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Lipopolysaccharide-induced Apoptosis of Macrophages Determines the Up-regulation of Concentrative Nucleoside Transporters Cnt1 and Cnt2 through Tumor Necrosis Factor-α-dependent and -independent Mechanisms*

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In murine bone marrow macrophages, lipopolysaccharide (LPS) induces apoptosis through the autocrine production of tumor necrosis factor-α (TNF-α), as demonstrated by the fact that macrophages from TNF-α receptor I knock-out mice did not undergo early apoptosis. In these conditions LPS up-regulated the two concentrative high affinity nucleoside transporters here shown to be expressed in murine bone marrow macrophages, concentrative nucleoside transporter (CNT) 1 and 2, in a rapid manner that is nevertheless consistent with the *de novo* synthesis of carrier proteins. This effect was not dependent on the presence of macrophage colony-stimulating factor, although LPS blocked the macrophage colony-stimulating factor-mediated up-regulation of the equilibrative nucleoside transport system *es*. TNF-α mimicked the regulatory response of nucleoside transporters triggered by LPS, but macrophages isolated from TNF-α receptor I knock-out mice similarly up-regulated nucleoside transport after LPS treatment. Although NO is produced by macrophages after LPS treatment, NO is not involved in these regulatory responses because LPS up-regulated CNT1 and CNT2 transport activity and expression in macrophages from inducible nitric oxide synthase and cationic amino acid transporter (CAT) 2 knock-out mice, both of which lack inducible nitric oxide synthesis. These data indicate that the early proapoptotic responses of macrophages, involving the up-regulation of CNT transporters, follow redundant regulatory pathways in which TNF-α-dependent- and -independent mechanisms are involved. These observations also support a role for CNT transporters in determining extracellular nucleoside availability and modulating macrophage apoptosis.

Macrophages play a key role in the pathogenesis of endotoxic shock by synthesizing NO and TNF-α[1] (1–4), which induce apoptosis in a variety of cell types, including macrophages themselves (1, 2, 4–6). Macrophage activation and apoptosis appear to be modulated by extracellular nucleosides and nucleotides (7–12). These effects are partially explained by their interaction with adenosine and P2 receptors, respectively. Although high concentrations of adenosine seem to favor apoptosis in a variety of cell types, including leukemia cells, endothelial cells, smooth muscle cells, and cells from the central nervous system (13–18), adenosine receptor agonists, when added to the macrophage cell line RAW264.7, inhibit the LPS-induced production of TNF-α and NO (19). The effect on TNF-α production appears to be exerted at the transcriptional level and is partly specific because it does not modify the production of other cytokines such as interleukin-6 and interleukin-8 (8). In primary cultures of murine bone marrow macrophages, adenosine, through its interaction with A2B receptors, blocks the IFN-γ-induced expression of inducible nitric oxide synthase (iNOS) and TNF-α (20). However, the expression of this specific receptor is also up-regulated by IFN-γ, thus contributing to a feedback mechanism for the fine tuning of macrophage activation/deactivation processes (20). A similar complex network may also apply to the actions of nucleotides. ATP can also block the release of TNF-α from macrophages activated by LPS and IFN-γ (7, 9–11, 21). Nevertheless, these two agents also up-regulate the synthesis of the P2Z/P2X7 receptor for ATP (22), which is again consistent with the idea of feedback mechanisms

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[1] The abbreviations used are: TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; IFN, interferon; MCSF, macrophage colony-stimulating factor; ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CAT, cationic amino acid transporter; ELISA, enzyme-linked immunosorbent assay.

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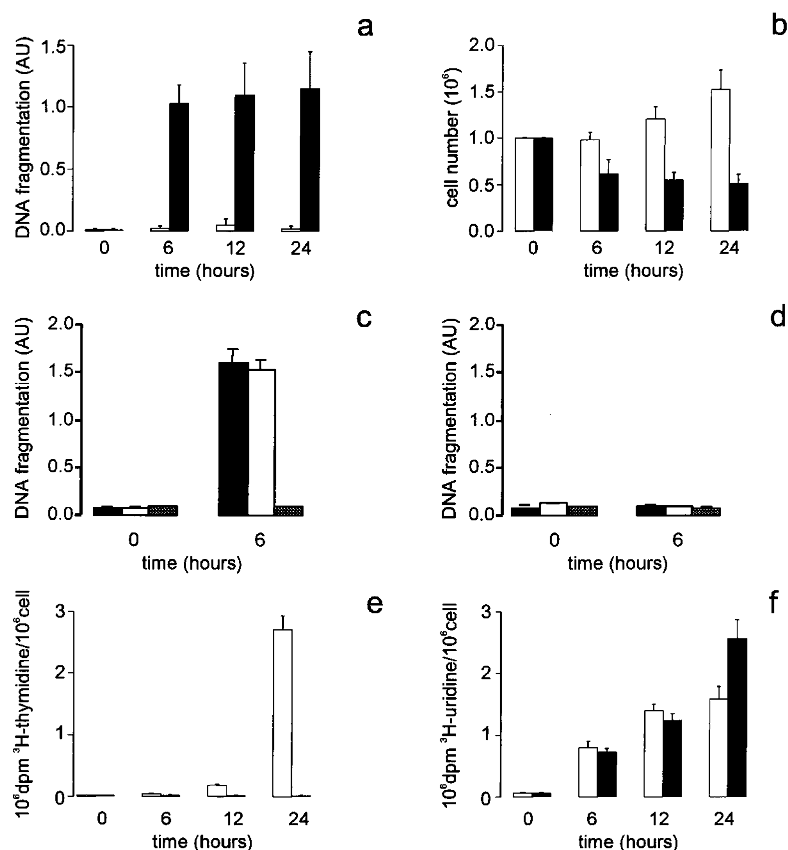


