

The *LMO2* T-Cell Oncogene Is Activated via Chromosomal Translocations or Retroviral Insertion during Gene Therapy but Has No Mandatory Role in Normal T-Cell Development

Matthew P. McCormack, Alan Forster, Lesley Drynan, Richard Pannell, and Terence H. Rabbitts*

MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom

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The *LMO2* gene encodes a LIM-only protein and is a target of chromosomal translocations in human T-cell leukemia. Recently, two X-SCID patients treated by gene therapy to rescue T-cell lymphopoiesis developed T-cell leukemias with retroviral insertion into the *LMO2* gene causing clonal T-cell proliferation. In view of the specificity of *LMO2* in T-cell tumorigenesis, we investigated a possible role for *Lmo2* in T-lymphopoiesis, using conditional knockout of mouse *Lmo2* with *loxP*-flanked *Lmo2* and *Cre* recombinase alleles driven by the promoters of the lymphoid-specific genes *Rag1*, *CD19*, and *Lck*. While efficient deletion of *Lmo2* was observed, even in the earliest detectable lymphoid cell progenitors of the bone marrow, there was no disturbance of lymphopoiesis in either T- or B-cell lineages, and in contrast to *Lmo2* transgenic mice, there were normal distributions of CD4⁺ CD8⁺ thymocytes. We conclude that there is no mandatory role for *LMO2* in lymphoid development, implying that its specific role in T-cell tumorigenesis results from a reprogramming of gene expression after enforced expression in T-cell precursors.

Cancer formation is frequently the result of alteration of the normal cellular functions of proto-oncogenes (10). The T-cell oncogene *LMO2* was first identified as a target of chromosomal translocations in human T-cell acute leukemias (2, 26). The gene encodes a LIM domain-only protein comprising two LIM domains (24) whose function is in protein interaction, binding to proteins such as TAL1/SCL and LDB1 in DNA-binding complexes that bind distinct bipartite target sites in normal hematopoietic (31) or T-cell tumor cells (7).

A remarkable feature of the *LMO2* gene is its involvement exclusively in T-cell malignancies when abnormally expressed (24). There are three lines of evidence supporting this contention. First, chromosomal translocations activating *LMO2* have only been observed in T-cell tumors. Indeed, a case of human T-cell acute leukemia has been described with both the Philadelphia chromosome (resulting in *BCR-ABL* fusion), normally the hallmark of myelogenous leukemia, and the *LMO2*-specific translocation t(11;14)(p13;q11) (6). Further, transgenic mice expressing *Lmo2* in the lymphoid lineage or with overexpression in all tissues (16, 18, 22, 23) only developed T-cell malignancies, indicating that *LMO2*-mediated tumorigenesis is specific to the T-cell lineage. Finally, an unfortunate outcome of a recent gene therapy trial with retrovirally delivered interleukin-2 receptor γ c subunit in X-linked severe combined immunodeficiency (X-SCID) patients (8) was the development of T-cell leukemia in two cases, following specific clonal expansion of T cells with retroviral insertion in the *LMO2* gene (9, 9a). The specific outcome of T-cell leukemia in both these X-SCID patients, despite the use in the clinical

protocol of the retroviral transduction of CD34-positive bone marrow progenitors, suggests that *LMO2* may influence T-cell differentiation.

Additional evidence for a such role in T-cell lymphopoiesis comes from the finding that *Lmo2* is normally expressed in immature CD4/CD8 double-negative thymocytes, before being downregulated as T cells mature (13). Moreover, transgenic mice with enforced *Lmo2* expression show a differentiation block at the same CD4/CD8 double-negative thymocyte stage, preceding the appearance of clonal T-cell tumors (17, 18).

Gene targeting experiments have addressed the question of a putative role for *Lmo2* in hematopoiesis (32, 35) but have been unable to answer whether *Lmo2* plays a role in T-cell development. Null mutation of *Lmo2* in mice causes embryonic lethality at around 10 days after inception, due to a failure of embryonic erythropoiesis (32). Thus, effects on lymphoid development could not be investigated. Similarly, the use of *Lmo2* null embryonic stem cells to make chimeric mice showed that there was no embryonic stem cell-derived contribution to adult hematopoiesis in these animals (35), indicating that *Lmo2* functions in the stem cells (i.e., the equivalent of the repopulating cells used in the X-SCID gene therapy) or their precursors. In both gene targeting strategies, any possible role for *Lmo2* in lymphopoiesis could not be determined because the null cells fail to contribute to hematopoietic subcompartments.

An approach to assess a role for *Lmo2* in lymphopoiesis is to use a conditional knockout strategy in which the *Lmo2* gene is deleted in specific cells in the hematopoietic lineage. We used a deletional strategy based on flanking the *Lmo2* gene with *Cre* recombinase recognition sites (*loxP* sites) and expressing *Cre* under the control of lymphoid-specific promoters (*Rag1*, *CD19*, or *Lck*). No changes in lymphoid development were found in the absence of the *Lmo2* gene with this approach.

* Corresponding author. Mailing address: MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, United Kingdom. Phone: 1223-402286. Fax: 1223-412178. E-mail: thr@mrc-lmb.cam.ac.uk.

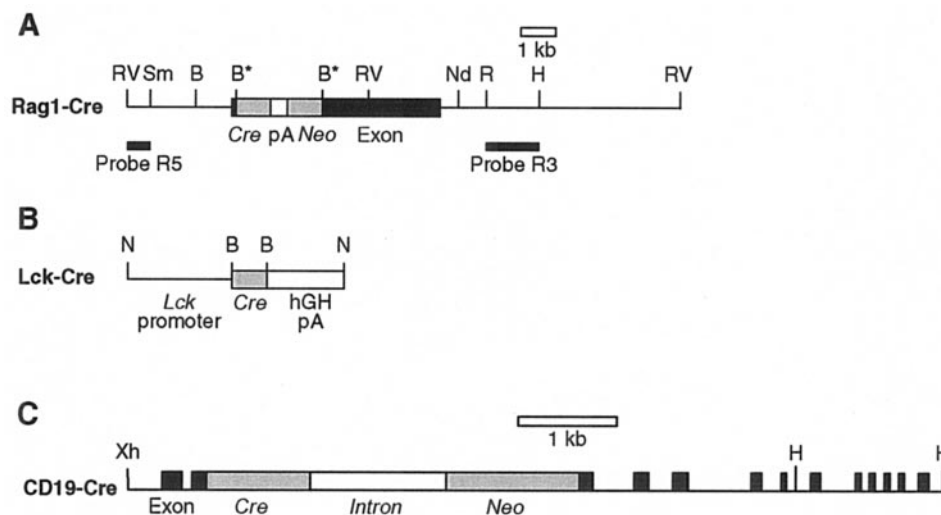


FIG. 1. Generation of cell-specific Cre expressing mouse lines. (A) *Rag1-Cre* was made by gene targeting knock-in of *Cre* into the *Rag1* exon. A *Rag1* targeting vector was constructed with a *SmaI-NdeI* fragment, encompassing the *Rag1* coding exon, a *BamHI* site was created in exon 1 of *Rag1* by mutation (B^*), and the *Cre* cDNA was cloned in-frame with the *Rag1* coding sequence. The simian virus 40 (SV40) polyadenylation site was cloned 3' of *Cre*, and the neomycin resistance cassette MC1-neo-pA (*Neo*) was cloned downstream of this. This construct was used to target an endogenous *Rag1* allele by homologous recombination to make embryonic stem cells with one targeted *Rag1* allele. Mice were made from these embryonic stem cells with germ line transmission of the *Rag1-Cre* allele. These mice showed no variation in hematopoiesis, and homozygous *Rag1-Cre* mice failed to develop lymphocytes, as previously found for *Rag1* knockout mice (21). (B) Generation of the *Lck-Cre* transgene. A *Cre* cDNA fragment was cloned into the unique *BamHI* site of a *Lck* promoter cassette (33). (C) *CD19-Cre* comprises a knock-in of *Cre* into exon 2 of the mouse *CD19* gene (25). Restriction sites are B, *BamHI*; H, *HindIII*; Nd, *NdeI*; N, *NotI*; R, *EcoRI*; RV, *EcoRV*; Sm, *SmaI*; S, *SacI*; X, *XbaI*; and Xh, *XhoI*.

