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Expression of LIF in transgenic mice results in altered thymic epithelium and apparent interconversion of thymic and lymph node morphologies

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Leukemia inhibitory factor (LIF) is a cytokine involved in embryonic and hematopoietic development. To investigate the effects of LIF on the lymphoid system, we generated a line of transgenic mice that expresses diffusible LIF protein specifically in T cells. These mice display two categories of phenotype that were not previously attributed to LIF overexpression. First, they display B cell hyperplasia, polyclonal hypergammaglobulinemia and mesangial proliferative glomerulonephritis, defects similar to those described for transgenic mice overexpressing the functionally related cytokine, interleukin-6. Secondly, the LIF transgenic mice display novel thymic and lymph node abnormalities. In the thymus, cortical CD4$^+$CD8$^+$ lymphocytes are lost, while numerous B cell follicles develop. Peripheral lymph nodes contain a vastly expanded CD4$^+$CD8$^+$ lymphocyte population. Furthermore, the thymic epithelium is profoundly disorganized, suggesting that disruption of stroma-lymphocyte interactions is responsible for many observed defects. Transplantation of transgenic bone marrow into wild type recipients transfers both the thymic and lymph node defects. However, transplantation of wild type marrow into transgenic recipients rescues the lymph node abnormality, but not the thymic defect, indicating the thymic epithelium is irreversibly altered. Our observations are consistent with a role for LIF in maintaining a functional thymic epithelium that will support proper T cell maturation.

Key words: B cell hyperplasia/CD4$^+$CD8$^+$ T cells/leukemia inhibitory factor/stroma-lymphocyte interactions/transgenic mouse

Introduction

The development and regulation of the lymphoid system requires a series of cytokine-mediated cellular interactions that influence lymphocyte differentiation, migration and immune responses. One cytokine that may be involved in these processes is leukemia inhibitory factor (LIF), which has several known activities within the hematopoietic system (reviewed by Hilton and Gough, 1991). LIF is a member of a family of related growth factors, including interleukin-6 (IL-6), oncostatin M and ciliary neurotrophic factor (Bazan, 1991; Rose and Bruce, 1991; Patterson and Nawa, 1993), which bind to receptors that share the common signal-transducing subunit gp130 (Gearing *et al.*, 1992; Ip *et al.*, 1992; Davis *et al.*, 1993). Members of this growth factor family have many similar activities on a wide range of hematopoietic, neuronal and embryonic cells (Patterson and Nawa, 1993).

While LIF can affect the proliferation or differentiation of several distinct hematopoietic cell types, previous studies have not characterized activities of LIF within the lymphoid system (Hilton and Gough, 1991). However, LIF has several activities outside of the lymphoid system, including its originally described ability to induce differentiation of the M1 myeloid cell line (Gearing *et al.*, 1987). LIF can also support proliferation of the DA-1a myeloid cell line (Moreau *et al.*, 1988), and can aid in the maintenance of hematopoietic progenitor cells (Fletcher *et al.*, 1990; Leary *et al.*, 1990). Furthermore, injection of large doses of LIF into mice leads to increased numbers of splenic megakaryocytes and elevated platelet levels (Metcalf *et al.*, 1990, 1991). Outside of the hematopoietic system, activities of LIF include induction of an acute phase response in cultured hepatocytes (Baumann and Wong, 1989), stimulation of cholinergic differentiation of post-mitotic sympathetic neurons (Yamamori *et al.*, 1989) and inhibition of embryonic stem cell differentiation (Smith *et al.*, 1988; Williams *et al.*, 1988). It is interesting to note that the range of LIF actions may be modulated by the differential expression of two transcripts which encode either freely diffusible or extracellular-matrix associated forms (Rathjen *et al.*, 1990).

Despite these diverse activities, LIF is not expressed at high levels in adult tissues (Bhatt *et al.*, 1991; Shen and Leder, 1992). In the hematopoietic system, LIF transcripts are produced by concanavalin A activated T lymphocytes (Gearing *et al.*, 1987), by alloreactive T cell clones (Moreau *et al.*, 1987), by cell lines established from bone marrow stroma (Wetzler *et al.*, 1991) and thymic epithelium (Le *et al.*, 1990; Sakata *et al.*, 1992), and at very low levels by the thymus itself (Bhatt *et al.*, 1991; Shen and Leder, 1992). Receptors for LIF are also relatively limited in their distribution, as shown from binding studies using ^{125}I-labeled LIF. High affinity receptors are localized on megakaryocytes (Hilton *et al.*, 1991b; Metcalf *et al.*, 1991), on cells of the monocyte/macrophage lineage (Hilton *et al.*, 1988), on osteoblasts (Allan *et al.*, 1990) and on hepatocytes (Hilton *et al.*, 1991a,b). Most notably, LIF can also bind to an uncharacterized subpopulation of lymphoid cells in the bone marrow, spleen, thymus and peritoneum, but does not bind to the majority of lymphocytes (Hilton *et al.*, 1991a).

Given the expression of LIF and LIF receptors within the lymphoid system, we were interested in investigating whether

LIF can affect lymphoid development *in vivo*. Previous studies showed that transplantation of LIF-expressing cells or intraperitoneal injection of recombinant LIF protein result in pleiotropic abnormalities and subsequent death of the recipient mouse (Metcalf and Gearing, 1989a,b; Metcalf *et al.*, 1990; Mori *et al.*, 1991). Depending to some extent on the method of LIF overexpression, the resulting pathology included cachexia, loss of both subcutaneous and intra-abdominal fat, splenomegaly, extramedullary hematopoiesis, behavioral changes, excess new bone formation, calcification of cardiac and skeletal muscle, and gonadal dysgenesis. In addition, a thymic atrophy, marked by a loss of cortical lymphocytes, was noted but not further characterized (Metcalf and Gearing, 1989a; Metcalf *et al.*, 1990).

Based on these studies, we anticipated that widespread expression of LIF in transgenic mice might be highly deleterious. To express LIF in a more regulated manner, and to determine whether it has a role in lymphoid development, we have constructed a line of transgenic mice (TG.OM) that overexpresses LIF specifically in T lymphocytes. This line of mice develops multiple severe perturbations in both the B and T cell compartments, with profound effects on cell populations that reside in the thymus and lymph nodes. In particular, we find that the $CD4^+CD8^+$ T lymphocyte subset is missing from the thymus, while it is greatly expanded in lymph nodes. Furthermore, the thymic epithelium is disrupted as a consequence of LIF expression by T cells.

Our results with transgenic mice overexpressing LIF complement recent studies on mice homozygous for a LIF null mutation (Escary *et al.*, 1993). These LIF-deficient mice possess reduced numbers of myeloid progenitors in the spleen, and are defective for thymic T cell activation, although no abnormalities in thymocyte populations were revealed by flow cytometry. These defects in T cell responsiveness and stem cell numbers were both due to a requirement for LIF expression by the hematopoietic microenvironment, as shown by bone marrow transplantation (Escary *et al.*, 1993). LIF expressed by the thymic stroma probably acts indirectly to permit T cell activation, since most T lymphocytes do not express LIF receptors (Hilton *et al.*, 1988, 1991a). Such an indirect action of LIF is consistent with the phenotype of our LIF transgenic mice. While the absence of LIF from the thymic stroma leads to defective T cell activation, we find that overexpression of LIF by T cells induces extensive epithelial disorganization. As will be discussed, our work further supports a role for LIF in mediating stroma−lymphocyte interactions.

Results

Generation of LIF-expressing transgenic mice

To direct LIF expression to T cells of transgenic mice, we generated the PμLIFCD2 construct (Figure 1A). This construct contains basal promoter (Pμ) elements derived from the human immunoglobulin μ gene, a cDNA/genomic hybrid mini-gene encoding the freely diffusible form of the LIF protein, and tissue-specific enhancer elements derived from the human CD2 gene. These enhancer elements are known to direct heterologous transgene expression to T lymphocytes (Lang *et al.*, 1988; Greaves *et al.*, 1989). We generated six live-born pups carrying the PμLIFCD2 transgene. Consistent with the predicted deleterious effects of LIF over-expression, five out of the six transgenic pups died shortly after birth. One transgenic founder survived, and was bred to establish the TG.OM line described here.

