Supporting Information

Schlecht-Louf et al. 10.1073/pnas.0913122107

SI Materials and Methods

Plasmids. p57-IRES-*gfp* plasmids were derived from the F-MLV containing p57 plasmid (as in the text) by introducing an IRES-*gfp* sequence 3′ to the F-MLV *env* gene. Both the WT and immunosuppression-negative DM versions were constructed by first introducing SalI and MluI restriction sites 3′ to the *env* gene (via a three-fragment ligation between WT or DM p57 opened by XhoI and BsmI and two PCR products generated with primer pairs S1–S2 and S3–S4; Table S1). These modified p57 plasmids were opened by SalI-MluI, Klenow-treated, and ligated with a blunt-ended PCR fragment amplified from p-MIGR1 (1) using primer pair S5–S6 to introduce the IRES-*gfp* unit. These plasmids were then used as in Fig. 1 to generate the WT and DM GFP-marked F-MLV virions. The pET28-based and pDFG-based expression vectors for the WT and DM ectodomain of syncytin-1 have been described (2).

To generate pDGF expressing WT and DM F-MLV Env ectodomains, PCR amplification was performed using primer pair S7 and S8 and WT and DM phCMV-envF-MLV plasmids as templates. SfiI/MluI-digested PCR fragments were then introduced into the pDFG vector opened with the same enzymes.

The XMRV Env ectodomain encoding the DNA sequence was generated by ligation of three sets of paired 70–75 oligomers with 4-nt cohesive ends for each oligomer, including SfiI and MluI restriction sites for the external fragments. The ligation product was introduced into the SfiI/MluI-opened pDFG vector. This plasmid was used as a template for PCR amplification of the XMRV Env ectodomain with primer pair S11–S12 to introduce NcoI and XhoI restriction sites, and the PCR fragment was introduced into the NcoI/XhoI-digested pET28 plasmid. Mutations were then introduced into this plasmid using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) to generate the DM pET28 counterpart using primer pair S13–S14. The DM XMRV Env ectodomain was introduced back into pDFG after PCR amplification with primer pair S9–S10.

pCMV-HTLVenv was a gift from C. Pique (Institut Cochin, Paris, France). The DM pCMV-HTLVenv mutant was generated by triple ligation of PCR fragments generated with primer pairs S15–S16 and S17–S18 digested with KpnI and NsiI, respectively, and the opened KpnI-NsiI-restricted pCMV-HTLVenv. The WT and DM HTLV Env ectodomains were then PCR-amplified with primer pair S19–S20, digested with SfiI-MluI and introduced into pDFG opened with the same enzymes. pMal bacterial expression vectors for fusion proteins with *E. coli* maltose-binding protein (MBP) were constructed by three-fragment ligation of pMal-c2x opened with BglII and HindIII, a BglII and PstI cut PCR fragment generated with primer pair S21 and 22 using pMal-c2x as a template and a PstI and HindIII cut PCR fragment generated with primer pair S23–24 using pCMV-HTLVenv as a template. These vectors encode an 84-residue long HTLV ectodomain fused to the C terminus of MBP through a trialanine linker identical to the fusion protein that has been crystallized (3). The empty pMal-c2x vector encodes the 85-residue long α-subunit of *E. coli* β-galactosidase fused to the C terminus of MBP and was used as a control protein. MBP fusion proteins were produced as indicated in *Materials and Methods* and were purified on cross-linked amylose resin (New England Biolabs) with PBS as a binding and washing buffer and 20 mM Tris-Cl, 5 mM maltose, pH 7.5, as an elution buffer. A second purification step on a MonoQ 5/50 GL column (Amersham) in 20 mM Tris-Cl, pH 7.5, with a 0–1-M NaCl linear gradient was performed to separate the fusion proteins from endogenous *E. coli* MBP. The fusion proteins were ultimately purified on a Superdex 200 HR 10/30 column (Amersham) to isolate the trimeric form.