Thus, despite its being a T-cell-specific oncogene, functional *Lmo2* is not required for normal T-cell development.

MATERIALS AND METHODS

Conditional targeting of *Lmo2*. Gene targeting was carried out with embryonic stem genomic DNA clones and homologous recombination after transfection into the CCB embryonic stem cell line as described (32, 35). The floxed *Lmo2* allele in embryonic stem cells was made in two stages. In the first step, a targeting vector (pLmo2pLXP) was constructed which comprised the DNA extending from the 5' end of exon 6 (which had an artificial *XhoI* site derived from a λ 2001 genomic *Lmo2* clone) to a *PstI* site \approx 300 bp on the 5' side of the most 3' *EcoRI* site (Fig. 5A). pLmo2pLXP was assembled by cloning the *XhoI-PstI* fragment into the *XhoI* and *XbaI* sites of pBluescript after filling in the *PstI* and *XbaI* sites. A *loxP*-puromycin cassette (3) was cloned (*BglII* fragment with filled-in ends) into the unique *XbaI* site of the pBluescript subclone, after filling in the *XbaI* site. The MC1-tk fragment was added at the *XhoI* site to complete the pLmo2pLXP construction.

Embryonic stem cells were transfected with pLmo2pLXP, and puromycin-resistant, ganciclovir-sensitive clones were selected. Gene targeted events were identified with genomic DNA digested with *XbaI* and filter hybridized by standard methods as described with the *Lmo2* genomic probe A (32). The targeting event was verified with a 300-bp *PstI-EcoRI* fragment (probe D) outside the 3' side of the targeting vector with *SphI* digests of genomic DNA. This identified targeted embryonic stem clones with a *loxP* site downstream of *Lmo2* exon 6. A verified, targeted embryonic stem clone (KO3PU/D3) was transfected in a second step to add a *loxP* site upstream of exon 5 to create the floxed *Lmo2* allele.

A targeting vector (pKO73hyg) was constructed with the *XbaI-EcoRI* fragment spanning exon 5 of *Lmo2* (see Fig. 5A) from a λ 2001 clone isolated from an embryonic stem cell DNA-derived genomic library (32). This fragment was cloned into the *XbaI* and *EcoRV* sites of pBluescript (after filling in the *EcoRV* site of the genomic fragment) and a *loxP*-hygromycin cassette (3) was cloned into the unique *EcoRV* genomic site. MC1-tk (29) was cloned into the *XbaI* site to give the pKO73hyg targeting clone.

The embryonic stem clone KO3PU/D3 was transfected with pKO73hyg, and hygromycin-resistant, ganciclovir-sensitive clones were selected. Homologous recombination targeted cells were identified with genomic DNA digested with

SacI, amplified by PCR, and including a nuclear localisation signal, an SV40 polyadenylation signal and MC1-neo-polyA (29), was cloned into the *BamHI* site to yield an in-frame fusion of the N-terminal three amino acids of *Rag1* with *Cre* (Met-Ala-Gly-Cre).

Lck-Cre was made by PCR amplifying the whole *Cre* cDNA sequence with primers including terminal *NcoI* restriction sites; the PCR product was cleaved with *NcoI*, the ends filled in and cloned into the blunt-ended *BamHI* site of the *Lck* transgenic expression clone (33). The transgenic fragment was excised with *NotI* and injected into pronuclei of fertilised eggs and transgenic strains were developed. One line in which detectable *Cre* protein was found was selected for further use.

The *CD19-Cre* targeting construct (a gift from Jurgen Roes and Klaus Rajewsky) has been described before (25). The plasmid was transfected into CCB embryonic stem cells, and targeted clones were identified by hybridization of *BamHI* digested DNA with a 0.9-kb *BamHI-PstI* probe located about 2 kb 5' of *CD19* exon 1. Targeted embryonic stem clones were injected into C57BL6 blastocysts and chimeric mice bred to give germ line transmission of the *CD19-Cre* targeted allele.

Filter hybridization. Genomic DNA was prepared from mouse tissues and 10 μ g amounts were digested to completion with *BamHI*, separated on 0.8% agarose gels and transferred to nylon membranes after denaturation (19, 28). The membranes were hybridized with the *Lmo2* genomic probes A or B (32) with random labeling (4) with [32 P]dCTP.

Genomic PCR. For genomic PCR of hematopoietic cells, 1000 cells were sorted with a MoFlo cell sorter (Dako Cytomation), directly into PCR tubes and analyzed with the following primers specific for *Lmo2* genomic sequences; Primers for detection of deleted *Lmo2* A5, 5'-GATGAATTCGTGCTCTTTCTCC TGCAACTA-3' A3, 5'-GATGGATCCAGGACGGTGGTTTGAGAACTGTT G-3' Primers for detection of *Lmo2* exon 5 B5, 5'-GTGGATGAGGTGCTGC AGATA-3' B3, 5'-TCCGTCCCAGCTGTAGTAGAG-3'.

PCR conditions used were 95°C for 2 min, followed by 30 cycles at 95°C for 30 s, 63°C for 30 secs and 72°C for 1 min and a final incubation at 72°C for 10 min. For multiplex PCR, it was necessary to add primers A5 and A3 in 4x excess to B5 and B3 to obtain equivalent amplifications for the deleted and undelated *Lmo2* alleles, respectively. A probe for detecting the deleted PCR product was made by amplification of a fully internal, 106bp fragment from the product of B5 plus B3 with primers KO73P plus KO73BN. The sequences of these primers

