Three day neonatal thymectomy selectively depletes NK1.1+ T cells

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Abstract

Neonatal thymectomy of mice 3 days after birth but not at birth leads to T cell-mediated, organ-specific, autoimmune disease in a strain-dependent manner. The mechanisms that lead to disease in this model remain unknown, but the answer may lie in a deficiency of thymus-dependent cells or factors. One candidate is the relatively rare population of NK1.1+ T cells (NKT cells). Conventional αβ T cells appear in the thymus from days 17–18 of embryogenesis and start emigrating to the periphery around birth, whereas the development of NKT cells is thought to be delayed until at least 1 week after birth. We have confirmed this to be the case in both (BALB/c × C57BL/6)F1 (autoimmune susceptible) and C57BL/6 (autoimmune resistant) mice. Moreover, examination of T cells (in spleen, lymph nodes, liver and bone marrow) from mice following 3 day neonatal thymectomy revealed a significant reduction in the presence of NKT cells in all tissues. However, the extent of depletion was generally more pronounced in (BALB/c × C57BL/6)F1 than in C57BL/6 mice, and the few remaining NKT cells in C57BL/6 mice were enriched for a CD4– CD8int subset which is absent from the thymus and may represent a distinct lineage of thymus-independent NKT cells. Given mounting evidence of a role for NKT cells in protection from autoimmune disease, it is possible that their specific removal by neonatal thymectomy may contribute to the susceptibility of these mice to autoimmune disease.

Introduction

NKT cells are a relatively rare population of T cells with a range of characteristics that distinguish them phenotypically and functionally from conventional T cells (reviewed in 1–5). They share several cell surface molecules with NK cells, including NK1.1, CD122 (IL-2Rβ), CD16 (FcR) and asialo-GM1. However, their identity as T cells is indicated by expression of the CD3–TCR complex. Unlike conventional T cells, however, NKT cells display a highly restricted TCR repertoire, in that ~50% of them express Vγ8.2 and the majority Vα14 (6,7). These cells are heterogeneous, including CD4+CD8– and CD4+CD8– subsets. Some studies have also described a small subset of NKT cells in the liver with a CD4+CD8+int phenotype (8,9 and Hammond et al., in preparation). It is likely that most NKT cells are restricted by, and responsive to, the MHC class I-like molecule CD1, rather than conventional MHC plus peptide (10–13). Perhaps the most significant feature of these cells is their ability to make high levels of immunoregulatory cytokines including IL-4, IL-10 and IFN-γ in response to TCR stimulation (14,15). This property in particular has led to the suggestion that NKT cells may be involved in controlling certain aspects of the immune response, including induction of some Th2 responses (16), although they are probably not essential for all Th2 responses (reviewed in 17). There is also a growing body of evidence to indicate that a deficiency of NKT cells is associated with susceptibility to autoimmune disease in several distinct mouse models (18–23) as well as in humans with systemic sclerosis (24) and type 1 diabetes (25). Moreover, in at least two of these models (21–23), enrichment of NKT cell numbers was shown to be associated with protection from autoimmune disease.

An interesting feature of NKT cells is their relatively late appearance during ontogeny (7,8,26). Whereas conventional T cells appear in the thymus just before birth, NK cells...
are not found until 1–2 weeks after birth. Thus, neonatal thymectomy performed 3 days after birth has the potential to induce a selective deficiency in NKT cells, which may be associated with the susceptibility of these mice to autoimmune disease.

Three day neonatal thymectomy in mice is known to result in T cell-mediated organ-specific tissue destruction in a strain-dependent manner (reviewed in 27,28). For example, BALB/c develop a high frequency of autoimmune gastritis (30–90%), whereas C57BL/6 mice are resistant to this disease. (BALB/c×C57BL/6)F1 mice are also susceptible to disease, but to a slightly lesser extent (30%) than inbred BALB/c mice. (BALB/c×C57BL/6)F1 mice were used in the present study so that the allelic marker NK1.1 could be used to specifically identify NKT cells in these mice. Studies in the 3 day neonatal thymectomy model using BALB/c mice have shown autoimmune gastritis to be mediated by CD4 T cells with specificity for the β subunit of the gastric parietal cell H/K ATPase, since these cells, but not autoantibodies, can transfer disease to T cell-deficient nude or SCID mice. The inflammatory lesion in the stomach appears to be associated with an IFN-γ-dependent T\(_{1,1}\)-type response (29).