T cell-specific expression of the LIF transgene

To determine the tissue sources of the LIF produced in the TG.OM mice, we analyzed the pattern of transgene expression using a sensitive RNase protection assay (Figure 1B). In this experiment, we used a LIF probe which detects both endogenous and transgenic messages (Figure 1A, probe A), and a ribosomal protein L32 probe as an internal control. Because most normal adult tissues express very little or no endogenous LIF as judged by the same assay (Bhatt *et al.*, 1991; Shen and Leder, 1992), all of the LIF transcripts detected are encoded by the transgene. We found LIF expression in the thymus, spleen and lung, with highest levels in the axillary and mesenteric lymph nodes (Figure 1B). In contrast, no expression was detected in brain, kidney, liver, stomach and bone marrow. RNase

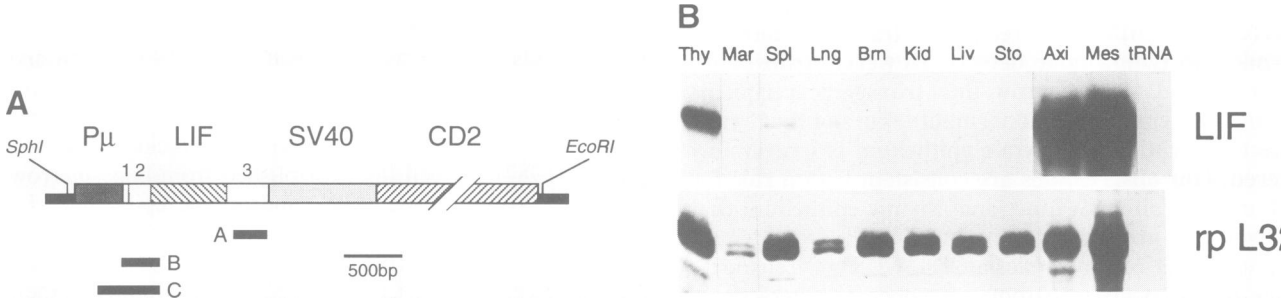

Fig. 1. (**A**) The PμLIFCD2 construct used to generate LIF transgenic mice. The three coding exons of LIF are labeled above the LIF mini-gene, which contains the intron (hatched segment) between exons 2 and 3 (Shen and Leder, 1992). The LIF mini-gene encodes freely diffusible LIF protein, which differs from the form associated with the extracellular matrix by usage of an alternative first exon encoding the leader peptide sequence (Rathjen *et al.*, 1990). The three probes (labeled A, B and C) used in RNase protection experiments are depicted below the construct, and are described in Materials and methods. Also shown are the positions of the *Sph*I and *Eco*RI restriction sites used to isolate the DNA fragment for microinjection. Abbreviations: Pμ, the human immunoglobulin heavy chain promoter derived from the pIgTE/N expression vector (Shipp *et al.*, 1989); LIF, a mini-gene encoding diffusible LIF; SV40, splice and polyadenylation sequences from pIgTE/N; CD2, a 5 kb genomic fragment containing enhancer elements from the CD2 gene (Greaves *et al.*, 1989). (**B**) RNase protection analysis of transgene expression in tissues of adult LIF transgenic mice. The LIF probe (probe A) detects both diffusible and matrix-associated LIF transcripts, while the ribosomal protein L32 probe was used as an internal control for RNA loading (Shen and Leder, 1992). 20 μg of total RNA were used in each hybridization; exposure times were 3 days for the LIF probe and 1 day for the ribosomal protein probe. Abbreviations: Thy, thymus; Mar, bone marrow; Spl, spleen; Lng, lung; Brn, brain; Kid, kidney; Liv, liver; Sto, stomach; Axi, axillary lymph node; Mes, mesenteric lymph node; rp, ribosomal protein.

protection experiments using two additional probes (Figure 1A, probes B and C) with the same RNA samples indicate that these LIF transcripts correspond to the diffusible form of LIF, and are correctly initiated in the immunoglobulin promoter (data not shown). The low levels of transgene expression in the spleen suggest that little or no transgene expression is derived from B lymphocytes. Thus, the tissue distribution and the levels of transgene expression parallel the presence of T cells, and correlate best with the subpopulation of $CD4^+CD8^+$ T cells, as will be discussed below.

Pathology of LIF transgenic mice

All of the transgenic offspring from the TG.OM founder mouse developed severe abnormalities, with 50% of animals dying by 75 days of age (Figure 2). Significant respiratory distress was observed in the terminal stages for many animals. In addition to early death, LIF transgenic males appeared to be sterile, an abnormality which may be related to the spermatogenesis defect noted for mice engrafted with LIF-expressing FDC-P1 cells (Hilton and Gough, 1991). Although we observed pregnancies in several transgenic females, none of these animals survived long enough to give birth. Consequently, we were only able to maintain the TG.OM line by ovarian transplantation for a few generations.

Necropsy of LIF transgenic mice at five to 12 weeks of age showed pulmonary congestion, pleural effusions, ascites, splenomegaly and mesenteric and peripheral lymphadenopathy. In addition, mesenteric fat was completely absent, while other fat pads, such as ovarian, testicular and mammary, appeared normal. Overall, we did not observe any significant weight loss.

Examination of histological sections from various tissues of LIF transgenic mice revealed further pleiotropic effects of the transgene, with consistent findings between different animals (14 mice examined). For example, these mice exhibited pulmonary edema, thickened alveoli and lymphoid infiltration of the lung (Figure 3A), evidence that respiratory defects may be a cause of death. In the spleen, histological examination revealed a 2- to 3-fold increase in megakaryocytes, a proliferation of stromal cells, and an increased number of primitive cells (Figure 3B). The lymph nodes showed a decreased number of follicles, an expansion of the paracortical zone (not shown), and a marked plasmacytosis of the medullary cords with many Russell bodies in the plasma cells (Figure 3C). The liver was histologically normal except for occasional areas of extramedullary hematopoiesis (Figure 3D). Finally, the glomeruli of the kidneys displayed a proliferative mesangial glomerulonephropathy with extensive hyaline deposits (Figure 3E).

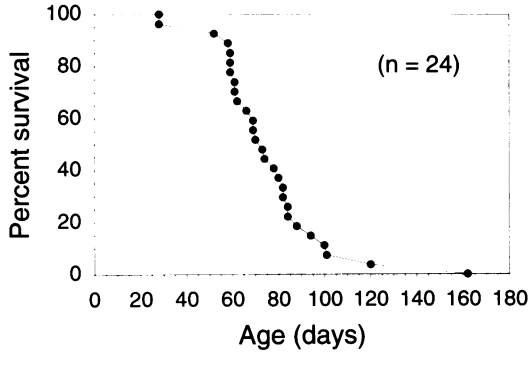

Fig. 2. Survival curve for LIF transgenic (TG.OM) mice. The percentage of mice surviving at a given age is plotted for 24 transgenic mice.

Abnormal thymic architecture in LIF transgenic mice

The most dramatic histological findings were observed in the thymus, which displayed severe cortical atrophy and the presence of apparent B cell follicles (Figure 3F) resembling those normally found in lymph nodes. Despite the loss of cortical thymocytes, the transgenic thymuses were similar in size to those of wild type littermates. Immunohistochemical analysis using anti-B220 antibodies showed that these thymic follicles did indeed contain B cells (Figure 4C and D). Flow cytometric analysis of transgenic thymocytes demonstrated that this B lymphocyte population is $B220^+$, κ^+ and μ^+ (Figures 5 and 7, Table I). In addition, we confirmed the striking absence of the immature $CD4^+CD8^+$ subpopulation of T lymphocytes that are normally found in the cortex (Figures 6 and 7, Table I). Thus, the LIF transgenic thymus resembles a lymph node, consisting of mature T cells and B cell follicles.

