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Expression of the TrkB neurotrophin receptor by thymic macrophages

O. GARCÍA-SUÁREZ*, J. HANNESTAD*, I. ESTEBAN, R. SAINZ, F. J. NAVES & J. A. VEGA *Departamento de Morfología y Biología Celular, Facultad de Medicina, Universidad de Oviedo, Oviedo, Spain*

SUMMARY

Increasing evidence suggests that some members of the neurotrophic factor family of neurotrophins could be implicated in the regulation of immune responses. Neurotrophins, as well as their tyrosine kinase signal-transducing receptors (the so-called Trk neurotrophin receptors), have been detected in different lymphoid tissues, although their cellular localization is not well known. In this study we used single and double immunohistochemistry to localize TrkB *in situ* in the rat thymus (in animals from 0 days to 2 years of age), in cytospin preparations of rat thymic cells, and in two mouse monocyte–macrophage cell lines (RAW 264.7 and J774A.1). We found TrkB protein expression in a subpopulation of cells in the corticomedullary junction, which simultaneously expressed the rat macrophage marker ED1. The density of TrkB-expressing cells increased with age, reaching maximal values at 2 years. Conversely, no evidence of TrkB protein expression could be found in dendritic cells, epithelial cells or thymocytes. Thymic macrophages in cytospin preparations, as well as in the mouse monocyte–macrophage cell lines, also expressed TrkB protein. Although the possible function of TrkB in the thymic macrophage remains to be clarified, present findings add further evidence to the proposed role of neurotrophins in the immune system.

INTRODUCTION

The neurotrophins are a group of trophic factors that are essential for development and maintenance of certain neuronal populations.[1,2] Since the mid-1990s, however, emerging evidence has suggested a possible role for these peptides in the immune system.[3,4] The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), NT-4/5 and NT-6.[2] Two different kinds of neurotrophin receptors have been described. The low-affinity neurotrophic receptor (or $p75^{LNGFR}$) binds all neurotrophins with similar affinity, although its functional role remains uncertain.[5,6] Conversely, the effects of neurotrophins on responding cells are mediated by the Trk family of high-affinity receptors (TrkA, B and C), which all possess tyrosine kinase activity that is considered to be essential for signal transduction.[7] TrkA binds preferentially NGF, TrkB binds BDNF and NT-4/5, while TrkC binds NT-3.[2]

Neurotrophins and their receptors have been detected at the mRNA or protein level in different mammalian and avian lymphoid organs[8–14] as well as in isolated lymphocytes,[15,16] and, *in vitro*, multiple effects of neurotrophins on lymphocytes have been reported, including cell proliferation and differentiation (reviewed in reference 3). The rat thymus contains mRNA for full-length and truncated TrkB.[8,11] Nevertheless, data available on the cell type expressing TrkB mRNA or TrkB are controversial. One group found TrkB mRNA in immature, cortical thymocytes,[15] while others observed it to be restricted mainly to stromal cells.[8,11]

The thymic stromal cell compartment comprises epithelial cells, dendritic cells, macrophages and cells derived from the neural crest mesenchyma.[17] As each of these cell types has a specific function in thymocyte maturation, it is of outermost interest to identify the thymic cells which express TrkB in order to elucidate the possible role of this receptor and its ligands in thymus physiology. Previous studies from our group,[9,18] and others[14] reported the expression of TrkB protein in macrophages of secondary lymphoid organs, although peritoneal macrophages lack TrkB mRNA.[8]

In this report we used single and double immunohistochemistry to localize and characterize the cells expressing TrkB protein in the rat thymus (in rats from 0 days to 2 years of age). We studied the expression of TrkB in cytospin preparations of rat thymic cells and in two mouse monocyte–macrophage cell lines (RAW 264.7 and J774A.1). TrkB was mainly detected in cells seen in the cortico-medullary border, which were identified as macrophages based on their morphology and expression of the rat macrophage marker ED1.[19] The density of these cells increased with rat age and reached maximum density at 2 years. Macrophages in cytospin preparations, as well as in the two cell lines analysed, also expressed TrkB. Conversely, no evidence of TrkB expression in thymic dendritic cells, epithelial cells or thymocytes could be found.