In this study, we provide data confirming the thymic dependence of NKT cells, in that neonatal thymectomy leads to their selective deficiency in all organs tested. This was observed in both autoimmune susceptible (BALB/c×C57BL/6)F1 mice and autoimmune resistant C57BL/6 mice, although to a lesser extent in the latter. This study therefore provides a basis for further investigation into the possibility that the deficiency in these cells may be a contributing factor to the susceptibility of these mice to the development of autoimmune disorders.

**Methods**

**Mice**

Adult mice were purchased from the Monash University Animal Facility and housed at the Monash Medical School Animal Facility. Mice were neonatally thymectomized at day 3 as described previously (30). Between 6 and 12 weeks of age, mice were killed at Monash Medical School for removal of spleen, lymph nodes, livers, femurs and thymuses (in sham-thymectomized animals). These organs were then couriered, in PBS on ice, to the Centenary Institute for analysis. For the ontogenic analysis, pregnant (BALB/c×C57BL/6)F1 or C57BL/6 mice were purchased from Animal Resources Centre (Canning Vale, Western Australia), and mothers and pups kept in the Centenary Institute Animal House.

**Cell suspensions**

Cell suspensions of thymus, spleen and lymph nodes were prepared by gently grinding the organs between the frosted ends of glass microscope slides in PBS containing 2% FCS and 0.02% azide (PBS/FCS/Az). Spleen cells were subsequently treated with red cell removal buffer (7.8 mg/ml NH\(_4\)Cl, 0.037 mg/ml EDTA, 1 mg/ml NaHCO\(_3\), adjusted to pH 7.3) prior to staining. Bone marrow cells were harvested from femurs by flushing with PBS/FCS/Az. Hepatic leukocytes were isolated by cutting individual livers into small pieces and gently pressing through 200 gauge wire mesh. The meshed suspension was digested for 10–15 min at 37°C with collagenase (Type II; Boehringer Mannheim, Castle Hill, NSW, Australia) and DNase I (Sigma, St Louis, MO). Hepatic lymphocytes were separated from hepatocyte nuclei and other cellular debris by spinning through a Percoll density gradient (Pharmacia, Uppsala, Sweden). Recovered leukocytes were treated with red cell removal buffer. Bone and liver samples were sometimes stored in PBS/FCS/Az overnight at 4°C and prepared and analysed on the following day, due to the large number of tests to be carried out. This did not significantly affect cell viability or subset representation.

**Flow cytometric analysis**

Cells were first incubated with anti-FcR (clone 2.4G2, grown in-house at Centenary Institute) to prevent non-specific binding by subsequent mAb. Cells were labelled with a cocktail of mAb consisting of anti-αβTCR–FITC (clone H57-597; PharMingen, San Diego, CA), anti-NK1.1–phycoerythrin (PE) (clone PK136; PharMingen), anti-CD4–biotin (clone CT-CD4; Caltag, San Francisco, CA) and anti-CD\(_{8α}\)–allophycocyanin (APC) (clone 53-6.7; PharMingen). Streptavidin–Texas Red was purchased from Molecular Probes (Eugene, OR). An irrelevant mouse IgG2a–PE (anti-rat CD44, clone OX49; PharMingen) mAb was used as an isotype control for anti-NK1.1–PE. Gates for analysis were set based on cell populations occurring in normal adult mice, taking care to exclude low background staining as indicated by isotype (negative) control. Four-colour analysis was performed using a FACStar plus flow cytometer (Becton Dickinson, San Jose, CA) interfaced to a Hewlett Packard Computer (model 340). The machine was calibrated by eye with total thymocytes unstained or stained with either anti-αβTCR–FITC, anti-CD4–PE (clone RM4-5; PharMingen), anti-CD\(_{8α}\)–APC or anti-CD4–biotin followed by streptavidin–Texas Red. Lysys II and CellQuest software (Becton Dickinson) were used for analysis; 3×10\(^4\) events were acquired and then an acquisition gate was applied to generate a large file of NK1.1\(^+\) cells. All plots show forward versus side light scatter lymphoid gated cells.

