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Intact noradrenaline transporter is needed for the sympathetic fine-tuning of cytokine balance

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Abstract

Earlier studies demonstrated that cytokine production is under the tonic control of noradrenaline. As the level and/or the duration of noradrenaline action is regulated by the noradrenaline transporter (NET), which is also a target of antidepressant treatment, we studied its role in the regulation of the cytokine response during inflammation. The endotoxin-evoked tumour necrosis factor-alpha (TNF-α) and interleukin-10 response was studied in genetically produced noradrenaline transporter-deficient (NET-KO) mice and by treatment with desipramine, a monoamine uptake-blocker antidepressant. NET-KO mice responded to endotoxin with significantly lower TNF-α and interleukin-10 production in comparison to their wild-type counterparts. Functional involvement of both α- and β-adrenoceptors could be demonstrated in our model systems, using 7,8-methylenedioxy-14α-hydroxy-alloberbane·HCl (CH-38083) and propranolol; however, the differences between the two phenotypes remained, suggesting a limited role of α-adrenoceptors in the observed changes. Acute treatment of both wild-type and NET-KO mice with desipramine significantly decreased the TNF-α response and significantly increased interleukin-10 production, indicating the role of an intact noradrenaline transporter in anti-inflammatory responses.
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Keywords: Noradrenaline uptake; Cytokine production; Antidepressants; NET-KO, mouse

1. Introduction

Cytokines are involved in a number of physiological and pathological processes, including cell–cell communication and promotion of cell proliferation, survival, cell death. Although the major source of cytokines is attributed to cells of the immune system, cytokines are also produced by neuronal cells in the central nervous system (CNS) (Aloisi et al., 1992; Szelenyi, 2001; Turrin and Plata-Salaman, 2000). Pro-inflammatory cytokine production, including tumour necrosis factor-alpha (TNF-α), might be induced by infection (due to the bacterial endotoxin, lipopolysaccharide) or by cell damage. The production of TNF-α is normally under the restraining influence of anti-inflammatory mechanisms and is regulated by other cytokines such as interleukin-10 (Barsig et al., 1995). The sympathetic nervous system, i.e., the level of monoamines, is also known to be a potent regulator of TNF-α and interleukin-10 production in the organism (Elenkov et al., 1995; Hasko et al., 1995; Szelenyi et al., 2000a,b,c). Sympathetic nerve-endings innervating both primary and secondary lymphoid organs (Felten et al., 1985) release mainly noradrenaline into the surroundings of immune cells and TNF-α production is under the tonic control of noradrenaline released from these sympathetic varicosities (Elenkov et al., 2000; Vizi, 1998).

The modulatory effect of noradrenaline on cytokine production is mediated mainly via β_2-adrenoceptors expressed on macrophages (Elenkov et al., 1995). This action of noradrenaline is highly dependent on its biophase concentration around the targets, i.e., on the release and disappearance of noradrenaline, similar to the activatory/restraining mechanisms of cytokine production. This is regulated by negative feedback modulation via presynaptic α_2-adrenoceptors (Elenkov and Vizi, 1991; Vizi and Elenkov, 2002; Vizi and Labos, 1991). The action of noradrenaline is terminated in part by its disappearance by uptake into presynaptic noradrenergic neurons by the plasma membrane noradrenaline transporter (NET) (Amara and Kuhar, 1993).

In our previous work, we studied the modulatory role of noradrenaline in the lipopolysaccharide-induced cytokine response on the release side in normal and reserpine-pre-

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treated mice (Szelenyi et al., 2000a,b). It was found that the reduction in sympathetic outflow induced by reserpine, which is in fact an animal model of depression, dramatically increased lipopolysaccharide-induced TNF-α production. As the level and/or the duration of noradrenaline action are regulated by the noradrenaline transporter on the uptake side, we theorized that the noradrenaline transporter plays a crucial role in the regulation of the cytokine response during inflammation. For this reason, in the present paper, we studied the role of noradrenaline reuptake in two model systems: in noradrenaline transporter-deficient mice, where the NET gene was genetically disrupted (NET-KO or $NET^{-/-}$ mice), and in mice in which the action of NET was blocked by pretreatment with desipramine, a NET inhibitor psychostimulant.