FIG. 1. **Effect of LPS treatment on cell number, DNA fragmentation, and radiolabeled nucleoside incorporation into DNA and RNA.** Murine bone marrow macrophages growing in the presence of MCSF were treated with LPS (100 ng/ml), and DNA fragmentation (*a*) and cell number (*b*) were assessed at the indicated times of treatment. *Solid bars* correspond to LPS-treated macrophages, whereas *open bars* depict data obtained in cell cultures in the absence of LPS. The role of TNF-α in macrophage apoptosis was analyzed in cells isolated either from wild type (*c*) or from TNF-α receptor I knock-out mice (*d*) cultured in the presence of either LPS (*solid bars*), TNF-α (*open bars*), or phosphate-buffered saline as control (*dashed bars*). DNA fragmentation was measured 6 h after treatments. Tritiated thymidine and uridine incorporation into nucleic acids was also assessed. The first measurement corresponds to incorporation into DNA (*e*), whereas the second parameter is essentially a measurement of nucleoside incorporation into RNA (*f*). *Open bars* correspond to cells cultured in the absence of LPS, whereas *solid bars* correspond to LPS-treated macrophages. All these observations are the mean ± S.E. of triplicate measurements made in at least three independent experiments. *AU*, absorbance unit.

contributing to deactivation in macrophages.

The actions of extracellular nucleotides may be partially mediated by their catabolites, which are generated by the action of extracellular nucleotidases and lead to the synthesis of adenosine. Similarly, adenosine catabolites such as inosine may mimic some of the effects of this nucleoside (23). Although some of these actions may require the extracellular presence of nucleosides, others appear to involve uptake into the cell (12, 17). Considering that nucleoside transport is necessary either to generate active metabolites intracellularly or to modulate adenosine availability to its receptors, nucleoside transporters may be elements of these regulatory networks, which are key determinants of macrophage activation during septic shock.

Recently we have characterized the nucleoside transporters in murine bone marrow macrophages (50). This cell type basically expresses an *es*-type nucleoside transport system, which presumably corresponds to the expression of the murine *ENT1* gene, and two concentrative nucleoside transporters of the *cit* and *cib* type, which are the result of the co-expression of the pyrimidine- and purine-preferring transporters CNT1 and CNT2. Most of the kinetic and molecular properties of these and other nucleoside transporters cloned so far have recently been reviewed (24–29). Nucleoside transporter expression is highly regulated in epithelial cell types, such as hepatocytes, by cell cycle progression and differentiation (29–31). In murine bone marrow macrophages, the stimulation of cell proliferation by MCSF is related to a selective up-regulation of the ENT1 transporter, whereas treatment of these cells with IFN-γ results in the inhibition of cell proliferation and ENT1 induction at a time when this triggers the expression of the two CNT transporters, CNT1 and CNT2 (50).

On the basis of this evidence, we wondered whether treatment of murine bone marrow macrophages with the proapoptotic agent LPS would modulate nucleoside transporter expression. If this were the case, it might help us understand how these plasma membrane proteins contribute to the regulation of macrophage activation and apoptosis. Essentially, this study shows that LPS selectively up-regulates the CNT1 and CNT2 transporters. This response is not dependent on the presence of MCSF, although LPS blocks the up-regulation of the *es* transport system it triggers. LPS treatment of murine bone marrow macrophages resulted in the early expression of TNF-α and the subsequent synthesis of iNOS. TNF-α triggers early apoptotic events in these primary cultures because early programmed cell death is blocked in LPS-treated macrophages isolated from TNF-α receptor I knock-out mice. NO does not appear to be involved in the up-regulation of CNT transporters because macrophages isolated from mice lacking inducible NO synthesis (iNOS and CAT2 knock-out mice) still respond to LPS. However, TNF-α was shown to mimic the effects of LPS on nucleoside transporters. Nevertheless, the evidence that macrophages isolated from TNF-α receptor I knock-out mice still respond to LPS treatment indicates that the early pro-apoptotic responses of macrophages involving CNT nucleoside transporters are regulated by redundant pathways in which TNF-α-dependent and -independent mechanisms participate.