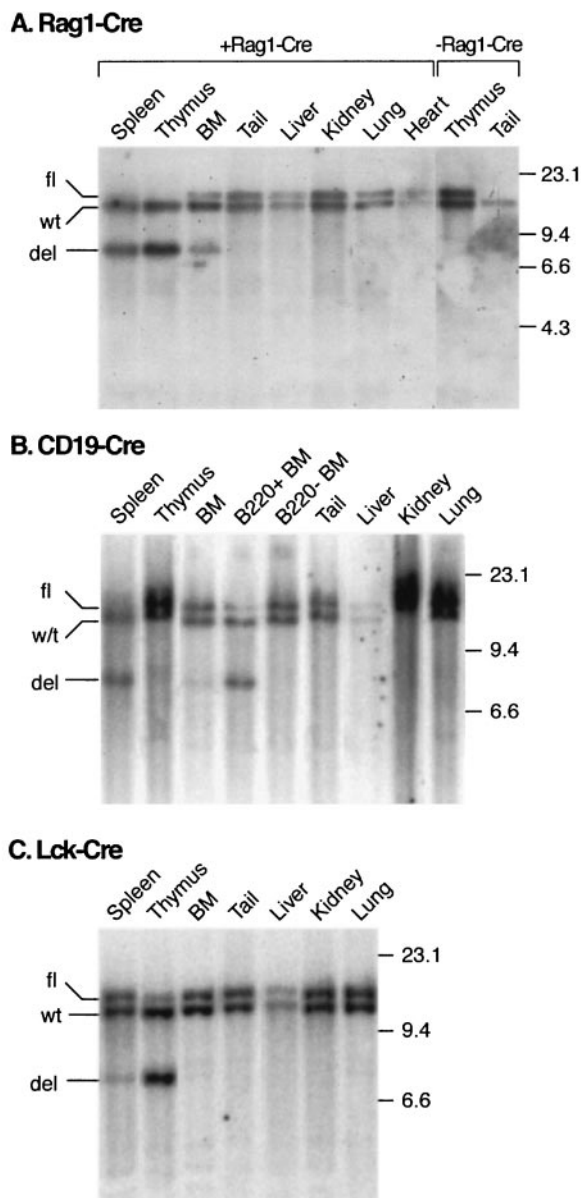


FIG. 2. Detection of *Lmo2* deletion in mouse tissues. Mice which were heterozygous for the floxed *Lmo2* allele (*Lmo2^{fl/wt}* mice) and which carried one of the three *Cre* alleles were euthanized, and genomic DNA was prepared from the indicated tissues. The DNA was digested with *Bam*HI, fractionated on agarose gels, immobilized on nylon and hybridized with *Lmo2* probe B (32) (Fig. 5A). Sizes of hybridizing bands were estimated by comigration of λ DNA cleaved with *Hind*III (shown in kilobases). The hybridizing bands are indicated corresponding to the wild-type *Lmo2* allele (wt), the floxed *Lmo2* allele (fl), and the deleted allele (del). (A) *Lmo2^{fl/wt};Rag1-Cre* mice. DNA from various tissues of *Lmo2^{fl/wt};Rag1-Cre* and *Lmo2^{fl/wt}* mice were examined. DNA from tail biopsy material serves as a control for detection of the wild type (wt) allele and the targeted (i.e., floxed) allele. Rearrangements (deletions, del) were restricted to hematopoietic organs spleen, bone marrow (BM), and thymus. (B) *Lmo2^{fl/wt};CD19-Cre* mice. DNAs from various tissues of *Lmo2^{fl/wt};CD19-Cre* were analyzed together with DNA prepared from purified B220⁺ cells selected from bone marrow with anti-B220 Dynabeads (B220⁺ bone marrow). B220⁻ bone marrow corresponds to DNA prepared from B220-depleted cells. (C) *Lmo2^{fl/wt};Lck-Cre* mice. DNAs from various tissues of *Lmo2^{fl/wt};Lck-Cre* mice were analyzed as shown. Clear evidence of rearrangements (deletions, del) is restricted to thymus and spleen DNA.

were KO73P, 5'-GGAGATCTGACTGCTTAAGGTGGC-3', and KO73BN, 5'-AGTAGAGCAAACATTCCTCTGAG-3'.

Western detection of proteins. Single cell suspensions were lysed by boiling in Laemmli sample buffer (0.25 M Tris-HCl, pH 6.8, 5% SDS, 10% glycerol, 100 mM dithiothreitol, 0.05% bromophenol blue) and protein equivalent to 10^6 cells was fractionated by 4 to 20% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride filter. The filter was washed for 1 h in TBS-T (Tris-buffered saline with 0.1% Tween-20) with 4% powdered skimmed milk at room temperature before 1 h of incubation with a 1:1,000 dilution of anti-*Cre* (Novagen). The filter was washed six times with TBS-T, incubated with a 1:5000 dilution of horseradish peroxidase conjugated anti-rabbit antibody (Amersham) and washed six times with TBS-T. The filter was subjected to enhanced chemiluminescence analysis (Amersham) according to the manufacturer's recommendations.

Flow cytometry. Single cell suspensions were prepared from the indicated tissues and preblocked with purified anti-CD16 (Fc-block); 10^6 cells were placed in FACS tubes and stained with phycoerythrin (PE)-conjugated anti-CD4, fluorescein isothiocyanate (FITC)-conjugated anti-CD8, PE-anti-T-cell receptor β subunit, PE-anti-CD2, PE-anti-CD3, PE-anti-Thy 1.2, PE-anti-B220, FITC-anti-IgM, biotinylated anti-IgG1, PE-anti-Ter119, PE-anti-Mac1, PE-anti-Sca1, PE-anti-CD19 and FITC-anti-CD34 antibodies. CD4-CD8 double-negative thymocytes were prepared as described below and incubated with PE-anti-CD44, FITC-anti-CD25, and FITC-anti-T-cell receptor γ subunit antibodies.

Bone marrow B-cell development was monitored according to the protocol of Hardy et al. (11, 12); 10^7 cells were immunostained with allophycocyanin (APC)-conjugated anti-B220 and biotinylated anti-CD43 antibodies, combined with FITC-anti-CD24 and PE-anti-Ly51/BP-1 for stages A to C or FITC-anti-IgM and PE-anti-IgD for stages D to F. Biotinylated antibodies were detected by subsequent staining with a streptavidin-cytochrome conjugate. All antibodies were from BD Pharmingen. Cells were analyzed with a FACScan flow cytometer (BD Immunocytometry Systems) with dual color excitation when detecting APC-conjugated antibodies, or sorted with a MoFlo cell sorter (Dako Cytomation).

Cell selection with Dynabeads. To perform cell enrichments or depletions, 5×10^7 cells were suspended in 1 ml of PBS containing 1% fetal bovine serum, incubated with 25 μ l (10^7) each of CD4-, CD8-, or B220-linked Dynabeads (DynaL Biotech), or combinations, for 20 min at 4°C. Target cells were isolated on a magnetic particle concentrator, and the procedure repeated on the supernatant. In the case of positive selections, and bound cells were washed three times in the above medium before use.

RESULTS

Conditional gene deletion in lymphopoiesis. The *Cre-loxP* recombination system is an ideal tool for tissue specific gene deletion. Homologous recombination is used to add *loxP* sites flanking the gene of interest in mouse embryonic stem cells and later mice are generated which contain the floxed gene and express the *Cre* recombinase in specific cells. To facilitate lymphoid-specific deletion we used the promoters of three lymphoid specific genes, viz., *Rag1*, which is expressed early in lymphopoiesis and during development of both T and B-cell lineages, *Lck* which is expressed specifically in T cells and the B-cell gene *CD19*. We generated alleles of *Rag1* and *Lck* controlling *Cre* expression, as well as utilizing a previously described *CD19-Cre* allele (25) (Fig. 1 A to C). In the case of the *Rag1-Cre* and *CD19-Cre* (25), homologous recombination was used to knock-in the *Cre* gene into the respective genes in embryonic stem cells, followed by generation of mice carrying the allele in their germ line (Fig. 1). An *Lck* promoter fragment (33) was used in the case of *Lck-Cre* to generate transgenic mice (see Materials and Methods).

The specificity of the three *Cre* lines was established with the *Lmo2^{fl}* locus as a reporter in mice. A conditional null *Lmo2* allele was made in embryonic stem cells with homologous recombination to add *loxP* sites within introns flanking the bulk of the coding region (exons 5 and 6, encoding codon 15 to the end of *Lmo2*) (Fig. 5A). Transient expression of *Cre* recom-

