Ablation of TrkA function in the immune system causes B cell abnormalities

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Summary

The nerve growth factor (NGF) receptor TrkA is widely expressed in non-neural tissues suggesting pleiotropic functions outside the nervous system. Based on pharmacological and immuno-depletion experiments, it has been hypothesized that NGF plays an important role in the normal development and function of the immune system. However, attempts to unravel these functions by conventional gene targeting in mice have been hampered by the early postnatal lethality caused by null mutations. We have developed a novel 'reverse conditional' gene targeting strategy by which TrkA function is restored specifically in the nervous system. Mice lacking TrkA in non-neuronal tissues are viable and appear grossly normal. All major immune system cell populations are present in normal numbers and distributions. However, mutant mice have elevated serum levels of certain immunoglobulin classes and accumulate B1 cells with aging. These data, confirmed in a classical reconstitution model using embryonic fetal liver from TrkA-null mice, demonstrate that endogenous NGF modulates B cell development through TrkA in vivo. Furthermore, they demonstrate that many of the dramatic effects previously reported by pharmacological or immuno-depletion approaches do not reflect physiological developmental roles of TrkA in the immune system.

Key words: TrkA, NGF, Immune system, Mouse

Introduction

Neurotrophins are soluble growth factors known mainly for their roles in regulating the development of the mammalian nervous system. Two types of receptors mediate the actions of these polypeptides: the Trk family of tyrosine kinase receptors and the p75 nerve growth factor (NGF) receptor (Bibel and Barde, 2000; Hempstead, 2002; Huang and Reichardt, 2001; Kaplan and Miller, 2000). Gene targeting approaches in mice have uncovered some functions of these genes in promoting survival and developmental maturation of certain types of neurons of the peripheral and central nervous system, thus confirming their critical role in neural development (Huang and Reichardt, 2001). Furthermore, the phenotypes observed in these mutants have demonstrated the specificity of the interactions between neurotrophins and their Trk receptors. For example, both NGF- and TrkA-deficient mice have similar phenotypes. Ablation of either gene results in the virtual absence of the superior cervical ganglia neurons by the first postnatal week, confirming the essential role of NGF/TrkA signaling in the development of sympathetic neurons. Furthermore, both mutant mice are insensitive to pain, because of the loss of small-diameter nociceptive sensory neurons (Huang and Reichardt, 2001; Tessarollo, 1998).

It has long been hypothesized that neurotrophic factors, particularly NGF, play a role in the development and function of structures outside the nervous system (Levi-Montalcini, 1987). Indeed, neurotrophins and their receptors are widely expressed in a variety of non-neuronal tissues throughout development, including the cardiovascular, endocrine, reproductive, and immune systems (reviewed by Aloe et al., 1999; Tessarollo, 1998; Vega et al., 2003). However, our knowledge of the function of neurotrophins in non-neuronal tissues is still fragmented. Evidence for such role was provided by mice lacking NT3 (Ntf3 – Mouse Genome Informatics) and BDNF, which revealed the requirement of these ligands and their receptors for the normal development of the cardiovascular system (Donovan et al., 1996; Donovan et al., 2000; Tessarollo et al., 1997). In addition, neurotrophin functions outside the nervous system have been suggested from phenotypes identified in hair follicles and thymus of neurotrophin and Trk receptor mutant mice (Botchkarev et al., 2004; Garcia-Suarez et al., 2002; Garcia-Suarez et al., 2000). However, one can not exclude that some of these deficits were indirect effects of the severe nervous system defects. Moreover, mice lacking neurotrophin functions, with the exception of p75 and NT5 null mice, die soon after birth thus precluding analysis of phenotypes in the adult animal.

Within the immune system, p75 and TrkA receptors are widely distributed and there is an increasing body of evidence

suggesting that NGF can regulate immune cell functions including inflammatory responses (Aloe et al., 1999; Micera et al., 2003; Tessarollo, 1998; Vega et al., 2003). For example, NGF blood levels are increased in several autoimmune and allergic human diseases (Aloe et al., 1997; Aloe et al., 1994). Furthermore, NGF can accelerate wound healing by modulating the inflammatory response in mice (Matsuda et al., 1998; Micera et al., 2001). However, the most intriguing data concern the effects of NGF on B-cell (Brodie and Gelfand, 1994; Brodie et al., 1996; Otten et al., 1989) and mast cell function (Leon et al., 1994). NGF can regulate immunoglobulin (Ig) production in vitro and serves as a survival factor for memory B cells (Brodie and Gelfand, 1994; Brodie et al., 1996; Torcia et al., 1996). Furthermore, NGF is synthesized, stored and released by mast cells (Leon et al., 1994). Yet, the physiological rather than pharmacological relevance of these effects is not clear (Tessarollo, 1998).

P75 mutant mice are viable and therefore amenable to investigation of non-neuronal structures including the immune system in the adult (Lee et al., 1992). However, a recent investigation has not found major deficits in the spleen and thymus of p75 mutant mice (Garcia-Suarez et al., 2001) and our own preliminary analysis has failed to unveil any overt immunological deficits (V.C. and L.T., unpublished). Therefore, we hypothesized that TrkA mediates most of the NGF functions described in the immune system likewise in the nervous system. To test this hypothesis in vivo, we decided to generate and characterize mice with a conditional null allele of the TrkA gene.

The classic approach to study the function of a gene in a specific organ includes the generation of a mouse strain with loxP sites flanking the gene of interest (floxed) and another mouse strain with the Cre recombinase under the transcriptional control of a promoter expressed in the organ of interest. By crossing these two strains, deletion of the gene flanked by loxP sequences should be achieved in an organ site-specific manner. However, one major limitation of this approach is the fact that deletion of the floxed gene is not always complete in all cells where Cre should be active (Rickert et al., 1997). Thus, the residual activity of the gene can potentially confound the analysis. This is particularly critical for the immune system in which clonal expansion of cells with a functional floxed allele may compensate for certain phenotypes. To obviate this problem we have reversed the strategy and activated TrkA specifically in neuronal structures within a null mutation background. This approach provides a means to distinguish between an intrinsic role of TrkA in the immune system versus defects that are caused by nervous system deficiencies in the complete knockout mouse model.

Using this strategy, we found that on the whole, the TrkA-deficient immune system develops normally. This result demonstrates that many of the dramatic effects previously reported by pharmacological or immuno-depletion approaches do not reflect physiological roles of TrkA in the immune system during homeostasis. However, we also identified disregulation of Ig production and accumulation of a B-cell subset. These data are the first demonstration that NGF/TrkA indeed modulates immunological functions not mediated by the nervous system in vivo.

Materials and methods

Mice

$TrkA^{neo/neo}$ mice were generated by a gene targeting approach using the CJ7 embryonic stem cell line as previously described (Bonin et al., 2001; Tessarollo, 2001). The nervous system-specific Tα1-Cre transgenic mouse line was generated by microinjection of fertilized eggs (Osborn et al., 1987) of a vector generated by fusion of the Tα-1 promoter region (a gift from Freda Miller) with the coding sequence of the Cre recombinase and the SV40 polyadenylation signal (Gloster et al., 1994). TrkA-deficient mice used for the reconstitution experiment were previously described (Liebl et al., 2000).