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*Both of these authors contributed equally to this paper.

Correspondence: J. A. Vega, Departamento de Morfología y, Biología Celular, Facultad de Medicina, Universidad de Oviedo, C/Julián Clavería, s/n, E-33006 Oviedo, Spain.

MATERIAL AND METHODS

Tissue treatment

Male Wistar rats of the following ages were used: embryonic day 18 (E18, $n=3$); 0 ($n=2$), 7 ($n=2$), 15 ($n=4$) and 30 ($n=5$) days; and 2 ($n=5$), 3 ($n=5$), 6 ($n=4$), 12 ($n=2$), 18 ($n=3$) and 24 ($n=4$) months. The thymuses were removed under deep chloral hydrate anaesthesia (350 mg/kg) and were fixed in Bouin's fixative for 24 hr. Paraffin-embedded tissue was cut in serial sections, 10 μm thick, mounted on microscope slides and processed for methylene-blue staining or indirect peroxidase immunohistochemistry.

One half of the thymus of one animal from the groups: 7 days, 30 days, 3 months and 6 months, was homogenized for cytospin immunohistochemistry as follows: the thymus tissue was minced with scissors and filtered through a stainless-steel screen. The cell suspension obtained was washed twice in RPMI-1640 (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (Sigma, Poole, Dorset, UK), and resuspended in the same medium. The presence of cells displaying TrkB and/or ED1 was analysed by immunohistochemistry on cytospin preparations (250 μl of a 10^6 cells/ml suspension).

Immunohistochemistry

Deparaffined and rehydrated sections were rinsed in 0·05 M Tris-buffered saline (TBS; pH 7·5) containing 0·1% bovine serum albumin and 0·2% Triton X-100. Endogenous peroxidase activity and non-specific binding were blocked with 3% H_2O_2 and 50% fetal calf serum (Sigma), respectively, and the sections were incubated overnight with the primary antibody in a humid chamber at 4°. We used a polyclonal anti-TrkB rabbit antibody, diluted 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA) which recognizes a sequence of the intracytoplasmic tyrosine kinase domain of the TrkB molecule (residues 794–808). To characterize the stromal cell of the rat thymus and to identify TrkB-expressing cells we used the following antibodies in serial sections: a rabbit polyclonal anti-S-100 protein antibody (Sigma; 1 μg/ml), as a marker of dendritic cells;[20] a mouse monoclonal antibody against ED1 (Serotec, Oxford, UK; 1:100) to identify macrophages;[19] a rabbit polyclonal antibody against PGP 9.5 (Biogenesis, Poole, UK; 1:1000) for neural crest-derived cells;[21] and a mouse monoclonal anti-pancytokeratins antibody (Sigma; 1:500) to identify epithelial cells.[22] Representative sections (three sections per animal, 100 μm apart) and cytospin preparations (six slides per animal) were processed for double immunolabelling of TrkB and ED1, incubating them with a mixture of the two antibodies (10 μl of each) overnight at 4°.

After incubation with the primary antibody, sections were incubated with peroxidase-labelled sheep anti-mouse or anti-rabbit antibody (Amersham, Little Chalfont, Bucks, UK) both diluted 1:100. For double immunostaining, sections were incubated for 1 hr at room temperature, with Texas red-labelled sheep anti-rabbit immunoglobulin G (IgG) and fluorescein isothiocyanate-conjugated sheep anti-mouse IgG, both diluted 1:100 (Amersham). Finally, tissue sections, cytospin preparations and cell lines were counterstained with haema-