2. Materials and methods

2.1. Animals

Wild-type mice (WT-129) and their genetically modified $NET^{-/-}$ counterparts (NET-KO) were obtained from the laboratory of M.G. Caron (Xu et al., 2000). For some experiments, Balb/c mice were purchased from Charles River Laboratories (Budapest). Mice were kept in individual cages in the Animal Unit for at least 7 days before use. Animals received food and water ad libitum and lighting was maintained on a 12-h cycle. All procedures were carried out in accordance with the European Community guidelines for the use of experimental animals and those of the institutional ethics committee.

2.2. Experimental design

One day before the experiment, mice were weighed and placed in individual cages in the experimental room. On the day of the experiment, animals were injected intraperitoneally (i.p.) with drugs at 1 ml/100 g body weight, 60 min before the administration of lipopolysaccharide (10 mg/kg i.p.). Control animals received the same diluent as vehicle and the same volume of saline. Groups were formed of five to eight mice and five independent experiments were carried out. Animals were killed 90 min after lipopolysaccharide administration. Blood was collected in ice-cold Eppendorf tubes containing heparin and centrifuged for 10 min at 4 °C at 1500 rpm. The plasma was stored at − 70 °C until analysed.

2.3. Drugs and reagents

Lipopolysaccharide from *Escherichia coli* 055:B5 (Sigma) was dissolved in distilled water; the mixture was sonicated for at least 3 min in a sonicating bath and aliquots were stored at − 70 °C until use. On the day of the experiment, appropriate dilutions were made in saline. Adrenoceptor antagonists, unless otherwise stated, were injected i.p. at doses of 10 mg/kg. 7,8-Methylenedioxy-14α-hydroxy-alloberbane·HCl (CH-38083) was obtained from Chinoin (Budapest) and used as a selective α_2-adrenoceptor antagonist (Vizi et al., 1986). The β-adrenoceptor antagonist propranolol and the tricyclic antidepressant desipramine were purchased from Sigma.

2.4. Analysis of plasma TNF-α and interleukin-10 levels

Solid-phase enzyme immunoassay that specifically detects murine TNF-α or interleukin-10 (R&D Systems, USA) was used with a detection limit of ≤ 16 pg/ml. Absorbance was read by Bio-Rad Microplate Reader Model 450 and calculated as concentration (pg/ml), using a standard curve by Microplate Manager/PC Data Analysis Software.

2.5. Statistical evaluation

Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test or by two-tailed Student's *t*-test where appropriate $P < 0.05$ was considered as statistically significant ($^*P < 0.05$).

3. Results

3.1. Lipopolysaccharide-induced cytokine production in NET-KO mice

In the series of experiments in which the effect of the targeted disruption of the NET-gene on cytokine production was studied, we administered lipopolysaccharide i.p. to wild-type and $NET^{-/-}$ mice. Injection of lipopolysaccharide caused an elevation of plasma TNF-α and interleukin-10 levels from their initial, undetectable levels (not shown) in both genotypes but to a different extent (Fig. 1). We could

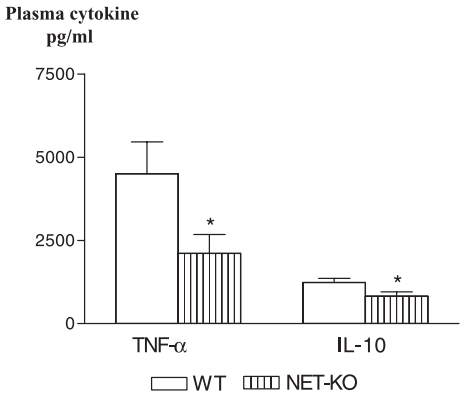

Fig. 1. Lipopolysaccharide-induced cytokine production in NET-KO mice. Cytokine response was compared 90 min after i.p. injection of lipopolysaccharide (10 mg/kg) into WT-129 and NET-KO mice. Data are expressed as means ± S.E.M. for 8–10 animals per group. *Indicates significance at level $P < 0.05$.

