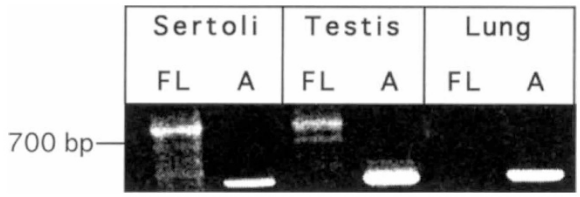

FIG. 2 CD95-ligand mRNA is expressed in Sertoli cells. RT–PCR analysis was performed on RNA isolated from mouse Sertoli cells, testis and lung. CD95L (FasL: FL) was expressed in testis and isolated Sertoli cells from testis, but not in lung. Actin (A) was used to normalize for RT–PCR analysis of the isolated RNA.
METHODS. Total RNA was isolated from C57Bl/6 testis, thymus, liver, lung and Sertoli cells using TRIzol reagent (Gibco/BRL), according to the manufacturer's instructions. First strand complementary DNA synthesis was performed using 5 μg of total RNA in the presence of Superscript II reverse transcriptase and oligo $(dT)_{12-18}$ primer. Reaction mixtures for PCR contained cDNA template, 1 μM each of sense and antisense primers, and a combination of 2.5 U *Taq* DNA polymerase (Perkin-Elmer/Cetus) and Vent (exo^+) DNA polymerase (NEB) in a ratio of 40:1 to ensure PCR fidelity. The CD95L sense primer (CGGGATCCATGCAGCAGCCCTTCAATTAC) flanked the ATG initiation codon and included a *Bam*H1 recognition site at the 5′ end for cloning; the antisense primer (CGGAATTCCTCTTAGAGCTTATATAAGCC) flanked the TAA termination codon including an *Eco*R1 recognition site. The upstream nested primer sequence (CGGGATCCATGCAGCTCTTCCACCTACAG), with an added *Bam*H1 recognition site, initiates at nucleotide 426 following the transmembrane region of the CD95L gene product. Amplification consisted of 30 cycles of denaturation (72 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 1 min). The PCR products were resolved on 0.8% agarose gels. Positive controls included full-length CD95L cDNA prepared from RNA isolated from mouse CTL stimulated with PMA and ionomycin. Verification of CD95L PCR product specificity was accomplished by nested PCR amplification of full-length reaction products and by restriction enzyme analysis. Controls for RNA integrity and tissue RNA normalization consisted of PCR amplification of F-Actin 3′ untranslated region sequence, using the 5′ primer (GATGCATTGTTACAGGAAGT) and 3′ primer (TCATACATCTCAAGTTGGGGG), producing a 240-bp fragment corresponding to nucleotides 3260–3500 of the gene, from oligo (dT)-primed cDNA.

shown previously by northern analysis[7], CD95L expression was observed in testis but not in lung. Relevant to the results presented above, CD95L transcripts were detected in freshly isolated Sertoli cells as well as in Sertoli cells cultured at 32 °C and 37 °C (data not shown) in consideration of the temperatures found in the scrotal and abdominal positions for transplantation. Our results indicate that at least part of the reported expression of CD95 ligand in rodent testis[5,7] can be attributed to Sertoli cells.

In summary, our findings suggest that expression of functional CD95L by Sertoli cells accounts for the immune-privileged nature of testis. The link between the failure of B6-*gld* Sertoli cells to protect against allograft immunity (Fig. 1) and the inability of B6-*gld* T cells to mediate *in vitro* cytotoxicity against syngeneic activated T-cell targets[10] is due to the loss of critical interactions between CD95 and its ligand. Consistent with this model, preliminary studies have shown that BALB/c testis was rejected by B6-*lpr* mice (data not shown).