**Results**

**NKT cells show delayed intrathymic development**

The development of NKT cells was monitored and compared to that of conventional T cells in the thymus and spleen (Fig. 1), from both C57BL/6 (autoimmune resistant) and (BALB/c×C57BL/6)F1 (autoimmune susceptible) strains of mice. Ontogenic analysis at several timepoints, ranging from neonatal day 1 to day 14, clearly showed that conventional NK1.1\(^+\) T cells were already present at day 1, whereas a significant population of NKT cells did not appear until between 7 and 14 days after birth, regardless of the strain examined. Even at day 14, the proportion of NKT cells was well below adult levels (see Fig. 2). This confirms and extends published data (2,26) indicating that
Three day NTx selectively depletes NK1.1$^+$ T cells

Fig. 1. NK1.1 versus $\alpha\beta$TCR expression on thymocytes and splenocytes derived from C57BL/6 or (BALB/c × C57BL/6)F1 mice at various timepoints after birth. The proportion of NK1.1$^+$ $\alpha\beta$TCR$^+$ cells (NKT cells) and NK1.1$^-$ $\alpha\beta$TCR$^+$ cells (conventional T cells) is shown as a percentage of lymphocyte gated cells beside the relevant region. Regions were set based on the location of these populations in adult mice, which were analysed in parallel (not shown). Care was taken to exclude low background staining as identified by the isotype control. These results are representative of three separate mice per group.
Fig. 2. NK1.1 versus αβ TCR expression on lymphocytes from adult mice (A, C57BL/6; B, (BALB/c×C57BL/6)F1) that were sham or neonatally thymectomized at day 3. The intensity of NK1.1 expression was always lower in the F1 mice, presumably due to their heterozygosity for the NK1.1 gene. Quadrants were set based on isotype control labelling. The percentages of each population are included in the corner of each quadrant. The profiles shown are representative of at least eight different mice per group. NTx, neonatally thymectomized.

NK cells show delayed ontogenic development compared to conventional NK1.1+ T cells. The proportion of total αβ TCR+ cells in spleens, even by day 14, was lower than that normally detected in adult mice. This observation has been previously reported (31,32).

NK cells are selectively diminished by neonatal thymectomy. C57BL/6 and (BALB/c×C57BL/6)F1 mice were thymectomized or sham-thymectomized postnatally on day 3. At between 6 and 12 weeks of age, they were killed for organ removal. In all tissues examined including spleen, liver and bone marrow, NK cells were significantly reduced following neonatal thymectomy, particularly in (BALB/c×C57BL/6)F1 mice and to a lesser degree in C57BL/6 mice (Table 1, and Figs 2 and 3). Conventional T cells were also reduced as previously reported (reviewed in 27,28), although not to the same extent as NK cells (Table 1). Lymph nodes were examined in parallel, but were virtually devoid of NK cells both in the 3 day neonatally thymectomized and sham-thymectomized controls (not shown), as is the case in normal mice (1,8). Although T cells were reduced in NTx mice, considering that other cell populations comprise a
greater component of these organs, and the inherent variability in cell counts, a significant reduction in the total cell counts was not observed (Table 1). In order to determine whether NKT cells were relatively reduced compared to conventional T cells, the ratio of NKT cells to T cells was calculated for each organ from each group of mice (Table 1 and Fig. 3). Although the proportion of NKT cells in the thymus remained low at day 7, thymectomy at this time point may have resulted in a greater component of these organs, and the inherent variability in cell counts, a significant reduction in the total cell counts was not observed (Table 1). In order to determine whether NKT cells were relatively reduced compared to conventional T cells, the ratio of NKT cells to T cells was calculated for each organ from each group of mice (Table 1 and Fig. 3). Although the proportion of NKT cells in the thymus remained low at day 7, thymectomy at this time point may have resulted in a greater component of these organs, and the inherent variability in cell counts, a significant reduction in the total cell counts was not observed. In tissues where NKT cells were still detectable, thymectomy led to a comparable reduction in all of these subsets. In tissues where NKT cells were still detectable, such as the liver of C57BL/6 and (BALB/c × C57BL/6)F1, and spleen of C57BL/6 mice, a consistent increase in the representation of CD4+CD8− CD4+CD8+ NK cell populations, whereas the CD4+CD8− Nikt cells seemed to be thymus independent. The relative proportions of CD4+CD8+ and CD4+CD8− conventional (NK1.1+) T cells were not altered by neonatal thymectomy.