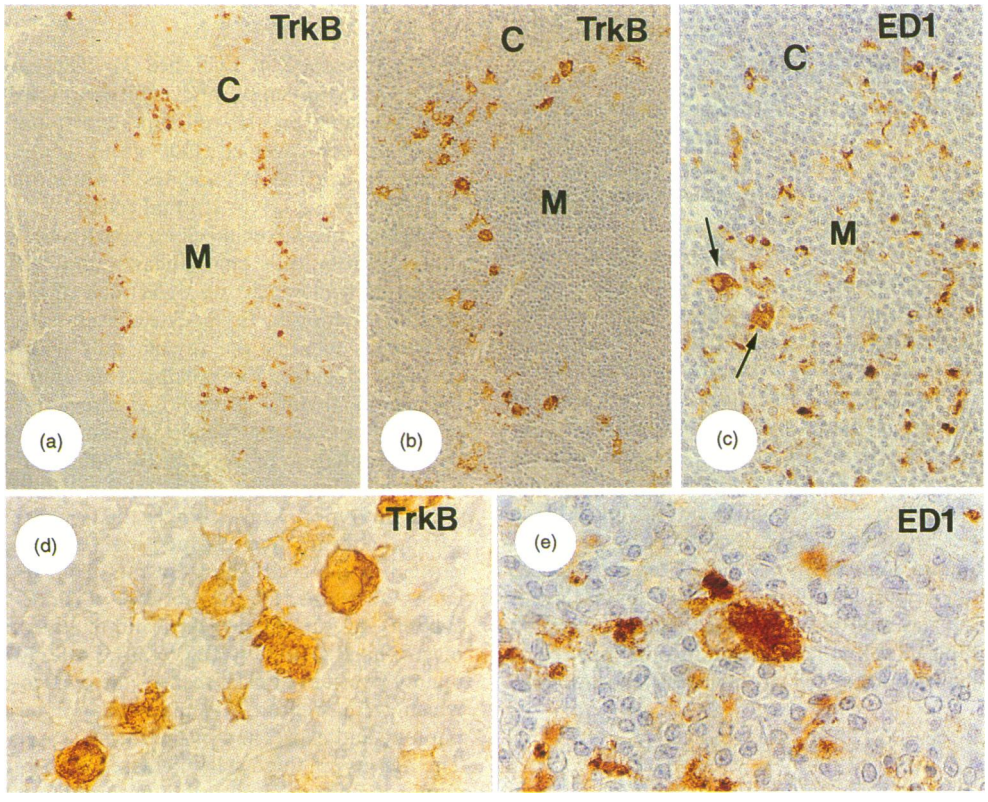


Figure 1. Immunohistochemical localization of TrkB-like protein (a, b, d) and ED1 (c, e) in the thymus of a 6-month-old rat. TrkB-like protein immunoreactive cells were large, irregular in shape and located mainly in the corticomedullary border. (a) ×70 magnification; (b, c) ×80 magnification; (d, e) ×720 magnification.

toxylin, and were photographed using a light microscope (Orthoplan, Leitz) or a confocal-laser scanning microscope (Bio-Rad MR-600; Servicio de Proceso de Imágenes de la Universidad de Oviedo).

Quantitative analysis

The area occupied by ED1 or TrkB immunoreactive cells was evaluated using a quantitative automatic image analysis system (MIP System, Servicio de Análisis de Imágenes, Universidad de Oviedo). Measurements were made on three sections, 200 μm apart, per specimen. The cortical and medullary areas in the entire sections were delimited manually and evaluated separately; the cortico-medullary border was included in the medulla. The percentage of the immunoreactive area was calculated automatically and the data expressed as mean ± standard error of the mean (SEM).

Monocyte–macrophage cell lines

The mouse monocyte–macrophage cell lines, RAW 264.7 and J774A.1[23] were used in order to investigate whether they