The Rosa-26 reporter mouse strain was kindly provided by Phil Soriano (Soriano, 1999). Adult C57Bl/6-SCID mice (Bosma et al., 1983) used in reconstitution experiments were purchased from Charles River (Frederick, MD, USA) and reconstituted by tail-vein injection with 5×10^6 E14.5 fetal liver cells.

All animals were treated in accordance with the guidelines provided by the Animal Care and Use Committee of the National Cancer Institute at Frederick (MD, USA).

Immunization protocols

To test T-cell-dependent (TD) humoral responses, 3-6-month-old mice were subjected to retro-ocular bleeding to determine basal Ig levels and then were injected intraperitoneally with 100 μg KLH-TNP (keyhole limpet hemocyanin-2,4,6,-trinitrophenyl; Biosearch Technologies, Novato, CA, USA) in PBS (day 0). After 40 days, mice were given a boosting dose of the same Ag and blood was drawn again after 1 week (day 47) and 2 weeks (day 54) from the boost. To test T-cell-independent (TI) type I and type II responses, mice were pre-bled and immunized with 20 μg of lipolysaccaride (LPS)-TNP or 25 μg of Ficoll-TNP (Biosearch Technologies), respectively. Blood was collected one and two weeks after challenge.

Northern blot and RT-PCR

Total RNA was isolated from different tissues by a single-step method (RNA STAT-60, Tel-Test ‘B’, Friendswood, TX, USA) and analyzed by classical northern blot analysis. TrkA transcripts were detected by using a rat kinase-specific TrkA probe (spanning exon 12 to exon 17). RT-PCR was performed using SuperScript™ RT-PCR System (GIBCO-Invitrogen, Grand Island, NY, USA) according to manufacturer instructions with the following primers: TrkA Exon10-Exon14 fragment: F primer: 5′-ACG GTA ACA GCA CAT CAA GAG-3′; R primer: 5′-CGG AGG AAA CGG TTG AGG TC-3′. β-actin (F primer: 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′; R primer: 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′).

Histological procedures

Sections (5 μm) from paraffin-embedded tissues were stained with anti-CD45R, 1:200 (B220, RA3-6B2 BD-Pharmingen, San Jose, CA, USA), anti-CD3, 1:600 (Dako, Carpinteria, CA, USA), and anti-mouse F4/80, 1:25 (Caltag, Burlingame, CA, USA) for B-cell, T-cell and macrophage-specific immunostaining, respectively. Apoptotic cells were detected with the Apoptag kit (Intergen, Purchase, NY, USA) following the manufacturer’s recommendations. For β-galactosidase staining, E11.5 embryos were fixed in PBS without Mg^{2+}/Ca^{2+}, 1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40 for 30 minutes at 4°C, washed twice in PBS without Mg^{2+}/Ca^{2+} at RT for 20 minutes, and stained overnight at 30°C with PBS without Mg^{2+}/Ca^{2+}, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg/ml X-gal. For adult tissue staining, 20 μm frozen sections were post-fixed with 4% phosphate-buffered paraformaldehyde for 5 minutes, washed three times for 5 minutes with 100 mM sodium phosphate buffer, 20 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, then stained overnight as above, rinsed in dH_2O and counterstained with neutral red for 40 seconds. After three rinses in 100% ethanol, cover slips were mounted in xylene.

Automated blood cell counts were performed using the HEMAVET® 850 apparatus (CDC Technologies, Oxford, CT, USA).

FACS and ELISA Ig analysis

For FACS analysis, samples were prepared as previously described (Coppola et al., 1998). All the unlabelled antibodies, phycoerythrin (PE), fluorescein isothiocyanate (FITC) or PerCP-conjugated were purchased from BD Pharmingen (San Jose, CA, USA). Elisa Ig analysis was performed using a modified protocol from BD Pharmingen. Briefly, plates (Dynex-Immulon®) were coated overnight with capture anti-Ig-specific Ab in PBS, washed once with PBS-0.05% Tween® 20, blocked for 1 hour at RT with PBS-3% BSA and incubated with serial dilutions of sera overnight at 4°C. The following day, the plates were washed five times and incubated with a secondary Ab Ig conjugated to HRP diluted 1:1000 for 1 hour at RT. After six further washes, substrate (ABTS Microwell Peroxidase Substrate System, Gaithersburg, MD, USA) was added. Capture mouse anti-IgM, anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, anti-IgA, and the HRP-conjugated Abs were purchased from Southern Biotech (Birmingham, AL, USA). All standards for the different Ig classes, capture anti-IgE, and biotin-conjugated anti-IgE were purchased from BD Pharmingen. For the IgE Elisa, HRP-streptavidin was purchased from Jackson ImmunoResearch (West Grove, PA, USA). For anti-TNP-specific Ig dosage, the plates were coated overnight at 4°C with OVA-TNP (Biosearch Technologies) at 10 μg/ml in carbonate/bicarbonate buffer pH 9.6. The following steps were similar to the dosage of total sera Ig. Plates were read using a Benchmark Bio-Rad Elisa reader at 405 nm wavelength (Software: Microplate Manager III®, Bio-Rad, Hercules, CA, USA). Data were analyzed and plotted using GraphPad PRISM® 3.0 (GraphPad Software, San Diego, CA, USA).

Passive cutaneous anaphylaxis

IgE-dependent passive cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-dinitrophenyl (DNP) IgE, followed 18-20 hours later by an injection of DNP-albumin (100 μl of a 1 μg/μl solution in saline) into the mouse tail vein. The right ear was injected intradermally with 25 ng of anti-DNP IgE and the left ear received 25 μl of saline to serve as control. Ear thickness was measured with a caliper (Dyer Company, Lancaster, PA, USA) 15 minutes before and 15 minutes after the antigen challenge.