MATERIALS AND METHODS

Mice, Cells, and Culture Conditions—Bone marrow macrophages were obtained as described previously (32) from the femurs of 6–10-week-old BALB/c mice. The ends of the bones were cut, and the marrow was irrigated with culture medium to obtain a suspension, which was then passed through an 18-gauge needle to desegregate the cells for subsequent culture. Cell culture was performed on 150-mm Petri dishes in 40 ml of high glucose Dulbecco's modified Eagle's medium containing 20% fetal calf serum (Sigma) and 30% L cell-conditioned medium as a source of MCSF. Dishes were cultured at 37 °C in a humidified 5% CO_2 atmosphere until confluence for 7–8 days. These cultures turned out to be fully differentiated homogeneous populations of adherent macrophages suitable for further experiments in which cells were either cultured in the same medium or starved of MCSF by keeping them in Dulbecco's modified Eagle's medium supplemented with 10% fetal bo-

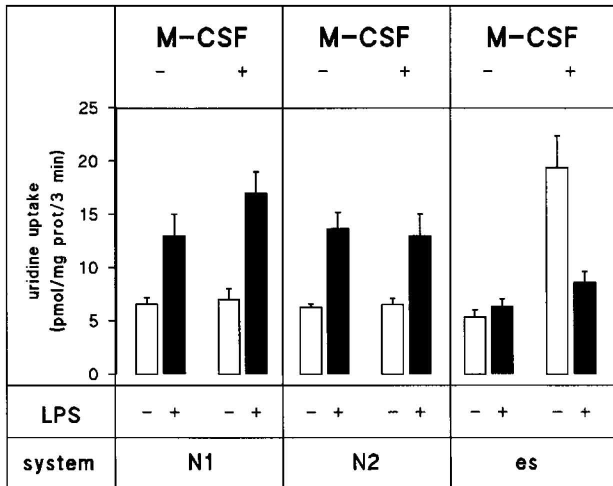


FIG. 2. **Up-regulation of CNT-type nucleoside transport activity and down-regulation of *es* transport in LPS-treated macrophages.** Macrophages were growth-arrested by culturing them for 18 h in the absence of MCSF. Cells were then induced to proliferate by MCSF or incubated in the absence of the growth factor for 24 h, either in the absence (*open bars*) or in the presence (*solid bars*) of LPS. At this point, Na^+-dependent uridine (1 μM) transport activity associated with N1 and N2 transport systems (CNT2 and CNT1 transporters, respectively), as well as the Na^+-independent ENT1-related transport activity *es,* were measured, as indicated under "Materials and Methods." Data are the mean ± S.E. of triplicate observations in three independent experiments. *prot*, protein.

vine serum for at least 18 h. In these conditions the cell cycle was arrested at G_0. Cells were then incubated either with or without MCSF (1200 units/ml) either in the presence or in the absence of LPS (100 ng/ml) for the indicated times (see below). In some experiments, cells cultured either in the presence or in the absence of MCSF were exposed to TNF-α (100 ng/ml). Recombinant murine TNF-α was obtained from PrepoTech EC (London, UK). LPS was purchased from Sigma and recombinant murine MCSF from R&D Systems (Minneapolis, MN).

Bone marrow macrophages isolated from three knock-out mouse strains were also used in this study. iNOS knock-out mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The TNF-α receptor I knock-out mice were generated and characterized as reported (33). Functional inducible NO knock-out mice generated by disrupting the arginine transporter *CAT2* gene (34) were also used to complement the data obtained with the iNOS knock-out animals.

Analysis of Apoptosis—Low molecular weight apoptotic DNA, obtained as described previously (4), was measured by an enzyme-linked immunosorbent assay (ELISA) (Cell Death Detection, ELISA Plus; Roche Molecular Biochemicals) that is directed against cytoplasmic histone-associated DNA fragments. Each point was performed in triplicate, and the results are expressed as the mean ± S. D.

