Retroviral Insertional Mutagenesis Can Contribute to Immortalization of Mature T Lymphocytes

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Several cases of T-cell leukemia caused by gammaretroviral insertional mutagenesis in children treated for x-linked severe combined immunodeficiency (SCID) by transplantation of autologous gene-modified stem cells were reported. In a comparative analysis, we recently showed that mature T cells, on the contrary, are highly resistant to transformation by gammaretroviral gene transfer. In the present study, we observed immortalization of a single T-cell clone *in vitro* after gammaretroviral transduction of the T-cell protooncogene *LMO2*. This clone was CD4/CD8 double-negative, but expressed a single rearranged T-cell receptor. The clone was able to overgrow nonmanipulated competitor T-cell populations *in vitro*, but no tumor formation was observed after transplantation into Rag-1 deficient recipients. The retroviral integration site (RIS) was found to be near the *IL2RA* and *IL15RA* genes. As a consequence, both receptors were constitutively upregulated on the RNA and protein level and the immortalized cell clone was highly IL-2 dependent. Ectopic expression of both, the *IL2RA* chain and *LMO2*, induced long-term growth in cultured primary T cells. This study demonstrates that insertional mutagenesis can contribute to immortalization of mature T cells, although this is a rare event. Furthermore, the results show that signaling of the IL-2 receptor and the protooncogene *LMO2* can act synergistically in malignant transformation of mature T lymphocytes.

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INTRODUCTION

Myeloid malignancies induced by gammaretroviral vector transfer were reported in several animal models and in a human gene therapy trial (1–3). T-cell malignancies were seen in five patients after stem cell gene therapy for severe combined immunodeficiency-X1 (SCID-X1) (4–7). Most of the latter clinical cases were induced by vector-mediated insertional transactivation of the protooncogene *LMO2*. The gene therapy vectors commonly used in the named studies were based on the murine leukemia virus (MLV). Those vectors have the propensity to integrate into active chromatin structures nearby promoter regions (8,9). The MLV-based vectors contained full-length long terminal repeats (LTRs) with strong promoter and enhancer activity, not only driving the expression of the delivered transgene, but potentially host genes flanking the integration sites as well (2,7).

Currently, it is believed that the target cell most vulnerable to insertional mutagenesis-mediated transformation is a primitive progenitor cell, and that more mature cells are less prone to this adverse event (10,11). Furthermore, in clinical trials dealing with gene transfer into mature T cells, malignant transformation has not been observed so far (12–15). In a previous study, we addressed this issue by the gammaretroviral transduction of potent (proto)oncogenes into hematopoietic stem cells (HSCs) and mature T lymphocytes. For that experimental setting, we clearly demonstrated that mature T-cell populations are less susceptible to transformation than HSCs (16).

Nevertheless, several types of T-cell leukemia or lymphoma with a mature phenotype exist, although these are rare diseases. So far, it has not been shown conclusively if those malignancies are really initiated in mature T cells or rather in an immature thymocyte population that retains the ability to differentiate into mature T cells. Mature T cells claim a special position in hematopoiesis, as they show long life spans and a high capacity of self-renewal.

In contrast, differentiated cells generally are limited in their proliferation capacity *in vitro* and *in vivo*. The resulting limited life span protects against an accumulation of mutations and a potential transformation.

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Here, we describe the *in vitro* immortalization of a T-cell clone after gammaretroviral gene transfer of the T-cell protooncogene *LMO2*, due to the insertional transactivation of the genes for the α chains of the Interleukin-2 and Interleukin-15 receptor (*IL2RA* and *IL15RA*).

MATERIALS AND METHODS

Retroviral Vectors/Cloning and Reverse Transcription for *IL2RA* cDNA Generation

MP91-EGFP and MP91-LMO2-EGFP were described previously (16). The cDNA of the murine *IL2RA* was generated via reverse transcription (SuperScript II Reverse Transcriptase, Invitrogen, Carlsbad, CA, USA). Total RNA was isolated (RNeasy Mini Kit, Qiagen, Hilden, Germany) from stimulated murine, mature T cells and used for reverse transcription with an *IL2RA* specific primer (IL2Ra-RT-Rev: CGTCTCAGAT TTGGCTTGAG). Generated *IL2RA-cDNA* was furthermore amplified (with following primers: IL2Ra-Forw: GTGCCAGGAAGATGGAG; IL2Ra-Rev: CATCCGCTTGCCTGGGCTC) and the PCR product then was cloned into MP91-EGFP in front of an internal ribosome entry site (IRES). The cDNA of the murine *IL15RA* was obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung (ImaGenes, Berlin, Germany) and also cloned into MP91-EGFP as described for *IL2RA*. To enable the detection of triple transduced cells (*IL2RA, IL15RA, LMO2*), we substituted EGFP with the fluorescent marker Venus (17) in the *IL2RA* encoding vector and with the fluorescent marker Cerulean (18) in the *IL15RA* encoding vector, respectively.

Retroviral Particle Production

Vector supernatants were produced in Dulbecco's modified Eagle medium (Lonza, Rockville, MD, USA) supplemented with 10% fetal calf serum (Pan Biotech, Aidenbach, Germany), 2% L-glutamine (Lonza), and 1% Pen/Strep (PAA Laboratories, Coelbe, Germany). Ecotropic supernatant was produced in a split genome approach by calcium-phosphate-mediated transient transfection of 293T human embryonic kidney producer cells. After 24, 48 and 60 h, supernatant was collected, filtered (0.45 μm), and stored at 4°C for a maximum of 1 wk. All supernatants were pooled and titrated on the embryonic murine fibroblast SC-1 cell line. After titration, supernatant was used directly for transduction.