Generation of bone marrow derived mast cells and degranulation assay

Bone marrow derived mast cells (BMMC) were generated by culturing femoral bone marrow cells from 6- to 8-week-old mice. Mice were sacrificed by CO_2 asphyxiation and intact femurs were removed. Bone marrow cells were harvested by flushing the bone shaft with DMEM (GIBCO-Invitrogen) medium with 2% FBS. Red blood cells were lysed by incubation with 0.8% ammonium chloride with 0.1 mM EDTA. Bone marrow cell cultures were established at a density of 1×10^6 cells/ml in DMEM containing 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 ng/ml recombinant mouse stem cell factor (SCF), 10 ng/ml recombinant mouse IL-3. Non-adherent cells were transferred to fresh culture plates once a week and fed by replacing 50% of the medium twice a week. After 4 to 6 weeks of culture, the purity of BMMC culture was greater that 95% as confirmed by FACS analysis for FcεRI and c-*kit* expression. BMMC were activated via FcεRI stimulation by first sensitizing overnight with anti-DNP IgE antibody (Sigma Aldrich, St Louis, MO, USA) at 1 μg/ml in culture medium. Sensitized cells were washed and stimulated for 30 minutes at 37°C with 100 ng/ml DNP conjugate of human serum albumin (DNP-HSA) (Sigma) in Hanks' Balance Salt Solution supplemented with 2 mg/ml glucose and 0.03% BSA. Cell supernatants were harvested and the cell pellets were lysed by freeze-and-thaw cycle. β-hexosaminidase was quantified in the supernatants and cell lysates by spectrophotometric analysis of hydrolysis of p-NAG (p-hitrophenyl-N-acetyl-D-glucosaminidine) (Sigma). β-hexosaminidase release was calculated as the percentage of β-hexosaminidase presented in the supernatants relative to the total amount of β-hexosaminidase present in the supernatant and cell pellet.

Results

Mice lacking the TrkA receptor in non-neuronal tissues are viable and display no overt phenotype

NGF and TrkA knockout mice have similar phenotypes. Ablation of either gene results in severe losses of peripheral sensory and sympathetic neurons and early post-natal lethality (Crowley et al., 1994; Smeyne et al., 1994). Even though TrkA is widely expressed outside the nervous system, the early demise of these mutant mice has been attributed to the severe neuronal deficits. We hypothesized that restoration of TrkA function in the nervous system would rescue the lethality associated with the general deletion of TrkA and would allow us to investigate TrkA-mediated NGF functions in the many non-neuronal organs in which its expression has been reported (Aloe et al., 1999; Tessarollo, 1998; Vega et al., 2003). To this end, we devised the following 'reverse conditional knockout' strategy. By gene targeting, we first generated an allele that harbors a loxP-flanked neomycin resistance gene in intron 11, upstream of the tyrosine kinase encoding exons (*TrkA*$^{neo/neo}$). Mice with the neo gene intact within the *TrkA* locus develop a phenotype identical to that of TrkA null mice (data not shown) (Smeyne et al., 1994). Northern blot and RT-PCR analysis verified that no RNA capable of producing a functional TrkA protein is generated by the mutant locus (Fig. 1C,D and data not shown). Thus, the insertion of the loxP-flanked neo-containing cassette into an intron of the *TrkA* locus disrupts expression of the gene creating a true null mutation. However, removal of the neo cassette by ubiquitous Cre-mediated recombination can restore TrkA function and rescues the lethality demonstrating that the residual loxP site does not interfere with *TrkA* expression (TrkA$^{loxP/loxP}$) (Fig. 1A,C). We then developed a transgenic mouse line using the Tα-1 tubulin promoter (Gloster et al., 1994) to express the Cre recombinase exclusively in the nervous system (Tα-1-*Cre*) (Fig. 1E,F). Tα-1-*Cre* mice do not show any obvious phenotype and they are indistinguishable from non-transgenic littermate controls (not shown). To confirm that Tα-1-*Cre* expression is specific to the nervous system and no ectopic cre expression was present in organs of the immune system, we crossed the Tα-1-*Cre* transgenic mouse to the Rosa-26 reporter strain (Soriano, 1999) (Fig. 1E). β-galactosidase staining of several lymphoid organs failed to detect any Cre recombinase activity (Fig. 1F), confirming the specificity of the Tα-1-*Cre* transgenic mouse line.

Crossing of the Tα-1-*Cre* allele into the *TrkA*$^{neo/neo}$ mutation generated mice that were viable and showed no overt phenotype. However, the presence of the neo cassette outside the nervous system completely inactivates TrkA (Fig. 1C,D) and thus allows the investigation of non-neuronal tissues lacking this gene function. Lack of neo-excision in several non-neuronal organs including spleen, thymus, bone marrow and lymph nodes of mice was further confirmed by PCR analysis (not shown). These data provide definitive evidence that TrkA function in the nervous system is the cause of the lethality observed in the TrkA knockout mice.