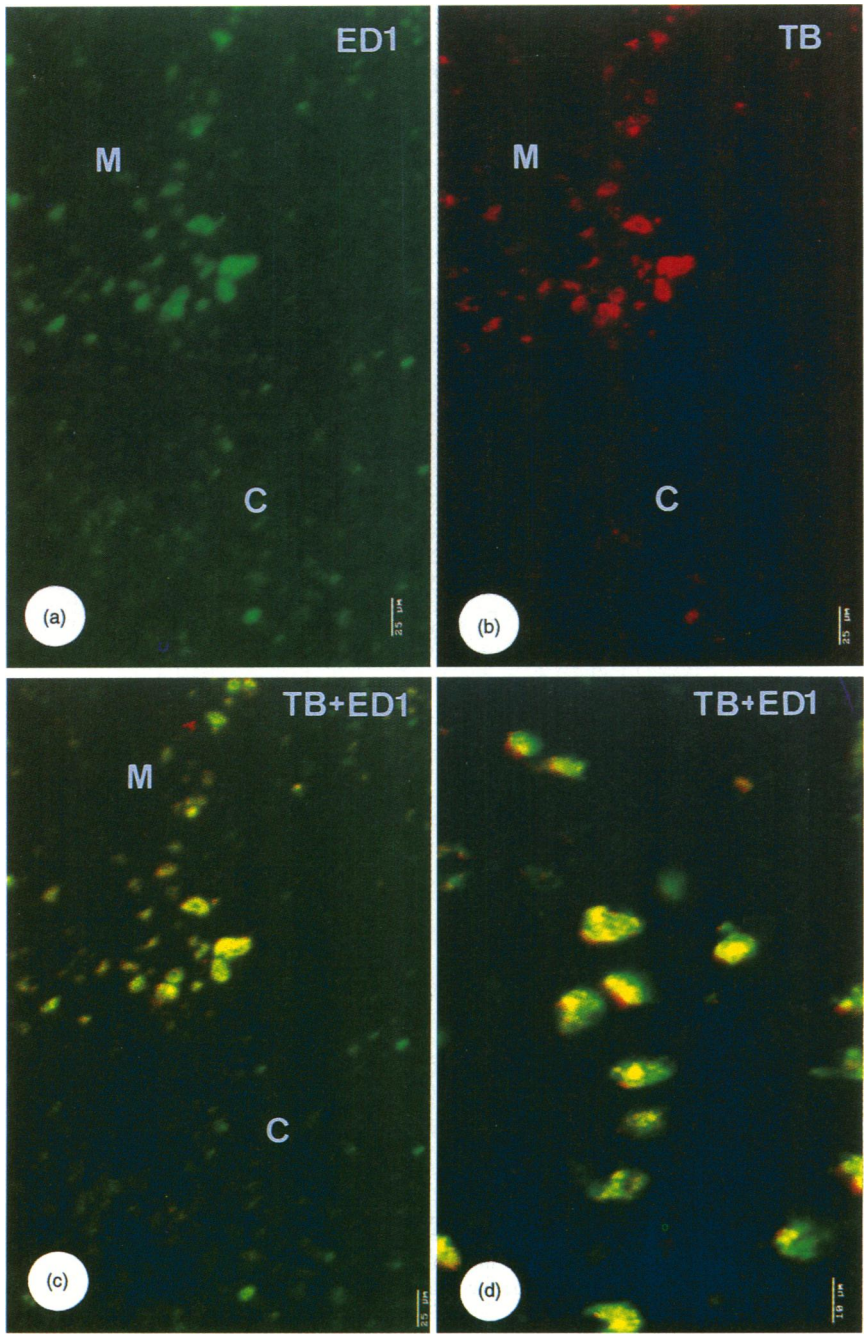


Figure 2. Immunohistochemical localization of ED1 (a), TrkB-like protein (b) and ED1 + TrkB-like protein (c, d) in the thymus of a 3-month-old rat. Immunoreactivity for both proteins was co-localized in a subset of ED1 + cells located in the corticomedullary border. Scale bar = 25 μm.

expressed the TrkB receptor. These cell lines were cultured in RPMI-1640 medium (Sigma) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown on 24-well plates (5×10^5 cells/well) at 37° in a 5% CO_2–95% air atmosphere. Finally, 10 μl of the cell suspension was pipetted onto gelatin-coated microscope slides, fixed with 10% formaldehyde, washed in 0·05 M TBS (pH 7·5) containing 0·1% bovine serum albumin and 0·2% Triton X-100, and processed for immunohistochemistry, as described above.

RESULTS

Tissue sections

Specific immunoreactivity for TrkB protein was observed in scattered cells in the thymus of all ages of rat studied. The TrkB+ cells were heterogeneous in both morphology and size, and were located mainly in the corticomedullary junction, and, to a lesser extent, in both the cortex and medulla, as well as in the trabecular and perivascular connective tissue (Fig. 1). The TrkB immunoreactive cells were much larger than the surrounding thymocytes, round or irregular in shape, and showed a granular cytoplasm with engulfed material and, occasionally, pyknotic nuclei (Fig. 1).