To confirm the thymic origin of this tissue, we analyzed cytokeratin expression by immunohistochemistry. A pan-cytokeratin antibody demonstrated the presence of keratin-positive epithelial cells (Figure 4A and B), which are not found in lymph nodes. However, the fine network characteristic of the normal thymic epithelium (Figure 4A) is replaced by disorganized clusters of cells around and below the B cell follicles (Figure 4B). These results indicate that expression of the LIF transgene perturbs the thymic architecture, profoundly affecting epithelial cells and disrupting the normal distribution of both T and B lymphocytes.

$CD4^+CD8^+$ T lymphocytes in lymph nodes of LIF transgenic mice

As shown by flow cytometry, the mesenteric lymph nodes of LIF transgenic mice consisted mainly of double-positive $CD4^+CD8^+$ T lymphocytes, cells which are not found outside of the thymic cortex in normal mice (Figures 6 and 7, Table I). Despite this vastly increased population of double-positive T cells, the overall architecture of the lymph nodes appeared normal (data not shown). B cell follicles were found at the periphery, although they tended to be displaced by the pericortical and medullary expansion of T cells. Similar, though less severe, lymphadenopathy was also observed in peripheral lymph nodes such as the axillary and mammary nodes.

Interestingly, we found a correlation between the early death of LIF transgenic mice and a high percentage of $CD4^+CD8^+$ T lymphocytes in their lymph nodes. While most transgenic mice died by 90 days, a small number survived for longer than 120 days (Figure 2). These mice displayed less severe lymphadenopathy and a significantly lower percentage of double-positive T cells, as compared with younger mice (Figure 7, Table I). This correlation is probably related to the observation that $CD4^+CD8^+$ T cells display the highest levels of transgene expression. As shown by a comparison of flow cytometry (Figure 7, Table I) and RNase protection data (Figure 1B), the pattern of transgene expression correlates with the distribution of double-positive T cells. Thus, very high LIF expression levels are found

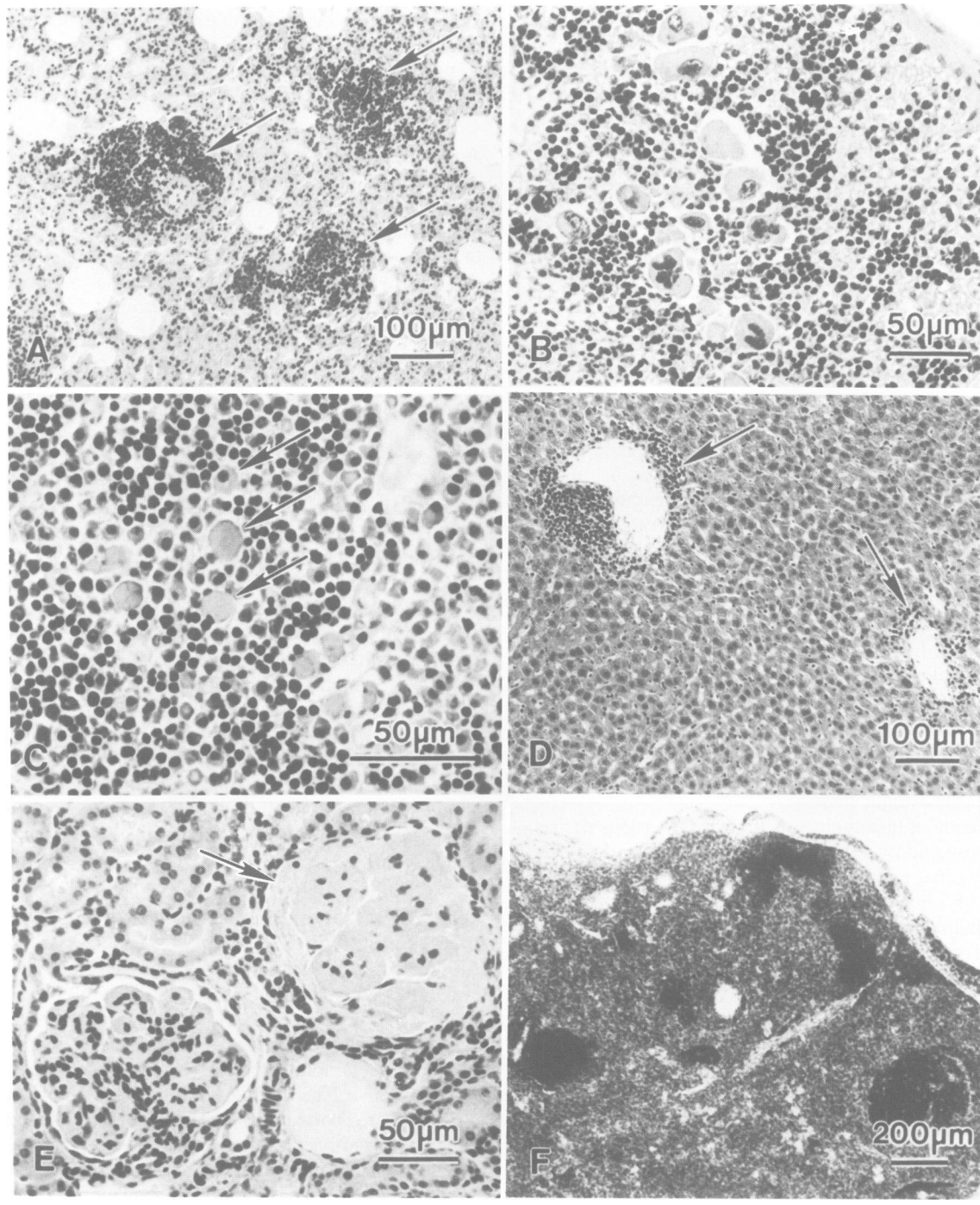

Fig. 3. Representative photomicrographs of hematoxylin and eosin-stained paraffin sections of tissues from LIF transgenic mice. (**A**) Photomicrograph of a lung, showing the dense, dark perivascular lymphoid infiltrates (arrows) and gross congestion of the alveoli (95×). (**B**) High magnification view of an area of a spleen with increased megakaryocytes and immature cells (240×). (**C**) High magnification photomicrograph of an area of a mesenteric lymph node, illustrating the medullary plasmacytosis with numerous intracytoplasmic inclusions (Russell bodies) (arrows) characteristic of the LIF transgenic mice (380×). (**D**) Photomicrograph of a liver, demonstrating several small areas of extramedullary hematopoiesis seen as dense, darkly staining perivascular zones (arrows) (95×). (**E**) Two glomeruli from a transgenic kidney. Both glomeruli are involved with a membrano-proliferative process. The larger glomerulus is almost completely obliterated by the hyaline material. Note also that the tubules are atrophic (240×). (**F**) Low power view of a thymus. Note the loss of cortex and the appearance of follicles (dark staining areas) (38×).

in the mesenteric and axillary lymph nodes, whose T cell population is almost exclusively $CD4^+CD8^+$ (Table I). Given the lower levels of transgene expression in thymus and spleen, it is also likely that LIF is produced at lower levels in single-positive $CD4^+CD8^-$ or $CD4^-CD8^+$ T cells. This pattern of expression of the PμLIFCD2 transgene differs somewhat from the normal *in vivo* distribution of the CD2 antigen, which is found at highest levels in mature single-positive T cells, and at intermediate levels in double-positives (Duplay *et al.*, 1989).