CD95 ligand-mediated immunosuppression would be expected to primarily target activated effector cells rather than the activation steps that produce them. The most commonly used immunosuppressive agents, such as cyclosporin A, target T-cell lymphokine production but need not block effector function. It is not surprising that these agents might be less effective against previously activated T cells[30]. By targeting only activated T lymphocytes, grafted cell-associated CD95L may provide a highly specific form of immunosuppression for ameliorating T-cell-dependent graft rejection. □

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Protection against mycoplasma infection using expression-library immunization

Michael A. Barry*, Wayne C. Lai† & Stephen Albert Johnston*‡§

Departments of *Medicine and ‡Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235–8573, USA
†Division of Comparative Medicine, Department of Pathology, University of Texas Southwestern Medical Center, Dallas, Texas 75235–9073, USA

As is evident from the human immunodeficiency virus epidemic, there is no systematic method for producing a vaccine. Genetic immunization[1] is a new approach to vaccine production that has many of the advantages of live/attenuated pathogens but no risk of infection. It involves introducing DNA encoding a pathogen protein into host cells and has shown promise in several disease models[2–13]. Here we describe a new method for vaccine development, expression-library immunization, which makes use of the technique of genetic immunization and the fact that all the antigens of a pathogen are encoded in its DNA. An expression library of pathogen DNA is used to immunize a host thereby producing the effects of antigen presentation of a live vaccine without the risk. We show that even partial expression libraries made from the DNA of *Mycoplasma pulmonis*, a natural pathogen in rodents, provide protection against challenge from the pathogen. Expression library immunization may prove to be a general method for vaccination against any pathogen.

§To whom correspondence should be addressed.

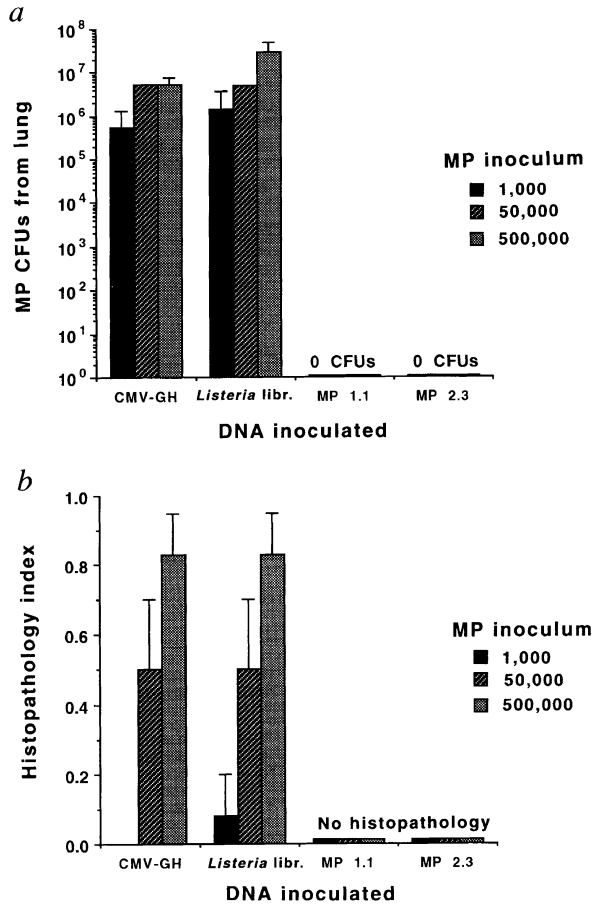


FIG. 1 MP titres and histopathology from ELI-immunized, MP-challenged mice. Mice were immunized with the indicated DNA and challenged with the indicated number of MP. *CMV-GH* expresses hGH. *Listeria* library is a hGH ELI library constructed using *Listeria monocytogenes* DNA. MP1.1 and MP2.3 are 3,000 transformant sibs of the mycoplasma library in which MP DNA was fused to coding frames 1 and 2, respectively, of hGH. *a*, MP titres. MP CFUs from lung represents the number of MP colony-forming units from each group of mice. *b*, Histopathology: the histopathological lesions induced by MP infection were scored from lung sections of mice by a histopathology index. An index of 1.0 represents the maximum number of lesions observed in infected mice. An index of zero indicates normal morphology. Each bar represents the mean from 2 to 4 mice. Error bars represent the standard deviation for each group.
METHODS. CMV-GH-F1 and -F3 and CMV-GH-F2 were constructed by inserting GATCTTGGATCCTAAGTAAGTA and GATCGGATCCTAAGTAAGTA, respectively, at the endogenous *Bgl*II site in exon 5 of hGH and *Hind*III site of the vector CMV-GH, a derivative of CMV1 (ref. 27) containing hGH from CMV-GH-ori[28]. Genomic DNA from MP or *Listeria* was digested with *Mbo*I to a median size of 0.5 kb and ligated into the *Bam*HI or *Bgl*II sites of CMV-GH-F1 and -F3 and CMV-GH-F2. TG1 bacteria were transformed and plasmid DNA was purified from pools of 3,000 transformants using Qiagen columns. 6-week-old female BALB/c mice were genetically immunized as described[28] on day 1 with 10 μg DNA and 5 μg on days 8, 21 and 52. Mice were challenged on day 64, and lung lavage and sectioning was done 14 days later as described[22].