In order to identify the TrkB+ cells, we compared their pattern of distribution and morphological characteristics, in serial sections, with that of other cell types recognized by their expression of specific antigens. Cells displaying S-100 protein, a marker of dendritic cells,[20] were restricted to the medulla and showed a very irregular morphology and occasionally cytoplasmic processes. PGP 9.5 IR labelled cortical epithelial cells, while the antibody against cytokeratins marked mainly medullary and subcapsular epithelial cells. Sections stained with methylene-blue contained metacromasic cells located in the connective tissue, but never inside the thymic parenchyma. These cells represent mast cells. Comparison of morphology and distribution in serial sections indicated that none of these cell types corresponded to the TrkB-expressing cells (data not shown).

ED1+ cells were widely distributed throughout the thymus, primarily in the medulla and corticomedullary junc-

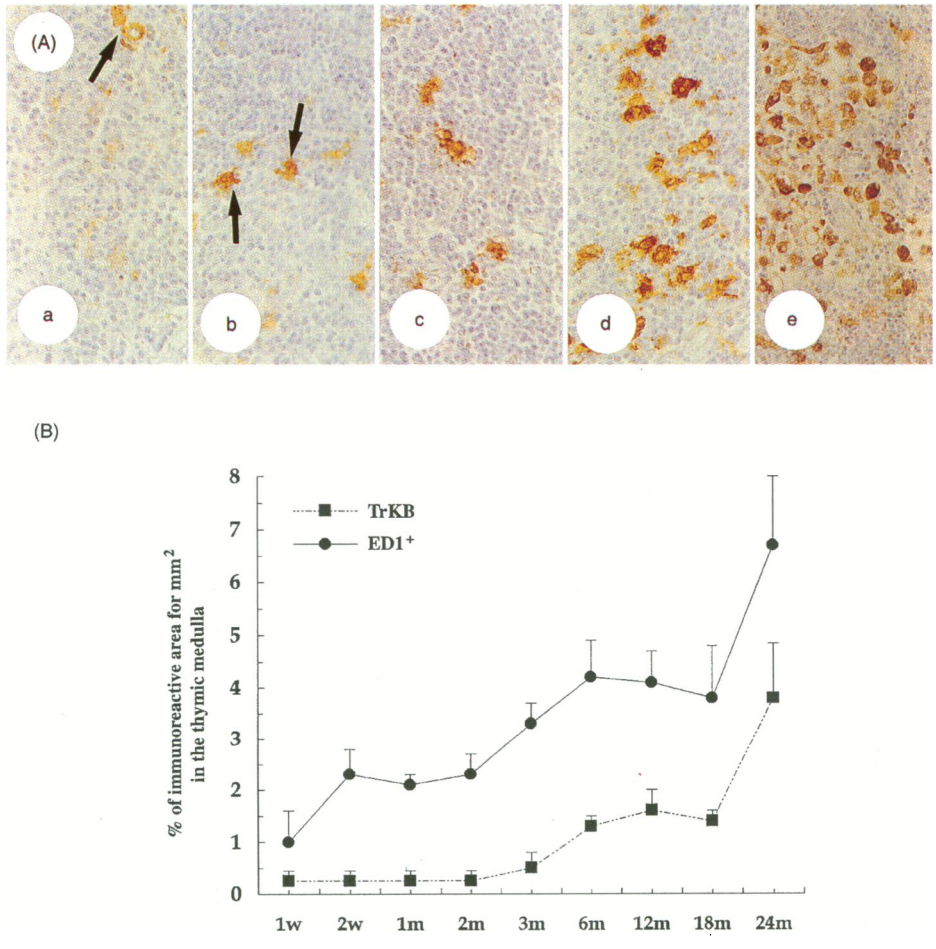


Figure 3. Age-dependent changes in the density of TrkB immunoreactive cells in the rat thymus. A. Scattered cells displaying TrkB-like protein immunoreactivity were observed in the medulla of newborn (a) and 1-week-old (b) rats; in 3-month (c) and 6-month (d) -old rats the immunoreactive cells were concentrated in the corticomedullary border; in older animals (24 months) some parts of the thymus showing signs of involution contained a high density of TrkB-like protein immunoreactive cells (e) (all magnifications ×180). B. Density of ED1+ and TrkB-like protein immunoreactive cells in the medulla or thymic lobules from rats of different ages. Between 1 week and 2 months, immunoreactive cells were observed, but their density was too low to be quantified.