Nucleic Acid Synthesis and Cell Counting—DNA synthesis was quantified by measuring the incorporation of tritiated thymidine (Amersham Pharmacia Biotech) into DNA as described previously (35). For this purpose cells were grown on 24-well tissue culture plates in 1 ml of medium without MCSF for at least 18 h. The cells were then cultured in the presence of MCSF and, at the desired times, the medium was removed and replaced by 0.5 ml of the same buffer but containing 1 μCi/ml [^{3}H]thymidine. Cells were incubated for 3 h and then fixed in 70% methanol, washed three times in ice-cold 10% trichloroacetic acid, and solubilized in 1% SDS and 0.3% NaOH prior to radioactivity counting. RNA synthesis was monitored in a similar way but with tritiated uridine as substrate. The number of viable cells was assessed by trypan blue exclusion with a hemocytometer.

Transport Experiments—Transport experiments were performed on 35-mm tissue culture dishes in which macrophages had been seeded after growing for 7 days in the 150-mm flasks. Uptake measurements were performed as described previously (31) with minor modifications. [5,6-^{3}H]uridine (Amersham Pharmacia Biotech) was routinely used as substrate at a concentration of 1 μM. Na^+-dependent transport was monitored by determining uridine uptake in either 137 mM NaCl or 137 mM choline chloride uptake medium as described (31). The relative contribution of the N1 transport system (CNT2-related) to the Na^+-dependent component of transport was assessed by inhibiting uridine uptake by saturating concentrations of formycin B, an N2 substrate. The formycin B-resistant component of the Na^+-dependent uridine transport is accounted for by the N2 transport system (CNT1-related). Three-min incubations were routinely performed because they reflect linear velocity conditions and provide enough sensitivity for *cis*-inhibition analysis. The dishes were washed three times in 2 ml of a cold stop buffer consisting of 137 mM NaCl and 10 mM HEPES, pH 7.4. Cells were then incubated with 0.5 ml of 0.5% Triton X-100 to allow disaggregation, and the resulting extract was used for radioactivity counting and protein determination following the Bradford technique (Bio-Rad).

RNA Isolation and Northern Blot Analysis—Total RNA (20 μg) isolated from macrophages using the Rneasy Mini kit from Qiagen (Hilden, Germany) was separated in 1% agarose with 20 mM MOPS, 1 mM EDTA, pH 7.4, and 3% formaldehyde buffer. The RNA was transferred overnight onto an Immobilon filter (Amersham Pharmacia Biotech) and cross-linked by irradiation with UV light. Filters were treated, prehybridized, and hybridized as described previously (31, 36, 37) using CNT2, ENT1, TNF-α, and 18 S cDNAs. The CNT2 and ENT1 probes corresponded to the rat cDNA fragments comprising nucleotides 776–1465 and 5–1375, respectively. Labeled probes were generated by random priming using [α-^{32}P]dCTP and routinely used at 10^6 cpm/ml. After high stringency washing (31, 36, 37), filters were exposed, and films were processed for densitometric analysis.

Loading and transfer controls were routinely used in all the experiments. Loading was assessed by including ethidium bromide in the samples to be run on the gels, whereas the 18 S RNA was used as a transfer control, as shown below.

Western Blot Analysis—Cell extracts for Western blot analysis were obtained after washing the cells twice in cold phosphate-buffered saline followed by lysis in 1% Nonidet P-40, 10% glycerol, 50 mM HEPES, pH 7.5, and 150 mM NaCl, supplemented with a protease inhibitor mixture consisting of 1 μg/ml aprotinin, 1 μg/ml leupeptin, 86 μg/ml iodoacetamide, and 1 mM phenylmethylsulfonyl fluoride. Protein was determined following the Bradford technique (Bio-Rad). Protein samples (20 μg) were incubated for 6 min at 95 °C prior to 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto filters (Immobilon-P, Millipore, New Bedford, MA), which were then blocked in a 5% dry milk-supplemented 0.2% Tween 20, phosphate-buffered saline prior to immunoreaction. To monitor CNT1 expression, a monospecific rabbit polyclonal antibody raised against an N terminus fragment (residues 45–67) of the corresponding rat horthologue was used (37, 38). This antibody specifically cross-reacted with the murine transporter as described previously (50). To monitor the expression of iNOS, a commercial rabbit antibody against mouse iNOS (M-19, Santa Cruz Biotechnology, Santa Cruz, CA) was used. As a loading and transfer control for this technique, a mouse anti-mouse β-actin antibody (Sigma) was used.

RESULTS

Effect of LPS Treatment on the Proliferation, Nucleic Acid Synthesis, and DNA Fragmentation of Murine Bone Marrow Macrophages—Treatment of murine bone marrow macrophages with LPS significantly decreased the cell number and induced the fragmentation of DNA and blockade of thymidine incorporation into DNA in 6 h (Fig. 1). The early apoptotic events triggered by LPS were mediated by the autocrine production of TNF-α by macrophages because cells isolated from TNF-α receptor I knock-out mice were protected from apoptosis after LPS treatment (Fig. 1). The late phase of apoptosis after 12 h of LPS treatment was shown previously to be NO-dependent (4) and occurs more slowly than the initial one. Both proliferating and apoptosing macrophages required extracellular nucleosides for RNA synthesis (Fig. 1).