We tested expression-library immunization (ELI) against *Mycoplasma pulmonis* (MP) because it is a normal lung pathogen in rodents[14,15], has a relatively small genome ($\sim 1 \times 10^6$ base pairs[16]), and has an unusual codon usage (for example, its tryptophan codon is a stop codon in eukaryotes[17,18]). An ELI library was made by fusing digested *M. pulmonis* DNA onto the last exon of the gene encoding human growth hormone (hGH), such that hGH-MP antigens might be secreted. To capture all antigens, MP DNA was fused into three frames of hGH and nine libraries of about 3,000 members (sibs) were prepared. Theoretically, a library expressing the entire genome of a pathogen could be used as a vaccine, but we found that ~1 ng DNA was required to produce an immune response by genetic immunization into the skin (data not shown and ref. 19). As 1–4 μg DNA is delivered per inoculation, libraries of 10^3–10^4 members were tested.

Two sib libraries, MP1.1 and MP2.3, were inoculated into separate groups of mice over 60 days with four inoculums totalling 25 μg DNA. This inoculum includes ~2 μg of MP DNA, representing $\sim 1 \times 10^9$ MP genomes or 10^6-fold more than introduced in a normal MP infection. As negative controls, the hGH plasmid or an hGH *Listeria monocytogenes* ELI library was also inoculated. After sixty days, the immunized mice were challenged with MP and tested for infection two weeks later (Fig. 1*a*). Control mice had 10^5–10^7 mycoplasma in lung lavages even after the lowest (10^3) challenge. Lung sections from these mice showed significant lesions (Figs 1*b* and 2). By contrast, mice inoculated with MP libraries had no culturable mycoplasma and no lung lesions (Figs 1 and 2). Protection by the MP libraries was pathogen-specific, because there was no protection by the *Listeria* library. This experiment was repeated with three inoculations over 30 days, followed by challenge with a more virulent isolate of the pathogen, and gave ~4 logs of protection as compared with control mice (results not shown). This indicates that library screening could be performed over shorter periods.

Figure 3 displays several important features of this technology. First, protective immunity conferred by ELI increases over time. Mice challenged with a more pathogenic isolate of MP had only partial protection after 60 days, but were fully protected after 100 days (Fig. 3 and data not shown). Second, a single immunization with an ELI library can confer lasting immunity.

TABLE 1. Immune responses induced by ELI libraries

	Anti-hGH antibodies*	Anti-MP antidbodies†	MP-specific DTH (mm)‡	MP-specific MMI (%)§
Control	−	−	2.3 ± 0.4	0
MP 1.1	+	+	16.4 ± 0.4	71.8
MP 2.3	+	+	19.8 ± 0.3	73.3

Mice were immunized as described for Fig. 1. Sera for antibody tests were recovered 10 days after the second inoculation. Two mice from each group were tested for DTH and inhibition of macrophage migration (MMI) 12 days after the last immunization. Control refers to unimmunized mice.

*Antibody levels against hGH protein: −, designates no antibodies; +, levels detectable by enzyme-linked immunosorbent assay (ELISA) only at dilutions of 1/250.

†Antibody levels against whole mycoplasma antigens: −, no antibodies; +, levels detectable by ELISA only at dilutions of 1/50.

‡MP-specific delayed-type hypersensitivity. Delayed-type hypersensitivity was evaluated as described[22] by injecting PBS buffer into the right rear footpad, and PBS containing 50 μg sonicated MP-cell protein into the left rear footpad. A dial-gauge calliper was used to measure the change in footpad thickness induced 24 h after injection. Three readings were measured and averaged. Measurements indicate the change in footpad thickness induced by injection of MP antigens in PBS minus that of PBS alone. Net footpad thickness (×100 mm) = ((mm post-MP injection − mm pre-MP injection) − mm post-PBS injection).

