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Regulatory Effects of Novel Neurotrophin-1/B Cell-Stimulating Factor-3 (Cardiotrophin-Like Cytokine) on B Cell Function

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We describe regulatory effects that a novel neurotrophin-1/B cell-stimulating factor-3 (NNT-1/BSF-3), also reported as cardiotrophin-like cytokine (CLC), is the most recently identified member of the IL-6 family of cytokines (1, 2). NNT-1/BSF-3 is a 225-aa protein with the highest homology to cardiotrophin-1 and ciliary neurotrophic factor (CNTF) (1, 2). We cloned NNT-1/BSF-3 cDNA from activated human T cells and found that NNT-1/BSF-3 mRNA is mainly expressed in lymph nodes and spleen (1). NNT-1/BSF-3 shows activities typical of IL-6 family members, proving to be both a neurotrophic factor and a BSF (1). NNT-1/BSF-3 stimulates B cell proliferation and Ig production in vitro. NNT-1/BSF-3 induces tyrosine phosphorylation of gp130 (1, 2), leukemia inhibitory factor receptor-β (LIF-Rβ) (1), and STAT-3 (1). In vitro, it supports the survival of chicken and rat embryo neurons (1, 3, 4). In mice, it induces serum amyloid A (SAA), attenuates the induction of corticosterone and IL-6 by IL-1, and causes body weight loss and B cell hyperplasia with serum IgG and IgM increase (1). NNT-1/BSF-3 can be secreted as a heterocomplex with cytokine-like factor (CLF) (5), also reported as NR6 (6), and uses the receptor complex for CNTF, which consists of CNTF-Rα, gp130, and LIF-Rβ (7), to initiate signal transduction (3, 8). Alternatively, NNT-1/BSF-3 can be secreted as a heterocomplex with soluble CNTF-Rα and uses the gp130/LIF-Rβ receptor dimer for signaling (4). Since mice lacking CLF or CNTF-Rα, unlike those lacking CNTF, show a lethal motor neuron defect (6, 9), NNT-1/BSF-3 is postulated to be the elusive CNTF II, a developmentally important second ligand for the receptor for CNTF (10).

To gain knowledge of the functional role of NNT-1/BSF-3 in immunity we have conducted in vitro and in vivo experiments that involved the generation of NNT-1/BSF-3-transgenic mice. The results of these experiments indicate that NNT-1/BSF-3 regulates immunity by stimulating B cell function and Ab production, with preference for Th2 over Th1 Ig types. The Journal of Immunology, 2002, 168: 5690–5698.

Materials and Methods

Mice and reagents

Transgenic mice with expression targeted to the liver were prepared as previously described (11). Briefly, the sequence encoding human NNT-1/BSF-3 (1) was subcloned into the apolipoprotein E expression vector, which is liver specific (11). The resultant plasmid was microinjected into single-cell embryos from BDF1 mice following published procedures (12, 13). After overnight culture, 15–20 two-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice. Following term pregnancy, offspring were screened by PCR for the presence of the integrated transgene and bred to generate transgenic heterozygotes. These heterozygotes (positive for the NNT-1/BSF-3 transgene), males and females, were studied between 6 and 28 wk of age together with sex- and age-matched littermate controls (negative for the NNT-1/BSF-3 transgene). Expression of the transgene was assessed by Northern blot of liver RNA and quantified with a PhosphorImager and the ImageQuant program (Molecular Dynamics, Sunnyvale, CA). Normal mice (BALB/c females, 9–12 wk old) were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed in rooms at constant temperature and humidity under 12-h light/dark cycles and fed with standard laboratory diet and water ad libitum. Procedures involving animals and their care were conducted observing institutional guidelines that are in compliance with national and international laws and policies. Recombinant human NNT-1/BSF-3 was produced in Escherichia coli as previously described (1). Before use in vitro or in vivo, all preparations of NNT-1/BSF-3 were checked for the presence of endotoxin and were always found to contain <4.4 endotoxin U/mg protein.