Effect of LPS on the Nucleoside Transporter Activity and Expression in Murine Bone Marrow Macrophages—Murine bone marrow macrophages showed at least three nucleoside transport activities that correspond to the co-expression of the *es*-related transporter ENT1 and the Na^+-dependent transporters N1 (CNT2) and N2 (CNT1). This was assessed not only by the detection of the biological activity of these systems but also by the presence of either the mRNA (as for ENT1 and CNT2) or the protein (as for CNT1) (see Fig. 3). LPS selectively up-regulated the N1 and N2 transport systems irrespective of whether the macrophages had been MCSF-starved (Fig. 2).

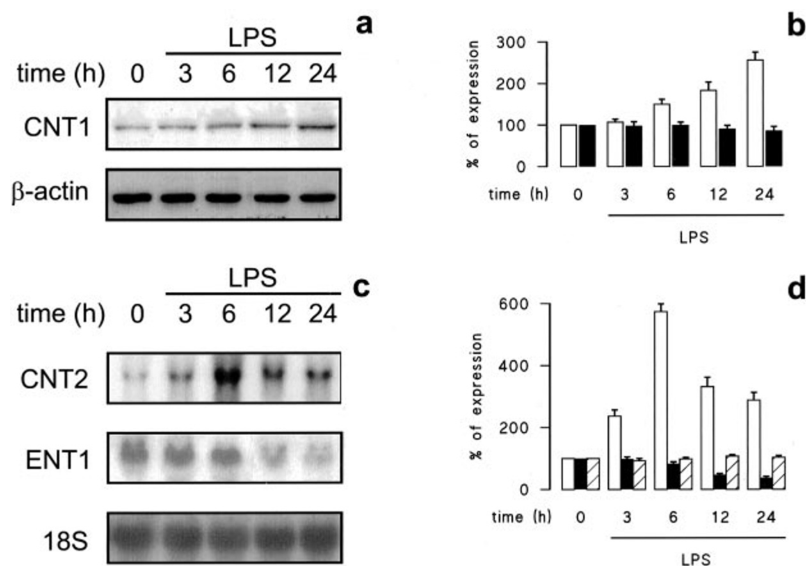
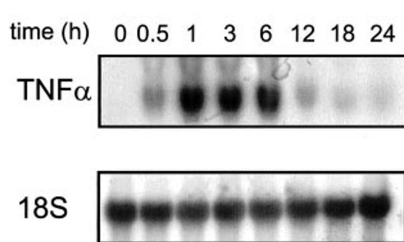


FIG. 3. **Time course of the effects of LPS on CNT1 protein and CNT2 and ENT1 mRNA amounts in macrophages.** Eighteen-h MCSF-starved macrophages were cultured in the presence of the growth factor either with or without LPS for the indicated times. *a*, the time course of CNT1 protein changes, as measured by Western blot, after the addition of LPS. The amounts of β-actin protein were also monitored as loading and transfer control. A representative experiment is shown. The densitometric data obtained from three independent experiments are shown in *b*. *Open bars* correspond to CNT1, whereas *solid bars* correspond to the β-actin control. *c*, the changes observed in CNT2 and ENT1 mRNA levels, as measured by Northern blot analysis, after LPS addition, including the 18 S RNA levels as loading and transfer controls. Similarly, the densitometric data obtained from four independent experiments are shown in *d* (*open bars*, CNT2 mRNA; *solid bars*, ENT1 mRNA; *dashed bars*, 18 S RNA).



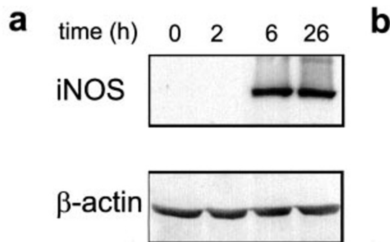
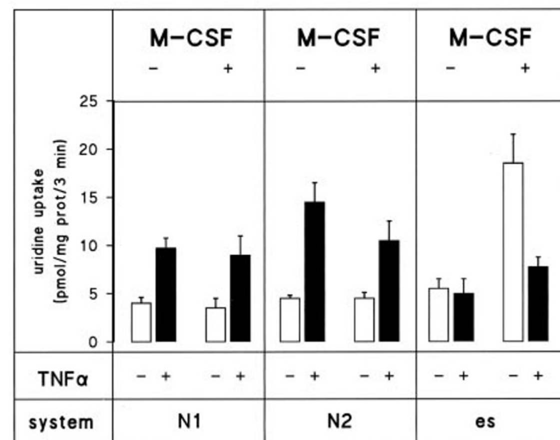


FIG. 4. **LPS triggered TNF-α expression and iNOS synthesis in macrophages.** In the culture conditions indicated in the legend for Fig. 3, the TNF-α mRNA levels and the amount of iNOS protein were analyzed by Northern and Western blots at the indicated times after LPS addition. As in Fig. 3, the 18 S RNA and the β-actin protein were used as controls. A representative experiment is shown; similar observations were obtained in three independent experiments.