§MP-specific macrophage migration inhibition. Macrophage migration inhibition was evaluated as described[22] by packing a glass capillary tube with 100 μl spleen-cell suspension (1×10^8 cells per ml) from each mouse and placing it horizontally in a well of a 24-well plate immersed in RPMI medium in the absence or presence of 50 μg ml^{-1} sonicated MP protein. After 24 h, the area of cell migration out of the tube was measured by digital imaging. Lower migration is indicative of release of macrophage migration inhibition factor (MIF) from T cells previously activated against MP antigens by immunization. Release of MIF in this assay results in reduced area of migrated cells from the capillary. Per cent inhibition was calculated from the formula: $(A-B)/A \times 100$, where A is the area of macrophage migration in medium and B is the area of macrophage migration in medium containing MP antigen.

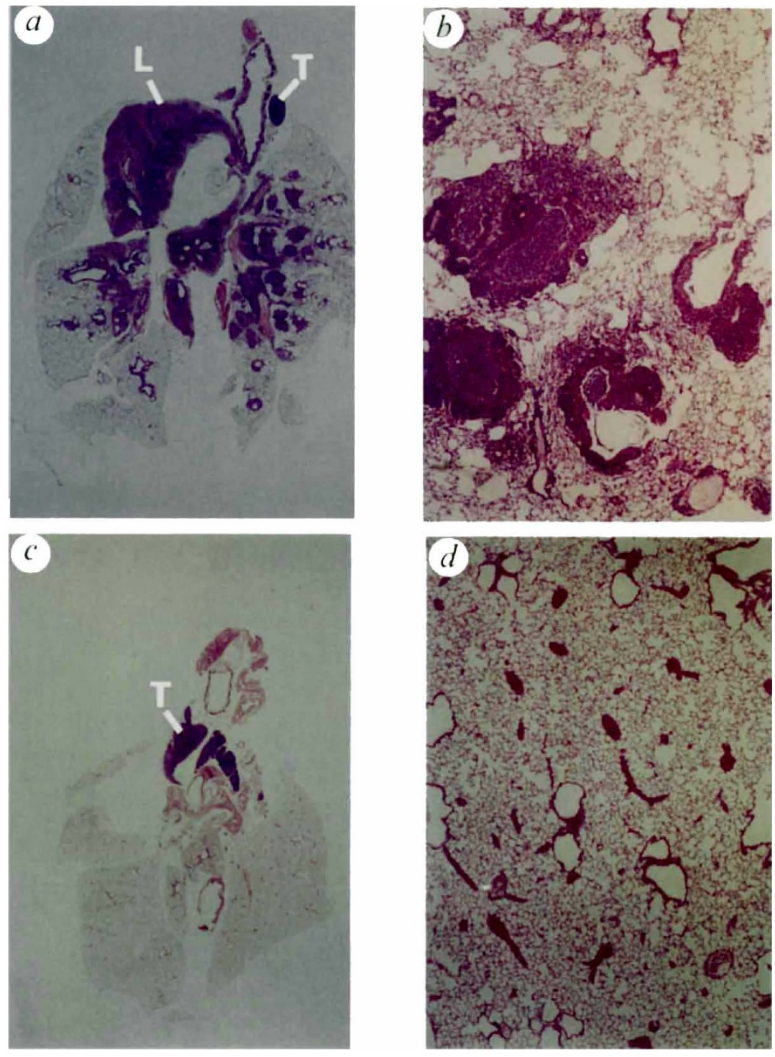
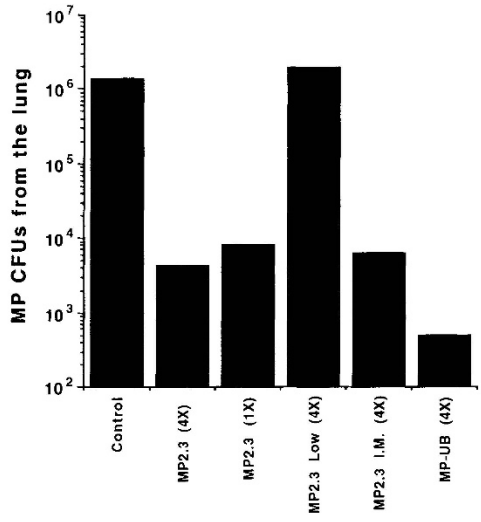