Retroviral Transduction and Culture Conditions

Murine mononuclear cells were isolated from the spleen and the lymph nodes (mesenteric and superficial inguinal) of C57BL/6J.Ly5.2 mice (Charles River Laboratories, Sulzfeld, Germany) and stimulated by anti-CD3 (clone 145–2C11), anti-CD28 monoclonal antibody (mAb, clone 37.51; both from BD Biosciences PharMingen) coated paramagnetic beads (Invitrogen) for 4 d to obtain stimulated mature T cells. The use of paramagnetic beads conjugated with mAb has been described previously (19). At d 4 after isolation, cells were transduced on vector supernatant-preloaded culture plates (BD), precoated with 50 μg/mL retronectin (Takara, Kyoto, Japan). Stimulated mature T cells were kept in RPMI 1640 (Lonza), supplemented with 10% fetal calf serum (Pan Biotech), 2% L-glutamine (Lonza), 1% Pen/Strep (PAA Laboratories), 1% sodium pyruvate (Invitrogen), 1% nonessential amino acids (Invitrogen) and 0.1% β-mercaptoethanol (Invitrogen) throughout the entire cultivation time. Culture conditions also included human IL-2 (Roche Diagnostics, Mannheim, Germany) at 100 U/mL for stimulation.

LM-PCR

Ligation-mediated polymerase chain reaction (LM-PCR) was performed as previously described (20). Genomic DNA was prepared, using the DNeasy Blood & Tissue Kit (Qiagen). After LM-PCR and subsequent sequencing, the identified integrations, which contained at least the LTR or polylinker sequence, were BLAST aligned using the NCBI36 mouse genome build (accessed October 2010).

Genes within 200kb upstream and downstream of the vector integrations as well as the genes closest to the integration sites were identified using NCBI map view data (accessed October 2010).

Integration-Site Specific PCR

To analyze clonality after limited dilution, integration-site specific PCR (and subsequent Nested-PCR) of 14 established clones was performed. Vector specific primers: Vector 1: 5′-CCATGCCTTG CAAAATGGC, Vector_Nested: 5′-CTTGC AAAATGGCGTTAC. Integration specific primers for *Hod1* on chromosome 5: Hod1_1: 5′-GGCTGTTGGATATTATGGAT GC, Hod1_Nested: 5′-CATGCTGACC TTTGGAGTGA; for *IL2RA/IL15RA* on chromosome 2: IL2RA/IL15RA_1: 5′-CCTGACTACCAGAATAGTGCAAAA, IL2RA/IL15RA_Nested: 5′-GAGCCCC CATATCTCTCTCC.

Microarray Analysis

Miltenyi Biotech performed Microarray ratio experiments commercially. RNA was isolated from fresh murine T lymphocytes, thymocytes and the immortalized T-cell population (each 1×10^7 cells from 8-wk-old C57BL/6 wild type (WT) donor animals) using standard RNA extraction protocols (NucleoSpin RNA II, Macherey-Nagel, Düren, Germany). The RNA samples were quality-checked via the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Santa Clara, CA, USA). For the linear T7–based amplification step, 0.5 μg of each total RNA sample was used. To produce Cy3–labeled cRNA, the RNA samples were amplified and labeled using the Agilent Low RNA Input Linear Amp Kit (Agilent Technologies) following the manufacturer's protocol. Yields of cRNA and the dye incorporation rate were measured with the ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The hybridization procedure was performed according to the Agilent 60-mer oligo-microarray processing protocol using the Agilent Gene Expression Hybridization

Kit (Agilent Technologies). Briefly, 1.65 μg Cy3-labeled fragmented cRNA in hybridization buffer was hybridized overnight (17 h, 65°C) to Agilent Whole Mouse Genome Oligo Microarrays 4 × 44K using Agilent's recommended hybridization chamber and oven. Finally, the microarrays were washed once with 6 × SSPE buffer containing 0.005% N-lauroylsarcosine for 1 min at room temperature followed by a second wash with preheated 0.06 × SSPE buffer (37°C) containing 0.005% N-lauroylsarcosine for 1 min. The last washing step was performed with acetonitrile for 30 s. Fluorescence signals of the hybridized Agilent Microarrays were detected using Agilent's Microarray Scanner System (Agilent Technologies). The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. For determination of differential gene expression FES derived output data files were analyzed further using the Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware, Seattle, WA, USA). Microarray raw data can be found under GEO-ID: GSE30349.

Flow Cytometric Analyses

Flow cytometric analyses were carried out on freshly isolated and long-term cultured murine T cells. The following anti-mouse monoclonal antibodies were used for staining: rat anti–mouse monoclonal antibodies (Invitrogen): phycoerythrin (PE)-conjugated CD8a (CT-CD8a), CD44 (IM7.8.1) and CD62L (MEL-14), (PE-Cy5.5)-conjugated CD4 (RM4–5) and CD19 (6D5), allophycocyanin conjugated CD25 (PC61 5.3), (PE-Cy5)-conjugated CD3 (145–2C11), (BD Biosciences PharMingen): (PE)-conjugated CD2 (RM2–5), CD11a (2D7) and Sca1 (E13–161.7), (PE-Cy5)-conjugated CD5 (53–7.3), allophycocyanin (APC)-conjugated CD117 (2B8), (eBioscience, San Diego, CA, USA): (PE-Cy5)-conjugated CD24 (M1/69); mouse anti–mouse monoclonal antibodies (BD Biosciences PharMingen): (PE)-conjugated CD45.1 (A20) and unconjugated pre–T-cell receptor α-chain (2F5), (ImmunoTools, Friesoythe, Germany): (PE)-conjugated CD90 (MRC OX-7), (eBioscience): allophycocyanin conjugated NK1.1 (PK136); hamster anti–mouse monoclonal antibodies (BD Biosciences PharMingen): allophycocyanin conjugated T-cell receptor β (TCRβ; H57–597), (PE)-conjugated CD27 (LG.3A10) and TCRγ/δ (GL3). Fluorescence-activated cell sorting (FACS)-based analysis of the expressed Vβ-TCR repertoire was performed with the Mouse Vβ TCR screening panel of BD Biosciences. Following secondary antibodies were used from Invitrogen: allophycocyanin conjugated goat anti–mouse IgG, (PE)-conjugated goat anti–hamster IgG and (PE-Cy5)-conjugated goat anti–rat IgG and IgM. To prevent nonspecific binding to Fc receptors, samples were incubated with mouse seroblock FcR (Serotec, Oxford, United Kingdom) or CD16/CD32 mAbs (2,4G2; BD Biosciences PharMingen). Analyses were performed on a FACScalibur using the CellquestPro software (both from BD Biosciences PharMingen). All cell counts were performed on a CASYCell Counter (Schärfe System, Reutlingen, Germany). Detection of T cells transduced with multiple colors (EGFP, Venus and Cerulean) was performed on a BD FACS Canto II and analyzed using BD FACSDiva 6.0 software.