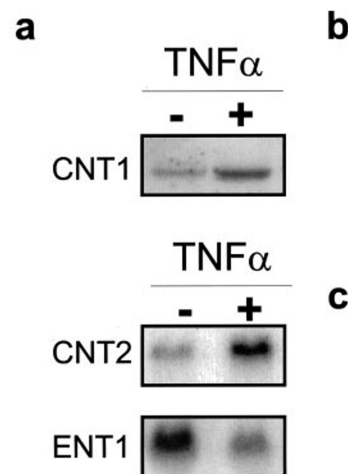


FIG. 5. **TNF-α mimicked the effects of LPS on CNT and ENT expression and related transport activities.** *a*, macrophages were again growth-arrested by culturing them for 18 h in the absence of MCSF, as described in the legend for Fig. 2. Cells were then induced to proliferate by MCSF or incubated in the absence of the growth factor for 24 h, either with (*solid bars*) or without (*open bars*) TNF-α (100 ng/ml). At this point, Na^+-dependent uridine (1 μM) transport activity associated with N1 and N2 transport systems (CNT2 and CNT1 transporters, respectively), as well as the Na^+-independent ENT1-related transport activity *es*, were measured. The amounts of CNT1 protein (*b*) and those of CNT2 and ENT1 mRNA (*c*) after 24 h of TNF-α treatment were also monitored. A representative experiment is shown, although identical observations were made in three independent experiments.

This suggests that the up-regulation of the concentrative nucleoside transporters is independent of the proliferative stimulus triggered by MCSF. However, the up-regulation of the *es* transport system triggered by MCSF was almost completely blocked by LPS, thus suggesting that the inhibition of macrophage proliferation associated with cell activation and apoptosis does not require enhanced *es* transport activity, although cell proliferation does, as described previously (50).

The time course of these events is relatively short (Fig. 3). CNT2 mRNA amounts increased only 3 h after LPS treatment (Fig. 3, *c* and *d*), which suggests that *CNT2* is one of the genes that may switch on during the early phase of LPS-induced apoptosis of murine bone marrow macrophages. The transient accumulation of CNT2 mRNA shown in Fig. 3, *c* and *d* is consistent with the transcriptional activation of this gene resulting in *de novo* synthesis of CNT2 transporters and high activity of the N1 transport system, as detected 24 h after LPS treatment (Fig. 2). Similarly, the amounts of CNT1 protein are significantly higher than control macrophages as early as 6 h after LPS treatment (Fig. 3, *a* and *b*), thus supporting the view that the two CNT transporters expressed in murine bone marrow macrophages are highly responsive to LPS-induced apoptosis and, if these regulatory responses were transcriptionally mediated, their genes should switch on relatively quickly after treatment with the lipopolysaccharide. The decrease in *es* transport activity found in LPS-treated macrophages in the presence of MCSF correlated with a progressive decrease in the ENT1 mRNA that occurred much more slowly than the changes recorded for the CNT2 mRNA (Fig. 3, *c* and *d*), which suggests that the regulatory response of the equilibrative nucleoside transporter mostly occurs during the second phase of LPS-induced apoptosis.