demonstrate significantly lower TNF-α production in the NET-KO genotype in comparison to their wild-type counterparts. The TNF-α levels in WT-129 mice corresponded to our previous data, 4500.77 ± 956 pg/ml in plasma 90 min after administration of 10 mg/ml lipopolysaccharide, while under the same conditions the plasma level in the NET-KO mice reached only 2106.75 ± 569 pg/ml. A significant difference was also observed in the lipopolysaccharide-evoked interleukin-10 production of the two genetic variants (1231.84 ± 129.79 pg/ml for the WT and 824.35 ± 126.47 pg/ml for the NET-KO variant (Fig. 1)).

3.2. Modulation of TNF-α and interleukin-10 production with α_2- and β-adrenoceptor antagonists (CH-38083 and propranolol) in WT-129 and NET-KO mice

The functional integrity of α_2-adrenoceptors is crucial for the regulation of noradrenaline release. To examine whether α_2-adrenoceptors are functioning normally in NET-KO mice, we compared the effect of the selective α_2-adrenoceptor antagonist CH-38083 on lipopolysaccharide-evoked cytokine production in the wild-type and in the genetically modified groups of mice. CH-38083 was administered to WT-129 and NET-KO mice 60 min before the lipopolysaccharide challenge. In the WT-129 group, the inhibition of the negative feedback regulation of noradrenaline by CH-38083 resulted in a significant decrease in TNF-α production accompanied by a significant increase in interleukin-10 production, similar to that in Balb/c mice (data not shown). The same results were obtained in the NET-KO group for both cytokines, indicating that in this respect the α_2-adrenoceptors were functionally intact (Table 1).

The regulation of cytokine production in macrophages by the sympathetic nervous system is achieved via the β_2-adrenoceptors expressed on the surface of these cells (Elenkov et al., 1995). In the next series of experiments, we tested whether this type of regulation is unaffected or changed in the genetically manipulated NET-KO mice. Administration of 10 mg/kg propranolol to the WT-129 and NET-KO mice resulted in a significant increase in lipopolysaccharide-induced TNF-α production in both groups. It is remarkable

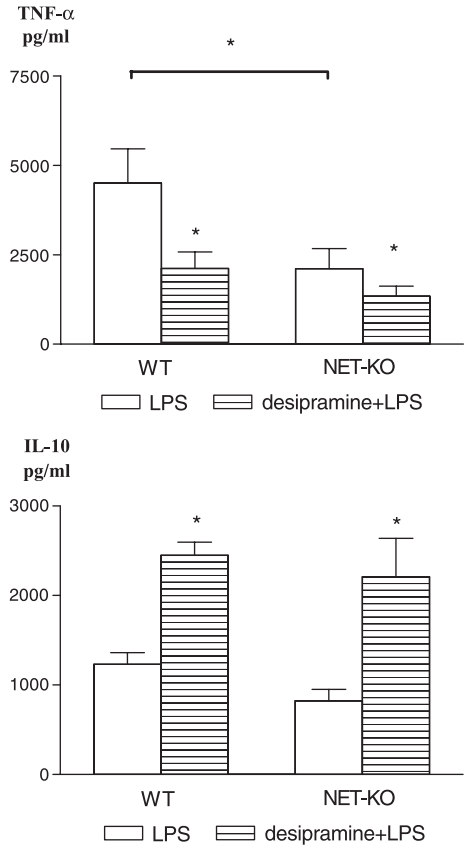

Fig. 2. The effect of acute administration of the monoamine uptake blocker desipramine on the cytokine response of WT-129 and NET-KO mice. Desipramine (10 mg/kg) was administered i.p. to WT-129 and to NET-KO mice 60 min before lipopolysaccharide treatment. Cytokine levels were measured 90 min after the lipopolysaccharide injection. Data are expressed as means ± S.E.M. for 8–10 animals per group. *Indicates significance at level $P < 0.05$.