tion and, to a lesser extent, in the cortex. These cells were very heterogeneous in shape and size. In serial sections, some of the ED1+ cells, especially those located in the corticomedullary border, displayed a morphology and distribution very similar to that of TrkB+ cells. To confirm that the TrkB-expressing cells were also ED1+ double immunohistochemistry was performed (Fig. 2). The results demonstrated that ED1+ cells located in the corticomedullary border showed simultaneous immunostaining for TrkB-like protein. A very scarce number of TrkB+ cells (<0·1%) were negative for ED1+ expression.

The thymus undergoes age-dependent changes, which involves both lymphoid and stromal cells.[24] We therefore analysed the expression of ED1 and TrkB-like expression in rats of different ages from E18 to 24 months. The results are represented in Fig. 3. We observed an increase in the number of ED1+ cells with age. This was also true for TrkB+ cells, although the density of ED1+ cells was much higher than that of TrkB+ cells at all ages of rat. In the oldest animals, the surface area occupied by TrkB+ cells reached 28% in areas with signs of active involution (Fig. 3, a–e).

Cytospin preparations

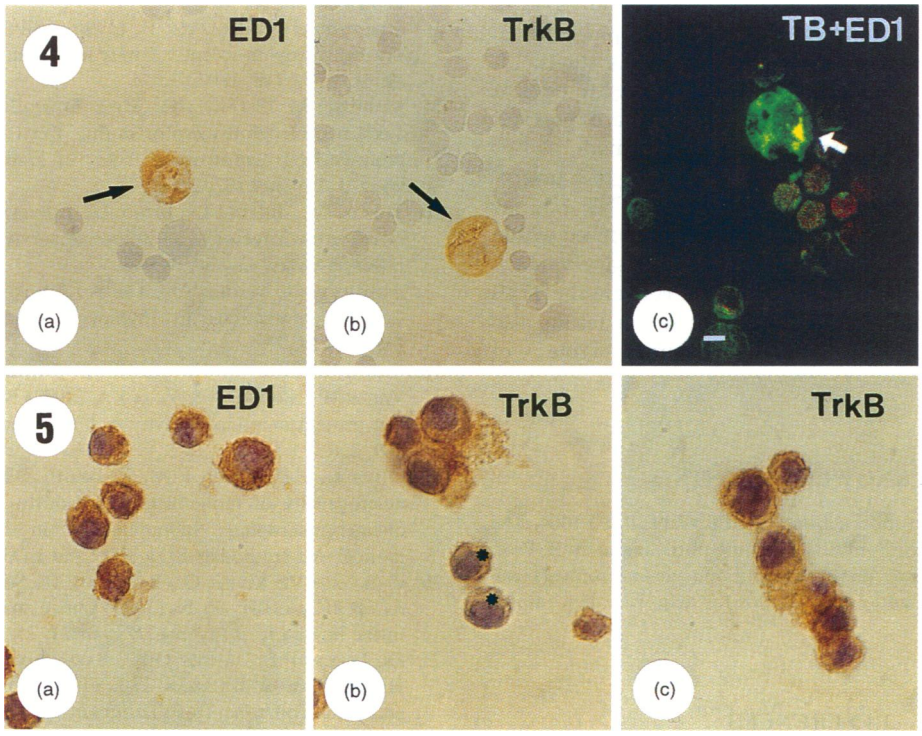
As identification of cell type by morphology in tissue sections is often difficult, we also processed cytospin preparations of homogenized thymuses for immunohistochemistry. ED1+ cells (Fig. 4a) were found to be scattered among lymphocytes and they showed morphological features typical of thymic macrophages.[25] The pattern of immunostaining was cytoplasmic and showed a faint granular aspect. Some of these ED1+ cells contained engulfed thymocytes, as observed in the tissue sections. The cells that expressed TrkB-like protein in cytospin preparations were very similar in morphology, although they were much rarer (Fig. 4b and 4c). To confirm that the TrkB+ cells were macrophages, double immunohistochemistry was performed as in tissue sections, and this confirmed that TrkB+ cells were also ED1+ (Fig. 4).