Peripheral blood of LIF transgenic mice

Examination of peripheral blood showed that a population of atypical lymphoid cells with abundant cytoplasm and large nuclei was present (17% ± 7%; $n = 3$). This population is not found in normal peripheral blood and presumably

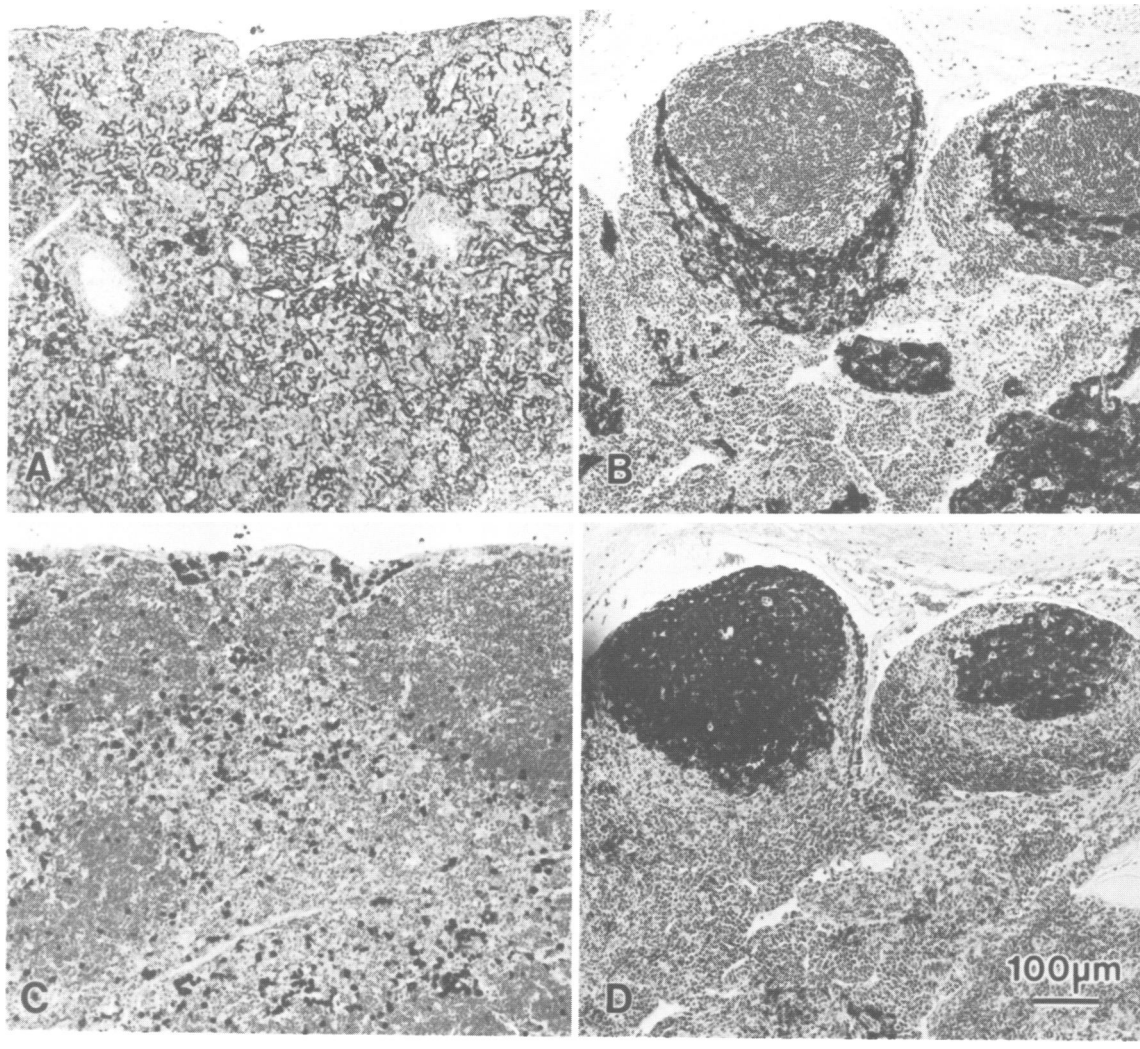

Fig. 4. Immunohistochemical analysis of cytokeratin (panels **A** and **B**) and B220 (**C** and **D**) expression in the thymi of wild type (A and C) and LIF transgenic (B and D) mice. Paraffin sections of the thymus were stained using the immunoperoxidase technique with polyclonal anti-keratin and monoclonal anti-B220 antibodies. Sections were counterstained with hematoxylin to demonstrate nuclei. Note that the keratin is distributed in epithelial cells forming a diffuse network in the normal thymus (A), but that the keratin-expressing cells form dark-staining aggregates around follicles or medullary clusters in the LIF transgenic thymus (B). While only a small number of B lymphocytes is found scattered throughout the wild type thymus (C), there are apparent follicles in the transgenic thymus (D).

represents activated lymphocytes. While the total white cell count in LIF transgenic mice was reduced, it was within normal limits (Table II), and blood smears revealed only a slight reduction in mature lymphocytes (42% ± 8%; n = 3) relative to wild type values. Together with the blood counts, flow cytometry analyses showed that the peripheral T cells are reduced in number, and that this reduction mostly affected the $CD4^+$ subset (Table I). Analyses of blood smears also revealed a mild monocytosis (20% ± 6%; n = 3) and a very mild thrombocytosis (Table II), which was less pronounced than that observed in mice injected with recombinant LIF (Metcalf *et al.*, 1990). In addition, LIF transgenic mice displayed a significant increase in mean platelet volume (Table II), suggesting that there is more rapid platelet turnover.

Polyclonal hypergammaglobulinemia in LIF transgenic mice

Cellulose acetate electrophoresis of serum proteins showed two greatly expanded peaks in transgenic animals when compared with controls (Figure 8). The increased α_2 globulin peak (15.4% in transgenic versus 6.6% in wild type) is characteristic of an acute phase response, consistent with the previous identification of LIF as a hepatocyte-stimulating factor (Baumann and Wong, 1989; Hilton *et al.*, 1991a; Kordula *et al.*, 1991), and with experiments on LIF-injected mice (Metcalf *et al.*, 1990). The broad γ globulin peak (51.2% in transgenic versus 11.4% in wild type) is characteristic of a polyclonal hypergammaglobulinemia. Radial immunodiffusion assays confirmed that the sera contained high levels of all immunoglobulins (data not shown). The hypergammaglobulinemia may be a manifestation of the B cell hyperplasia observed in the lungs and the thymus (Table I), and the plasmacytosis observed in the lymph nodes (Figure 3C).

To test their immunocompetence, we immunized LIF transgenic mice with keyhole limpet hemocyanin (KLH), a T cell-dependent antigen. Following immunization of either transgenic or wild type mice, we harvested serum for analysis. Both control and transgenic mice generated antibodies against KLH as assayed by the Ouchterlony double diffusion test. A precipitation arc was detectable at

up to a 1:9 serum dilution in mice immunized with KLH (data not shown). This result indicates that the LIF transgenic mice can generate a humoral immune response, despite their lymphoid abnormalities.

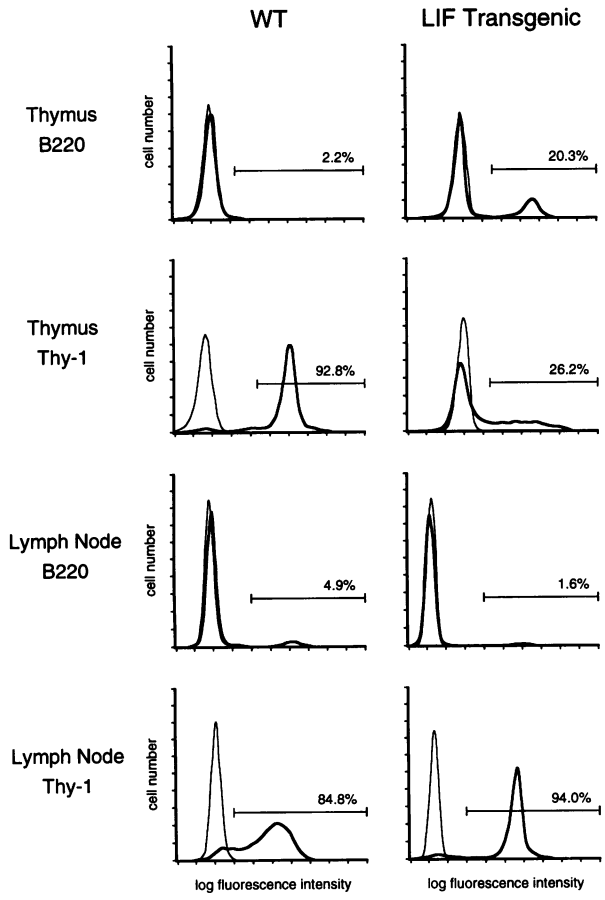

Fig. 5. Histograms of representative flow cytometry data. Cell preparations from thymus and mesenteric lymph node of wild type and LIF transgenic mice were stained with fluorescent-tagged antibodies recognizing murine B220 or Thy-1. Each histogram compares experimental staining (thick line) with control staining (thin line). Fluorescence intensity of staining (x-axis, where 3.2 divisions represent one log difference in intensity) is plotted against relative cell number (y-axis). The percentage of positively stained cells within the indicated window (horizontal bar) is given.