### Discussion

Three day neonatal thymectomy of mice results in the occurrence of organ-specific autoimmune diseases. The range of diseases induced in various mouse strains includes gastritis, thyroiditis and autoimmune diseases of the reproductive organs (reviewed in 27,28). Several factors have been suggested to play a role in development of autoimmune disease in this model. The neonatal thymus is known to be inefficient at mediating clonal deletion, resulting in escape of autoreactive cells to the periphery during the early days after birth (33-36). Lymphopenia also occurs as a result of thymectomy, which may contribute to their susceptibility to autoimmune disease (27,28,37) due to the presence of a higher pathogen load, which in turn would result in higher numbers of activated APC carrying both self and foreign peptides. As T cells encounter and are activated by these APC, homeostatic pressures would force their expansion, leading to an abnormally high representation of autoreactive T cells in such neonatally thymectomized mice. Conversely, in normal (non-thymectomized) mice, the constant and increasing output from the thymus would greatly enrich both the number and diversity of T cells, thereby alleviating the homeostatic pressure applied to the very early thymic emigrants. The genesis and maintenance of a pathological autoimmune lesion also requires an environment that permits the development of an inflammatory response, such as mediated by Th1-type T cells. In this respect, a deficiency in immunoregulatory populations such as NKT cells may be of relevance.

Evidence of the potential immunoregulatory role of NKT cells, when combined with previous data suggesting that NKT cells arise somewhat later in ontogeny than conventional T cells, prompted us to investigate whether there was a selective deficiency in NKT cells in mice thymectomized 3 days after birth. Here, we report that NKT cells do not appear in significant numbers in C57BL/6 mice and (BALB/c × C57BL/6)F1 mice until between 7 and 14 days after birth. Presumably due to their delayed appearance,