B cell proliferation and Ig production in vitro

The Raji human Burkitt’s B cell lymphoma cells (American Type Culture Collection, Manassas, VA) were cultured in complete RPMI 1640 medium (Life Technologies, Grand Island, NY) with 10% FBS (HyClone, Logan, UT) and 25 × 10⁻³ M HEPES in the presence or the absence of different concentrations of NNT-1/BSF-3, IL-6 (Amgen), and soluble CNTF-Rα.
Necropsy, histological examination, and counts of spleen, lymph node, and peripheral blood cells of NNT-1/BSF-3-transgenic mice

NNT-1/BSF-3-transgenic mice were sacrificed for necropsy analysis at different ages, ranging between 6 and 28 wk. Organs were weighed and fixed in formalin, embedded in paraffin, sectioned, and stained with H&E or periodic acid-Schiff (PAS) using standard histochimical techniques. Livers and kidneys were also collected in OCT (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and cut in a cryostat. Livers were stained by an indirect immunoperoxidase technique using mAb against B220 and CD3 (BD PharMingen, San Diego, CA) and avidin-biotin-peroxidase complexes. Sections were finally counterstained with hematoxyl. Kidneys were stained by direct immunofluorescence technique using FITC-conjugated polyclonal Ab (goat anti-mouse IgM and horse anti-mouse IgG from Vector (Burlingame, CA) and goat anti-mouse C3 from Cappel (Aurora, OH)).

For transmission electron microscopy (TEM), kidneys were fixed by perfusion or immersion. For perfusion fixation, a transcardiac catheter was employed. After a brief initial flush with saline, the vasculature was perfused for 20 min with a fixative consisting of 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. Immediately after perfusion, the kidneys were removed, thinly sliced, and immersion fixed at 4°C overnight. For immersion fixation, blocks approximately 1 mm³ were placed directly into fixative overnight. The specimens were trimmed into blocks and rinsed for 24 h in 0.1 M sodium cacodylate buffer at 4°C. The kidneys were then postfixed in 1% aqueous osmium tetroxide for 1 h, rinsed in distilled water, and dehydrated in ethanol. Following a transition to propylene oxide, the tissue blocks were infiltrated and embedded with an Eponate-12 epoxy resin mixture (Ted Pella, Redding, CA) and goat F(ab')2 anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA). Mouse B cell proliferation was estimated after 4 days of culture studying [3H]thymidine incorporation, for which [3H]thymidine was added to the cells during the final 18 h of culture. Mouse B cell Ig production was estimated after 7 days of culture measuring IgM, IgG, and IgA in culture supernatants.

Induction of Ag-specific Ab

NNT-1/BSF-3-transgenic mice were immunized on day 0 by s.c. injection of 100 μg keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL) in CFA along the flanks of the abdomen or by i.p. injection of 115 μg Pneumovax (Merck, West Point, PA). Mice were bled to detect Ag-specific Ab immediately before and 7, 14, and 21 days after immunization. Normal mice were immunized and bled as described above and also given NNT-1/BSF-3 (3 mg/Kg) or NNT-1/BSF-3 vehicle as a control i.p. for 7 consecutive days starting on day 0. Anti-KLH IgM, IgG, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE were measured in serum by ELISA. Briefly, plates were coated with KLH in PBS, blocked, and added with dilutions of test samples. Captured anti-KLH Ab were revealed using biotinylated Ab specific for Ig classes and subclasses (all from Southern Biotechnology Associates (Birmingham, AL), except anti-IgE, which was from BD PharMingen (San Diego, CA)), nevirudin-conjugated peroxidase (Pierce), and TMB microwell peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD). Plates were also coated with standard preparations (IgM and IgG from Calbiochem (La Jolla, CA); IgG1, IgG2a, IgG2b, and IgG3 from Southern Biotechnology Associates; and IgA and IgE from PharMingen) in PBS, blocked, and further processed using biotinylated Ab and other reagents as described above. Anti-Pneumovax IgM were also measured in serum by ELISA. Briefly, plates were coated with Pneumovax using poly-l-lysine, blocked, and added with dilutions of test samples. Captured anti-Pneumovax IgM were revealed using a biotinylated anti-IgM Ab, nevirudin-conjugated peroxidase, and peroxidase substrate as described above. Anti-Pneumovax IgM and IgG were also measured by ELISA. Briefly, plates were coated with a DNA coating solution (DNA-3000, Immunovision, Springdale, ARZ) diluted in PBS, blocked, and added with dilutions of test samples. Captured anti-Pneumovax IgM and IgG were revealed as described above for Ig. For the measurement of both Ig and anti-dsDNA IgM and IgG, plates were also coated with standard preparations and further processed as described above. IgA and IgM were measured in serum using commercially available kits (BioSource, Camarillo, CA). OD was quantitated as described above.