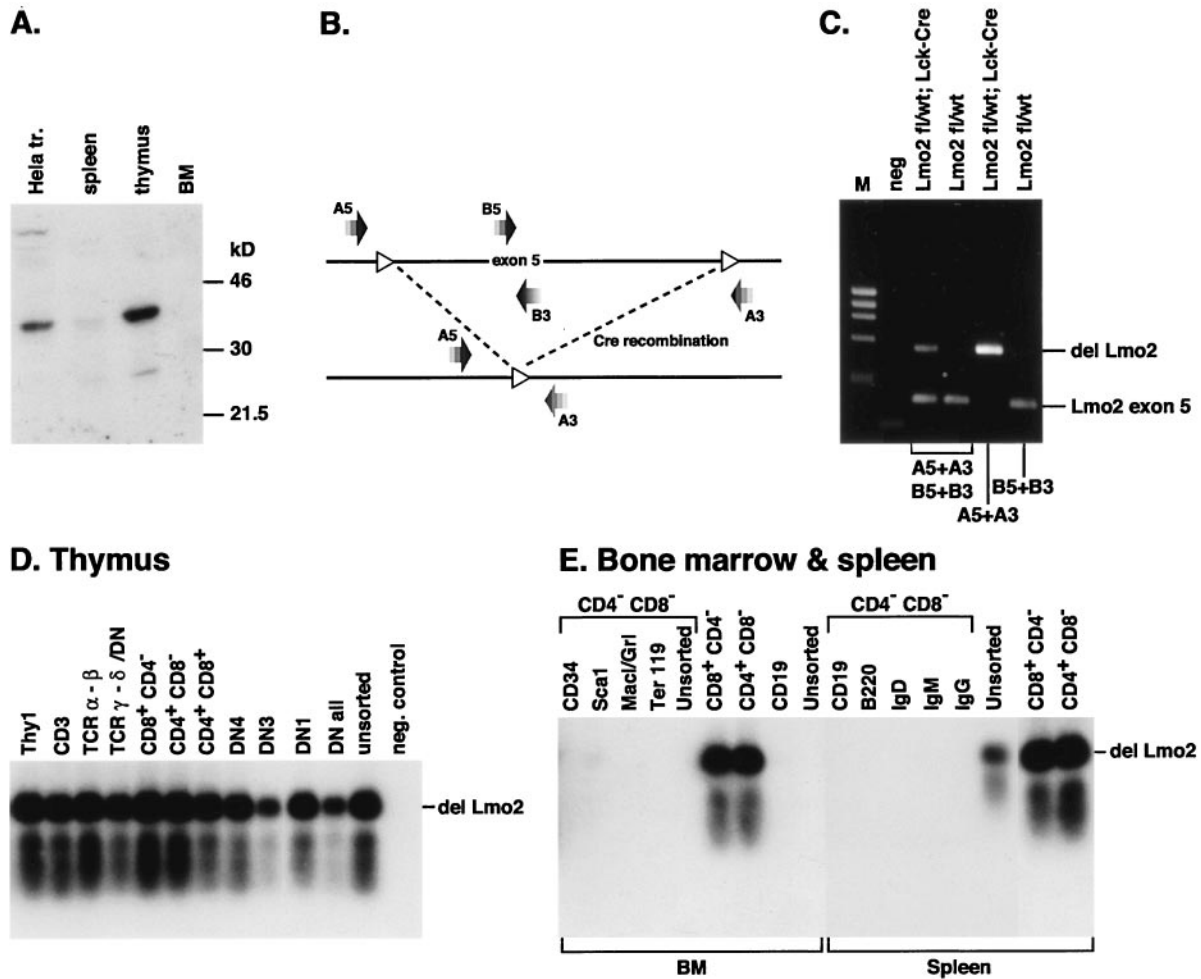


FIG. 3. Specificity of Cre recombinase expression in *Lck-Cre* mice. Transgenic *Lck-Cre* mice were bred with floxed *Lmo2* mice, and deletion of the floxed allele was used as a reporter for Cre activity. Protein or DNA was isolated from the various sources indicated for Western detection analysis of Cre protein expression or genomic PCR analysis of *Lmo2* deletion due to Cre activity. (A) Western blot analysis of Cre recombinase protein. Whole cell lysates from the indicated tissues of an *Lck-Cre* mouse or from HeLa cells transfected with a Cre expression construct (pGK-Cre, a gift from A. McKenzie) (30) (HeLa tr.), were prepared and subjected to Western detection analysis as described in Materials and Methods. BM, bone marrow. (B) Diagrammatic representation of *Lmo2* PCR primer locations (see also Materials and Methods) with respect to the *Lmo2* gene. Primers A5 plus A3 flank the loxP sites in the floxed *Lmo2* allele and do not yield a PCR product from the intact floxed *Lmo2* allele but do so when a deletion has occurred as the primers are brought into proximity of each other, yielding a 500-bp PCR fragment. Primers B5 and B3 are located in *Lmo2* exon 5 and give rise to a 184-bp PCR fragment from intact *Lmo2* (germ line or floxed). (C) Multiple PCR detection of *Lmo2* and deleted *Lmo2* alleles. Genomic DNA was prepared from the thymus of the indicated mice and used for PCR analysis with the indicated primers. PCR products were fractionated on 1.3% agarose gels and stained with ethidium bromide for photography. Lane M, ϕ X174 DNA markers cut with *Hae*III. (D and E) PCR analysis of genomic DNA from different subsets from thymus (D) and bone marrow or spleen (E) of an *Lmo2*^{fl/wt};*Lck-Cre* mouse. One thousand cells were sorted according to surface marker expression by FACS and directly used for multiplex PCR analysis, with primers A5, A3, B5, and B3. In some cases, to reduce potential background amplification from contaminating cells, cell populations were depleted with Dynabeads prior to sorting with specific antibody (CD4⁻ CD8⁻ bone marrow and spleen). DN represents CD4⁻ CD8⁻ double-negative thymocytes, and DN1, DN2, and DN3 are subsets of double-negative T cells defined by expression of CD44 and/or CD25 (see Fig. 5). PCR products were fractionated on agarose, transferred to nylon, and hybridized with the probe internal to the *Lmo2*^{del}-specific PCR product of primers A5 and A3. As an internal control for amplification, all PCRs also gave rise to the *Lmo2* exon 5 product as in C (data not shown).

binase in these floxed embryonic stem cells (designated *Lmo2*^{fl}) caused deletion in the *Lmo2* coding region, as judged by a PCR based assay (data not shown). Mice were generated with the floxed *Lmo2* allele and were bred to homozygosity, and maintained as such, with no detectable phenotypic alteration. These mice were used as a source of conditional null *Lmo2* and were crossed with mice carrying a constitutive null

Lmo2 allele, in which the *lacZ* gene had been knocked into *Lmo2* exon 5 (34) (*Lmo2*^{lacZ}), to give mice designated *Lmo2*^{fl/lacZ}. In this line of mice, the *Lmo2*^{fl/lacZ} genotype produces no phenotypic change (i.e., these mice are heterozygous *Lmo2* null) but cells become homozygous *Lmo2* null when Cre-mediated deletion of the *Lmo2*^{fl} allele occurs. Thus, when Cre expression is restricted to any particular cell type, such as