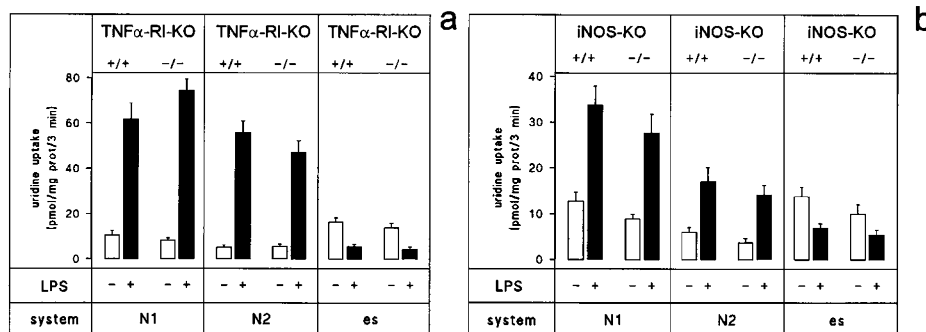


FIG. 6. **Evidence for TNF-α and iNOS-independent mechanisms mediating the regulatory responses of nucleoside transport systems to LPS in macrophages.** N1, N2, and *es* nucleoside transport activities were measured in macrophages that had been starved for 18 h in the absence of MCSF and then incubated for a further 24 h in the presence of the growth factor, either with (*solid bars*) or without (*open bars*) LPS. To determine whether TNF-α and/or iNOS mediate the effects of LPS on these transport systems, macrophages used in these experiments were obtained from knock-out mice lacking the p55 receptor for TNF-α (*TNF-α-RI-KO*) or iNOS (*iNOS-KO*). Data are the mean ± S.E. of triplicate observations in three independent experiments. RI, receptor I; *prot*, protein.

Characterization of TNF-α and iNOS Production after LPS Treatment of Murine Bone Marrow Macrophages—LPS rapidly increased the expression of TNF-α in macrophages (Fig. 4*a*). As previously reported, this is immediately followed by the secretion of TNF-α, as deduced from the measurement of this cytokine in supernatants from LPS-treated macrophages (4). However, iNOS synthesis takes longer to occur, which is in agreement with its role during the second phase of the LPS-induced apoptosis (Fig. 4*b*). In fact, the iNOS protein was not detected 2 h after LPS treatment, which in principle would rule out a putative role for NO in the regulatory responses of the concentrative nucleoside transporters because the two CNT proteins appear to be up-regulated before iNOS synthesis occurs.

Role of TNF-α in the LPS-mediated Regulation of Nucleoside Transporters in Murine Bone Marrow Macrophages—The immediate autocrine production of TNF-α by LPS-stimulated macrophages suggests that this cytokine is involved in the regulatory responses of nucleoside transporters during the early phase of LPS-induced apoptosis. Thus, the activity of the three macrophage nucleoside transport systems was measured after treatment of the cells with TNF-α either in the presence or in the absence of MCSF. TNF-α alone mimicked the LPS-induced changes in the activity of nucleoside transport systems N1, N2, and *es* (Fig. 5*a*). In fact, TNF-α blocked the up-regulation of the *es* transport system triggered by MCSF. TNF-α also increased the amounts of CNT1 protein (Fig. 5*b*) and the expression of CNT2 mRNA (Fig. 5*c*). In contrast, ENT1 mRNA amounts decreased (Fig. 5*d*). These observations, taken together, would suggest that TNF-α may be the only mediator of the LPS-induced effects on nucleoside transporter expression. To test this hypothesis, similar experiments were performed using bone marrow macrophages derived from TNF-α receptor I knock-out mice (Fig. 6*a*). As previously reported (4), the type I p55 TNF-α receptor is solely responsible for TNF-α-induced apoptosis in macrophages. In fact, the early apoptotic events triggered by the autocrine production of this cytokine are completely blocked in LPS-stimulated macrophages from TNF-α receptor I knock-out mice. Interestingly, the regulatory responses of nucleoside transport systems N1, N2, and *es* were unchanged in LPS-treated macrophages (Fig. 6*a*), which rules out the idea that TNF-α is the only mediator of the action of LPS on nucleoside transport performance. Although iNOS is unlikely to be involved in the up-regulation of CNT-related transport systems because there is a lack of concordance between the time course of their up-regulation, it was possible that NO could be involved in the slow down-regulation of the *es* transport system. Thus, the response of these transport systems to LPS was also investigated in bone marrow macrophages derived from iNOS knock-out mice. No changes in the up-regulation of N1 and N2 transport systems were observed in the iNOS−/− macrophages after exposure to LPS nor was the decrease in *es* transport activity affected (Fig. 6*b*). Similar results were obtained in macrophages derived from CAT2 knock-out mice (not shown).

DISCUSSION

Although LPS is an activator of macrophages that induces the secretion of several cytokines (including TNF-α, interleukin-1, and interleukin-6), it is also a potent apoptotic agent (4). This activity occurs mostly in the early phase of interaction with LPS and after the release of TNF-α. In fact, macrophages synthesize and secrete proinflammatory cytokines during septic shock (4, 39, 40), and thus treatment of primary cultures of murine bone marrow macrophages with LPS results in a two-step process leading to apoptosis, triggered initially by the autocrine production of TNF-α and followed by the generation of NO as a consequence of the *de novo* synthesis of iNOS (Ref. 4; this study).