Cell lines

Finally, the two mouse monocyte–macrophage cell lines, RAW 264.7 and J774A.1 both displayed immunoreactivity for TrkB-like protein: 82% and 100% of the total number of cells, respectively (Fig. 5b and c).

DISCUSSION

It is generally accepted that the thymus is a part of the immunoneuroendocrine system.[26,27] A great variety of substances act on thymic lymphocytes and stromal cells, forming a complex network of autocrine, paracrine and endocrine signals that involves classic hormones, cytokines, thymic hormones and growth factors.[28] The rat thymus contains a heterogeneous stromal cell population,[17] which is influenced by these substances.[29] A putative role for neurotrophins in the control of thymic stromal cells arises from the expression of the TrkA neurotrophin-receptor protein in mammalian thymic



Figures 4 and 5. Fig. 4. Immunodetection of ED1 (a, arrow) and TrkB (b, c, arrows) in cytospin preparations of thymic cells that contain engulfed lymphocytes; the pattern of immunostaining for TrkB-like protein was granular and cytoplasmic (all magnifications ×280). Fig. 5. Immunodetection of ED1 (a) and TrkB (b, c) in the cell lines RAW 264·7 (b) and J774A (c). Some RAW 264·7 cells (asterisks in b) lacked TrkB-like protein immunoreactivity, while all J774A.1 cells displayed immunoreactivity (all magnifications ×280).

epithelial cells,[10,14] and of TrkA, TrkB and TrkC in different types of thymic stromal cells in birds.[12] TrkB mRNA has also been detected in the rat thymus.[8,11]

In this report we describe the expression of TrkB protein by rat thymic macrophages, identified by their morphology and their ED1 expression. In addition, TrkB was detected in two well-characterized mouse monocyte–macrophage cell lines. The number of TrkB-like protein immunoreactive cells in the thymus was much lower than that of ED1+ macrophages, a marker which recognizes the majority of macrophage subtypes,[19] thus indicating that only a subpopulation of thymic macrophages expresses the TrkB-receptor protein. On the other hand, virtually all TrkB+ cells co-expressed ED1, suggesting that neither other types of stromal cells nor thymocytes express TrkB. Finally, we observed that the expression of TrkB underwent age-dependent changes, reaching maximal density during thymic involution. Similarly, the number of macrophages increased with age, as reported elsewhere.[30,31] Our findings are in agreement with previous studies describing TrkB expression in a subpopulation of macrophages in secondary lymphoid organs.[9,14,18] Recently, Maroder *et al.*[15] reported that immature, double-negative thymocytes express the TrkB receptor. However, we were unable to detect any TrkB-like expression in thymocytes. This discordance may be caused by the different sensitivity of the methods used.

Macrophages play an important role in both normal and pathophysiological conditions and secrete numerous active substances, influencing many aspects of the immune response.[32,33] The secreted substances, as well as the receptors they express, comprise molecules that are considered classically as specific for either the neuroendocrine or the immune system. This has led to the hypothesis that the macrophage could represent an evolutionary link between these two systems.[34] It is therefore not surprising that this cell type expresses a neurotrophin receptor, as the neurotrophins are peptides shared by the neuroendocrine and immune systems.[3]

Taken together, our results suggest that thymic macrophages could be influenced by BDNF and/or NT-4/5, the physiological ligands of the TrkB,[2] which are produced within the thymus itself.[8,15,35] Further studies are necessary to elucidate whether these neurotrophins bind and activate the TrkB receptor present in macrophages. In any case our results also support the proposed immunoregulatory role of neurotrophins.

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