FIG. 2 Lung-tissue sections from ELI-immunized, MP-challenged mice. Lungs from the mice used for Fig. 1 were fixed in formalin, sectioned, and stained with eosin and haematoxylin. *a* and *b*, Lung section from a mouse immunized with CMV-GH and challenged with 5×10^5 MP viewed at 1.5× and 10× magnification, respectively. The dark purple staining of mononuclear cell infiltration around bronchiole and blood vessels of the lungs (marked as L) indicates the lesions induced by massive infection with MP. The thymus is also evident and is marked as T. *c* and *d*, Lung section from a mouse immunized with library MP1.1 and challenged with 5×10^5 MP viewed at 1.5× and 10× magnification, respectively. No mononuclear cell infiltration is observed, indicating the absence of MP infection. The thymus is marked as T.

FIG. 3 Comparison of the route, dosage, length of protection and number of inoculations required to protect against a highly pathogenic isolate of MP. Mice were immunized over 60 days before challenge with a more virulent isolate of MP than that described in Fig. 1 legend. (1X), A single inoculation of MP2.3 60 days before challenge; (4X), 4 immunizations over 60 days, as for Fig. 1; MP2.3 Low, inoculation with 1 μg at each immunization (total, 4 μg), compared to a normal total inoculum of 25 μg; MP2.3 I.M., mice immunized by intramuscular injection 4× with 100 μg of MP2.3; MP-UB, mice immunized with a total of 25 μg of a library created by fusing MP DNA to mouse ubiquitin rather than to GH. Each bar represents the average for two mice. Although only a small number of mice were used in this screening experiment, the relative differences in MP titres between groups were substantial. The specific number of MP from the lung for each mouse were: control, 3.0×10^5 and 2.2×10^6; MP2.3 (4X), 7.7×10^3 and 3.3×10^3; MP2.3 (1X), 5.0×10^2 and 1.1×10^3; MP2.3 Low (4X), 4.9×10^5 and 3.4×10^6; MP2.3 I.M. (4X), 4.0×10^2 and 1.2×10^4; MP-UB (4X), 1.0×10^3 and 0. METHODS. Experiments were performed as for Fig. 1 except where indicated. The gene for mouse ubiquitin was amplified by PCR from BALB/c genomic DNA with two mutagenic oligonucleotides. One places a *Bam*HI 5′ to the gene, supplies a Kozak start ATG, and knocks out the endogenous *Bgl*II site at amino acid 3 (CTCAGGATCCCACCATGCAGATTTTCGTGAAG). The other changes the final amino acid from Gly to Ala to inhibit cleavage of proteins fused to the carboxy terminus of ubiquitin[29] and adds a *Bgl*II site 3′ to the coding sequence (GCTCAAGATCTGCACCTCTCAGGCG). This PCR product was digested with *Bam*HI and *Bgl*II, then cloned into the *Bgl*II site of CMVB. Frame-insertion sites were added as described for the GH library in Fig. 1 legend.



A single inoculation of 10 μg MP2.3 60 days before challenge gave protection comparable to four inoculations totalling 25 μg over the same time (Fig. 3). Third, a threshold of antigen expression must be exceeded to produce protection. Four inoculations of only 1 μg gave no protection (Fig. 3). Finally, ELI libraries confer protective immunity when delivered in larger amounts by intramuscular injection, another method of genetic immunization. Injection of 100 μg of MP2.3 by this route four times over 60 days conferred a level of protection against the pathogenic strain similar to that achieved using 16-fold less DNA with the gene gun (Fig. 3).

Antibody and cellular immune responses induced by conventional vaccines have variable protective effects against MP in rodents[15,20–24]. T cells from the library-immunized mice were primed against MP, as demonstrated by positive inhibition of macrophage migration and by strong delayed-type hypersensitivity (DTH) reactions (Table 1). Sera from library-immunized mice had low titres of antibodies against hGH and mycoplasma proteins. The low hGH antibodies suggest that some antigens fused to hGH may block normal secretion of hGH. Preliminary experiments indicate that ubiquitin-MP libraries give similar or better protection than hGH-MP libraries (Fig. 3), suggesting that a protective cellular immunity is elicited by ELI vaccines, because ubiquitin should drive antigens to the proteasome[25] for subsequent major histocompatibility complex class I presentation[26].