Nucleosides, and particularly adenosine, can inhibit TNF-α and NO synthesis in LPS-stimulated and IFN-γ-induced macrophages (7–12, 19) by mechanisms that rely, at least partially, upon the interaction of adenosine with membrane receptors. The long term control of these regulatory interactions appears to involve receptor synthesis and insertion into the plasma membrane of the activated macrophage, as suggested by evidence that IFN-γ can up-regulate the synthesis of adenosine receptors of the A2B type (20). This sort of feedback regulatory mechanism may also apply to ATP and P2 receptors, as indicated above (7, 9–11, 21, 22).

This study demonstrates that LPS-induced macrophages differentially regulate nucleoside transporter activity and expression and that this occurs in two steps; rapid up-regulation of the nucleoside transporters of CNT type N1 and N2 is followed by a slower decrease in ENT1 expression, thus resulting in low *es* transport activity. The inhibition of *es* transport activity and ENT1 mRNA levels by LPS is evident only when macrophages are actively proliferating in the presence of MCSF, which further supports the recently described role of the ENT1 transporter in macrophage proliferation and DNA synthesis (50). Consequently, the blocking of MCSF-induced proliferation results in *es* down-regulation.

In general terms CNT-type transporters show a much higher affinity for nucleosides than the equilibrative ENT-type carriers (24–26). In addition, it has recently been shown that cells with high endogenous equilibrative transport activities can be

highly sensitized to nucleoside-derived cytotoxic drugs simply by heterologously expressing a CNT isoform, irrespective of whether they were also substrates for the ENT carriers already expressed (41). Moreover, it is well established that high affinity neurotransmitter transporters are involved in the modulation of neurotransmitter availability to receptors in synaptic transmission (42, 43), which further supports the view that high affinity transporters are better regulators of agonist availability than low affinity carriers. This is consistent with the need of very low concentrations of agonists to induce the receptor-mediated responses, which may be much lower than most reported apparent K_m values of other transporters with similar substrate specificities but are not involved in synapsis. If CNT transporters determine adenosine availability to those receptors expressed in macrophages, the up-regulation of CNT transporter expression and activity by either LPS or IFN-γ may contribute to the feedback mechanisms that determine the complex regulatory network of activation/deactivation and apoptosis pathways in macrophages. This is consistent with the consideration of the two CNT genes as "early response" genes during LPS-induced apoptosis. The possibility that up-regulation of nucleoside transporters also affects the intracellular nucleoside/nucleotide pool in a way that may modulate the apoptotic response of macrophages should not be ruled out and requires further research.

The evidence that CNT transporters are involved in the early apoptotic events in LPS-induced macrophages also suggests that they are putative targets in anti-inflammatory therapy. Although no effective irreversible inhibitors have been described for the CNT-type transporters, these carriers have broad substrate specificity, as demonstrated when analyzing the routes for the cell uptake of nucleoside derivatives used in antiviral and anticancer therapy (44–46). This suggests that the transporter tolerance of chemical modification of the natural substrates may ultimately result in the design of suitable transporter inhibitors of pharmacological interest.

This study has also addressed the role of TNF-α and NO as mediators of the effects of LPS on CNT and ENT transporters. NO was initially a poor candidate for mediating LPS action based upon the time course of CNT induction and that of iNOS synthesis (Ref. 4; this study). Nevertheless, NO could play a role in the longer term down-regulation of ENT1, as demonstrated in B cells (47). This is not the case according to the results obtained using the iNOS and CAT2 knock-out macrophages. The possibility that TNF-α mediates the phorbol 12-myristate 13-acetate-induced up-regulation of N-type transporters in human B cells was indicated by the finding that it could increase the concentrative nucleoside transport activity in Raji cells (36). However, from this study it appears that, although TNF-α may contribute to the LPS-induced early apoptotic events that lead to the up-regulation of the CNT-transporters, this cytokine is not required for this specific action because macrophages from TNF-α receptor I knock-out mice still respond to LPS treatment by increasing CNT1 and CNT2 transporter activity and expression, although activation of p55 type I TNF-α receptor is necessary and sufficient for apoptosis in a variety of cell types (4–6, 48, 49).

In summary, this study shows that LPS-induced apoptosis of murine bone marrow macrophages is characterized by a rapid increase in the concentrative nucleoside high affinity transport systems N1 and N2, a feature that is consistent with the *de novo* synthesis of CNT1 and CNT2 carrier proteins. This regulatory response occurs during a TNF-α-dependent early phase of LPS-induced apoptosis in macrophages, but although TNF-α by itself can also up-regulate CNT transporters and thus may be considered as a mediator of LPS action, a TNF-α-independent pathway could also be involved. Work is in progress to elucidate the TNF-α-independent pathway of LPS activation of concentrative nucleoside transporters. These data, together with previous observations linking nucleoside and nucleotide receptors with cytokine production by macrophages, suggest that nucleoside transporters are also elements of this complex regulatory network that determines macrophage activation/deactivation and apoptosis processes.

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