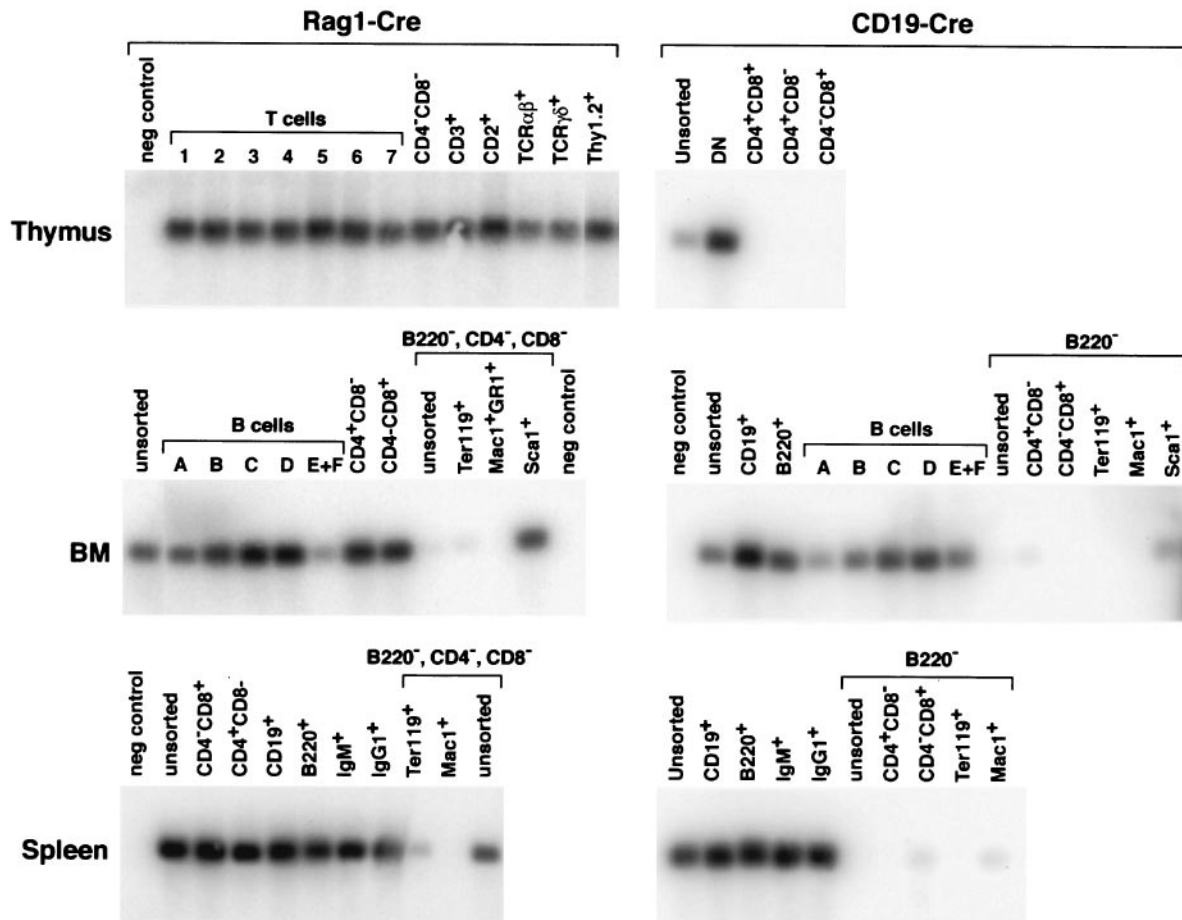


FIG. 4. Cell type specificity of Cre expression in *Rag1-Cre* and *CD19-Cre* mice. *CD19-Cre* or *Rag1-Cre* mice were bred with floxed *Lmo2* mice to use the deletion of the floxed allele as a measure of Cre activity; 1,000 cells from thymus (top), bone marrow (BM, middle), or spleen (bottom) were obtained with the surface markers indicated and used directly for PCR analysis. The right-hand panels show data from *Lmo2^{fl/wt};CD19-Cre* mice, and the left-hand panels show data from the *Lmo2^{fl/wt};Rag1-Cre* mice. PCRs were performed with the same approach as in Fig. 3, and the products hybridized with the probe specific for the *Lmo2^{del}* PCR product. B220-, CD4-, CD8-, or B220-depleted populations were used for FACS sorting from bone marrow or spleen where indicated. T-cell stages are labeled numerically, and B-cell stages are labeled alphabetically (12). As an internal control for amplification, all PCRs gave rise to the *Lmo2* exon 2 PCR product as in Fig. 3C.

hematopoietic subsets, consequences of *Lmo2* null mutation for those specific cells (and their descendants) can be monitored.

Lmo2^{fl};Cre mice were generated for each lymphoid Cre expressing line and initially whole tissue DNA genomic filter hybridization was used to compare *Lmo2* gene deletion in hematopoietic tissues (i.e., spleen, thymus, or bone marrow). This showed that *Lmo2^{fl}* deletion was specifically confined to the target lymphopoietic organs (Fig. 2). The presence of the *Rag1-Cre* allele caused essentially complete loss of the *Lmo2^{fl}* allele in the spleen and thymus giving rise to a deleted allele (termed *Lmo2^{del}*) but only partial deletion in bone marrow, implying that the *Rag1-Cre* allele causes complete deletion of *Lmo2^{fl}* in developing lymphoid cells (Fig. 2A). The *CD19-Cre* allele caused significant deletion in spleen, but very little in thymus and bone marrow, consistent with activity in B cells (25). Indeed, purified B220⁺ B cells from bone marrow showed ≈80% deletion of the floxed *Lmo2* allele (Fig. 2B). Conversely, the *Lck-Cre* allele caused ≈90% deletion in thymus

but little deletion in bone marrow, implying that Cre function is restricted to T cells.

More detailed analyses of the cell specificity of the Cre expression was carried out with PCR based approaches (Fig. 3 and 4). A multiplex genomic PCR assay was developed to allow detection of the undeleted and deleted forms of *Lmo2* by designing primers which either amplify an *Lmo2* exon 5 fragment (from undeleted *Lmo2*) or the fused intronic sequences after deletion of the floxed *Lmo2* allele (Fig. 3, B and C). Specific subsets of cells were selected based on surface marker expression, with a fluorescence activated cell sorter, from thymus (Fig. 3, D) or bone marrow and spleen (Fig. 3, E) of *Lmo2^{fl};Lck-Cre* mice and subjected to the PCR analysis. This showed *Lmo2^{fl}* gene deletion in T cells of all maturities but no detectable deletion in B cells (B220, CD19, Ig⁺), myeloid cells (Mac1/Gr1⁺), erythroid (Ter119⁺) or progenitor cells (CD34⁺ or Sca1⁻), corroborating the T-cell specificity of the *Lck-Cre* allele.

A similar analysis was performed on *Lmo2^{fl/wt}* mice bearing

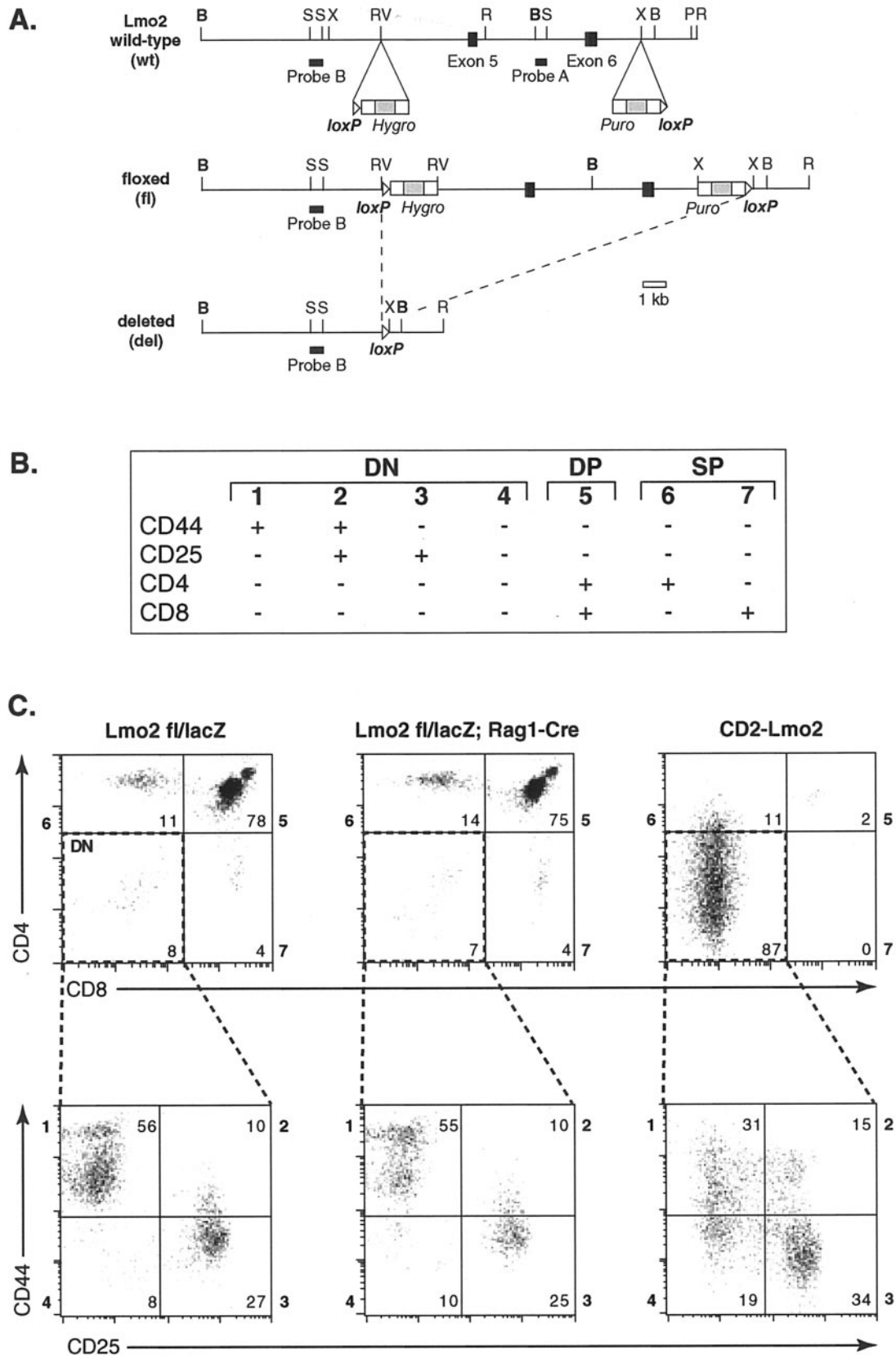


FIG. 5. Conditional deletion of the *Lmo2* gene has no effect on T-cell development. A floxed *Lmo2* allele was made in embryonic stem cells with homologous recombination to add *loxP* sites flanking exons 5 and 6 of *Lmo2* (exons encoding most of the *Lmo2* protein). Mice were made with the floxed *Lmo2* allele (*Lmo2*^{fl}), and compound genotype individuals were generated carrying *Lmo2*^{fl} on one chromosome and a null *Lmo2*