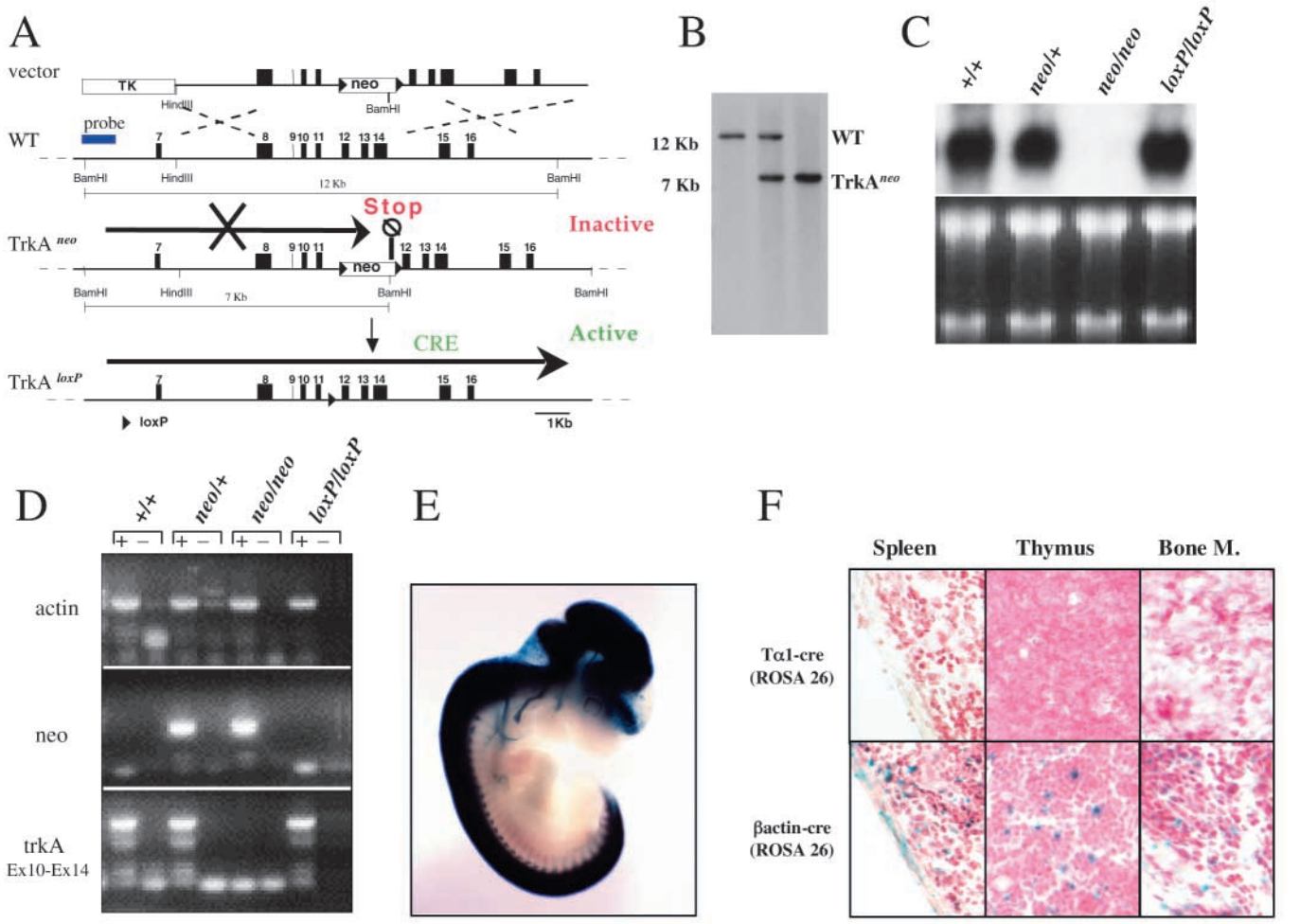

Fig. 1. Conditional inactivation of TrkA. (A) Diagram of the replacement vector strategy used to generate the TrkA^{neo} allele and re-activation of TrkA expression by Cre recombinase. The pGKneobpA cassette flanked by loxP sites was placed into intron 11. (B) Southern blot analysis of tail DNA from three-week-old mice with a 5′ external probe shows the switch of the wild-type (WT) BamHI fragment from 12 kb (WT) to a 7 kb restriction fragment (TrkA^{neo}) after neo insertion. (C) Northern blot analysis of total RNA extracted from Dorsal Root Ganglia (DRG) neurons of E13.5 embryos either WT (+/+), heterozygous for the neo insertion (+/neo), homozygous for the neo insertion (neo/neo), or homozygous for the allele after neo removal with β-actin cre (loxP/loxP), hybridized with a TrkA-kinase domain-specific probe (exon 14-17). Note that neo insertion completely eliminates TrkA-specific transcripts whereas TrkA-transcription is restored to WT levels after neo-excision by Cre recombination. (D) RT-PCR analysis of the same samples analyzed by northern blot. Specific primers for actin, neo, and TrkA exon 10 and 14 were used. Samples were treated with (+) and without (–) RT. Note the complete absence of the 609 bp TrkA-specific PCR product in TrkA$^{neo/neo}$ mice. (E) Wholemount *lacZ* staining of a Rosa-26 E11.5 embryo with theTα1-cre transgene. Note the specific staining in both central and peripheral nervous system. (F) *lacZ* staining of sections from spleen, thymus and bone marrow of adult Rosa-26 mice carrying the Tα1-*Cre* (top panels) or a β-actin-Cre transgene with mosaic expression used as control (bottom panels) (Ma et al., 2003). Cre recombinase under the control of the Tα-1 promoter is only expressed in the nervous system (E) and not in organs of the immune system (F).

TrkA is dispensable for the development of immune system organs

Over the years, the NGF/TrkA ligand/receptor system has been extensively implicated in regulating the development and function of the immune system. However, the lack of suitable in vivo models has hampered the investigation toward the dissection of a direct role of NGF/TrkA in this system. For example, systemic knockouts or models based on immunodepletion of endogenous NGF affect all organs including the nervous system, which may be required for normal development of the lymphoid organs. Our model inactivates the NGF receptor TrkA only in non-neuronal tissues and thus allows us to investigate the intrinsic function of this receptor in structures including the organs of the immune system. Anatomical and histological analysis of thymus, spleen and lymph nodes did not reveal any significant difference between TrkA-deficient and control tissues. For example, cellularity and morphology of the spleen of mutant mice were similar to control littermates (not shown). In addition, lymphoid follicles (Fig. 2A,B) and distribution of apoptotic cells (Fig. 2C,D) were also present in similar numbers. Spleen sections immunostained with the B-cell-specific B220 and T-cell-specific anti-CD3 antibodies did not detect any difference in number or distribution of B- and T-cells in the Tα-1-*Cre*;TrkA$^{neo/neo}$ mutants compared with control littermates. (Fig. 2E-H). Similarly, lymph nodes, thymus and bone marrow

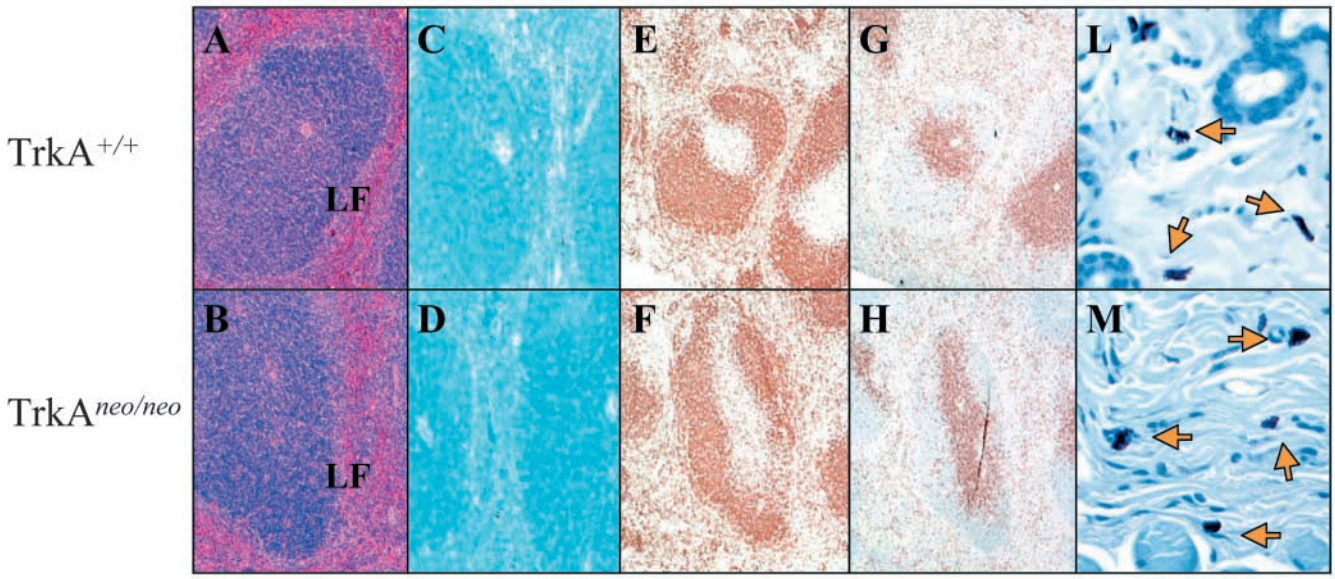

Fig. 2. TrkA deficiency does not affect spleen development and skin mast cells. Spleens of wild-type (A,C,E,G) and mutant (B,D,F,H) mice were stained with hematoxylin/eosin (A,B), Apoptag (C,D), and B cell- (anti-B220; E,F) and T cell-specific (anti-CD3; G,H) antibodies. Note the absence of any remarkable difference between mutant and control organs. (L,M) Toluidine-blue staining of mutant (M) skin samples shows presence of mast cells (arrows) in number and distribution similar to wild-type littermate controls (L). LF, lymphoid follicle.

seemed unaffected by TrkA loss at the anatomical and histological level (data not shown). To assess the major cell populations of the immune system organs, we performed cytofluorimetric analysis on thymus, spleen and bone marrow cells with anti-B220, -IgM, -IgD, -CD19, -CD3, -CD4, -CD8, -Mac-1, -Gr1 and -CD5 antibodies. No quantitative or qualitative abnormalities in the B- and T-cell populations, macrophages and granulocytes (not shown) were detected in mutant mice. Furthermore, blood cell counts of mutant mice were also comparable to littermate controls (Table 1).