PCR Analysis of the Expressed Vβ-TCR Repertoire

Analysis of the diversity in the T-cell antigen receptor (TCR) repertoire of polyclonal WT (C57BL6J.Ly5.2) T cells and the immortalized T-cell population was essentially performed as described (21). Shortly, after isolating total RNA (RNeasy Mini Kit, Qiagen) from 5×10^6 cells of the immortalized T-cell clone or from 5×10^6 C57BL/6 WT T cells (8 wk old donor animal) and subsequent RT-PCR reaction (SuperScript II Reverse Transcriptase, Invitrogen) amplification of expressed Vβ-TCR repertoire was performed using the following primer sets: murine C-gene-specific 3′ primer (constant region primer), MuTCB3C AAGCA CACGAGGGTAGCCT; panel of murine Vβ-gene-specific 5′ primers (variable TCR-β-chain region primers), MuBV1 CTGAATGCCCAGACAGCTCCAAGC, MuBV2 TCACTGATAC GGAGCTGAGGC, MuBV3.1 CCTTGCAGCCTAGAA ATTCAGT, MuBV4 GCCTCAAGTC GCTTCCAACCTC, MuBV5.1 CATTA TGATAAAATGGAG AGAGAT, MuBV5.2 AAGGTGGAGAGAGAC AAAGGATTC, MuBV5.3 AGAAA GGAAACCTGCCTGGTT, MuBV6 CTCTCACTGTGACATCTGCCC, MuBV7 TACAGGGTCTCACGGAAGAAGC, MuBV8.1N GGCTGATCC ATTACTCATA TGTC, MuBV8.2N TCATATGGTGCTGGC AGCACTG, MuBV8.3 TGCTGGCAAC CTTCGAATAGGA, MuBV9 TCTCT CTACA TTGGCTCTGCAGGC, MuBV10 ATCAAGTCTGTAGAGCCGGAGGA, MuBV11 GCACTCAACTCTGAAGATCC AGAGC, MuBV12 GATGGTGGG GCTTT CAAGGATC, MuBV13N AGGCCTAAAG GAACTAACTCCACT, MuBV14 ACGAC CAATTCATCCTAAGCAC, MuBV15 CCCATCAG TCATCCCAACTTATCC, MuBV16 CACTCTGAAAATCCAACCCAC, MuBV17N CTAAGTGTTCCTCGA ACTCAC, MuBV18 CAGCCGGCCA AA CCTAACATTCTC, MuBV19 CTGCT AAGAAACCATGTACCA, MuBV20 TCTGCAGCCTGGGAATCAGAA.

Western Blotting

For Western blot expression studies of IL15RA we prepared cell lysates from freshly isolated mature T cells, 4 d anti-CD3/CD28 stimulated T cells (from 8-wk-old WT C57BL/6 donors) and the immortalized T-cell clone (each 5×10^6 cells). As a primary antibody we used rat anti-IL15RA (AZ-12) in a 1:200 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Western blotting was performed according to the manufacturer's instructions; the primary antibody was detected with horseradish peroxidase–conjugated secondary antibody: goat anti–rat HRP in a 1:10000 dilution (Santa Cruz Biotechnology).

Annexin-V staining

Apoptosis induction of the immortalized T-cell population was verified by

staining with the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences) following the manufacturer's instructions. Immortalized cells were cultured in the presence of different interleukin-2 concentrations (400 U/mL, 100 U/mL, 50 U/mL, 0 U/mL) for 48 h and used in the apoptosis assay. Analysis was performed on a FACScalibur (BD Biosciences).

All supplementary materials are available online at www.molmed.org.

RESULTS

Immortalization of Primary Murine T Cells after Retroviral Transduction with the T-Cell Protooncogene *LMO2*

To investigate the susceptibility of mature T cells to transformation *in vitro*, we utilized gammaretroviral gene transfer of the potent T-cell protooncogene *LMO2*. The gammaretroviral vector MP91-LMO2 coexpresses EGFP as a marker gene via an IRES element. A vector encoding EGFP only (MP91-EGFP) served as a control (Figure 1A).

T cells were isolated from lymph nodes and spleens of adult C57BL/6J.Ly5.2 mice and transduced following 4 d of anti-CD3/28 prestimulation. High multiplicities of infection (MOI) between 50 and 300 were used to achieve high transduction efficacies with multiple integrations. Per vector, we initiated 53 independent cultures (a total of 106) with cell counts in the range of 3×10^4 to 1×10^6, each. Fifty to 60% of the cells were gene modified in the cultures as determined by EGFP expression and the majority of the cells were of a CD8+ T-cell phenotype. One culture per vector is shown representatively in Figure 1B. The cells then were passaged for at least 12 wks, with supplementation of 100 U/mL interleukin-2 (IL-2) to assess the potential outgrowth of immortalized cell clones.

In a pilot experiment, clonal dynamics were analyzed by LM-PCR of 7 MP91-EGFP-transduced T-cell cultures 4, 11 and 84 d after transduction. As early as 11 d after transduction, certain clones began to dominate (one culture is shown representatively in Supplementary Figure 1). However, these T-cell cultures did not survive beyond wk 12 and no obviously growth-promoting genes flanked the retroviral integration sites, even in a 400 kb window of analysis (data not shown).