that under the same conditions the effect of propranolol was significantly greater in the NET-KO mice (389.8%) than in their wild-type counterparts (151.2%), or in the Balb/c strain (data not shown). As was expected, propranolol attenuated the lipopolysaccharide-evoked interleukin-10 response in both groups. The inhibitory effect of propranolol on interleukin-10 production was much more pronounced in the

Table 1
Modulation of lipopolysaccharide-evoked TNF-α and interleukin-10 production by α_2- and β-adrenoceptor antagonists (CH-38083 and propranolol) in WT-129 and NET-KO mice

	TNF-α (pg/ml)		Interleukin-10 (pg/ml)	
	WT	NET-KO	WT	NET-KO
Control	4500.77 ± 956.0	2106.75 ± 569.0	1231.85 ± 129.8	824.35 ± 126.5
CH-38083	1621.30 ± 170.2[c]	601.75 ± 189.9[b]	3277.75 ± 401.8[b]	2910.93 ± 736.8[b]
Propranolol	6808.90 ± 1147.3[a]	8210.00 ± 1349.4[a]	520.56 ± 146.8[b]	570.80 ± 90.2

CH-38083 (10 mg/kg) was injected i.p. 60 min before lipopolysaccharide injection. Propranolol (10 mg/kg) was injected i.p. 60 min before lipopolysaccharide injection. TNF-α and interleukin-10 were measured in the blood plasma 90 min after lipopolysaccharide administration. Data are expressed as means ± S.E.M. for 8–10 animals per group.

[a] Indicates significance at level $P < 0.05$.

[b] Indicates significance at level $P < 0.01$.

[c] Indicates significance at level $P < 0.005$.

WT-129 group than in the NET-KO group (61.5% and 34.3%, respectively) (Table 1).

3.3. The effect of acute administration of the monoamine uptake blocker desipramine on the cytokine response of WT-129 and NET-KO mice

A single dose of the tricyclic antidepressant desipramine was administered to the WT-129 and NET-KO mice i.p. 60 min before the lipopolysaccharide challenge. This inhibition of the NET resulted in a significant decrease in the TNF-α response in both groups; however, the blockade of noradrenaline reuptake by desipramine resulted in a more pronounced decrease in the WT-129 group than in the NET-KO group (53.1% vs. 66.4%) (Fig. 2). Interleukin-10 production was significantly increased by desipramine treatment both in the wild-type (181.3%) and in the genetically modified NET-KO groups (254.5%). As shown in Fig. 2, a tendency for a more pronounced, but not significantly higher, interleukin-10 response in the NET-KO group could also be observed.

4. Discussion

The main finding of this study is that lipopolysaccharide-induced cytokine production is impaired in mice when the noradrenaline transporter is missing or blocked (Fig. 3). This result confirms our previous hypothesis that, besides noradrenaline release, the duration of the extracellular presence of noradrenaline is also an important factor in its immunomodulatory effect. As noradrenaline uptake is frequently blocked during treatment of patients with psychological disorders, the immunological consequences of these therapies cannot be neglected. In our studies, the two model systems, i.e., the noradrenaline transporter deficient mice, where the noradrenaline gene was genetically disrupted, and the desipramine-treated mice, where the action of NET was blocked chemically, showed considerable correspondence although there were some dissimilarities that added further points to the understanding of the underlying mechanisms.