### Table 1. The effect of neonatal thymectomy on the proportion of NK1.1+ T (NKT) cells

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group</th>
<th>n</th>
<th>NK1+</th>
<th>NKT</th>
<th>T</th>
<th>NKT/T (%)</th>
<th>Cells (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>B6 sham</td>
<td>10</td>
<td>3 ± 1</td>
<td>1.0 ± 0.4</td>
<td>18 ± 6</td>
<td>5 ± 2</td>
<td>140 ± 80</td>
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<tr>
<td></td>
<td>B6 NTx</td>
<td>12</td>
<td>3 ± 2</td>
<td>0.2 ± 0.1</td>
<td>5 ± 4</td>
<td>3 ± 2</td>
<td>80 ± 30</td>
</tr>
<tr>
<td></td>
<td>F1 sham</td>
<td>11</td>
<td>3.3 ± 0.6</td>
<td>0.7 ± 0.1</td>
<td>25 ± 3</td>
<td>3.0 ± 0.8</td>
<td>130 ± 80</td>
</tr>
<tr>
<td></td>
<td>F1 NTx</td>
<td>10</td>
<td>5 ± 1</td>
<td>0.09 ± 0.06</td>
<td>9 ± 3</td>
<td>1.0 ± 0.8</td>
<td>120 ± 80</td>
</tr>
<tr>
<td>Liver</td>
<td>B6 sham</td>
<td>10</td>
<td>8 ± 2</td>
<td>18 ± 5</td>
<td>25 ± 7</td>
<td>40 ± 10</td>
<td>4 ± 2</td>
</tr>
<tr>
<td></td>
<td>B6 NTx</td>
<td>11</td>
<td>14 ± 4</td>
<td>2 ± 3</td>
<td>20 ± 12</td>
<td>10 ± 6</td>
<td>3 ± 2</td>
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<td>F1 sham</td>
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<td>6 ± 2</td>
<td>19 ± 4</td>
<td>26 ± 3</td>
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<td>8</td>
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<td>3 ± 2</td>
<td>13 ± 4</td>
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<td>3 ± 2</td>
</tr>
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<td>Bone marrow</td>
<td>B6 sham</td>
<td>10</td>
<td>0.5 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>1.0 ± 0.6</td>
<td>21 ± 9</td>
<td>13 ± 6</td>
</tr>
<tr>
<td></td>
<td>B6 NTx</td>
<td>11</td>
<td>0.4 ± 0.4</td>
<td>0.06 ± 0.08</td>
<td>0.5 ± 0.4</td>
<td>9 ± 6</td>
<td>10 ± 10</td>
</tr>
<tr>
<td></td>
<td>F1 sham</td>
<td>10</td>
<td>0.5 ± 0.1</td>
<td>0.14 ± 0.04</td>
<td>1.0 ± 0.3</td>
<td>12 ± 3</td>
<td>20 ± 10</td>
</tr>
<tr>
<td></td>
<td>F1 NTx</td>
<td>8</td>
<td>0.6 ± 0.2</td>
<td>0.03 ± 0.03</td>
<td>0.5 ± 0.2</td>
<td>7 ± 4</td>
<td>14 ± 5</td>
</tr>
</tbody>
</table>

- NK1.1+αβTCR− (NK) cells.
- Total cell yield per organ.
- Mean percentage ± SD.
- B6 = C57BL/6; F1 = (BALB/c × C57BL/6)F1; NTx = neonatally thymectomised; sham = sham thymectomised.
NKT cells were selectively reduced in overall number and as a proportion of total \( \alpha \beta \) T cells in mice thymectomized on day 3. This observation represents the first formal demonstration of prolonged deficiency of a distinct T cell lineage following neonatal thymectomy. Since NKT cells have the potential to be protective against \( T_\alpha \)1-type autoimmune tissue destruction (21–23), it is possible that their absence may contribute to the development of autoimmune disease in these mice.

Recently, cell transfer experiments have been used to define T cell subpopulations with the capacity to prevent inflammatory immune diseases in a number of model systems. These T cells include CD45RB\(^{low}\) (in mice) (38) or CD45RC\(^{low}\) (in rats) (39) and CD25\(^{high}\) CD4 T cells (40) in mice. At present, the precise mechanisms by which these T cell populations abrogate autoimmunity is unclear, although provision of regulatory cytokines or various competitive mechanisms are obvious possibilities. These populations have not been shown to be affected by neonatal thymectomy (40) and as these markers do not uniquely define NKT cells they have not been included in the current study. It should be emphasized that the development of an autoimmune disease from an initial self-recognition event to a destructive inflammatory lesion is almost certainly a
Three day NTx selectively depletes NK1.1+ T cells

In attempting to explain precisely how a deficiency in NKT cells might be linked with Th1-type autoimmunity, it is important to point out that the mechanism of activation of NKT cells in vivo is unknown. It is likely to involve an interaction between the TCR on these cells and their ligand CD1 (11), possibly with co-stimulation by accessory molecules such as B7/CD28 (42). CD1 is expressed by thymocytes, gastrointestinal epithelial cells, and at low levels on B and T cells (reviewed in 43), and therefore is likely to be present at sites of inflammation. If activated at these sites, NKT cells could produce cytokines such as IL-4 and IL-10, which have the potential to dampen the Th1-driven inflammatory response (44), thereby protecting the host from excessive tissue damage and autoimmune disease. This might apply regardless of whether the initial response was against a foreign or self antigen and would be consistent with a model where the absence of NKT cells alone does not trigger an autoimmune response, but rather their presence would protect from autoimmune tissue destruction. Clearly, now that some models exist for investigating the protective effect of NKT cells in vivo (21–23) the next important step will be to determine the mechanisms responsible for protection.