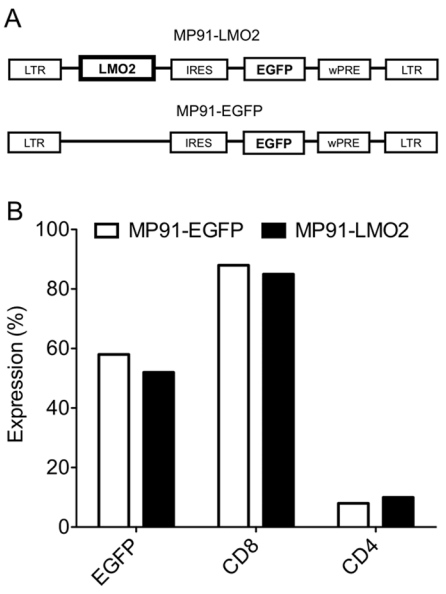

Figure 1. Retroviral vector design and phenotype of transduced primary T lymphocyte cultures. (A) A gammaretroviral vector coexpressing LMO2 and EGFP (MP91-LMO2) as well as a control vector expressing EGFP only (MP91-EGFP) were used in this study. IRES, internal ribosome entry site; wPRE, Woodchuck hepatitis virus posttranscriptional regulatory element. (B) Transduction efficacies (EGFP expression) as well as CD4 and CD8 expression levels were determined by flow cytometry 2 d after transduction and are shown for the vectors MP91-EGFP and MP91-LMO2.

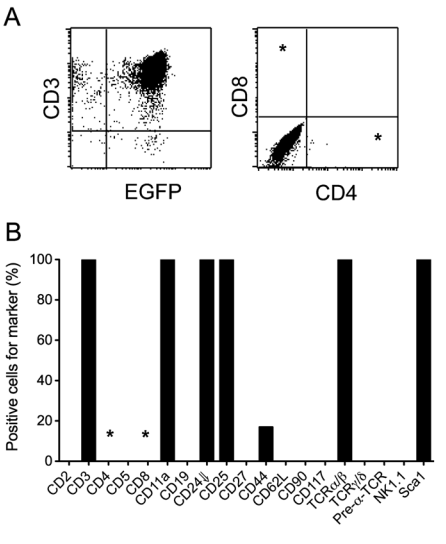

Figure 2. Marker profile. FACS analysis of the immortalized MP91-LMO2-transduced, mature T-cell population was performed after 6 wks of culture. (A) Over 90% of the cells expressed EGFP and the T-cell marker CD3. A loss in coreceptor (CD4 and CD8) expression, however, was observed (*). (B) The following mature T-cell markers were expressed: CD3, CD24 (low), TCR/β, Sca1 and CD25. No expression of the immature marker pre-TCR, however, was detected.

Only one *LMO2*-transduced T-cell culture of the 106 cultures initiated (frequency < 1%) had the ability to grow continuously for more than 12 wks. This culture was passaged for nearly 1 year, was > 98% EGFP positive with a CD4/CD8 double-negative phenotype (Figure 2A). Other mature T-cell markers, such as the T-cell receptor α/β chains and CD3, were expressed (Figure 2B). Particularly, high expression levels were seen for the T-cell-specific activation markers CD25 and Sca1. The low expression of the heat stable antigen (CD24) also was supportive of a mature T-cell phenotype. The leukocyte antigen CD11a was expressed on all cells. Markers for the following lineages were negative: B cells (CD19), natural killer cells (NK1.1), other T-cell types (TCRγ/δ), stem cells (CD90, CD117), some T-cell markers (CD2, CD5) and immature T-cell antigens (pre-α-TCR). As expected, CD27 was downregulated after prolonged activation *in vitro* (22). Taking the expression of CD44 and the lack of the homing marker L-selectin (CD62L) as well as the high CD25 expression into consideration, this profile resembles a memory T-cell phenotype, despite the absence of CD4 and CD8.

Immortalized T cells were cocultured on OP9–DL1 cells, which are known to support *in vitro* T-cell differentiation (23), with the aim to regain CD4 or CD8 coreceptor expression (data not shown). But, even after repeated attempts, the cells remained CD4 and $CD8^-$. This observation

supports the conclusion that the immortalized cells most closely resemble memory T cells and not double-negative thymocytes.

Growth Characteristics and Clonality of the *in vitro* Immortalized T-Cell Population

The immortalized T-cell population remained IL-2 dependent. Decreasing amounts of IL-2 in the culture resulted in massive apoptosis, which was detected by Annexin V staining (Figure 3A). To further address the growth properties of the immortalized cells, a competition experiment with nonmanipulated, primary T lymphocytes was initiated. Nontransduced primary T cells were stimulated for 4 d *in vitro* and mixed with the immortalized cells. In four independent experiments, the initial culture (d 0) was established with 10% cells from the immortalized T-cell culture and 90% freshly stimulated, nonmanipulated T cells. Cells were cultured in the presence of 100 U/mL IL-2 and on d 2, 6 and 8 EGFP expression was determined as an indicator for the proportion of immortalized cells and their competitiveness. The immortalized T-cell population clearly overgrew the competitor cells rapidly and dominated the culture on d 8 (Figure 3B).

We next assessed whether the immortalized T cells were capable of inducing T-cell leukemia/lymphoma in an immunodeficient mouse model. However, the immortalized T cells were not capable of giving rise to leukemia/lymphoma after transplantation into Rag-1 deficient syngeneic mice.