FIGURE 1. NNT-1/BSF-3 stimulates B cell proliferation and Ig production in vitro. a, NNT-1/BSF-3 stimulates the proliferation of Raji hu-

technology Associates; and IgA and IgE from PharMingen) in PBS,

and further processed as described above. OD were quantitated in a Thermomax ELISA reader (Molecular Devices, Menlo Park, CA).

Ig, anti-dsDNA Ab, and SAA Ig classes and subclasses were measured in serum and culture supernatants by ELISA. Briefly, plates were coated with capture Ab specific for Ig classes (all from Southern Biotechnology Associates, except anti-IgE, which was from BD PharMingen) in PBS, blocked, and added with dilutions of test samples. Captured anti-dsDNA IgM and IgG were revealed using biotinylated anti-IgM Ab, nevirudin-conjugated peroxidase, and peroxidase substrate as described above. Serum anti-dsDNA IgM and IgG were also measured by ELISA. Briefly, plates were coated with a DNA coating solution (DNA-3000, Immunovision, Springdale, ARZ) diluted in PBS, blocked, and added with dilutions of test samples. Captured anti-dsDNA IgM and IgG were revealed as described above for Ig. For the measurement of both Ig and anti-dsDNA IgM and IgG, plates were also coated with standard preparations and further processed as described above. SAA was measured in serum by ELISA using commercially available kits (BioSource, Camarillo, CA). OD was quantitated as described above.

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NNT-1/BSF-3 also costimulates the proliferation of murine primary B cells by potentiating the proliferation-inducing effect of an anti-IgM Ab (Fig. 1c). NNT-1/BSF-3 does not stimulate the proliferation of primary B cells either alone or in conjunction with LPS or IL-4 or an anti-CD40 Ab (data not shown). Alone, however, NNT-1/BSF-3 is able to stimulate primary B cells to produce IgM, IgG, and IgA (Fig. 1d). NNT-1/BSF-3 does not appear to affect the proliferation of murine primary T cells either alone or in conjunction with an anti-CD3 Ab (data not shown).

**Necropsy findings in NNT-1/BSF-3-transgenic mice**

The transgenic mice presented in this paper were engineered to express NNT-1/BSF-3 in the liver under control of the apolipoprotein E promoter and secrete it into the circulation together with plasma proteins (11, 13). Transgenic mice, i.e., positive by PCR for the NNT-1/BSF-3 transgene, were studied together with sex- and age-matched littermate controls, i.e., negative for the NNT-1/BSF-3 transgene. Transgenic mice show liver expression of NNT-1/BSF-3 mRNA (Fig. 2, a and b). This expression is variable, but much increased compared with that in littermate controls (Fig. 2, a and b) and is accompanied by the expression of the phenotype, as represented by high spleen B cell counts and high serum IgM levels (see below; Fig. 2, c and d).

NNT-1/BSF-3-transgenic mice show B cell hyperplasia. Spleens from NNT-1/BSF-3-transgenic mice are larger and have higher absolute counts of total cells and B cells, but not T cells, than spleens from littermate controls (Tables I and II). Compared

**Table III. Lymph node and peripheral blood cell counts in NNT-1/BSF-3-transgenic mice***

<table>
<thead>
<tr>
<th>Lymph nodes</th>
<th>NNT-1/BSF-3 TG</th>
<th>Control LM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells (no.)</td>
<td>8.6 ± 2.3</td>
<td>9.7 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>CD3+ cells (%)</td>
<td>46.6 ± 5.8</td>
<td>66.0 ± 5.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CD3+ cells (no.)</td>
<td>3.5 ± 0.6</td>
<td>6.1 ± 0.8</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>CD3+ B220+ cells (%)</td>
<td>50.0 ± 5.5</td>
<td>32.0 ± 5.4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CD3+ B220+ cells (no.)</td>
<td>4.8 ± 1.7</td>
<td>3.4 ± 1.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Peripher blood</th>
<th>NNT-1/BSF-3 TG</th>
<th>Control LM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes (no.)</td>
<td>10.9 ± 1.7</td>
<td>10.9 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>CD3- cells (%)</td>
<td>14.0 ± 2.1</td>
<td>21.2 ± 0.7</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>CD3- cells (no.)</td>
<td>1.5 ± 0.3</td>
<td>2.3 ± 0.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CD3- B220+ cells (%)</td>
<td>80.0 ± 2.1</td>
<td>72.8 ± 1.3</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>CD3- B220+ cells (no.)</td>
<td>8.8 ± 1.4</td>
<td>8.0 ± 0.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