Transplantation of the phenotype by transgenic bone marrow cells

To determine whether the phenotype depends on transgene expression in lymphoid cells, we transplanted bone marrow from LIF transgenic mice into lethally irradiated recipient mice (n = 15). In these transplant recipients, we observed the characteristic thymus and lymph node defects of LIF transgenic mice. Flow cytometry of transplant recipient tissues showed that $CD4^+CD8^+$ thymocytes do not repopulate the thymus, that B cells are present in the thymus, and that $CD4^+CD8^+$ T lymphocytes are found in the lymph nodes (Table III). In addition, histological and immunohistochemical analyses of one thymus from a transplant recipient revealed follicular structures and thymic epithelial defects similar to those of LIF transgenic mice (not shown). Surprisingly, many recipients (eight out of 15) of transgenic bone marrow remained healthy until necropsy, for up to 370 days after transplantation. Therefore, the lethality associated with LIF expression in transgenic mice was not completely transplanted, perhaps consistent with the relatively low levels of lymph node $CD4^+CD8^+$ T cells (22%, n = 4; Table III).

In addition to the lymphoid abnormalities, wild type mice transplanted with transgenic bone marrow displayed most of the other LIF transgenic defects. Histological examination of the kidneys of four transplanted animals revealed mesangial proliferative glomerulonephritis similar to that found in LIF transgenic mice (data not shown). Histology of the spleen (n = 4) also showed alterations similar to those observed in LIF transgenic mice. However, the thrombocytosis observed in LIF transgenic mice is more pronounced in the transplanted animals (Table II). As expected, Southern blot analysis of peripheral blood DNA using a transgene-specific probe confirmed that all transplanted mice were reconstituted with LIF transgene-containing bone marrow (data not shown).

We also determined whether transplantation of normal bone marrow could rescue the abnormalities of LIF transgenic recipients. For the bone marrow donor, we used a phenotypically normal mouse (TG.XA) carrying a *lacZ* transgene (R.C.Skoda, unpublished results), which was used to assess donor contribution to the hematopoietic system of transplanted mice by DNA, blot analysis. Thus, LIF transgenic mice were lethally irradiated and transplanted with

Table I. Flow cytometry of cell preparations from wild type and LIF transgenic tissues

Genotype	Tissue	n	Control	B220	κ	μ	Thy-1	$CD4^+$	$CD8^+$	$CD4^+CD8^+$	Mac-1
Wild type	spleen	4	0.3	30.6	39.1	30.3	45.7	31.0	10.1	0.1	1.8
LIF transgenic	spleen	4	2.9	41.7	56.3	46.9	14.0	8.9	5.0	0.2	6.5
Wild type	thymus	10	0.2	1.8	1.3	1.6	88.3	91.6	83.9	80.6	2.8
LIF transgenic	thymus	13	0.3	36.7	50.9	42.5	27.6	13.9	28.1	4.4	6.1
Wild type	mesenteric node	5	0.1	9.1	12.8	11.2	77.6	66.8	16.5	0.4	ND
LIF transgenic	mes. node (young)	5	0.0	4.7	8.4	8.1	73.9	84.8	83.9	81.6	ND
LIF transgenic	mes. node (old)	4	0.2	31.8	ND	46.0	38.3	38.8	32.5	22.9	ND
Wild type	bone marrow	1	0.4	22.9	6.3	7.6	0.6	0.5	0.7	0.1	0.2
LIF transgenic	bone marrow	2	0.8	5.8	9.2	10.9	1.9	0.6	0.2	0.0	0.9
Wild type	blood	2	0.8	12.6	11.1	12.2	73.0	64.1	10.0	0.2	3.3
LIF transgenic	blood	2	0.9	52.3	47.0	57.4	21.7	26.2	13.7	0.0	18.1
LIF transgenic	ascites	2	0.4	37.7	21.6	74.4	51.1	24.0	6.7	ND	20.0
LIF transgenic	lung	2	0.0	15.0	39.9	ND	14.1	16.2	10.4	0.4	1.2

Averages of the percentage of positively stained cells are shown. For lymph node experiments, 'young' animals were <70 days of age, 'old' animals were >115 days of age. ND = not determined.

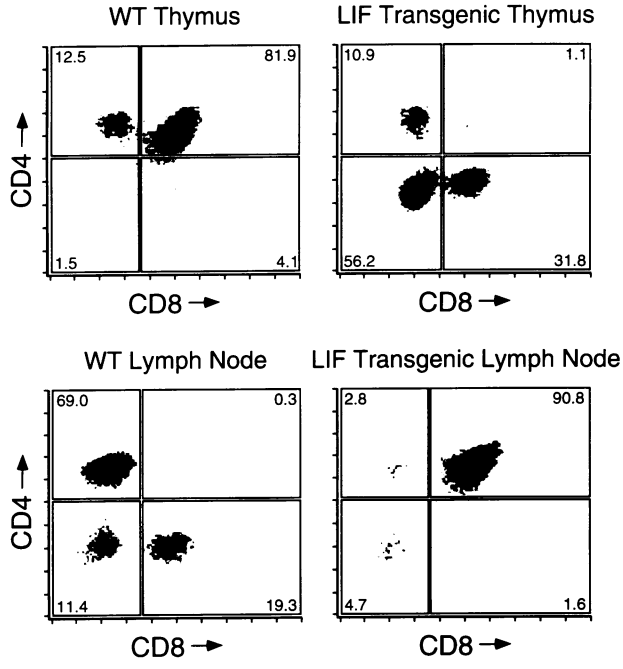

Fig. 6. CD4 and CD8 staining of cell populations, analyzed by two-color flow cytometry. Cells from thymus and mesenteric lymph node of wild type and LIF transgenic mice were stained with antibodies against CD4 and CD8. Fluorescence intensity is shown on log-log scatter plots, where 3.2 divisions represent one log difference in intensity. The percentage of cells within each quadrant is indicated.

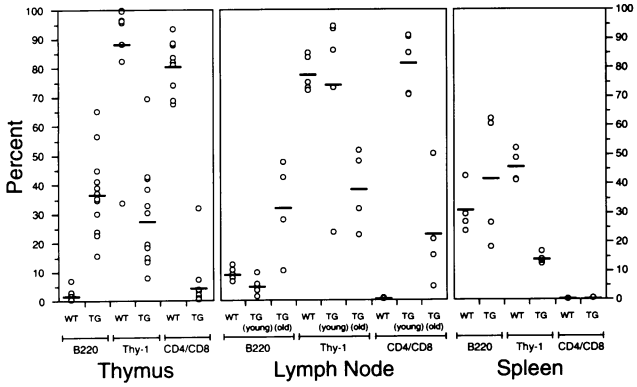

Fig. 7. Graphical representation of selected flow cytometry data from Table I. Cells prepared from wild type (WT) and LIF transgenic (TG) thymus, mesenteric lymph node, and spleen were analyzed using antibodies directed against murine B220, Thy-1, CD4 and CD8. Each column displays the values for the percentage of cells positively stained for B220 or Thy-1, or doubly positive for CD4 and CD8. The value obtained for each animal analyzed is shown as an open circle, while the mean of the sample values is depicted by a horizontal bar. For the lymph node experiments, young animals were <70 days of age, while old animals were >115 days of age.

normal (*lacZ* transgenic) bone marrow. In contrast to the failure of 95% of non-transplanted LIF transgenic mice to survive beyond 100 days, transplant recipients remained healthy until necropsy at 104 (n = 2) and 245 days (n = 2) after transplantation. Therefore, the lethal phenotype of LIF transgenic mice could be circumvented through transplantation of normal bone marrow. In addition, the lymph nodes from these transplant recipients appeared relatively normal, with slightly elevated levels of B cells and only single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) T cells (Table III).