In a recent study on the T cells present in neonatally thymectomized mice (45), increased numbers of TCRint, CD122 (IL-2Rβ)+ T cells were detected. As this phenotype includes that of NKT cells, the investigators proposed that neonatal thymectomy may lead to an increased

### Table 2. Phenotype of NK cells in neonatally thymectomized mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group</th>
<th>n</th>
<th>CD4+CD8+</th>
<th>CD4+CD8-</th>
<th>CD4-CD8+</th>
<th>CD4-CD8-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>B6 sham</td>
<td>10</td>
<td>60 ± 10b</td>
<td>29 ± 6</td>
<td>9 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6 NTx</td>
<td>6</td>
<td>40 ± 20</td>
<td>30 ± 10</td>
<td>30 ± 10</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>B6 sham</td>
<td>10</td>
<td>50 ± 10</td>
<td>42 ± 8</td>
<td>7 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6 NTx</td>
<td>11</td>
<td>20 ± 10</td>
<td>20 ± 10</td>
<td>40 ± 20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F1 sham</td>
<td>10</td>
<td>69 ± 5</td>
<td>28 ± 5</td>
<td>1.5 ± 0.6</td>
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<td>F1 NTx</td>
<td>8</td>
<td>40 ± 20</td>
<td>20 ± 3</td>
<td>30 ± 20</td>
<td></td>
</tr>
<tr>
<td>F1 sham</td>
<td>B6 sham</td>
<td>10</td>
<td>62 ± 5</td>
<td>4 ± 2</td>
<td>32 ± 5</td>
<td></td>
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<tr>
<td></td>
<td>B6 NTx</td>
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<td>64 ± 6</td>
<td>9 ± 7</td>
<td>30 ± 10</td>
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<td>F1 sham</td>
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<td>63 ± 3</td>
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<td>67 ± 6</td>
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<tr>
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<td>52 ± 9</td>
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<td>8</td>
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<td>11</td>
<td>59 ± 3</td>
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<td>48 ± 9</td>
<td>1.0 ± 0.9</td>
<td>49 ± 9</td>
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</table>

Table footnote:

aTissues where NKT cells were absent or not clearly detectable after neonatal thymectomy are not shown.
bAll numbers show the mean percentage ± SD.
cDefined as NK1.1–αβTCRint (conventional T) cells.

B6 = C57BL/6; F1 = (BALB/c × C57BL/6) F1; NTx = neonatally thymectomised; sham = sham thymectomised.

**Fig. 4.** CD4 and CD8 expression by NKT cells. Four-colour flow cytometry was used to examine subsets of NKT cells defined by CD4 and CD8 expression. NKT cells were identified using anti-NK1.1 and anti-αβTCR (as depicted in Figs 1 and 2; not shown here) and electronically gated for analysis of CD4 versus CD8 expression. Tissues from sham-thymectomized mice are shown in the top row, and tissues from neonatally thymectomized mice are shown in the bottom row. The CD4 intensity was usually reduced in the liver samples due to partial digestion of CD4 due to contaminating proteolytic enzymes during collagenase digestion. The percentages of each population are included in the corner of each plot. The profiles shown are representative of at least six separate samples per group. NTx: Neonatally thymectomized.
representation of NKT cells. However, as they were studying BALB/c mice, which do not express the NK1.1 marker, they were unable to confirm this hypothesis, whereas our results clearly show that the opposite is in fact the case. The simplest explanation for this apparent discrepancy is that NKT cells represent only a subset of TCRαβ+, CD122+ T cells in the periphery of normal mice (46) and were not represented amongst the cells with this phenotype in the neonatally thymectomized mice. Other markers for NKT cells such as Vα14, Vβ8.2 and Ly49 were not used in this study for several reasons. The Vα14 mAb is not widely available and its specificity has recently been questioned (2). The majority of Vβ8+ cells are conventional T cells, hence a deficiency in NKT cells would not be reflected by the percentage of cells expressing this marker. Finally, Ly49a is only expressed by a small subset (15%) of NKT cells (1). To summarize, NK1.1 remains the only widely available marker that accurately identifies NKT cells. It is unlikely neonatal thymectomy has a direct effect on the NK1.1 molecule itself as the level expressed by NK cells (NK1.1+CD8+) is normal.