***TG, transgenic mice; LM, littermate controls; %, percentage of total cells; no., count in millions of cells; n = 5.

---

**Table II. Counts of spleen B cell subsets in NNT-1/BSF-3-transgenic mice***

<table>
<thead>
<tr>
<th>NNT-1/BSF-3 TG</th>
<th>Control LM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells (no.)</td>
<td>226.4 ± 27.2</td>
<td>107.2 ± 13.4</td>
</tr>
<tr>
<td>IgM high IgD low cells (%)</td>
<td>2.5 ± 0.2</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>IgM high IgD low cells (no.)</td>
<td>5.7 ± 0.9</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>IgM high IgD high cells (%)</td>
<td>6.0 ± 0.5</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td>IgM high IgD high cells (no.)</td>
<td>13.8 ± 2.3</td>
<td>6.9 ± 1.1</td>
</tr>
<tr>
<td>IgM low IgD high cells (%)</td>
<td>39.8 ± 3.0</td>
<td>31.1 ± 1.4</td>
</tr>
<tr>
<td>IgM low IgD high cells (no.)</td>
<td>91.1 ± 16.4</td>
<td>33.7 ± 5.0</td>
</tr>
<tr>
<td>IgM CD5+ cells (%)</td>
<td>6.9 ± 0.9</td>
<td>6.9 ± 0.7</td>
</tr>
<tr>
<td>IgM CD5+ cells (no.)</td>
<td>10.3 ± 1.6</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td>IgM CD21+CD23- cells (%)</td>
<td>9.4 ± 1.2</td>
<td>8.7 ± 1.0</td>
</tr>
<tr>
<td>IgM CD21+CD23- cells (no.)</td>
<td>13.8 ± 2.3</td>
<td>6.1 ± 0.8</td>
</tr>
</tbody>
</table>

***TG, transgenic mice; LM, littermate controls; %, percentage of total cells; no., count in millions of cells; n = 7.

---

**Results**

**B cell proliferation and Ig production in vitro**

NNT-1/BSF-3 stimulates the proliferation of the Raji human Burkitt’s B cell lymphoma cells in culture dose-dependently and similarly to IL-6 (Fig. 1a). This stimulation is potentiated by soluble CNTF-Rα. This stimulation is potentiated by soluble CNTF-Rα. Likewise to IL-6 (Fig. 1).

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**Figure 2.** NNT-1/BSF-3-transgenic mice (TG) show liver expression of NNT-1/BSF-3 mRNA, which is increased compared with that in littermate controls (LM) and is accompanied by high counts of spleen B cells and high levels of serum IgM. a, Northern blot of liver RNA revealing NNT-1/BSF-3 mRNA. b, Levels of NNT-1/BSF-3 mRNA quantified with a PhosphorImager and the ImageQuant program. c, Counts of spleen B cells (IgM+). d, Levels of serum IgM.

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**Statistical analysis**

Results are expressed as the mean ± SE. Differences between groups were analyzed by Student’s t test.

**Table I. Spleen weight and cell counts in NNT-1/BSF-3-transgenic mice***

<table>
<thead>
<tr>
<th>NNT-1/BSF-3 TG</th>
<th>Control LM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mg)</td>
<td>275 ± 22</td>
<td>179 ± 17</td>
</tr>
<tr>
<td>Total cells (no.)</td>
<td>243.5 ± 30.2</td>
<td>134.6 ± 16.1</td>
</tr>
<tr>
<td>CD3+ cells (%)</td>
<td>13.4 ± 2.1</td>
<td>20.8 ± 3.4</td>
</tr>
<tr>
<td>CD3+ cells (no.)</td>
<td>29.3 ± 3.8</td>
<td>26.6 ± 5.4</td>
</tr>
<tr>
<td>CD3+ B220+ cells (%)</td>
<td>68.8 ± 2.7</td>
<td>62.8 ± 3.1</td>
</tr>
<tr>
<td>CD3+ B220+ cells (no.)</td>
<td>163.5 ± 25.1</td>
<td>81.1 ± 8.1</td>
</tr>
</tbody>
</table>