The level of certain neurotransmitters, like monoamines, might be elevated because of infections, and thus they are able to modulate the immune response (Dunn, 1992; Hall and Hodge, 1971). However, a decreased level of monoamines is causally related with depression (Leonard, 1993). The extracellular concentration of these neurotransmitters is fine-tuned by the balance between their release and uptake via the appropriate regulatory mechanisms present in the sympathetic nervous system. In previous studies, we demonstrated that abolishment of the sympathetic outflow by reserpine treatment of mice impaired their lipopolysaccharide-induced cytokine production (Szelenyi et al., 2000b,c).

One of the most characteristic events following lipopolysaccharide administration is the elevation of the level of pro-inflammatory cytokines, e.g., TNF-α, whereas previously these were present at an almost undetectable level in blood. Lipopolysaccharide treatment is accompanied by the induction of the anti-inflammatory cytokine interleukin-10 too. These two cytokines are able to regulate reciprocally the production of each other; i.e., TNF-α induces interleukin-10 production and the induced interleukin-10 is able to downregulate TNF-α production (Barsig et al., 1995). Data from the literature, and also our previous work (Elenkov et al., 1995; Hasko et al., 1995; Madden et al., 1995; Nelson, 1982; Severn et al., 1992; Szelenyi et al., 2000c), showed that the production of these cytokines is under the control of the cyclic AMP (cAMP) signalling pathway. Generally, an increased cAMP level results in a decreased pro-inflamma-

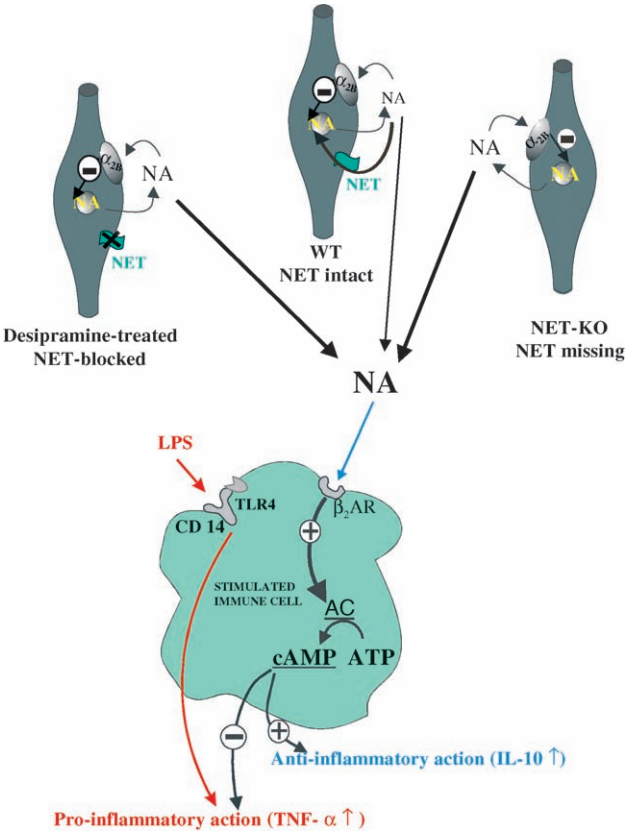

Fig. 3. Genetically or chemically disrupted noradrenaline uptake shifts the cytokine response. The biophase level of noradrenaline is fine-tuned by its release from non-synaptic varicosities and by its uptake through NET. This balance is demonstrated in the case of WT-129 mice where NET is intact. Genetically abolished (NET-KO) or chemically inhibited (desipramine treated) NET results in an increase in the extracellular level of noradrenaline, thereby prolonging its action (represented by larger letters and thicker arrows than in case of intact NET). Extracellular noradrenaline controls the equilibrium between pro-inflammatory and anti-inflammatory cytokines, mainly via the β_2-adrenoceptors expressed on the surface of stimulated immune cells, especially on macrophages. The higher the level of noradrenaline is, the lower is the production of TNF-α, accompanied by a higher level of interleukin-10. Abbreviations: NA, noradrenaline; NET, noradrenaline transporter; LPS, bacterial lipopolysaccharide; CD14, lipopolysaccharide receptor; TLR4, CD14 associated Toll-like receptor 4; β_2AR, β_2-adrenoceptor; AC, adenylate cyclase; cAMP, adenosine 3′,5′-cyclic monophosphate; ATP, adenosine triphosphate; TNF-α, tumour necrosis factor-alpha; IL-10, interleukin-10.