The clonality of the immortalized population was analyzed by PCR for the expressed T-cell receptor (TCR) Vβ-chain. To analyze the Vβ-chain expression via FACS, we used freshly isolated WT T cells as a positive control (data not shown). Compared to WT T cells, we could only detect a signal for the variable β-chain 13 (Vβ13) in the immortalized population (Figures 4A, B), which was indicative of a monoclonal T-cell population. Furthermore, only a limited number of integrations were detected by LM-PCR (Figure 5A). Among the integrations mapped for this immortalized T-cell population, one turned out to be very interesting. The integration was mapped closely to the gene cluster of the IL-2 receptor α *(IL2RA)* and IL-15 receptor α *(IL15RA)* genes (Figure 5B). The vector insertion was in reverse orientation in sufficiently close proximity to potentially transactivate these genes (23kb to *IL2RA* and 86kb to *IL15RA*, respectively). Especially, a subsequent overexpression of *IL2RA* appears to be a critical event, as it is enlisted in the retroviral tagged cancer gene database (RTCGD), see Table 1. A detailed list of all mapped genes flanking the retroviral integration sites in a 400 kb window, in the immortalized T-cell population is enclosed in Table 1.

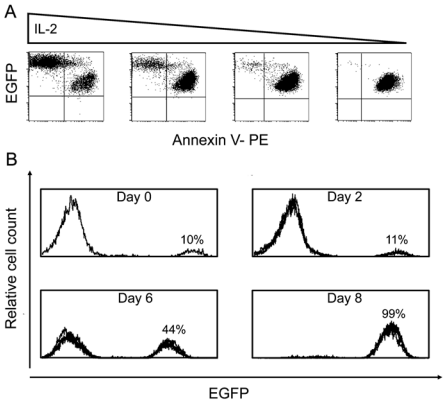

Figure 3. IL-2 dependence of the immortalized T-cell population and growth advantage of the immortalized T-cell clone over freshly stimulated T cells. (A) Immortalized T cells were cultured in the presence of different IL-2 concentrations (from left: 400 U/mL, 100 U/mL, 50 U/mL and 0 U/mL). After 2 d of culture, cells were stained for the apoptosis marker Annexin V. With declining IL-2 concentrations, the percentage of Annexin V expressing cells increased. (B) Competition experiment to analyze the growth behavior of the immortalized cell population. Primary, freshly stimulated T cells were chosen as a competitor population and set to 90% in the mixed culture, the remaining 10% were immortalized, EGFP-expressing cells (d 0). In total four independent experiments were performed, which are shown in overlay of the individual histogram blots on the day of measurement (d 2, 6 and 8). The immortalized T cells eventually overgrew the competitor cells on d 8 of culture.

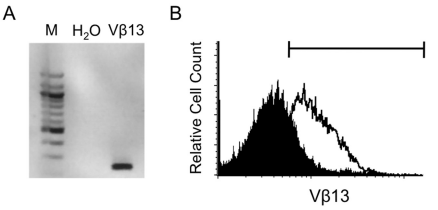

Figure 4. The immortalized T-cell population is monoclonal for the T-cell receptor (Vβ13). (A) Compared to a WT population, where all TCR Vβ variants were found (data not shown), only Vβ13 could be PCR amplified in immortalized cells from cDNA; M, Marker; H_2O, negative control. (B) This result was further verified by FACS-mediated detection of Vβ13 on the majority of cells, while all other Vβ antibodies were nonreactive.

The cells were cloned by limited dilution and integration-site-specific PCR was performed on 14 established clones. In all analyzed clones, we could reproducibly detect the investigated integrations on chromosome 2 (near *IL2RA/IL15RA*) and chromosome 5 (near *Hod1*) (Supplementary Figure S2). Moreover, we solely identified Vβ13–rearrangement in the obtained subclones (data not shown). Subsequently, based on these results, we declared the population as clonal. Further analyses were performed on clone 8.

Overexpression of *IL2RA* and *IL15RA* Promoted Immortalization

In a microarray ratio experiment, we compared the expression profile of the immortalized T-cell clone to that of mature and immature T lymphocytes. But, the pattern of up- or downregulated genes of the immortalized T-cell clone differed significantly from both cell types (Figure 6). The overexpression of the *IL2RA* (CD25) detected via FACS analysis (see Figure 2B) was verified by the microarray expression data. The immortalized T cells expressed 50- and/or 100-fold higher levels of the *IL2RA mRNA*, respectively, than the nonstimulated, mature T cells and/or the thymo-

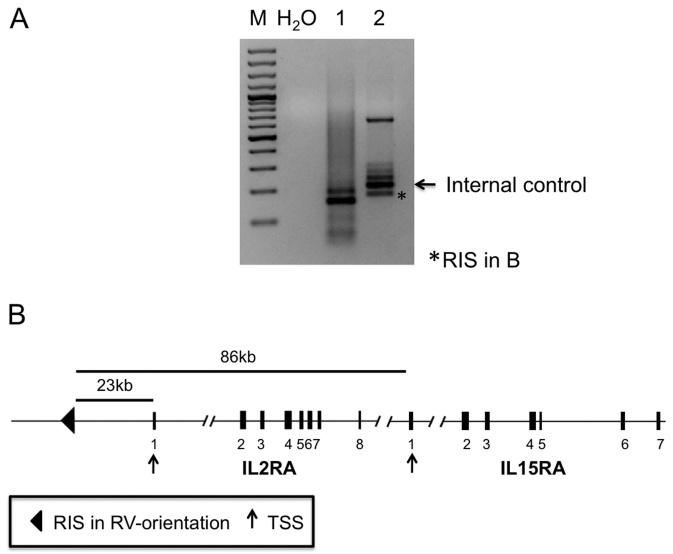

Figure 5. Retroviral integration site analysis of the immortalized T-cell population. (A) Compared to a freshly stimulated and transduced T-cell population (1) the immortalized cells (2) showed an oligo- to monoclonal integration pattern. One of the integrations of the immortalized population was mapped near the genes of *IL2RA* and *IL15RA* (*). Arrow marks the internal control band. M, Marker. (B) Scheme of retroviral integration site (RIS) mapped upstream of the *IL2RA* and the long isoform of the *IL15RA* loci. Black blocks indicate *IL2RA* and *IL15RA* exons. The arrowhead represents RIS in reverse (RV) vector orientation. Black arrows show transcription start sites (TSS).

cytes. Furthermore, *LMO2* and *IL15RA* showed a 4- and 17-fold increase in expression, respectively, in the immortalized clone. In addition, *IL15RA* overexpression was analyzed on protein level by Western blotting. Compared to freshly isolated, nonstimulated T cells, the immortalized T cells showed an increased expression of *IL15RA*, while stimulated WT T cells demonstrated the highest expression level (Supplementary Figure 3).