either *Rag1-Cre* or *CD19-Cre* alleles (Fig. 4). In the case of *Rag1-Cre*, *Lmo2^{del}*-derived PCR products were amplified from all stages of T cells (5) sorted from the thymus, from sorted spleen and bone marrow-derived mature T cells and from all B-cell stages (11) sorted from spleen and bone marrow (Fig. 4), whereas Ter119⁺ or Mac1/Gr1⁺ cells had little or no evidence of Cre activity. While some level of *Lmo2* deletion was observed in Sca1⁺ cells, neither myeloid (Mac1⁺) nor erythroid (Ter119⁺) cells showed this deletion, hence this result presumably reflects the fact that *Rag1* and *Sca1* are coexpressed in the earliest detectable lymphoid progenitor cells (14). These data are consistent with the lymphoid specificity of the *Rag1-Cre* allele. The B-cell specificity of the *CD19-Cre* (25) was confirmed, as *Lmo2^{del}* products were derived from all B-cell subsets of the bone marrow and spleen (Fig. 4). When these tissues were depleted of B cells, residual myeloid (Mac1/Gr1⁺), erythroid (Ter119⁺) and T cells (CD4 and/or CD8⁺) did not exhibit deletion of the floxed *Lmo2*.

Lmo2 has no mandatory role in lymphoid development. The consequence of *Lmo2* deletion in lymphoid cells was analyzed with *Lmo2^{fl/lacZ}* mice crossed with one of the three Cre expressing strains. This strategy for *Lmo2* gene deletion obviates any problem which might arise from inefficient Cre-mediated recombination since mice with one null *Lmo2* allele (*Lmo2^{lacZ}*) and one floxed *Lmo2* allele (*Lmo2^{fl}*) were used. Thus, all cells in which the floxed allele is deleted become homozygous for *Lmo2* null mutation. *Lmo2^{fl/lacZ};Cre* mice were viable and exhibited no obvious hematopoietic defects, with normal bone marrow, spleen and thymus cell counts (data not shown). The possible importance of *Lmo2* in T lymphopoiesis was examined by partitioning thymic T cells from *Lmo2^{fl/lacZ};Rag1-Cre* mice into various developmental stages with flow cytometry (illustrated in Fig. 5B, stages 1 to 7).

No significant alteration in T-cell stages 5, 6, or 7 was found in *Lmo2^{fl/lacZ};Rag1-Cre* mice compared with *Lmo2^{fl/lacZ}* littermates (Fig. 5C). When the CD4⁻ CD8⁻ double-negative T cells were further analyzed for CD44 and CD25 expression, subfractionating the cells into DN1 to DN4 subsets, we could not detect any significant shift in the four T-cell populations (Fig. 5C, bottom). By contrast, age-matched *CD2-Lmo2* transgenic mice, which have T-cell expression of *Lmo2*, showed an increase in CD4⁻ CD8⁻ double-negative thymocytes (17, 18), which display a predominant arrest at the stage DN3 of devel-

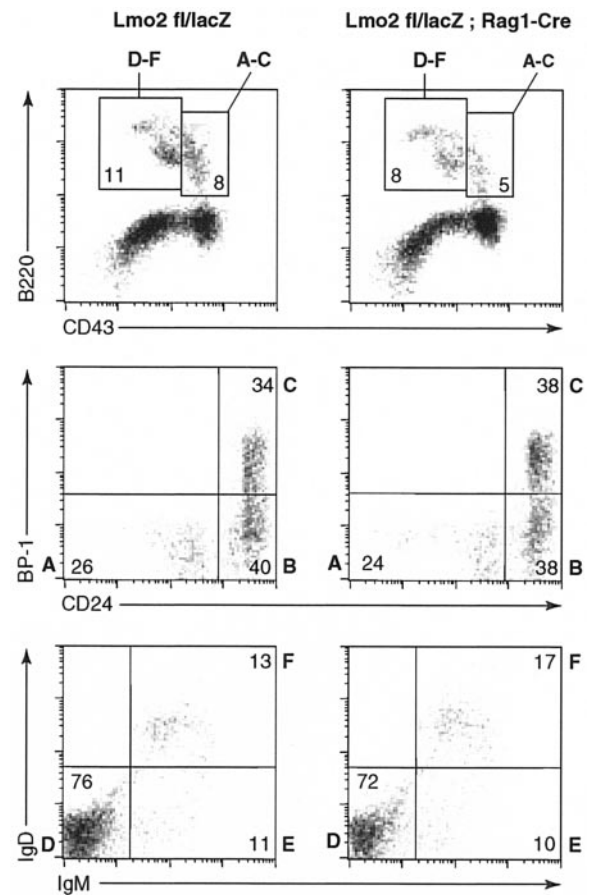


FIG. 6. Normal B-cell development occurs in the absence of *Lmo2*. Bone marrow cells were prepared from *Lmo2^{fl/lacZ}* and *Lmo2^{fl/lacZ};Rag1-Cre* littermates, and B cells were separated into six stages of development by four-color immunocytometry with antibodies binding to B220, CD43, Ly51/BP-1, CD24 (HSA), BP-1, IgM, and IgD (11, 12). B-cell stages are shown in bold alongside the quadrants to which they are ascribed, and the cells in each subpopulation are indicated in each quadrant. The top panels show un-gated B220 and CD43 double fluorescence profiles with the location of the six B-cell stages (A to F, ranging from most immature to most mature IgM, IgD double positive). The separate stages are shown as gated subpopulations in middle (A to C) and lower panels (D to F).

allele on the other (*Lmo2^{lacZ}*) and with a *Rag1-Cre* allele. Thymus cells were isolated from these mice at 8 to 16 weeks, and the surface marker profiles were examined by flow cytometry and compared with *Lmo2^{fl};Lmo2^{lacZ}* littermates and with *CD2-Lmo2* transgenic mice. (A) Restriction map of the floxed *Lmo2* locus. A partial restriction map of the genomic region of *Lmo2* is depicted in the top line, indicating the positions of *Lmo2* exons 5 and 6 and those of two probes used to detect targeting events in the embryonic stem cells and subsequently in genomic DNA of mouse tissues. A *loxP*-hygromycin resistance marker cassette (3) was introduced at the *EcoRV* site in intron 4, and a *loxP*-puromycin resistance marker cassette (3) was introduced at the *XbaI* site 3' of the last coding exon 6. The organization of the floxed *Lmo2* allele is shown in the middle line. After Cre-mediated deletion through the *loxP* sites, a deleted allele is made, from which the two selectable markers are deleted. Restriction sites are B, *BamHI*; P, *PstI*; R, *EcoRI*; RV, *EcoRV*; S, *SacI*; and X, *XbaI*. (B) A representation of the T-cell development stages. DN, DP, and SP represent the CD4/CD8 status of the different stages, where DN = CD4 and CD8 negative, DP = CD4 and CD8 positive, SP6 = CD4 positive (CD4⁺ CD8⁻), and SP7 = CD8 positive (CD4⁻ CD8⁺). (C) Thymocytes were prepared from mice of the indicated genotypes and labeled with fluorescent antibodies specific to the indicated T-cell markers. In the top panels, the distribution of CD4 and CD8 markers is shown. The quadrants representing T-cell stages 5 to 7 are indicated by the side of each quadrant and percent for each T-cell subpopulation within the quadrant. The double-negative T cells are in the bottom left quadrant in each case. For the lower panels, the double CD4⁻ and CD8⁻ cells were analyzed for CD44 and CD25 marker expression, to subdivide them into DN1 to DN4. For this analysis, the CD4⁺ CD8⁺ were first depleted with antibody-coated Dynabeads, resulting in CD4⁻ CD8⁻ double-negative thymocytes with purity of 98%, 96%, and 98% for *Lmo2^{fl/lacZ}*, *Lmo2^{fl/lacZ};Rag1-Cre*, and *CD2-Lmo2* mice, respectively. The double-negative T-cell stages are shown in bold alongside the FACS quadrants to which they are ascribed.