Taken together these data provide definitive evidence that TrkA-mediated NGF function is dispensable for normal development of the immune system. Furthermore, these findings suggest that immune system deficiencies reported in other models are most likely because of indirect effects caused by NGF/TrkA loss in the nervous system.

Table 1. TrkA$^{neo/neo}$ mice have a normal hematological profile

	WBC				RBC	PLT
	(10^3/μl)	% Ly	% Ne	% Mo	(10^6/μl)	(10^3/μl)
Males						
CTR	9.04±1.5	78.2±4.5	17.7±4.9	3.6±1.8	10.1±0.8	643±144
MUT	9.31±1.4	75.5±9.9	20.2±10.0	3.7±1.2	9.9±0.6	592±72
Females						
CTR	9.14±1.4	84.6±1.7	10.2±2.6	4.9±1.9	9.3±0.7	558±128
MUT	8.86±1.6	82.4±2.8	13.2±2.9	3.9±1.9	9.3±0.3	643±145

Absolute numbers of leukocytes (WBC), and erythrocytes (RBC) and platelets (PLT) in TrkA$^{neo/neo}$ mice (MUT) do not show any significant difference when compared with control littermates (CTR). In addition, percentages of blood lymphocytes (Ly), neutrophils (Ne) and monocytes (Mo) are not significantly different in either male or female TrkA$^{neo/neo}$ animals compared with controls.

Mast cells develop and function in TrkA mutant mice

Mast cells constitute another cell population for which there is ample literature suggesting a direct role of NGF in their development and function (Aloe et al., 1999; Tessarollo, 1998; Vega et al., 2003). Toluidine blue and alcian blue/safranin staining of histological preparations from skin, spleen and lymph node of Tα1-*Cre*;TrkA$^{neo/neo}$ mice showed that mast cells were present in comparable numbers and distribution to that of tissues from control littermates (Fig. 2L,M and data not shown). Thus, TrkA signaling is not required for mast cell differentiation in vivo. To begin to address whether mast cells lacking TrkA displayed functional abnormalities in vivo, we employed a wheal and flare paradigm. The skin of ears from mutant and control mice was sensitized with an intradermal injection of anti-DNP IgE followed by a systemic injection of DNP-albumin, as described. Compared with saline challenged skin, IgE/DNP intradermal injections caused prominent mast cell degranulation and mixed inflammatory cell infiltrates in both mutant and wild-type mice (Fig. 3A-F). However, no significant differences were observed in mast cell degranulation activity in the two genotypes (Fig. 3 and data not shown). It should be noted that this result does not preclude the presence of subtle defects caused by TrkA loss because we have observed great variability in the response among individuals of both control and mutant group. In addition, on the current genetic background, the response to DNP by mutant and control mice was approximately 20-fold lower than that of mice on a pure Balb/c background, making it difficult to assess the presence of subtle phenotypes in vivo (data not shown). Nevertheless, the in vivo degranulation response of mast cells lacking TrkA would suggest that this gene is dispensable for mast cells' activity. Because in vivo mast cells develop normally and can be activated by passive cutaneous reaction, we then derived mast

cells from bone marrow (BMMC) of TrkA mutant and control mice to investigate whether loss of TrkA had any effect at all on the differentiation and functional potential of this cell population. No apparent differences were observed in their capacity to differentiate in vitro. However, when BMMC were induced to degranulate by IgE cross-linking, the TrkA-deficient BMMC showed a modest but significant reduction in β-hexoseaminidase release in comparison with BMMC derived from control mice (P<0.01) (Fig. 3G). This result suggests that, although TrkA is not required for mast cell development in vivo, its loss may nevertheless impact their normal activity.

TrkA deficiency causes abnormal Ig serum levels

We found that lack of TrkA-mediated NGF signaling is compatible with the development of lymphoid organs. However, it has long been suggested that NGF signaling may also modulate specific cell functions. For example, in vitro experiments have shown that NGF can regulate Ig production (Brodie et al., 1996; Kimata et al., 1991a; Kimata et al., 1991b;