To directly assess the role of *IL2RA* and *IL15RA*, we finally expressed the two receptor chains ectopically. For this purpose, we cloned the murine cDNAs of the two chains each into the MP91-EGFP vector resulting in the vectors MP91-IL2RA and MP91-IL15RA. We replaced EGFP by fluorescent marker Venus in the *IL2RA*-encoding vector and by Cerulean in the *IL15RA*-encoding vector (Figure 7A). We transduced primary murine T cells with either *LMO2*, *IL2RA* or *IL15RA* alone or with all possible vector combinations: *LMO2* + *IL2RA*, *LMO2* + *IL15RA*, *IL2RA* + *IL15RA* and *LMO2* + *IL2RA* + *IL15RA*. As control populations, we initiated control vector MP91-EGFP-transduced and nonmodified T lymphocyte cultures.

To address the influence of the investigated transgenes exclusively, we minimized gammaretroviral insertional site effects by working with low copy numbers of the transferred vectors (0.2%–6% transduction efficacy). To ectopically mimic the previously observed insertional transactivation, we started with an absolute number of 4,700 triple-transduced (*LMO2* + *IL2RA* + *IL15RA*) T lymphocytes.

The cells were passaged under standard culture conditions. Over time the levels of gene marking increased from 6% to 100% in *IL2RA* cultures and from 3% to 100% in *LMO2* + *IL2RA* cultures, respectively (Figure 7B). Nevertheless, only T cells transduced with *IL2RA* in combination with *LMO2* demonstrated sustained growth after a critical *in vitro* cultivation time of 8 wks (Figure 7C). However, a full immortalization was not achieved, as we were not able to cultivate these cells as long as the immortalized T-cell clone (not longer than 12 wks). Even triple overexpression of *LMO2*, *IL2RA* and *IL15RA* in mature T lymphocytes did not induce any growth/proliferation-enhancing effect. This observation indicates that further survival-promoting events besides insertional mutagenesis may have contributed to immortalization of the original clone.

Table 1. Genes flanking the retroviral integration site (RIS) in the immortalized T-cell population.

Genes flanking the RIS	Gene ID	Chromosome	Distance to TSS[a]	Orientation of RIS[b]	RTCGD[c]
Spink2	69982	5	+117 kb	F	no
Hod1	74318	5	+8 kb	F	no
1700023E05Rik	71868	5	+78 kb	R	no
Arl9	384185	5	+91 kb	R	no
Srp72	66661	5	+120 kb	F	no
Cbara1	216001	10	−114 kb	R	no
D130073L02Rik	215999	10	−131 kb	F	no
Oit3	18302	10	−147 kb	F	no
Pla2g12b	69836	10	+185 kb	R	no
Rbm17	76938	2	+16 kb	R	no
Il2RA	16184	2	−23 kb	R	4
Il15RA	16169	2	−86 kb	R	no

[a]TSS, transcription start site.
[b]Relative orientation of integrated vector (F = Forward; R = Reverse).
[c]Hits in RTCGD.

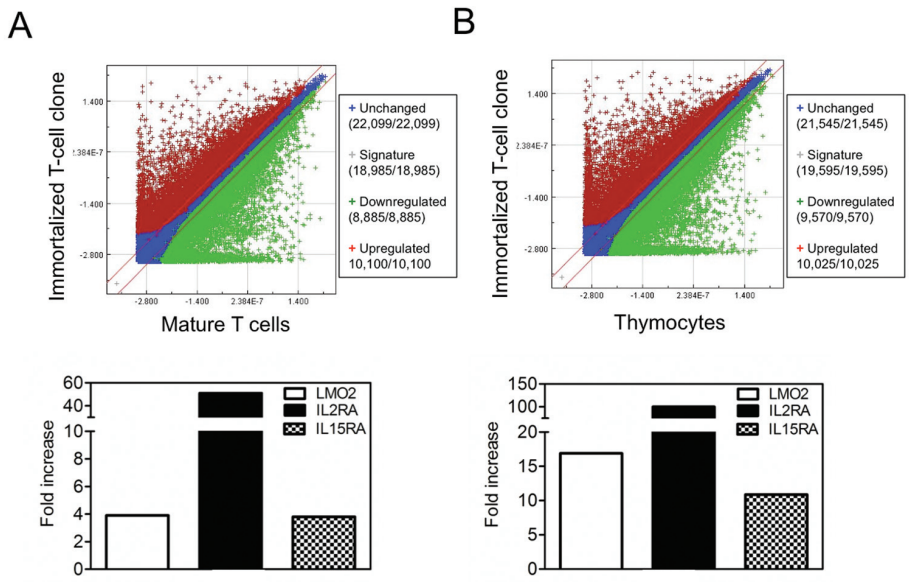

Figure 6. Expression profile of immortalized T cells compared with fresh mature and immature T cells. RNA was purified from freshly isolated T lymphocytes from spleen and lymph nodes and thymocytes from 8-wk-old WT donors and the immortalized T-cell clone. Miltenyi Biotech performed Microarray ratio experiments commercially. Immortalized T cells were compared with freshly isolated mature (A) and immature T cells (B). The signal intensities of each feature represented by a spot are shown in a double logarithmic scale as a scatter blot. *x* Axis: mature T cells (A) or thymocytes (B) log signal intensity; *y* axis: immortalized T-cell clone (A,B) log signal intensity. Red diagonal lines define the areas of 2-fold differential signal intensities. Blue crosses: unchanged genes. Red crosses: significantly upregulated genes (*P* value < 0.01). Green crosses: significantly downregulated genes (*P* value < 0.01). Grey cross in legend: summary of significantly up- and downregulated signatures. No similarities to one or the other cell type were observed for the investigated immortalized cells. As expected, the transduced *LMO2* gene and the retroviral integration site (RIS) flanking genes *IL2RA* and *IL15RA* showed increased expression in the immortalized cells relative to fresh mature and immature WT T cells.