***TG, transgenic mice; LM, littermate controls; %, percentage of total cells; no., count in millions of cells; n = 5.
FIGURE 3. NNT-1/BSF-3-transgenic mice show B cell hyperplasia. a and b, Spleens from an NNT-1/BSF-3-transgenic mouse and a littermate control. The spleen from the transgenic mouse shows follicular hyperplasia (arrows, a). c–f, Lymph nodes from an NNT-1/BSF-3-transgenic mouse (c) and a littermate control (d). The lymph node from the NNT-1/BSF-3-transgenic mouse shows sinusoidal hyperplasia (arrows, c) and medullary cord expansion with prominent presence of plasma cells (arrows, e), some of which contain eosinophilic cytoplasmic globules, also called Russell bodies (arrows, f). H&E staining. The microbar length is indicated in micrometers.

FIGURE 4. NNT-1/BSF-3-transgenic mice show anomalous lymphoid aggregates. a–c, Kidney (a), lung (b), and liver (c) from NNT-1/BSF-3-transgenic mice showing anomalous lymphoid aggregates. d, Liver from an NNT-1/BSF-3-transgenic mouse with a lymphoid aggregate containing numerous plasma cells (arrows). e and f, Liver from an NNT-1/BSF-3-transgenic mouse showing a lymphoid aggregate largely consisting of B cells (e) and only few T cells (f). a–d, H&E staining, e and f, Immunoperoxidase staining for B220 (e) and CD3 (f). The microbar length is indicated in micrometers.
with controls, spleens from the transgenic mice have higher absolute counts of all B cell subsets identified (B1 + MZ + T1, T2, B2, B1a, and MZ), higher percentages of B2 cells, and unchanged percentages of the other subsets (Table II). Thus, NNT-1/BSF-3-transgenic mice have an enlarged population of B2 B cells in both absolute and relative terms. Lymph nodes and peripheral blood from NNT-1/BSF-3-transgenic mice have higher percentages of B cells and lower percentages of T cells than littermate controls (Table III). Spleen and lymph nodes from NNT-1/BSF-3-transgenic mice show, respectively, follicular and sinusoidal lymphoid hyperplasia with the prominent presence of plasma cells (Fig. 3, a, c, e, and f). Some of these plasma cells contain eosinophilic cytoplasmic globules, also called Russell bodies (Fig. 3f). Various organs from NNT-1/BSF-3-transgenic mice, especially liver, kidneys, and lungs, show anomalous lymphoid aggregates (Fig. 4). These aggregates are found in the interstitium of kidneys (Fig. 4a) and lungs (Fig. 4b) and mostly in the liver periportal area (Fig. 4, c–f), consist of B cells with only a few T cells (Fig. 4, e and f), and contain a considerable number of plasma cells (Fig. 4d).

NNT-1/BSF-3-transgenic mice show hypergammaglobulinemia. NNT-1/BSF-3-transgenic mice have higher serum levels of IgM and IgE, but not total IgG and IgA, than controls (Fig. 5a). Considering the individual IgG subclasses, however, NNT-1/BSF-3-transgenic mice have higher serum levels of IgG2b and IgG3 than controls. Notably, in the NNT-1/BSF-3-transgenic mice the levels of IgM show a strong tendency to increase with age (Fig. 5b). NNT-1/BSF-3-transgenic mice also have higher serum levels of anti-dsDNA IgM and IgG than controls (Fig. 5c).

NNT-1/BSF-3-transgenic mice show conspicuous deposits of amorphous material in the kidney glomeruli, which are collected within the mesangium (Fig. 6, a and b). These deposits contain glycoproteins, as indicated by their cosinophilic nature (Fig. 6, a and b) and by staining with PAS (Fig. 6, c and d). IgM, IgG, and the complement component C3 accumulate within these deposits, as shown by immunofluorescence staining (Fig. 6, e–j). Surprisingly, these deposits do not seem to contain amyloid, as indicated by the fact that are negative for Congo Red-associated birefringence (data not shown), even though NNT-1/BSF-3-transgenic mice have high levels of serum SAA (5.77 ± 0.70 μg/ml in NNT-1/BSF-3-transgenic mice (n = 59) vs 1.12 ± 0.15 μg/ml in controls (n = 22); p < 0.001). TEM provided further evidence that these deposits do not contain amyloid. The deposits were found to consist of arrays of fibrils and tubular structures located in the mesangial and subendothelial sites within affected glomeruli (Fig. 7, a–c). Where present, the tubular structures were approximately 30–50 nm in diameter (Fig. 7d). The regular arrangement and approximate diameter of these structures contrast with the randomly arranged 10- to 12-nm diameter fibrils described for amyloid (17). Rather, the findings were reminiscent of a collection of rare diseases reported as fibrillar and immunotactoid glomerulopathies (18–20). The presence of these unusual glomerular deposits was especially prominent in NNT-1/BSF-3-transgenic mice ≥22 wk of age and with marked elevations of circulating IgM. NNT-1/BSF-3-transgenic mice show no other abnormalities other than those mentioned above.