In contrast, several LIF transgenic defects were not rescued by wild type bone marrow transplantation. In these transplant recipients (n = 4), the thymus possessed a large percentage of B cells, while the thymic cortex contained few $CD4^+CD8^+$ T cells (Table III). Furthermore, histological and immunohistochemical analyses demonstrated follicular structures and disrupted thymic epithelium in two thymuses from transplant recipients (not shown). It is likely that defective thymic epithelium is responsible for the inability of normal bone marrow to rescue the LIF transgenic thymic abnormalities. Surprisingly, LIF transgenic mice transplanted with normal bone marrow also retained elevated platelet counts (Table II), while the histology of the spleen revealed increased numbers of megakaryocytes (not shown). In addition, the kidneys of transplant recipients displayed a mesangial proliferative glomerulonephritis that probably existed at the time of transplantation, and might be irreversible. Finally, the lungs of transplant recipients also showed lymphoid infiltrates. The failure to reverse these LIF transgenic defects was not due to inefficient transplantation, as DNA blot analysis confirmed that the white blood cells in the transplant recipients were derived from the normal donor (*lacZ* transgenic) bone marrow (data not shown). As expected, the presence of donor-derived cells was detected in Southern analysis of lymph nodes and thymuses of these transplant recipients. Control experiments showed that the lymphoid populations of wild type mice were completely normal after transplantation with *lacZ* transgenic bone marrow (Table III).

Discussion

The pleiotropic transgenic phenotype results from LIF overexpression

The phenotypic alterations that we have described in LIF transgenic mice can be classified into three broad categories (Table IV). First, some aspects of the LIF transgenic phenotype have been previously described for mice engrafted with LIF-overexpressing FDC-P1 cells or injected with recombinant LIF protein (Metcalf and Gearing, 1989a; Metcalf *et al.*, 1990). Secondly, the B cell hyperplasia and hypergammaglobulinemia resemble those described for transgenic mice overexpressing the related cytokine IL-6 (Suematsu *et al.*, 1989, 1992). Finally, most of the thymic and lymph node abnormalities appear to be unique to LIF transgenic mice.

The first category of abnormalities encompasses those described in earlier studies of LIF overexpression (Metcalf and Gearing, 1989a; Metcalf *et al.*, 1990), such as moderate splenomegaly, increased numbers of splenic megakaryocytes, extramedullary hematopoiesis, an acute phase response and absence of mesenteric fat. In contrast to these studies, we failed to observe extensive loss of body fat or calcium deposits in cardiac and skeletal muscle. It is also unclear to what extent the cortical thymic atrophy mentioned in these studies might resemble the LIF transgenic thymic phenotype, since this thymic atrophy was not characterized in detail. These differing results might simply reflect relatively lower levels of LIF in our transgenic mice, although we have not measured levels of circulating LIF in the serum.

Since only a single line (TG.OM) carrying the LIF transgene has been established, it is important to determine

Table II. Analysis of peripheral blood

	WT	LIF transgenic	WT recipient/ *lacZ* donor	WT recipient/ LIF transgenic donor	LIF transgenic recipient/*lacZ* donor
Mice analyzed	4	5	4	9	2
WBC × 10^3/ml	8.0 ± 1	4.9 ± 2.9	3.4 ± 2.1	3.8 ± 2.6	3.3 ± 0.5
LUC (%)	1.6 ± 1.2	14 ± 5.1	3.1 ± 2	5.7 ± 4.8	5.6 ± 0.1
Platelets × 10^3/ml	1070 ± 133	1271 ± 183	1008 ± 310	1482 ± 202	1416 ± 107
MPV (fl)	4.2 ± 0.3	5.5 ± 0.3	4.7 ± 0.3	5.3 ± 0.4	5.2 ± 0.4
RBC × 10^6/ml	9.0 ± 0.7	8.5 ± 1.1	6.9 ± 1.6	8.0 ± 1.3	7.7 ± 0.8
Hematocrit (%)	45 ± 3	41 ± 5	33 ± 10	35 ± 6	38 ± 3
Hgb (g/100 ml)	14 ± 1	12 ± 1	11 ± 3	11 ± 2	12 ± 1
MCH (pg)	16 ± 0.5	15 ± 0.6	16 ± 0.5	14 ± 0.5	15 ± 0
MCV (fl)	50 ± 0.7	48 ± 1.4	48 ± 3.5	44 ± 2.2	49 ± 1.4
MCHC (g/100 ml)	31 ± 0.5	31 ± 1.1	33 ± 2.8	33 ± 1.1	30 ± 1.3

Blood counts were performed on a Technicon H1. Average values are given together with standard deviations. WBC, white blood cells; LUC, large unstained (peroxidase negative) cells; MPV, mean platelet volume; RBC, red blood cells; Hgb, hemoglobin; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration.

whether all of its pleiotropic defects are due to LIF overexpression. Several observations indicate that this is the case, and that the phenotype is not a consequence of disruption of an endogenous gene. First, previous results from studies on LIF overexpression are consistent with much of the transgenic phenotype. Secondly, we generated five other founder mice, but these died within 2 weeks of birth, indicating that expression of the LIF transgene is highly deleterious. Finally, the fact that the lymphoid defects could be transferred by bone marrow transplantation argues that hematopoietic cells carrying the LIF transgene are responsible for the effects observed. In particular, the finding of a disrupted thymic epithelium in these transplant recipients demonstrates the non-cell autonomous effect of the transgenic hematopoietic cells. While it remains a formal possibility that transgene insertion has activated an endogenous gene encoding a factor that can act in this fashion, this observation favors a causative role for transgene-encoded LIF.

Similarities in phenotype of LIF and IL-6 transgenic mice

A second category of abnormalities observed in LIF transgenic mice includes those described previously for transgenic mice that overexpress the related cytokine IL-6 (Suematsu *et al.*, 1989, 1992). Some shared defects, such as an acute phase response, splenomegaly and increased megakaryocyte levels, were also found in studies of LIF-overexpressing mice (Metcalf and Gearing, 1989a; Metcalf *et al.*, 1990). However, other abnormalities, such as B cell hyperplasia, polyclonal hypergammaglobulinemia and mesangial proliferative glomerulonephritis, are only observed in LIF and IL-6 transgenic mice.

The similar effects of LIF and IL-6 in transgenic mice may be a reflection of their functional relatedness, as indicated by a variety of *in vitro* assays (Baumann *et al.*, 1989; Metcalf, 1989; Bazan, 1991; Kordula *et al.*, 1991; Rose and Bruce, 1991; Burstein *et al.*, 1992). At the molecular level, these two cytokines bind receptor complexes that share the signal-transducing subunit gp130 (Gearing *et al.*, 1992; Ip *et al.*, 1992; Liu *et al.*, 1992), and induce similar downstream events (Hoffman-Liebermann and Liebermann, 1991; Lord *et al.*, 1991; Ip *et al.*, 1992). Alternatively, it is possible that LIF expression might stimulate the expression of IL-6 in the spleen and other

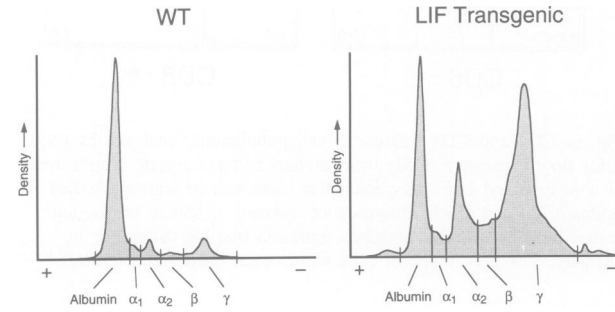

Fig. 8. Densitometer tracings of electrophoresis of serum from a wild type and a LIF transgenic mouse. Electrophorectic separation of serum proteins (*x*-axis, with the directions of the anode (+) and cathode (−) as indicated) is plotted against relative protein density (*y*-axis).

tissues. This might be consistent with the induction of IL-6 expression in M1 myeloid cells that differentiate in response to LIF treatment (Metcalf, 1989; Miyaura *et al.*, 1989; Gruss *et al.*, 1992).

