1 binding motifs were not found. In addition, analysis of the 5'-flanking sequence of human CNT1 and CNT2 genes shows putative binding sites for c/EBP β and STATs. c/EBP β is a pleiotropic transcription factor of the CCAAT family of transcription factors, up-regulated by IFN- γ [28,47], which can control gene transcription through the IFN- γ -activated transcription element. However, it appears that the transcriptional activity of c/EBP β requires STAT1 and extracellular-signal-regulated kinase phosphorylation at least to drive the expression of the IFN-stimulated response element 3 γ (p48) promoter [48]. Moreover, the 5'-flanking regions of these genes contain putative binding motifs for several transcription factors, such as nuclear factor κ B, cAMP-response-element-binding protein and activator protein 1, which are common targets for several cytokines and growth factors, depending on the regulation of nucleoside transporters by many stimuli, such as IFN- γ (Figure 3), IFN- α/β (Figure 4), M-CSF [21,49], TNF- α , LPS [19,22] or PMA [14,19,50]. Nevertheless, the mechanisms by which stimuli regulate nucleoside transport have not been characterized as yet. Studies are underway on cloned nucleoside transporter promoters to determine the molecular basis for the probable transcriptional regulation of these genes by cytokines and other agents.

NO is not involved in the STAT1-independent inhibition of ENT1 activity in response to IFN- γ in macrophages

The decrease in ENT1 activity but not the down-regulation of ENT1 mRNA levels triggered by PMA in B lymphocytes requires NO, indicating that NO can regulate the ENT1 transport system at the post-transcriptional level [50]. Therefore, to examine the STAT1-dependent mechanisms by which IFN- γ could regulate ENT1 activity, we tested the role of NO. There is an increasing number of proteins that are regulated at the post-transcriptional level by NO, which acts as a redox-based mechanism [51]. Furthermore, IFN- γ induces NO synthesis through induction of iNOS via a STAT1-dependent signalling mechanism [32]. The induction of iNOS by IFN- γ , which catalyses the production of NO from arginine, requires STAT1 (Figure 5A). Consequently, IFN- γ did not trigger an increase in NO production in STAT1 knockout macrophages, whereas it did in wild-type cells (Figure 5B). On the other hand, in STAT1 knockout macrophages, IFN- γ induced the expression [27] and activity of arginase (Figure 5C), an enzyme that shares the substrate arginine with NOS (nitric oxide synthase) and thus prevents NO production by substrate depletion [51]. Nevertheless, we did not detect a decrease in NO levels in IFN- γ -treated versus -untreated STAT1 knockout macrophages (Figure 5B), probably because of a lack of arginine for NO synthesis through other cellular NOSs. These results are in agreement with the absence of expression of other NOSs from bone-marrow macrophages (M. Modolell, unpublished work). Furthermore, arginase and iNOS do not share the same arginine intracellular pool [52]. Therefore NO synthesis may be regulated only by iNOS in bone-marrow macrophages. Therefore to establish whether the STAT1-dependent induction of NO by IFN- γ was required to inhibit ENT1 activity in macrophages, we examined its regulation in bone-marrow macrophages from iNOS knockout mice. No changes in the inhibition of ENT1 activity were observed in the iNOS knockout macrophages treated with IFN- γ (Figure 4D).

Figure 5 iNOS is not involved in IFN- γ inhibition of the ENT1 activity

Macrophages from wild-type mice, STAT1 knockout mice (A–C) and iNOS knockout mice (D) were incubated in the absence (open bars) or presence (black bars) of IFN- γ for the indicated times (A) or for 24 h (B–D). The expressions of iNOS and β -actin (β -act) were measured by Western-blot analysis (A). Production of NO (B), the arginase activity (C) and the ENT1-related transport activity (D) were measured as described in the Experimental section. A representative result from three independent experiments is shown.

Similar results were obtained with macrophages treated with LPS [22]. Therefore the induced synthesis of NO through iNOS by either IFN- γ or LPS did not modulate ENT1 activity in macrophages. It is not uncommon that signalling by the same stimuli differs depending on cell type. For instance, NO triggers apoptosis in macrophages [53], but inhibits this process in B-cells [54]. On the other hand, other post-translational modifications may regulate ENT1 activity, such as phosphorylation. In this regard, δ/ϵ isoforms of protein kinase C are apparently involved in the short-term-mediated activation of ENT1 proteins once inserted at the plasma membrane [55]. Our results indicate that IFN- γ regulates both ENT1 mRNA expression and ENT1 protein activity, independent of and dependent on STAT1 respectively. Similarly, IFN- γ regulates c/EBP β expression independent of STAT1 [28], but stimulates c/EBP β activity by phosphorylation dependent on STAT1 [48]. Therefore IFN- γ can regulate the expression and activity of many genes by both STAT1-dependent and -independent mechanisms. Thus responses to IFN- γ result from the integration of these multiple signalling pathways [25,29].

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