**FIGURE 5.** NNT-1/BSF-3-transgenic mice show high serum levels of IgM, IgE, IgG2b, IgG3, and anti-dsDNA Abs. a, NNT-1/BSF-3-transgenic mice (TG) have higher serum levels of IgM and IgE, but not IgG and IgA, than littermate controls (LM). Mice were bled at the age of 6–28 wk (n = 15). b, NNT-1/BSF-3-transgenic mice (TG) have serum IgM levels showing a stronger tendency to increase with age than littermate controls (LM; n = 5). c, NNT-1/BSF-3-transgenic mice (TG; n = 65) have higher serum levels of anti-dsDNA IgM and IgG than littermate controls (LM; n = 22). a–c, **p < 0.05; ***p < 0.01; ****p < 0.001.

Ag-specific Ab production in NNT-1/BSF-3-transgenic mice and in normal mice treated with NNT-1/BSF-3

Compared with littermate controls, NNT-1/BSF-3-transgenic mice showed increased production of Ag-specific Ab of the IgM, IgA, and IgE classes in response to immunization with the T cell-dependent Ag keyhole limpet hemocyanin (KLH; Fig. 8a). On the other hand, compared with controls, NNT-1/BSF-3-transgenic mice showed decreased production of anti-KLH IgG2a and IgG3 (Fig. 8a). The production of anti-KLH total IgG, IgG1, and IgG2b in NNT-1/BSF-3-transgenic mice was not significantly different from that in controls (Fig. 8a). Compared with controls, NNT-1/BSF-3-transgenic mice also showed increased production of Ag-specific IgM in response to immunization with the T cell-independent Ag Pneumovax (Fig. 8b). Normal mice treated with NNT-1/BSF-3 had increased production of anti-KLH IgE compared with control mice treated with NNT-1/BSF-3 vehicle (Fig. 8c). Mice were treated for 7 days starting on the day of immunization and showed increased anti-KLH IgE production on day 14, but not on day 21. The response to KLH, either proliferation or production of IL-2, IFN-γ, IL-4, and IL-10, of the lymph node cells from NNT-1/BSF-3-transgenic mice was not significantly different from that of controls (data not shown).
FIGURE 6. NNT-1/BSF-3-transgenic mice show mesangial deposits of IgM, IgG, and C3. a–j, Kidney glomeruli from NNT-1/BSF-3-transgenic mice (TG; right column) and littermate controls (LM; left column). a–d, Compared with the littermate control, the glomerulus from the transgenic mouse shows in the mesangium conspicuous deposits of amorphous eosinophilic material (a and b) that are PAS positive (c and d). e–j, The glomerulus from the transgenic mouse shows deposits of IgG (f), IgM (h), and C3 (j), while that from the littermate control is devoid of such deposits (e, g, and i). a and b, H&E staining. c and d, PAS staining. e–j, Immunofluorescence staining for IgG (e and f), IgM (g and h), and C3 (i and j). Microbars are 20 micrometers.
Discussion

This study illustrates the regulatory effects of NNT-1/BSF-3 on immunity, demonstrating that it is an integral molecule of the immune system. NNT-1/BSF-3 has effects on adaptive immunity, directly stimulating B cells to proliferate and produce Ab with preference of Th2 over Th1 Ig types.

NNT-1/BSF-3 is a cytokine of the IL-6 family of very recent identification (1, 2), and little is known of its physiological significance. NNT-1/BSF-3 is a gp130 activator with highest homology to cardiophrin-1 (1, 2), hence the denomination CLC (2). NNT-1/BSF-3 has been shown to have general activities typical of IL-6 family members in vivo, such as induction of SAA, potentiation of induction of corticosterone and IL-6 by IL-1, and induction of body weight loss (1, 21). More specifically, NNT-1/BSF-3 has been shown to have neurotrophic properties similar to CNTF in vitro (1, 3, 4, 22), which suggested the denomination NNT-1 (1), and to have effects reminiscent of IL-6 on B cells in vivo (1, 23), which suggested the other denomination, BSF-3 (1).