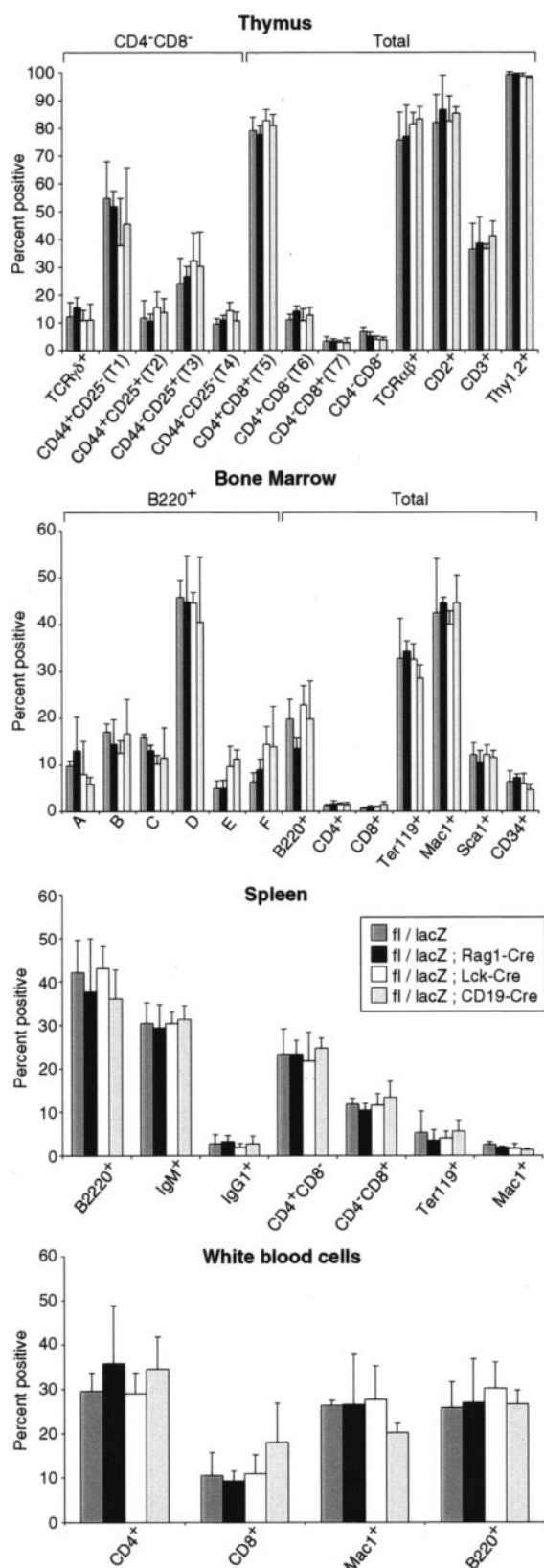


FIG. 7. Cumulative flow cytometric data of hematopoietic cells in mice lacking lymphoid *Lmo2* expression. Hematopoietic cells from *Lmo2^{fl/lacZ}* mice or *Lmo2^{fl/lacZ}* mice carrying a *Cre* allele were prepared from thymus, bone marrow, spleen, or peripheral blood and analyzed

as previously seen (7) (Fig. 5C). Thus, there is no detectable effect of *Lmo2* gene deletion on T-cell development, while, paradoxically, enforced expression of *Lmo2* in the T-cell lineage causes a profound developmental abnormality.

An analogous assessment of B-cell development was conducted on bone marrow-derived B cells (Fig. 6), as this is the site of B-cell lymphopoiesis in the adult mouse. When these were separated into B-cell subpopulations, by four-color immunocytometry with the established protocol (11), we found that the six B-cell stages of increasing maturity (Fig. 6A to F) were not significantly altered between *Lmo2^{fl/lacZ};Rag1-Cre* mice and *Lmo2^{fl/lacZ}* littermates. As the *Rag1* gene is expressed at early stages of both T and B lymphoid development, the lack of detectable effect on B lymphopoiesis argues that the presence of *Lmo2* protein is not necessary for B lymphopoiesis to proceed normally.

This analysis was taken further by comparing the proportions of hematopoietic cells with various surface marker phenotypes in mice with and without *Lmo2* deletion. *Lmo2^{fl/lacZ}* mice were produced containing either *Rag1-Cre*, *CD19-Cre*, or *Lck-Cre* alleles and various hematopoietic populations in thymus, spleen, bone marrow, or blood were compared with mice lacking *Cre* (Fig. 7). No significant alteration was observed in thymus, spleen, bone marrow, or white blood cell FACS profiles between the groups. These results argue against a compensatory molecule(s) present at specific stages of T- or B-cell development that could replace the functionality normally provided by *Lmo2*. The family of LIM-only proteins comprises four known members (24) whose sequence dissimilarities and expression patterns argue against their substituting for *Lmo2*.

We assessed the extent of *Lmo2* deletion in lymphoid cells of *Lmo2^{fl/lacZ};Cre* mice by Southern blotting (Fig. 8). Two hybridizing bands are found in *Lmo2^{fl/lacZ}* DNA, corresponding to floxed (Fig. 8A, fl) and null (Fig. 8A, lacZ) *Lmo2* alleles. Greater than 90% deletion was consistently found in thymus DNA of *Lmo2^{fl/lacZ};Rag1-Cre* and *Lmo2^{fl/lacZ};Lck-Cre* mice (Fig. 8B, C). The efficiency of *Lmo2* deletion by the *Rag1* controlled *Cre* in the B-cell lineage appears lower than in T cells, as the deletion in whole spleen DNA (predominantly B and T cells) of *Lmo2^{fl/lacZ};Rag1-Cre* mice was around 75%. Purified B220⁺ B cells from the spleen showed a similar level of *Lmo2* deletion (Fig. 8B). Deletion in the similar B220⁺ B-cell population in *Lmo2^{fl/lacZ};CD19-Cre* mice was in excess of 90% (Fig. 8D). Coupled with the analysis of hematopoiesis (Fig. 5 to 7), the high proportions of *Lmo2* homozygous null mutant cells supports the conclusion that both T and B lymphocytes can develop in the absence of *Lmo2*. Thus, while transgenic expression of *Lmo2* has severe consequences for T-cell development, this protein is not mandatory for normal development of the T lymphoid system.

by flow cytometry with antibodies against the indicated surface markers. The percentage of cells which were positive for each marker in each population are shown as the mean data from four mice in each group. Error bars denote one standard deviation of the mean.