This study is also significant with regard to the issue of the developmental origins of NKT cells. While some studies have suggested that NKT cells are a thymus-independent population that may develop in the liver, there is also evidence that these cells depend on the thymus for their development (reviewed in 1–5). One way of resolving this controversy is to suggest that NKT cells may be of both thymic and extrathymic origin. Here, the number of NKT cells was shown to be markedly diminished by 3 day neonatal thymectomy which strongly favours the thymus dependence of most NKT cells, including those found in the liver and bone marrow. There are two distinct possibilities that may explain this deficiency. NKT cells may develop in the thymus, hence its removal at day 3 removes the source of these cells; alternatively NKT cell generation may be extrathymic, but they require the thymus for their final development or selection. We believe the former possibility is more likely as NKT cells can develop in fetal thymic organ culture (26). In either case it is clear from these studies that the development of most NKT cells depends on the presence of a thymus.

However, a small minority of NKT cells, particularly those expressing a CD4+CD8− phenotype, the proportion of which was increased in neonatally thymectomized mice, may develop in the absence of the thymus. It is unlikely that this subset of NKT cells simply appears earlier in thymic development than the other NKT cells (and therefore may escape before day 3), as they were not detected in the thymus throughout ontogeny (not shown). A similar finding was reported in T cell factor-1 deficient mice (9). Whereas CD4+ and CD4+CD8− NKT cells were virtually absent in the liver and thymus of these mice, an unusual population of CD8+ NKT cells was detected in the liver but not the thymus. Taken together, these findings suggest that CD8+ NKT cells represent a distinct lineage from CD4+ and CD4− CD8− NKT cells.

NKT cells were significantly reduced following 3 day neonatal thymectomy in both autoimmune susceptible (BALB/c×C57BL/6)F1 and resistant C57BL/6 mice. However, an identifiable population of these cells usually remained in the spleens of C57BL/6 mice (Fig. 2). The reasons for this difference are unknown. Our ontogenic analysis of these cells showed no temporal difference in the appearance of NKT cells in the two types of mice. It may reflect a more active extrathymic pathway of their development in C57BL/6 mice. Moreover, it is an interesting possibility that this difference may be related to the relative resistance of C57BL/6 mice to 3 day neonatal thymectomy-induced autoimmune disease. The majority of (BALB/c×C57BL/6)F1 mice used in this study were 6 weeks of age (13 of 19 total mice). This age was chosen because the events which lead to gastritis occur in the first 6 weeks (28). As this is lower than the age at which autoimmune pathology is normally detected, we were not able to determine whether there was a fine association between residual NKT cells and autoimmune pathology.

In conclusion, this is the first paper to demonstrate that 3 day neonatal thymectomy causes a deficiency in a specific lineage of T cells (NKT cells) in the peripheral lymphoid organs. Given the role for these cells in immunoregulation and protection from autoimmune disease, we hypothesize that 3 day neonatal thymectomy-induced deficiency in this lineage might contribute to the events responsible for development of autoimmunity in this model.

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Abbreviations

APC aliphophycocyanin
NKT NK1.1+CD8− T cells
NTx neonatally thymectomized

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Three day NTx selectively depletes NK1.1$^+$ T cells