To acquire further information on NNT-1/BSF-3 functions, transgenic mice were engineered in this study to aberrantly express NNT-1/BSF-3 in the liver under control of the apolipoprotein E promoter and to secrete it into the circulation together with most plasma proteins (11). These NNT-1/BSF-3-transgenic mice show a phenotype consisting of B cell hyperplasia, hypergammaglobulinemia with anti-dsDNA Ab, and glomerulopathy with mesangial Ig deposition. Interestingly, B cell hyperplasia and hypergammaglobulinemia are also the main abnormalities seen in normal mice given a daily injection of recombinant NNT-1/BSF-3 for 7 days (1), indicating that the effects of NNT-1/BSF-3 supplementation, because of either transgenic expression or pharmacologic administration, are mainly confined to the immune system. Notably, however, NNT-1/BSF-3-injected mice also demonstrate body weight loss (1), a phenomenon not shown by NNT-1/BSF-3-transgenic mice, probably as a consequence of developmental compensatory mechanisms.

The fact that the effects of NNT-1/BSF-3 supplementation mainly concern the immune system does not exclude the possibility that this cytokine has functional roles within other systems, especially the nervous system. NNT-1/BSF-3 may have important functions within the nervous system, as indicated by its neurotrophic properties in vitro (1, 3, 4) and by the phenotype of mice lacking either CLF or CNTF-Rα, two molecules assisting NNT-1/BSF-3 secretion and signal transduction, but devoid of neurotrophic properties per se (3–5, 8, 24), which consists of lethal motor neuron deficiency (6, 9). The study of NNT-1/BSF-3-deficient mice appears at this point to be crucial to prove conclusively that NNT-1/BSF-3 is a factor necessary for the development of motor neurons (10). Studies using neutralizing Ab have the potential to uncover additional functions that NNT-1/BSF-3 may have in adult individuals.

We noticed that NNT-1/BSF-3, which has a conventional signal peptide (1, 2), is produced, but not readily secreted, in vitro by mammalian cells genetically engineered for its recombinant production (unpublished observations). It seems that NNT-1/BSF-3 needs assistance from a concomitantly expressed molecule, such as CLC (3) or CNTF-Rα (4), to be secreted. The finding in this study that mice engineered to express an NNT-1/BSF-3 transgene specifically in the liver and secrete NNT-1/BSF-3 into the general circulation show a systemic phenotype consistent with the phenotype observed in normal mice given i.p. E. coli-derived NNT-1/BSF-3 (1) indicates the possibility that NNT-1/BSF-3 is secreted, at least by the liver, independently of CLC or CNTF-Rα. In fact, neither CLC nor CNTF-Rα is expressed in the liver (5, 24). It remains to be determined whether this occurs because the expression of the transgene abnormally overrides a postulated retention mechanism (4) or because the liver is normally capable of secreting NNT-1/BSF-3, either alone or in conjunction with an as yet unknown molecule. Interestingly, we have noticed a nonnegligible amount of NNT-1/BSF-3 expression in normal mouse liver (1).

B cell hyperplasia, hypergammaglobulinemia, and glomerulopathy with mesangial Ig deposition are also the main abnormalities seen in IL-6-transgenic mice (25, 26), which significantly reinforces the functional connection between NNT-1/BSF-3 and IL-6 (1). However, beyond the similarities, there are differences between the phenotypes of NNT-1/BSF-3 and IL-6-transgenic mice. In NNT-1/BSF-3-transgenic mice B cell hyperplasia also involves cells at less mature stages than plasma cells and particularly concerns B2 (mature follicular) B cells, while in IL-6-transgenic mice...
it involves virtually only plasma cells (25, 26). In NNT-1/BSF-3-transgenic mice hypergammaglobulinemia is limited to a 4- to 6-fold increase in IgM and, interestingly, IgE, while in IL-6-transgenic mice it involves a characteristic increase in IgG1, up to 400-fold (25, 26).

These observations suggest that NNT-1/BSF-3 and IL-6 stimulate B cells at different developmental stages.