Alternatively, one might suggest that the levels of ectopic expression achieved by retroviral gene transfer differ significantly from those resulting from the transactivation event.

DISCUSSION

In the present study, we initiated a total of 106 primary murine T-cell cultures, half of them following retroviral transduction of the *LMO2* T-cell protooncogene. In one culture (approximately 1%), we observed a fully immortalized T-cell clone with unlimited growth potential. The immortalized cell clone was CD4 and CD8 double negative but expressed a rearranged, monoclonal T-cell receptor β-chain. The immortalized cells were not able to initiate leukemia/lymphoma after transplantation into syngeneic *Rag-1* deficient recipient mice. According to integration analysis and expression profiles, we found the immortalization to be caused by insertional transactivation of *IL2RA* and *IL15RA* in combination with the ectopic expression of *LMO2*. Here we report about the rare event of insertional mutagenesis-mediated T-cell immortalization.

The immortalized clone did not express the T-cell markers CD4 and/or CD8. Loss of T-cell identity due to oncogene expression is a phenomenon which also is sporadically observed in mature T-cell leukemia/lymphoma (24). Furthermore, dedifferentiation of malignant lymphocytes recently was described for mature B lymphocytes during the development of Hodgkin's lymphoma (25). Moreover, induced pluripotent stem cells (IPSCs) are generated by overexpression of self-renewal promoting genes in fully differentiated cell types (26,27). Thus, dedifferentiation and immortalization are often associated. Interestingly, the *IL2RA* is highly expressed in immature, developing lymphocytes. Therefore, the overexpression of the *IL2RA* in our clone is in line with a dedifferentiated T-cell phenotype and could even have contributed to the loss of mature T-cell markers.

Clearly, after ectopic expression, we observed a growth-enhancing effect in *IL2RA* and *LMO2* double-transduced T cells. This result is consistent with speculations that signaling of the IL-2 receptor and the protooncogene *LMO2* can act synergistically in malignization of T lymphocytes, as assumed for the adverse site effects observed in patients of the SCID-X1 gene therapy trial (28).

However, we were not able to induce an identically immortalized phenotype by the ectopic expression of *IL2RA* alone or in combination with *LMO2* and *IL15RA*. Therefore, we assume that overexpression of these genes is not sufficient to immortalize T lymphocytes. It might well be speculated that, in addition to overexpression of *LMO2* and *IL2RA*/*IL15RA*, further mutations acquired in the immortalized T-cell clone were involved in the full manifestation of the observed phenomenon.

The low immortalization rate found in this study confirms our previous finding that mature T cells are highly resistant to gammaretroviral insertional mutagenesis. This is unexpected as T lymphocytes present unique characteristics associated with susceptibility to malignant transformation (16,29). They have an impressive replicative capacity allowing them to respond to antigen challenges with dramatic proliferation bursts and they are capable of long-term self-renewal.

It is commonly accepted that complete malignant transformation requires multiple genetic lesions which deregulate cell differentiation and apoptosis and stimulate proliferation (30,31). *LMO2* overexpression alone, for instance, is not sufficient for the development of leukemia