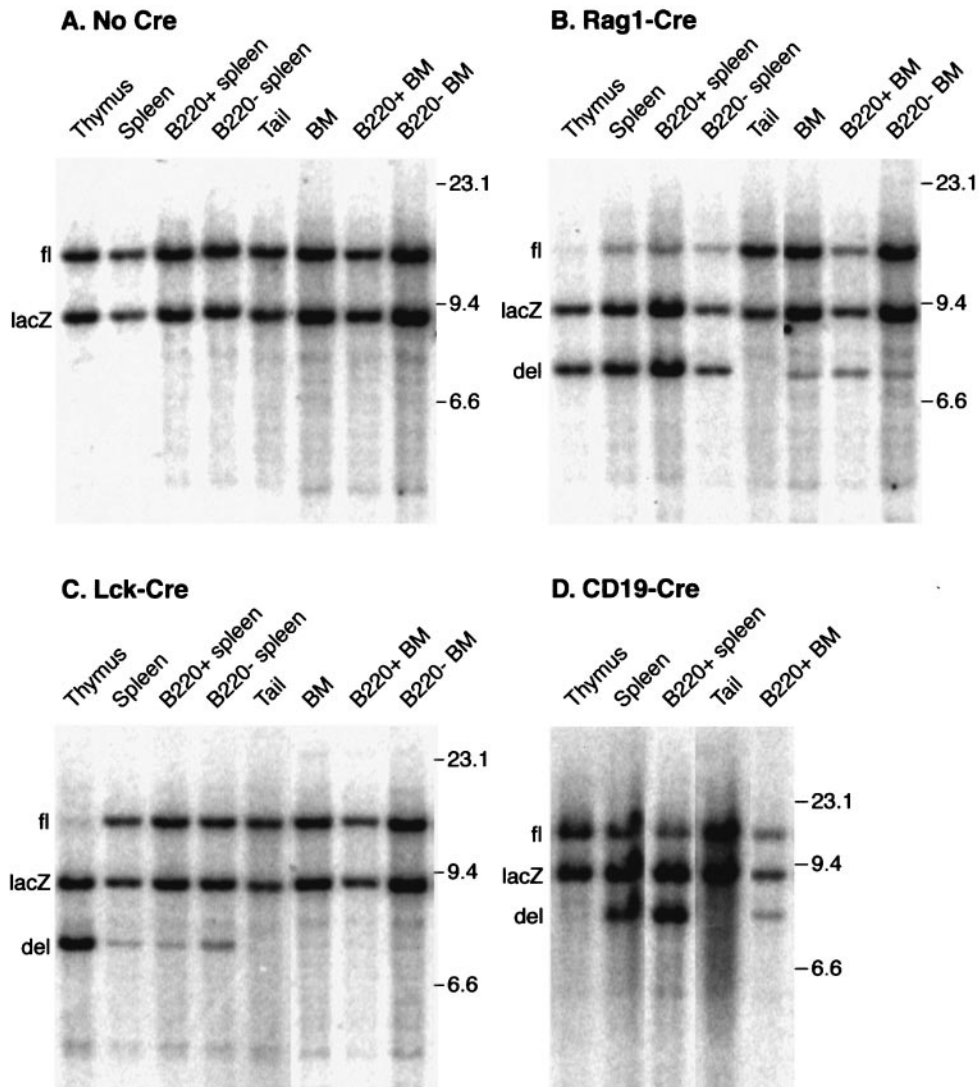


FIG. 8. Deletion of *Lmo2* in mouse lymphoid cells expressing Cre recombinase. Genomic DNA was extracted from the indicated tissues of *Lmo2^{fl/lacZ}* mice (No Cre) or from *Lmo2^{fl/lacZ};Rag1-Cre* (Rag1-Cre), *Lmo2^{fl/lacZ};Lck-Cre* (Lck-Cre), or *Lmo2^{fl/lacZ};CD19-Cre* (CD19-Cre) and digested with *Bam*HI followed by filter hybridization analysis with *Lmo2* probe B (Fig. 5A). In some cases of spleen and bone marrow, B220-expressing cells were purified with anti-B220 Dynabeads and DNA was prepared from these (B220⁺) and from the B220-depleted (B220⁻) populations. Genomic DNA bands are indicated, corresponding to *Lmo2* floxed (fl), wild-type (wt), *lacZ*, and deleted (del) alleles. Cells in which deletion of the floxed *Lmo2* occurs become homozygous null for *Lmo2*, as the second allele in these mice was a preexisting null allele (*lacZ* knock-in). Sizes (in kilobases) were estimated by coelectrophoresis of λ DNA cleaved with *Hind*III.

DISCUSSION

No obligatory requirement for *Lmo2* in lymphopoiesis. In this study we assessed the role of the acute T-cell leukemia oncogene *Lmo2* in normal lymphoid development in mice, by creating specific null mutations of the gene in lymphopoiesis. This was achieved with three Cre expressing mouse lines in which Cre was synthesized from *Rag1*, *Lck*, or *CD19* genes. We could find no evidence that loss of the *Lmo2* gene had any effect on lymphoid development. This contrasts with previous data with *Lmo2* null chimaeric mice in which no hematopoiesis was observed, implying a necessary role for *Lmo2* in hematopoietic stem cells or in the generation of those cells from precursors (35) but none in lymphoid development. A later

role in the myeloid-erythroid lineage has not yet been specifically examined.

The lack of an obligatory role for *Lmo2* in T-cell development is paradoxical in view of the specific association of activated LMO2 with T-cell tumors and the normal expression of the gene in DN1 and DN2 T cells (13, 15). It is especially intriguing because enforced expression of *Lmo2* in transgenic mice, under the control of the T-cell *CD2* promoter, causes a block of T-cell differentiation at the DN3 stage (7), which is the stage at which *Lmo2* is normally downregulated (13). This implies that the inability to shut off the function of *Lmo2* at the DN2/DN3 differentiation boundary inhibits further passage through the T-cell differ-

entiation pathway and in turn implies a normal role for Lmo2 at this T-cell differentiation stage. Our data show that there is no such discernible role for Lmo2.

LMO2 is a T-cell-specific oncogene. LMO2 has only been implicated thus far in T-cell malignancies. Its discovery by association with recurrent chromosomal translocations in T-cell acute leukemias (24) was followed by its verification as a T-cell-specific oncogene in transgenic models where enforced Lmo2 expression was not limited to T cells (23). The recent development of leukemia by LMO2 activation via retroviral insertion in two X-SCID gene therapy patients (9a) has reinforced this T-cell tropism, as both patients developed T-cell acute leukemias (9).

A possible role for LMO2 in guiding differentiation into the T-cell lineage is indicated by these findings because the source of the cells for the retroviral transduction were CD34⁺ multipotential bone marrow cells (8). If there is no mandatory role for LMO2 in T-lymphoid development, it suggests that its specific role in T-cell tumorigenesis results from a reprogramming of gene expression after enforced expression in T-cell precursors. Examination of Lmo2-associated DNA binding complexes in transgenic mouse leukemic cells showed that Lmo2 forms complexes which differ in composition from those found in erythroid cells (7). Such complexes could be involved in the transcriptional regulation of target genes leading to a block in T-cell development and onset of leukemia. However, our current results imply that such complexes do not play a role in normal lymphoid development, implying either that they do not form during normal T lymphopoiesis, or that they form but are not functional.

It has been shown that in *Lmo2-Tal1/Scl* double transgenic mice have increased efficacy in T-cell leukemia development (17). In addition, another member of the family of LIM-only T-cell oncogenes, *Lmo1*, cooperates with *Tal1/Scl* in T-cell tumorigenesis and causes an inhibition of the E2A-HEB-mediated expression of the pre-T-cell receptor α -chain. This is thought to contribute to the arrested thymic development seen in *Tal1/Scl* transgenic mice (13). Moreover, mice lacking *E2A* develop leukemia of similar phenotype to *CD2-Lmo2* transgenic mice (1). Hence, Lmo2-Tal1/Scl-induced leukemogenesis may be due to an abrogation of E2A protein function in T-cell development. By contrast, quantitative PCR analysis showed that regulation of pre-T-cell receptor α chain was normal in *Lmo2^{flacZ};Lck-Cre* mice, implying that Lmo2 does not regulate this receptor subunit during normal T-cell development (data not shown).

LMO2 and retroviral insertion in X-SCID gene therapy. The X-SCID gene therapy patients who developed leukemia as a result of retroviral activation of LMO2, had been infused with retrovirally transduced CD34⁺ progenitors to rescue the hematopoietic defect in these patients, with a retrovirus expressing the interleukin-2 receptor common γ chain which is absent in X-SCID (8). As a number of different chromosomal translocation genes have been identified which appear specific to T-cell acute leukemia (20), it is not clear why only LMO2 has been found in X-SCID leukemia cases.

One hypothesis is that there is cooperation between γ chain and LMO2 protein functions, perhaps with the former dictating cell division and the latter cell differentiation. In this situation, LMO2 expression is enforced by the presence of retro-

virus (9, 9a), in an analogous way to the transgenic *Lmo2* mouse models.

It is intriguing that the LMO2-associated chromosomal translocations in humans appear to be mediated by mistakes of the RAG recombinase (27). The *Rag1* gene is expressed in the DN2/DN3 stages of T-cell differentiation and furthermore transgenic enforced *Lmo2* expression results in accumulation of DN3 T cells. These combined observations suggest that the chromosomal translocations occur in humans by RAG-mediated errors in DN2/DN3 cells and the resultant enforced LMO2 expression leads to inhibition in T-cell development and onset of leukemia (24). By contrast, however, *Lmo2* expression early in normal T-cell development is likely to be a remnant of expression in hematopoietic precursors, as it is not required for normal lymphoid maturation.

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