In NNT-1/BSF-3-transgenic mice no amyloidosis is found in any organ including kidneys, even when these mice show conspicuous proteinaceous deposits in the mesangium and high levels of circulating SAA that may favor the deposition of AA amyloid. In contrast, in IL-6-transgenic mice, also engineered to express the transgene under control of a liver-specific promoter, i.e., metallothionein I (26), amyloid A amyloidosis is found in spleen, liver, and kidneys, including the mesangium, together with high levels of circulating SAA (27). On the other hand, NNT-1/BSF-3-transgenic mice show the presence in the mesangium of a peculiar type of fibrils of distinctive ultrastructure that has not been observed in IL-6-transgenic mice despite electron microscopic analysis (27). Intriguingly, the accumulation of fibrils and tubular structures in the glomeruli of NNT-1/BSF-3-transgenic mice is reminiscent of a group of rare glomerulopathies of unknown etiology described in patients with proteinuria and often the nephrotic syndrome and reported as fibrillary or immunotactoid glomerulopathies (17–20, 28, 29). Immunotactoid glomerulopathy is ultrastructurally distinguished from fibrillary glomerulopathy by the presence of tubular structures, although some controversy exists concerning whether these glomerulopathies should be considered distinct entities (20). The biopsy specimens from affected patients are often observed to contain Ig. These patients are sometimes found to have an underlying lymphoproliferative disorder possibly with dysproteinemia (18), suggesting a comparable pathogenesis of these types of human glomerulopathies to that of NNT-1/BSF-3-transgenic mice.

NNT-1/BSF-3 has stimulating effects on B cells by virtue of direct action, as shown by the experiments involving the use in vitro of the human B cell line Raji or primary B cells purified from mouse spleens. Two interesting observations were made using the Raji cells, i.e., that NNT-1/BSF-3 has growth-stimulating activity similar to that of IL-6 and that such activity can be increased by soluble CNTF-Rα. That soluble CNTF-Rα can increase NNT-1/BSF-3 activity is consistent with the recent reports that in addition to gp130 and LIF-Rβ, NNT-1/BSF-3 needs CNTF-Rα for signaling in either membrane-bound (3) or soluble form (4) and illustrates the possibility of modulating NNT-1/BSF-3 activity with soluble CNTF-Rα, as previously shown for CNTF (30). NNT-1/BSF-3 does not seem to be able to affect primary B cells independently of stimulation through their Ag receptor. Thus, NNT-1/BSF-3 is likely to act physiologically only as a B cell costimulatory agent. Primary B cells are much less sensitive to NNT-1/BSF-3 than Raji cells. This may depend on various factors, including differences in the expression of NNT-1/BSF-3 receptor components by these cell types.

An interesting finding of this study is the ability of NNT-1/BSF-3 to privilege a Th2 over Th1 type of Ig production. This is already apparent when total levels of circulating Ig of different postswitch classes (IgG, IgA, and IgE) are examined, since NNT-1/BSF-3-transgenic mice, in addition to pre-switch IgM, show higher levels of IgE than controls, but not of IgG or IgA (31). It becomes even more apparent when Ag-specific levels of circulating Ig classes and subclasses are examined, since NNT-1/BSF-3-transgenic mice not only show higher levels of Ag-specific IgE than controls, but also have lower levels of IgG2a. In fact, IgG2a is considered to be the expression of a Th1-type immune response and IgE of a Th2-type response (32, 33). Since lymph node cells from NNT-1/BSF-3-transgenic mice were not found to proliferate or produce Ig class switch-modulating cytokines, such as IFN-γ and IL-4 (34), under Ag-specific stimulation differently from controls, it seems that the ability of NNT-1/BSF-3 to privilege the Ig class switch of Th2 over Th1 type is independent of effects on T cells and, rather, reflects a direct action on B cells.
This is supported by the observation that NNT-1/BSF-3 stimulates B cell proliferation and Ig production in vitro. Other interesting findings of this study are that NNT-1/BSF-3 can stimulate the production of Ag-specific Ab of the IgA class and that it can stimulate Ab production against both T cell-dependent (like KLH) and independent Ag (such as Pneumovax) (35).

In conclusion, NNT-1/BSF-3-transgenic mice show a phenotype of immunological relevance. Since NNT-1/BSF-3 is strongly expressed in lymphoid tissues (1, 2), and since NNT-1/BSF-3-treated mice mainly show immune alterations (1), this new observation indicates that NNT-1/BSF-3 is an integral molecule of the immune system. Future studies, possibly involving deficient mice and neutralizing Abs, may reveal additional physiological roles.

References