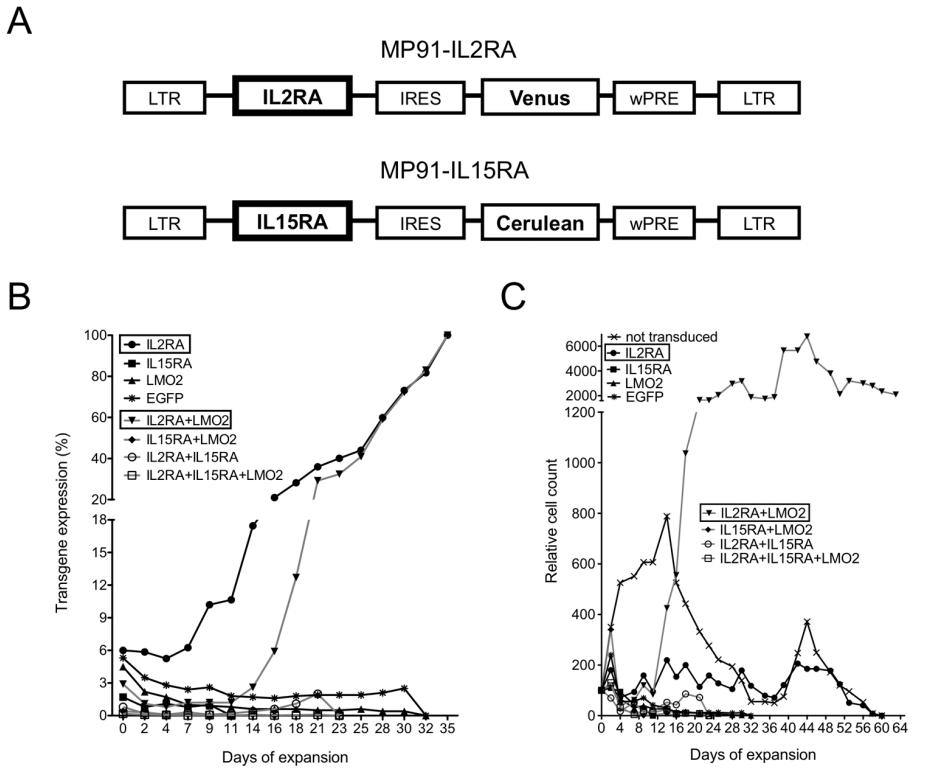

Figure 7. Ectopic expression of *IL2RA* and *IL15RA*. (A) The murine cDNAs for *IL2RA* and *IL15RA* were cloned into MP91-EGFP upstream of the IRES. IRES, internal ribosomal entry site; wPRE, Woodchuck hepatitis virus posttranscriptional regulatory element. To detect cells transduced with different vectors fluorescent marker Venus substituted for EGFP in MP91-IL2RA and Cerulean in MP91-IL15RA (B) Primary mature T cells were transduced either with *LMO2*, *IL2RA* or *IL15RA* alone or in combination: *LMO2 + IL2RA*, *LMO2 + IL15RA*, *IL2RA + IL15RA and LMO2 + IL2RA + IL15RA*. As control populations, control vector MP91-EGFP-transduced and nonmodified T lymphocyte cultures were initiated. During follow-up culture, marker gene expression served as the level of gene modification. Gene marking increased solely in *IL2RA*- (6% to 100%) and *LMO2 + IL2RA*-cultures (3% to 100%). (C) Gene modified T cells were kept under standard cell culture conditions for several weeks and cell counts determined regularly. MP91-EGFP and nonmodified T lymphocytes served as control populations. Only the combination of *IL2RA* with *LMO2* supported long-term growth of mature T lymphocytes. Relative cell counts over time are depicted.

(32). However, MLV-based vectors, such as the ones used in this study, are highly genotoxic and could add additional genetic lesions that allow full immortalization or transformation. As they integrate preferentially near accessible, open and transcribed promoters, and since T cells need some level of activation and must be cycling to be transduced with a gammaretroviral vector, integration near proliferation-supporting or antiapoptotic genes is obviously favored by MLV-based vectors (14). Transactivation of such genes could indeed act as cooperating transforming events in gammaretroviral gene transfer into mature T lymphocytes. Recently, it was reported that derepression of an endogenous LTR can activate a protooncogene in human lymphoma (33).

In accord with this prediction, the immortalized T-cell clone described in this study showed an insertional activation of the *IL2RA* and *IL15RA* genes. Both genes play a crucial role in T-cell proliferation and survival. *IL2RA* expression occurs in early T and B lymphocytes and is induced during activation of mature lymphocytes, including regulatory T cells. It acts as a cell cycle progression factor and promotes functional differentiation of T and B cells (34). In homeostasis, IL-2 levels and *IL2RA* expression control the balance between clonal expansion and cell death following immune activation (35). The immortalized T-cell clone still showed a dramatic IL-2-dependency (see Figure 3A). However, even in the presence of high IL-2 concentrations, a high percentage of apoptotic cells were always present in the culture. An explanation for this observation could be that *IL2RA* signaling not only provides a proliferation signal, but, depending on additional signals, also can drive T cells into apoptosis. However, the immortalized T-cell population was capable of exploiting the IL-2 resources very efficiently and thereby out competed freshly stimulated, cocultured T cells (see Figure 3B).

Along with the *IL2RA* upregulation, we observed a transactivation of the *IL15RA* gene. The α-chain of the IL-15 receptor shares the β- and γ-chains with the IL-2 receptor and plays a crucial role in T-cell homeostasis, expansion and survival (36). IL-15 transgenic animals develop fatal lymphocytic leukemia of a T-NK phenotype (37). The overexpression of the IL-15 receptor or a constitutive activation of the IL-15R signaling pathway can present important growth and survival factors in the leukemogenesis of T cells.

Interestingly, in a previous study (16), we observed a very similar retroviral integration site (Table S6A, clone GFPA8_161-PCR). The insertion site was located in even closer proximity to the *IL2RA*– and *IL15RA*–gene locus (distance of 1.8 kb). It was detected in *EGFP*-transduced, mature T cells, after long-term persistence of the cells *in vivo* (481 d after transplantation). Even after a long latency, the isolated T cells carrying this integration site were polyclonal and showed no signs of malignancy. This observation further supports our finding that at least ectopic coexpression of *LMO2* was required for full immortalization. Furthermore, additional mechanisms may control the immortalization and/or

transformation of mature T cells *in vivo* which are not active *in vitro* (for example, clonal competition).

The conclusion that the specific retroviral integration indeed contributed to the immortalization of the T-cell clone in this study is supported by the known growth-promoting activity of *IL2RA* and *IL15RA*, the fact that both genes were indeed persistently upregulated and the observation that the ectopic expression of *LMO2* in combination with *IL2RA* supported long-term growth of T cells *in vitro*. Others have reported previously such an immortalization of T lymphocytes. Retroviral gene transfer of *IL-15* was found to induce a cytokine-independent growth of primary human cytotoxic T cells (38) and the overexpression of the antiapoptotic gene *Bcl-2* enhanced the persistence of tumor-specific T cells *in vivo* (39). This report, however, shows that retroviral insertional mutagenesis can contribute to the immortalization of mature T cells. On the other hand, we found this to be an extremely rare event, full transformation with *in vivo* leukemogenicity was not observed and additional ectopic expression of a T-cell protooncogene was required for full immortalization. Moreover, this result could not be reproduced with human T cells, although we performed even more extensive and repeated (a total of several hundred cultures with a total of around 10^9 T cells) experiments with human peripheral blood mononuclear cells (PBMCs). Therefore, this study clearly shows that gammaretroviral insertional mutagenesis can alter the phenotype of mature T lymphocytes, however, the chance of transformation is extremely low. This reflects the absence of any genotoxic events in past clinical trials involving the genetic modification of mature T lymphocytes. Although, we can conclude that therapeutic retroviral gene transfer into mature T lymphocytes is not associated with a significant risk of insertional mutagenesis, our study clearly demonstrates, that under certain conditions, insertional genotoxicity can lead to survival-enhancing effects or even immortalization in mature T cells. Therefore, the presented *in vitro* system might be considered as a test procedure for therapeutic transgenes of T-cell based clinical gene therapy trials.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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