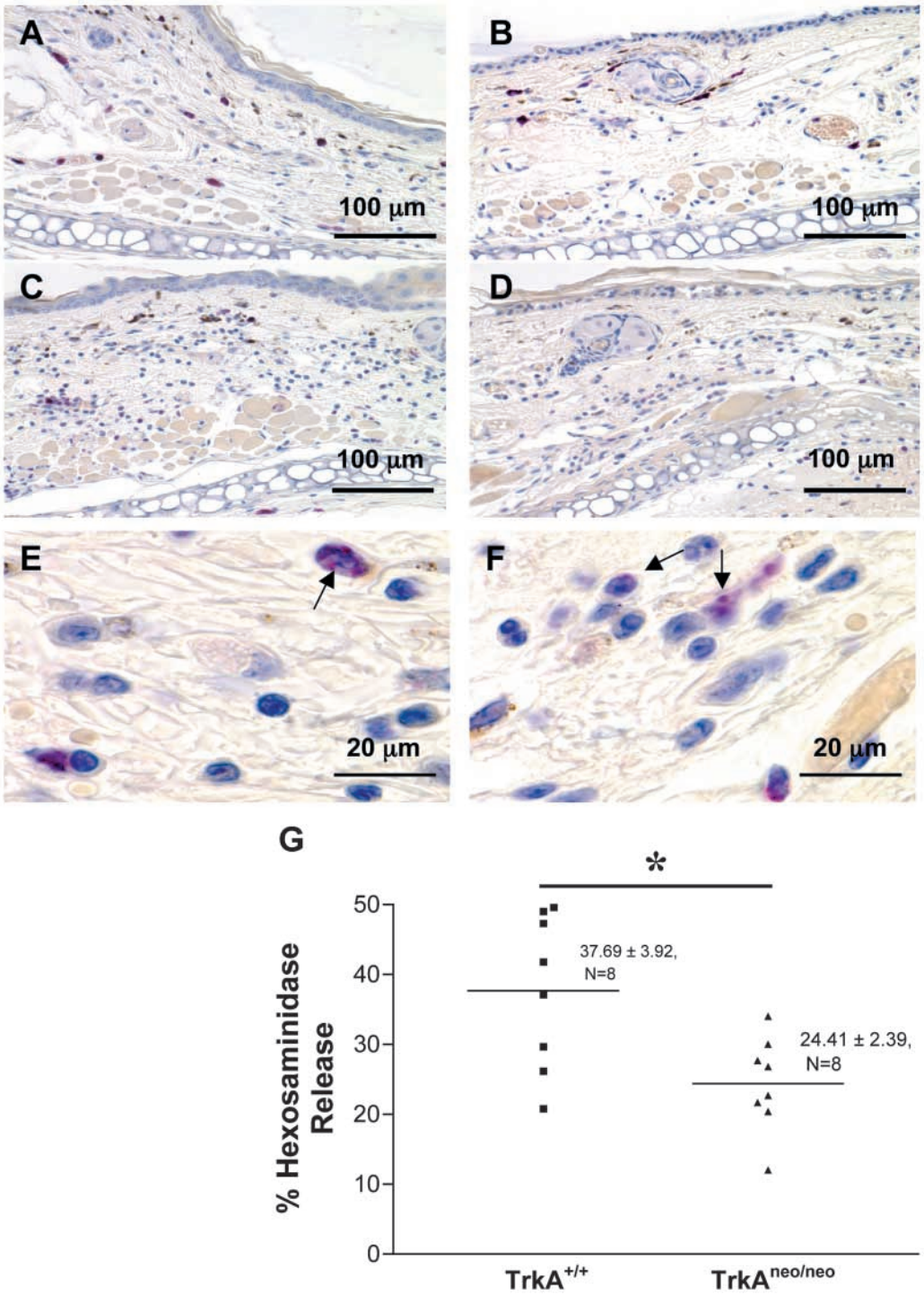

Fig. 3. Mast cells degranulate in TrkA-deficient mice. Histological assessment of passive cutaneous anaphylaxis in control (A,C,E) and TrkA mutant (B,D,F) mouse ear sections stained for naphthol AS-D chloroacetate esterase. Compared with saline challenged mice (A,B), the IgE/DNP-treated mice (C-F) exhibited prominent mast cell degranulation and mixed inflammatory cell infiltrates. Arrows are directed to degranulated mast cells. (G) β-hexoseaminidase release from bone marrow-derived mast cells obtained from TrkA mutant ($TrkA^{neo/neo}$) and control ($TrkA^{+/+}$) littermate mice was measured as described in Materials and methods. Mast cells obtained from the TrkA mutant mice show reduced β-hexoseaminidase release relative to those from controls (*P<0.01).

Kimata et al., 1991c). Indeed, serum analysis of Igs in Tα1-*Cre*;TrkA$^{neo/neo}$ mice revealed a specific increase in the levels of IgM, IgG2a and IgG1 (Fig. 4A). In contrast, IgG2b, IgG3, IgA and IgE (Fig. 4A and data not shown) were not significantly different from control littermates. Because these differences, albeit significant, were small, we next compared this genetic model with the 'classical reconstitution model' (Schlesinger et al., 1965). The immune system of adult C57Bl/6-SCID mice was reconstituted with fetal liver cells from a previously generated TrkA null mutant mouse line (Liebl et al., 2000). Reconstitution efficiency was monitored monthly by flow cytometry analysis of peripheral blood B- and T-cells. Mice reconstituted with TrkA-deficient cells showed the same B- and T-cell numbers compared with animals reconstituted with littermate wild-type cells (not shown). As observed in the 'reverse conditional TrkA mouse model', we found increased Ig levels in mice reconstituted with TrkA-deficient fetal cells (Fig. 4B). Importantly, these data validate our genetic model. Although the match is not perfect, perhaps because the stimulation condition is different in the two models, it remains interesting that both systems show an increase in Ig production, suggesting that overall, NGF exerts an inhibitory role on Ig production through TrkA in vivo. Therefore, detailed characterization of subtle phenotypes can be more appropriately studied in a genetic system that is less susceptible to the variations associated with reconstitution experiments.

TrkA deficiency does not impair B-cell memory and T-cell independent responses

Because it has been reported that NGF is a survival factor for memory B cells (Torcia et al., 1996), we next investigated B cell memory response in the absence of TrkA. Mice were injected with KLH-TNP [T-cell-dependent (TD)-antigen] and memory response was elicited after six weeks with a boost injection of the same antigen. Specific anti-TNP Ig were dosed one and two weeks after the boost. Mice lacking TrkA in the immune system produced Ig against the TNP antigen at even higher levels than controls, at least for the IgG2b class (Fig.

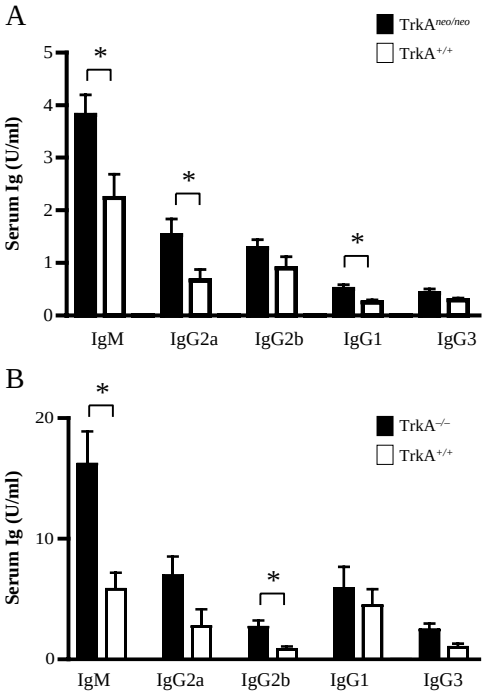

Fig. 4. TrkA deficiency in the immune system causes increased Ig production. (A) Tα1-*Cre*;TrkA$^{neo/neo}$ mice have increased levels of specific classes of Igs. IgM, IgG2a, IgG2b, IgG1 and IgG3 serum levels were measured by ELISA in 12-16-week-old mutants (KO, n=18) and control littermates (CTR, n=14). Data are expressed in Units/ml where 1 U=1 mg for IgM and 1 U=1 μg for IgG2a, IgG2b, IgG1 and IgG3. Bars: means±s.e.m. Data were evaluated using a two-tailed Student's t-test. *P<0.05. (B) C57Bl/6-SCID mice reconstituted with TrkA-deficient fetal liver cells (KO, n=10) have increased levels of serum Igs compared with TrkA wild-type-reconstituted controls (WT, n=6). C57Bl/6-SCID mice were reconstituted and monitored for Ig serum levels from 1 to 5 months after reconstitution. Data after two months from reconstitution are shown. All Ig classes are higher in TrkA-deficient animals, although only IgM and IgG2b are statistically different from controls (two-tailed Student's t-test. *P<0.05).