In summary, ELI can produce a non-infectious multipartite vaccine, even when little is known of the pathogen's biology. The primary limitation of genetic immunization—knowing which pathogen gene to use—is circumvented by ELI, which uses the immune system to screen candidate genes. MP1.1 and MP2.3 have $\sim 3 \times 10^3$ members, of which ~500 should be in-frame. The protection offered by both libraries indicates that there must be several different protective plasmids in each. Preliminary tests of two 69-member sibs derived from MP2.3 gave no protection, suggesting that the protective plasmid(s) in MP2.3 are located elsewhere in the library. We are currently testing the sibs from MP libraries to isolate these protective members. Once isolated, these genes could be used as genetic vaccines or to develop recombinant protein vaccines. ELI may be the fastest method to isolate protective genes from a pathogen, and, for pathogens that are difficult to grow or attenuate, it could provide the only avenue to an effective vaccine. Cancers or pathogens with larger genomes should be amenable to ELI for we have found a 27,000-member library that also confers protection against MP (results not shown). ELI should be readily applicable to viral pathogens, including HIV, considering that most have genomes that are 10- to 100-fold smaller than that of mycoplasma. □

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Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene

Jean-Christophe Bories*†, Dennis M. Willerford*, Delphine Grévin‡, Laurie Davidson*, Anne Camus§, Patrick Martin‡, Dominique Stéhelin‡ & Frederick W. Alt*‖

* Howard Hughes Medical Institute, The Children's Hospital, and
‖ The Department of Genetics, Harvard Medical School and
Center for Blood Research, Boston, Massachusetts 02115, USA
† INSERM U 93, Hospital Saint Louis, 1 Avenue Claude Vellefaux, 75475 Paris, France
‡ CNRS URA 1160, Laboratorie d'Oncologie Moleculaire, Institut Pasteur, 1 rue Calmette, 59019 Lille, France
§ CNRS URA 1960, Unité de Biologie du developement, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris, France

THE Ets-1 proto-oncogene is a member of a transcription factor family characterized by homology to the *v-ets* oncogene[1–4]. In adult mice, Ets-1 is expressed predominantly in lymphoid cells where it has been implicated in regulating transcription of lymphocyte-specific genes[5–7]. Following T-cell activation, the specific DNA binding activity of Ets-1 is inactivated by transient phosphorylation, suggesting a function in the transition from the resting to activated state[8,9]. Ets-1 has also been suggested to cooperate with the AP-1 transcription factor complex to mediate cellular growth factor responses[4]. Here we show, by using RAG-2-deficient blastocyst complementation[10], that Ets-1 deficiency has dramatic, but different, effects on development and function of T- and B-lineage cells. Ets-1-deficient T cells were present in reduced numbers and were highly susceptible to cell death *in vitro*. In contrast, Ets-1-deficient B cells were present in normal numbers but a large proportion were IgM plasma cells. Our data demonstrate that Ets-1 is essential for maintenance of the normal pool of resting T- and B-lineage cells.

To investigate Ets-1 function in the immune system, we assayed the ability of Ets-1-deficient embryonic stem (ES) cells to differentiate into B and T lymphocytes following introduction into *RAG-2*$^{-/-}$ blastocysts. In the resulting chimaeras, all peripheral B and T cells are derived from ES cells[10,11]. We used gene-targeted mutation[12] to replace a region of the *Ets-1* gene[13–15] encoding the DNA-binding domain (the last 110 amino acids of the protein) with a neomycin resistance gene (Fig. 1*a*). Heterozygous mutant (*Ets-1*$^{+/-}$) ES cells were selected for growth in high G418 concentrations to isolate homozygous mutant (*Ets-1*$^{-/-}$) ES cells[16]. *Ets-1*$^{-/-}$-*RAG-2*$^{-/-}$ chimaeric mice derived from two independently targeted *Ets-1*$^{-/-}$ ES cell clones contained substantial numbers of splenic B and T cells but severely reduced numbers of lymph-node lymphocytes. Ets-1-hybridizing transcripts were specifically lacking in RNA from