The B cell hyperplasia in LIF transgenic mice is manifested by significant populations of mature B cells ($B220^+$ and κ^+) in the lung and thymus (Table I). It is conceivable that this hyperplasia represents a direct proliferative response to LIF by LIF receptor-expressing lymphocytes (Hilton *et al.*, 1991a). The observed B cell follicles in the thymus might also be consistent with a LIF-induced auto-immune disorder. A similar follicular hyperplasia is observed in NZW × NZB auto-immune mice (Fariñas *et al.*, 1990), and human patients with myasthenia gravis (Leprince *et al.*, 1990). In either case, it is likely that this defect is relatively specific for LIF transgenic mice, since medullary follicles were not described in earlier studies of LIF overexpression (Metcalf and Gearing, 1989a; Metcalf *et al.*, 1990). If so, the novel transgenic lymphoid abnormalities might be a consequence of directing LIF expression predominantly to a specific T cell subtype, and/or might represent developmental defects that cannot easily be recapitulated through short term administration of LIF to adult mice.

Abnormal T lymphocyte development in LIF transgenic mice

The final category of abnormalities observed in LIF transgenic mice includes the altered distribution of cells in

Table III. Flow cytometry of cell preparations from tissues of bone marrow transplant recipients

Donor	recipient	Tissue	*n*	Control	B220	μ	Thy-1	CD4+	CD8+	CD4+CD8+
Normal (*lacZ*)	wild type	thymus	5	1.0	1.1	4.3	81.9	84.2	74.8	71.4
LIF transgenic	wild type	thymus	3	0.5	36.7	35.5	55.5	45.5	18.9	5.4
Normal (*lacZ*)	LIF transgenic	thymus	3	1.1	32.7	40.0	31.8	18.9	18.0	3.2
Normal (*lacZ*)	wild type	mesenteric node	5	0.4	11.2	17.7	83.5	71.1	10.6	0.4
LIF transgenic	wild type	mesenteric node	4	0.3	23.6	34.7	78.2	64.8	27.6	21.5
Normal (*lacZ*)	LIF transgenic	mesenteric node	4	0.3	26.1	22.0	50.5	40.9	13.7	0.4

Averages of the percentage of positively stained cells are shown.

Table IV. Summary of LIF and IL-6 induced abnormalities

Defect	LIF transgenic	LIF engrafted	LIF injected	IL-6 transgenic
CD4+CD8+ T cells in lymph nodes	+			
Thymic B cell follicles	+			
Disorganized thymic epithelium	+			
Lymphoid infiltrate in lungs	+			+
Hypergammaglobulinemia	+			+
Mesangial proliferative glomerulonephritis	+			+
Loss of CD4+CD8+ thymocytes	+	?	?	
Loss of body fat	(+)	+	+	
Extramedullary hematopoiesis	(+)	+		
Defective spermatogenesis	?	+		
Splenomegaly	+	+	(+)	+
Megakaryocyte hyperplasia	+	+	+	+
Acute phase response	+	+	+	+
Calcium deposits in tissues		+	(+)	
Pancreatitis		+		

+, defect observed; (+), defect variable or slight; ?, defect suspected but not examined (Metcalf and Gearing, 1989; Suematsu *et al.*, 1989; Metcalf *et al.*, 1990; Hilton and Gough, 1991; Suematsu *et al.*, 1992; this work).

the lymphoid system. Thus, the immature subpopulation of CD4+CD8+ T lymphocytes that are normally located in the thymic cortex are absent, and are now found exclusively in the lymph nodes. In addition, B cells populate medullary follicles in the thymus, and now account for a significant proportion of the lymphocytes present. These abnormalities may be related to the finding that LIF is required for thymic T cell activation (Escary *et al.*, 1993). This observation suggests that overexpression of LIF in transgenic mice might lead to premature maturation of T cells, perhaps occurring before negative selection takes place at the CD4+CD8+ developmental stage. If so, T lymphocytes might exit prematurely from the thymus, possibly leading to expansion of the lymph node CD4+CD8+ population and ultimately resulting in auto-immune disorders.

We believe that these lymphoid defects are a consequence of the severe disorganization of the thymic epithelium. Thus, disrupted stroma−lymphocyte interactions may be responsible for the absence of CD4+CD8+ T cells in the thymus. Such interactions are thought to be crucial for lymphocyte maturation and activation (Bauvois *et al.*, 1989; Gutierrez and Palacios, 1991; Hoffmann *et al.*, 1992). Consistent with such a defect, we were unable to rescue the LIF thymic phenotype by transplantation of wild type bone marrow. In addition, there might also be a defect in the splenic stroma, as indicated by histological observations and by the failure of transplanted wild type bone marrow to reverse the thrombocytosis in LIF transgenic recipients (Table II). These results suggest that the splenic stroma is also irreversibly altered by LIF overexpression, and is essential for the normal regulation of megakaryopoiesis.

A role for LIF in normal hematopoietic development

Our results complement recent studies of LIF-deficient mice, which have shown that LIF expression by the thymic microenvironment is required for T cell activation (Escary *et al.*, 1993). Such a requirement is consistent with the expression of LIF by cell lines isolated from thymic epithelium (Le *et al.*, 1990; Sakata *et al.*, 1992). Since most T lymphocytes do not express LIF receptors (Hilton *et al.*, 1988, 1991a), the requirement for LIF in T cell activation is likely to be indirect, involving the action of LIF on an as yet unidentified cell population. This population would in turn interact with T lymphocytes, or produce other cytokines required for T cell activation.

In this view, the thymic epithelial defect in LIF transgenic mice and in wild type recipients of transgenic bone marrow would result from either a direct or an indirect activity of LIF expressed by T lymphocytes. The overexpressed LIF could act directly upon the epithelial cells, thereby resulting in their disorganization and/or altered function. Alternatively, LIF might act in a paracrine manner upon lymphocytes. A population of lymphocytes that are capable of binding LIF has been identified by Hilton *et al.* (1991a). Such lymphocytes might then respond by producing a second cytokine that is capable of perturbing epithelial architecture. These observations are compatible with a bidirectional

interaction between T lymphocytes and the thymic epithelium.

Taken together, our data are consistent with two possible models for normal LIF function in wild type stroma–hematopoietic cell interactions. First, stromal cells and hematopoietic progenitors may interact bidirectionally, with stromal cells producing LIF to regulate the proliferation and differentiation of hematopoietic precursors, which in turn express other factors required for normal stromal function. In this model, stromal cells are not required to express LIF receptors, because LIF deregulation would alter the expression of other cytokines required for stromal function and organization. Alternatively, LIF expressed by thymic epithelium might act in an autocrine or paracrine fashion to regulate stromal function and organization. Loss of LIF activity would then lead to defective support for hematopoietic cells, whereas excess LIF activity might disrupt normal stromal architecture and function. This second model would predict that splenic and thymic stromal cells should express LIF receptors. Further investigations of these issues will require the continued characterization of LIF and LIF receptor expression and their gain- and loss-of-function phenotypes *in vivo*.

Materials and methods

Construction of plasmid vectors

The LIF mini-gene was constructed by using the polymerase chain reaction to amplify a cDNA/genomic hybrid that contained exon 1 (encoding the leader peptide of the diffusible form of LIF), fused to the common exon 2, followed by an intron and exon 3 (Shen and Leder, 1992). This 1197 bp mini-gene was inserted into the unique *Xho*I cloning site in the pIgTE/N expression vector (Shipp *et al.*, 1989). The resulting plasmid EμPμLIF contains a murine heavy chain immunoglobulin enhancer (Eμ) and human heavy chain immunoglobulin promoter (Pμ) upstream of the LIF mini-gene, followed by SV40 splice and polyadenylation sequences. The EμPμLIF plasmid was partially digested with *Xba*I, followed by complete digestion with *Bam*HI, to release a 2.65 kb *Xba*I–*Bam*HI fragment that deletes the immunoglobulin enhancer and retains the immunoglobulin promoter, LIF, and SV40 sequences. This *Xba*I–*Bam*HI fragment was ligated upstream of a 5 kb genomic fragment (*Xba*I–*Bam*HI) that contains the CD2 enhancer (Greaves *et al.*, 1989) in a *Xba*I linearized pGEM7zf vector (Promega Biotech), to yield the plasmid PμLIFCD2 shown in Figure 1A. A 7.65 kb *Sph*I–*Eco*RI fragment of PμLIFCD2, which contains no vector sequences, was used for oocyte microinjection.