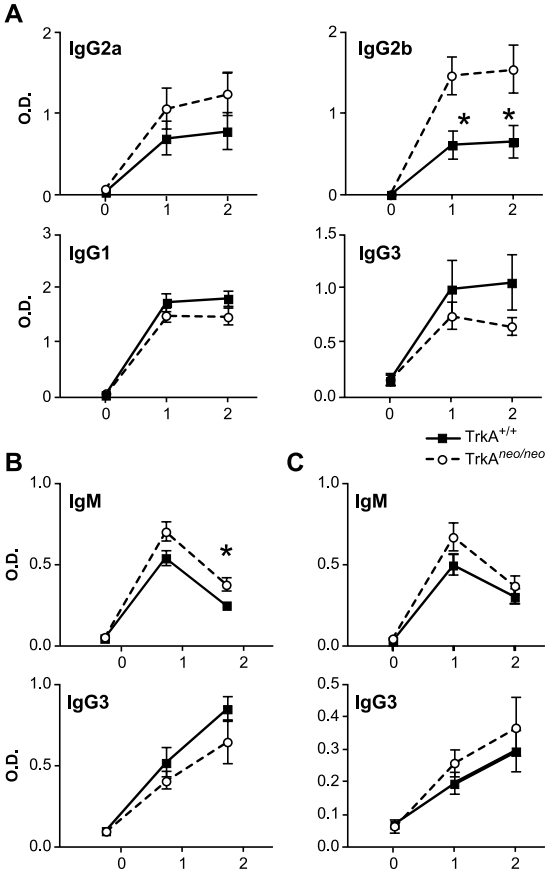

Fig. 5. Absence of TrkA does not impair T-cell-dependent memory nor T-cell-independent type I and II B responses. (A) For the TD memory response, Tα1-*Cre*;TrkA$^{neo/neo}$ (n=7) and control littermates (n=6) were immunized with KLH-TNP as described in Materials and methods. (B,C) for the TI type I and II responses, mice were immunized respectively with LPS-TNP (B, six mutants and five controls) or Ficoll-TNP (C, six mutants and seven controls). Serum levels of specific anti-TNP Igs were measured by ELISA before immunization (0), at one (1) and two (2) weeks after the challenge. Bars: means±s.e.m. Data were evaluated using a two-tailed Student's t-test. *P<0.05.

5A). Therefore, TrkA-deficient B cells can mount a humoral memory response. Next, we investigated whether the T-cell-independent (TI) type I and type II B-cell responses were affected by the TrkA deletion. Mice were immunized with LPS-TNP for the TI type I response or Ficoll-TNP for the TI type II response (Hasan et al., 2002). Again, anti-TNP-specific Ig levels in mutant mice after one and two weeks from the boost injection were comparable or even higher than those of control littermates, indicating that TI responses are mounted efficiently in the absence of TrkA (Fig. 5B,C). Instead, the higher levels of TNP-specific IgM observed in the absence of TrkA following LPS immunization suggest that the abnormal Ig production in the mutant mice is B-cell autonomous.

TrkA deficiency leads to accumulation of B1 lymphocytes

The increased Ig levels observed in TrkA mutant mice suggest the presence of B-cell abnormalities. Changes in Ig levels have also been observed in mice with defects in B Cell Receptor (BCR) signaling (Hardy and Hayakawa, 2001). In vivo alterations in BCR signaling are often associated with changes in B1 lymphocyte numbers (Hardy and Hayakawa, 2001; Hayakawa and Hardy, 2000). Thus, we used flow cytometry to analyze the peritoneal B1 cell population of four-, eight- and twelve-month-old mice. The absolute number of cells recovered from the peritoneal cavity was similar in mutant and control mice (data not shown). Four- and eight-month-old mutant mice harbored normal B1 cell numbers (data not shown). However, 12-months or older mutant mice (n=7) showed a 30% (P<0.05) increase of B1 cells compared with control littermates (n=6) (Fig. 6). This was demonstrated by an increased population of lymphocytes expressing $B220^{low}$, IgM^{high}, Mac-1^{+}, IgD^{low} and $CD5^{+}$, which represents the hallmark of B1 lymphocytes (Fig. 6) (Hardy et al., 2000; Hayakawa and Hardy, 2000). These data suggest that TrkA may control lymphoid function by modulating BCR signaling.

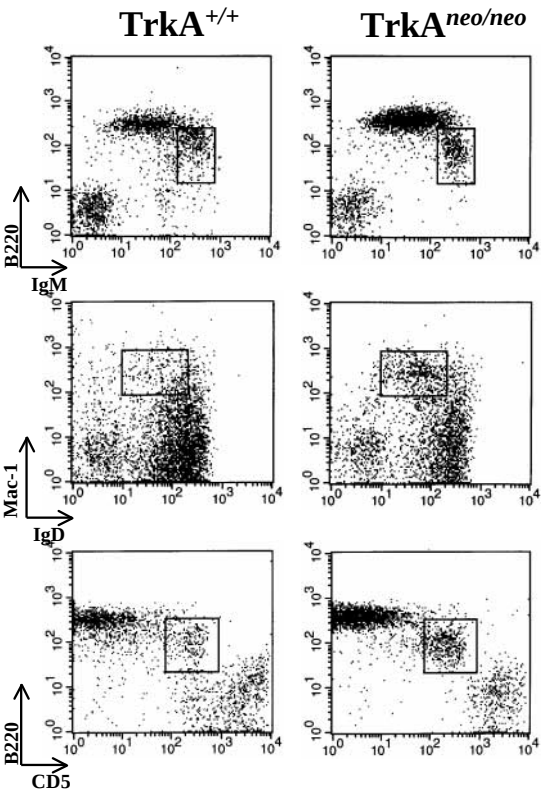

Fig. 6. Aged Tα1-*Cre*;$TrkA^{neo/neo}$ mice have increased levels of peritoneal B1 cells. Twelve- to eighteen-month-old mutant (n=6) and control (n=6) littermates were sacrificed and peritoneal cells were collected as previously described (32). Similar numbers of cells were recovered from both groups. Cells were stained for FACS analysis with anti-B220, -IgM, -CD5, IgD and -Mac-1 antibodies in triple staining combinations. Three staining combinations are shown (B220/IgM; Mac-1/IgD; B220/CD5). The B1 cell population is indicated by a box. Percentages of peritoneal B2 cells ($B220^{+}$/IgM^{low}/IgD^{high}/Mac-1^{-}) and macrophages ($B220^{-}$/Mac-1^{+}) were not different in mutant mice compared with controls.

Discussion

A role for NGF in the normal development of the immune system has long been hypothesized and yet there is no convincing data supporting this function in vivo. A major limitation in addressing this issue has been the lack of a suitable in vivo model to dissect direct versus indirect effects of NGF on lymphoid organs. For example, systemic ablation of NGF or its TrkA receptor causes such severe sensory and sympathetic neuronal losses that phenotypes observed in non-neuronal organs could be caused by innervation deficits rather than an intrinsic loss of NGF/TrkA function. We have generated a model that provides a means to address this question by rescuing TrkA function in the nervous system, but not in non-neuronal structures. We have shown that NGF/TrkA is dispensable for the development of the lymphoid organs and yet causes defects including increased Ig production and accumulation of peritoneal B1 cells. These data are consistent with a modulatory role of this ligand/receptor system on immunological functions.