tory cytokine production, accompanied by an elevation in the level of the anti-inflammatory cytokines, especially that of interleukin-10 (Benbernou et al., 1997).

It should be mentioned, however, that there are also some exceptions (e.g., in case of reserpine treatment (Szelenyi et al., 2000b) or for the dopamine D2 receptor (Hasko et al., 1996)) where a change in the cAMP level does not counter-regulate TNF-α and interleukin-10 production. These data indicate that lipopolysaccharide-induced cytokine production is under the control of a more complex interplay of neuroimmune pathways.

As the action of noradrenaline is realised dominantly via β_2-adrenoceptors in the monocyte/macrophage system (Elenkov et al., 1995), the prolonged presence of noradrenaline results in an increased cAMP level. The decrease in TNF-α production that is due to the inappropriate noradrenaline uptake, observed in both the NET-KO (Fig. 1) and in the desipramine-treated (Fig. 2) model systems, can easily be explained by this common mechanism. In this respect, the NET-KO mice, where the reuptake system was absent, behaved similarly to their acute antidepressant-treated wild-type counterparts. In contrast to lipopolysaccharide-induced TNF-α production, the interleukin-10 response in the NET-KO model and that in the desipramine model were different. Considering the increased extracellular noradrenaline level (Xu et al., 2000) and the decreased TNF-α production in the NET-KO strain, an elevated interleukin-10 production would have been predicted. But this was not the case. In contrast to this expectation, the interleukin-10 production in NET-KO mice was significantly less than that observed in their wild-type counterparts (Fig. 1).

Therefore, in order to interpret the difference that was observed between our two model systems, other viewpoints should also be taken into account. Because in genetically modified KO animals compensatory mechanisms are frequently observed, perturbation of other monoamine-uptake systems (e.g., the dopamine-transporter) cannot be ruled out in the NET-KO model (Moron et al., 2002). It has been shown that the absence of the NET gene is accompanied by supersensitivity of dopamine D2/D3 receptors (Xu et al., 2000), and a decrease in cAMP level due to the activation of dopamine D2/D3 receptors might explain their lower interleukin-10 response. Although the action of dopamine prevails partly through β_2-adrenoceptors, as was shown in certain cell lines in vitro (Hasko et al., 2002), the changes demonstrated in the NET-KO mice argue for the role of neuronal dopaminergic receptors in this case.

Another possible explanation for the interleukin-10 production being lower than expected in NET-KO mice is that the regulation of interleukin-10 production by cAMP might be realised through different mechanisms (Platzer et al., 2000). Finally, as TNF-α has a role in the initiation of interleukin-10 production, the lower TNF-α level might also contribute to the observed lower interleukin-10 production.

The functional input of both α_2- and β-adrenoceptors in the NET-KO mice could be demonstrated in our experiments. As shown in Table 1, the selective α_2-adrenoceptor antagonist CH-38083 was able to decrease the TNF-α response and increase the interleukin-10 response, in both the wild-type and in the NET-KO mice strains, although the phenotypic difference remained. Pretreatment of wild-type and NET-KO mice with the β-adrenoceptor antagonist propranolol resulted in an increased TNF-α and a decreased interleukin-10 response in both strains of animals. Propranolol, however, affected TNF-α production in the NET-KO mice much more intensively, and its effect on interleukin-10 production was only marginal, compared to that in the wild-type group. This supports our assumption that the regulation of the interleukin-10 response by the monoamine system is not completely clarified and is the result of a fine balance of the different mechanisms.