Generation and maintenance of transgenic mice

All animals were maintained under specific pathogen-free (SPF) conditions in micro-isolator cages and were handled in accordance with Harvard University guidelines. We produced transgenic mice in the FVB/N inbred strain (Taconic Farms) by standard oocyte injection methods (Hogan *et al.*, 1986). Of ~1500 eggs injected with either EμPμLIF or PμLIFCD2 DNA fragments, 12 pups survived to adulthood, and from these, a single transgenic founder was identified. Nine other pups that died prior to weaning were also recovered, five of which were found to be transgenic by DNA analysis. The single transgenic founder, designated TG.OM, was bred to establish a transgenic line. Given its apparently normal phenotype, this founder male was probably mosaic in its somatic tissues, although Mendelian inheritance ratios were observed for its transgenic progeny.

All of the heterozygous transgenic offspring from the founder developed similar severe defects, and most died between 2 and 4 months of age. Consequently, we maintained the TG.OM line by ovarian transplantation from transgenic females into FVB/N foster female mice. Due to these difficulties in maintainence, the TG.OM line is now extinct.

RNA isolation and RNase protection analysis

RNA samples were prepared from adult tissues by Polytron homogenization in 4 M guanidium isothiocyanate, followed by ultracentrifugation on a 5.7 M CsCl cushion (Chirgwin *et al.*, 1979). For RNase protection analysis, T3 and T7 antisense riboprobes were synthesized and hybridized to total RNA samples essentially as described (Krieg and Melton, 1987). To achieve comparable signals, we synthesized ribosomal protein L32 probes at one-tenth the specific activity of LIF probes. After hybridization at 50°C for 14–22 h, samples were digested with 40 μg/ml RNase A and 2 μg/ml RNase T1 (Sigma) for 15 min at 37°C. Protected fragments were separated on 6% polyacrylamide–8 M urea sequencing gels, which were then dried and exposed for autoradiography using Kodak XAR-5 film and an intensifying screen.

Antisense riboprobes that detect total LIF transcripts and that distinguish between the diffusible and extracellular matrix-associated forms of LIF have been previously described (Shen and Leder, 1992). The riboprobe for total LIF transcripts (Figure 1A, probe A) protects a 280 nt fragment, while the other riboprobe (Figure 1A, probe B) protects 209 nt from the transcript encoding the diffusible form, and 181 nt from the transcript encoding the extracellular matrix-associated form. To examine the site of transcript initiation from the PμLIFCD2 transgene, we subcloned a 555 bp *Hin*dIII–*Eco*47III fragment from EμPμLIF into Bluescript IIKS(+) (Stratagene Cloning Systems, La Jolla, CA), and linearized the resulting plasmid with *Hin*dIII. The riboprobe (Figure 1A, probe C) synthesized from this template protects fragments of ~270 nt, which indicates transcript initiation ~50 nt upstream of the pIgTE/N *Xho*I site. The antisense probe used to detect the ribosomal protein L32 transcript was derived from a clone for the processed pseudogene 4A (Dudov and Perry, 1984). The *Xho*II–*Pvu*II fragment of the pseudogene was subcloned into Bluescript IIKS(+), and linearized at the *Xba*I site, resulting in a riboprobe that protects 197 nt.

Histological analysis

Freshly dissected tissues were fixed in Optimal*Fix (American Histology Reagent Co., Stockton, CA). Fixed specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin by the Transgenic Pathology Laboratory at the University of California at Davis. For immunohistochemistry, sections were stained using a standard immunoperoxidase protocol (Harlow and Lane, 1988). The anti-cytokeratin antibody was a rabbit polyclonal antiserum (BioGenex Laboratories, San Ramon, CA) used at a dilution of 1:100.

Flow cytometry

Cell preparations for flow cytometry were obtained by crushing freshly dissected tissues between flat forceps in modified Hanks' balanced salt solution (mHBSS; Gibco). Debris was allowed to settle for 10 min, after which non-adherent cells were harvested and washed twice. Cells were concentrated by centrifugation and resuspended at ~2.7×10^7/ml in mHBSS. We incubated 50 μl of the cell suspension with the primary antibody, serum or buffer for 40 min at 4°C. When antibodies were directly conjugated to fluorescent dyes, cells were washed twice with mHBSS, resuspended in 400 μl of 1% formaldehyde in phosphate-buffered saline (PBS) and analyzed on a Cytofluorograf IIs flow cytometer (Ortho Diagnostic Systems, Inc., Westwood, MA). When secondary antibodies were required, cells were washed twice with mHBSS, incubated with a directly conjugated secondary antibody for 40 min at 4°C, washed twice with mHBSS and then resuspended in 1% formaldehyde for analysis.

Monoclonal antibodies used for analysis included: anti-mouse B220 (LY5, CD45R) as culture supernatant from hybridoma clone 6B2 (Coffman, 1982); fluorescein isothiocyanate (FITC) conjugated anti-mouse Thy-1.1 (New England Nuclear, Boston, MA); phycoerythrin-conjugated anti-mouse L3T4 (murine homolog of CD4) from hybridoma GK1.5 (Becton Dickinson, Mountain View, CA); FITC-conjugated anti-mouse Lyt-2 (murine homolog of human CD8) from hybridoma 53-6.7 (Becton Dickinson); and culture supernatant from a hybridoma producing anti-mouse Mac-1 (Springer *et al.*, 1979). Polyclonal antisera used were FITC-conjugated goat anti-mouse IgM (Southern Biotechnology Associates Inc., Birmingham, AL), and FITC-conjugated goat anti-mouse Ig κ light chain (Southern Biotechnology Associates Inc.). The secondary antibody was FITC-conjugated goat anti-rat IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Controls for staining with directly conjugated primaries were unstained cells, while controls for unconjugated primaries were cells incubated with secondary antibody alone.

Blood analysis and bone marrow transplantation

Blood was obtained either from the retrobulbar plexus from anesthetized mice or by cardiac puncture. Automated blood counts were performed with a Technicon H-1 analyzer (Technicon Co.). Blood smears were stained with May–Grünwald–Giemsa.

Bone marrow was harvested from the femurs of either TG.OM or TG.XA donor transgenic mice. The transgenic line TG.XA contains a *lacZ* transgene as a molecular marker for the transplanted tissue and possesses a completely wild type phenotype (R.C.Skoda, unpublished observations). We washed the harvested cells in mHBSS and injected 2×10^6 cells in a volume of 100 μl into the tail vein of recipient FVB/N or TG.OM mice that had been

irradiated with 1200 rad. Transplant recipients were maintained under specific pathogen-free conditions for 3–12 months, until they were sacrificed for necropsy and analysis.

Serum electrophoresis and analysis of immune responses

Serum electrophoresis was performed using a prepackaged kit (Beckman). Control and experimental samples were diluted 1:5 in buffer and electrophoresed at 100 V for 25 min. After acid alcohol fixation, the gels were stained and then analyzed by densitometry.

To assess their ability to mount an antibody response, mice were injected intraperitoneally with 100 μg of KLH (Calbiochem) suspended in Freund's complete adjuvant (Sigma). Four weeks later, the animals were re-immunized with 50 μg of KLH in incomplete Freund's adjuvant, and were sacrificed after two additional weeks to obtain serum for an Ouchterlony double diffusion test. For these experiments, 10 μl of KLH (2 mg/ml in PBS) was placed in the center well of an Ouchterlony plate, and 10 μl of serial 1:3 dilutions of serum was placed in the peripheral wells. Plates were incubated at 37°C overnight, stored at 4°C and photographed.

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