The phenotype observed in the mutant mouse used in our study is less severe than the one observed in the complete TrkA-deficient mice which have thymus abnormalities consistent with organogenesis defects (Garcia-Suarez et al., 2000). Because the difference between these two mutants is mainly the presence (this study) or the absence (Garcia-Suarez et al., 2000) of a fully developed nervous system, it is tempting to speculate that a cross-talk between the immune system and the nervous system is required not only for normal function but also development of the immune system. Similarly, less severe phenotypes were observed in our mutant compared with those of a transgenic mouse model producing anti-NGF neutralizing antibodies (Ruberti et al., 2000) which develops massive cell death in the spleen, skeletal dystrophy and body weight reduction resulting in mouse lethality. Although that model is of value for other studies (Capsoni et al., 2000), our data suggest that the non-neuronal phenotypes are most likely caused by abnormal innervation or a toxic effect of the anti-NGF antibodies on different tissues. Indeed, the massive cell death observed in the spleen is not easily reconcilable with the limited expression of NGF receptors in this organ.

NGF has been particularly studied for its effects on B lymphocytes. Several reports have suggested that in vitro NGF can modulate Ig production as well as B-cell proliferation and survival. However, these data often lack consistency and

depending on the model used, somewhat contrasting results were described (Brodie et al., 1996; Kimata et al., 1991a; Kimata et al., 1991b; Kimata et al., 1991c). Thus, to date it is still unclear what role NGF exerts in vivo. To a certain degree, the lack of consistency of those results may reflect differences between cell lines. In this study, we have found that although B lymphocytes appear to develop normally in the absence of TrkA, Ig production is affected. Some Ig classes are significantly increased. These increases were subtle, but could be confirmed in a 'classical reconstitution model'. Mice reconstituted with TrkA-deficient cells showed the same B- and T-cell numbers compared with animals reconstituted with littermate wild-type cells (not shown). As observed in the 'reverse conditional TrkA mouse model', we found increased Ig levels in mice reconstituted with TrkA-deficient fetal cells. Importantly, these data validate our genetic model.

It has been reported that antibody neutralization of NGF in vitro and in vivo affects the viability of B memory lymphocytes, suggesting an autocrine role for NGF on the survival of memory B lymphocytes (Torcia et al., 1996). Although the mechanism for this effect is still unknown, it has recently been suggested that inactivation of p38 MAPK, possibly through a TrkA-activated phosphatase, may be required to prevent B memory lymphocyte apoptosis (Torcia et al., 2001). In contrast, we have found that TrkA-deficient mice can mount a robust B memory response (Fig. 5A). We tested the response one and two weeks after the boost dose of antigen, whereas Torcia et al. (Torcia et al., 1996) analyzed the response after only four days from the recall-dose of immunogen (six days after anti-NGF treatment). Thus, we cannot exclude that the discrepancy is caused by different timing of the analysis. Alternatively, we have exclusively ablated TrkA function while anti-NGF antibodies were used in the previous study. Therefore, it is also possible that other players such as p75 (or other yet unknown NGF receptors) may be involved in the development of a B memory response. However, our preliminary data suggest that p75-deficient mice can also mount a humoral response.

Following immunization with a T-dependent antigen (KLH-TNP), TrkA-deficient mice are able to produce specific Ig at levels comparable to those of control littermates. Notably, following immunization the mutants produce more IgG2b, a class that is overall similarly represented in basal conditions. These data provide definitive evidence that NGF/TrkA does not regulate Ig class switch as previously suggested (Torcia et al., 1996).

In addition to disregulation of Ig levels, we have also found that TrkA deficiency causes an increase in peritoneal B1 cells with aging. Several studies have shown that B1 cells are increased in mouse models deficient for molecules that negatively regulate BCR signaling, whereas B1 are decreased when molecules positively regulating BCR signaling are inactivated (Hardy et al., 2000; Hayakawa and Hardy, 2000). Therefore, it appears that TrkA may play an inhibitory role on BCR signaling. A direct link between BCR- and TrkA-signaling has not yet been described. However, both receptors can independently affect vav, a Rac guanine nucleotide exchange factor expressed exclusively in hematopoietic cells (Melamed et al., 1999). Thus, it is tempting to speculate that TrkA may influence BCR signaling by acting on one of its downstream players such as vav. This effect on BCR signaling could ultimately be involved in dictating B-cell commitment to proliferation or differentiation.

Collectively, our data suggest that TrkA, although modulating immune system functions, is not required for its normal development. B- and T-cell number and distribution in spleen, thymus, bone marrow and blood appear unaffected by TrkA deletion. Similar results have been obtained for mast cells in skin, spleen and lymph nodes despite reports that NGF injections cause their increase in various tissues of neonatal rats (Aloe and Levi-Montalcini, 1977; Bienenstock et al., 1987; Bullock and Johnson, Jr, 1996). This is a striking result because many studies suggested that disruption of the NGF/TrkA system in the immune system would cause dramatic deficits. In our initial in vivo studies of TrkA-deficient mast cells, we have been unable to detect a clear phenotype probably because of compensatory mechanisms or vagaries associated with the mixed genetic background typical of targeted mutation mouse models (Fig. 3A-F). However, we have detected some abnormalities in their degranulation potential in vitro, suggesting that TrkA may have a role in mast cell function (Fig. 3G). Mast cell degranulation followed by mediator release is a well-described contributor to airway hyper-reactivity. It has been reported that NGF augments the allergic early-phase reaction in the lung (Path et al., 2002). In vivo experiments using this TrkA mutant mouse model in a pure genetic background will help elucidate the role and contributions of NGF/TrkA in mast cell function and their net contribution to mast cell-driven pathophysiology. It has been reported that plasma and/or serum levels of NGF are increased in several allergic disorders including asthma, urticaria-angioedema, allergic rhinoconjunctivitis and vernal keratoconjunctivitis (Aloe and Tuveri, 1997; Bonini et al., 1996; Lambiase et al., 1995). Because mast cells express exclusively TrkA and not p75, it is tempting to suggest that this receptor may provide a target for the management of inflammatory diseases (Micera et al., 2003). Yet, efforts toward this goal have not been vigorously pursued because the available data had suggested that tampering with the NGF/TrkA pathway would cause severe immunological deficits. However, the relatively limited impact on immune system development reported here indicates that TrkA could provide a useful target for the control of inflammatory diseases. Thus, this mouse model should provide a useful system to test the contribution of NGF/TrkA in the pathology of these disorders.

Finally, this novel approach by 'reverse conditional gene targeting', could be applied to many other systems in which complete inactivation of a gene in specific organs/tissues is required but cannot be achieved with the currently available recombinase and transgenic systems.

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