In contrast to the NET-KO model, the desipramine model conformed to expectations, i.e., the decreased TNF-α production was accompanied by a significantly higher amount of interleukin-10 in comparison to the appropriate control group, both in the wild-type and in the NET-KO mice (Fig. 2). It is also worth noticing that the same single-dose desipramine treatment increased the interleukin-10 production of the NET-KO mice to a similar, if not higher, extent, demonstrating that the inducibility of this cytokine under the conditions of blocked noradrenaline uptake remained intact in these animals. This finding is in good agreement with the increase in extracellular noradrenaline induced by i.p. administration of desipramine observed by Reith et al. (1997) and with the preventive effect of desipramine on the consequences of chemical sympathectomy induced by 6-hydroxydopamine administration (Madden et al., 1994).

The correlation between antidepressant treatment and immunological changes is the subject of many studies (Leonard, 2001b; Szelenyi and Selmeczy, 2002; Yirmiya, 2000). Most data available concern chronic (3–4 weeks long) treatment with tricyclic antidepressants, because it takes time for them to be effective (Kubera et al., 2000; Maes et al., 1999). In certain aspects, the permanent blockade of the monoamine uptake system could be better compared to the ablation of the NET gene. Daily administration of desipramine to mice for one week was shown to reduce secretion of the anti-inflammatory cytokine interleukin-4 (Kubera et al., 2000), which is in good accordance with the decreased interleukin-10 response observed by us in the NET-KO animals (Fig. 1).

It is remarkable that our data on the acute effect of desipramine are in good accordance with those available for single-dose or short-term tricyclic antidepressant treatments. A single i.p. injection of desipramine increased the ability of mice to produce interleukin-10 (Kubera et al., 1998), as was shown in our experiments too (Fig. 2). It should be taken into account, however, that treatment with tricyclic antidepressants has pleiotropic effects (e.g., anticholinergic, Hennings et al., 1999; α_2-adrenoceptor downregulation, Garcia-Sevilla and Zubieta, 1986; Nickola et al., 2001; Smith et al., 1981; etc.) in the CNS and block of the monoamine uptake

systems. Desipramine may exert its antidepressant action also by interacting with other monoamine uptake systems, e.g., the dopaminergic and serotoninergic systems (Maes and Meltzer, 1995).

No additional effect was expected from desipramine in NET-KO mice, provided that noradrenaline was taken up exclusively by NET. But this was not the case. Desipramine treatment resulted in an additional reduction of TNF-α production (Fig. 2) in the NET-KO mice. Since desipramine is not a selective blocker of noradrenaline uptake, it seems likely that noradrenaline is taken up not only by NET, but also by other monoamine transporters (Moron et al., 2002).

Taken together, our results demonstrate that the concentration of extracellular noradrenaline and the duration of its presence are among the key regulators of the lipopolysaccharide-evoked cytokine response. Our experiments revealed that the monoamine uptake systems also fulfil an important immunomodulatory role. We conclude that the decreased availability of catecholamines and the increased level of pro-inflammatory cytokines, e.g., TNF-α (Ignatowski and Spengler, 1994; Leonard, 2001a; Maes et al., 1999; Mikova et al., 2001) are both involved in the development of depression.

Our results may have clinically important implications. States associated with acute or chronic hypo- or hyperactivity of the sympathetic nervous system might influence the susceptibility of the organism to certain autoimmune, allergic, infectious or malignant diseases, besides alterations in the CNS (Elenkov et al., 2000). Accordingly, pharmacological interventions, which influence monoamine release or uptake, may be important in the treatment not only of various neurological and psychiatric diseases but of immune-mediated diseases too. It is also important to emphasise that such treatments may change the immunological responses of the depressed patient.

Acknowledgements

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