Detection of Neutralizing Antibodies. Neutralizing antibodies were tested by their ability to inhibit infection of NIH/3T3 target cells by F-MLV, as described for another retrovirus by Langhammer et al. (4). Fifty microliters of F-MLV (2×10^6 copies/mL) was incubated with serial dilutions of heat-inactivated serum for 45 min at 37 °C and then transferred to NIH/3T3 cells seeded at 5,000 cells per well into 96-well microplates. Three days postinfection, DNA was extracted (3 freeze-thaw cycles and proteinase K digestion) and F-MLV-integrated proviruses were quantified by real-time PCR with the 18S rRNA gene as a reference control (using the Applied BioSystems primer set).

1. Pear WS, et al. (1998) Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood* 92:3780–3792.
2. Mangeney M, et al. (2007) Placental syncytins: Genetic disjunction between the fusogenic and immunosuppressive activity of retroviral envelope proteins. *Proc Natl Acad Sci USA* 104:20534–20539.
3. Center RJ, et al. (1998) Crystallization of a trimeric human T cell leukemia virus type 1 gp21 ectodomain fragment as a chimera with maltose-binding protein. *Protein Sci* 7: 1612–1619.
4. Langhammer S, Hübner J, Kurth R, Denner J (2006) Antibodies neutralizing feline leukaemia virus (FeLV) in cats immunized with the transmembrane envelope protein p15E. *Immunology* 117:229–237.

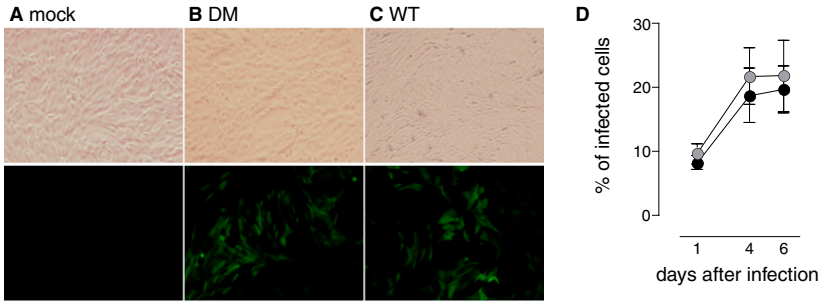

Fig. S1. Ex vivo viremia of WT and DM GFP-marked F-MLV. GFP-marked F-MLV virions were generated by transient transfection of 293T cells with p57-IRES-*gfp* plasmids (*SI Materials and Methods*), and the filtered supernatants were used to infect NIH/3T3 cells under conditions similar to those in Fig. 1. (*A*–*C*) Infected GFP-expressing cells were visualized by fluorescence (*Lower*) or phase-contrast (*Upper*) microscopy 4 days postinfection with no virus (mock transfected 293T-cell supernatant) (*A*), DM (*B*), and WT (*C*) GFP-marked F-MLV. (*D*) Compared in vitro propagation kinetics of WT (black circles) and DM (gray circles) GFP-marked F-MLV. The percentages of GFP^+ infected NIH/3T3 cells were determined by flow cytometry using a FACScalibur flow cytometer (BD Biosciences) at days 1, 4, and 6 postinfection (mean ± SD of three independent experiments).

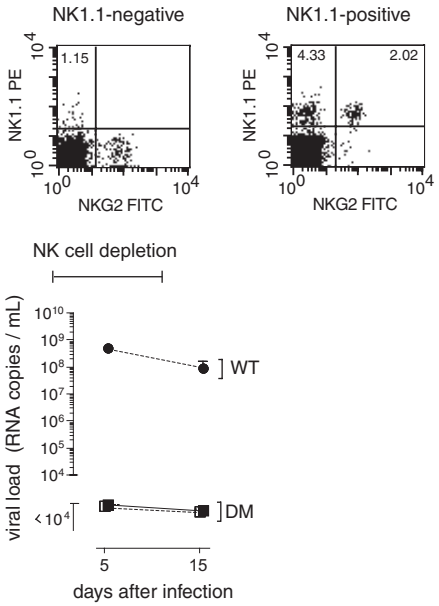

Fig. S2. Control injection of anti-NK1.1 antibodies into $NK1.1^-$ Swiss mice does not allow mutant F-MLV to propagate. (*Upper*) Phenotyping of $NK1.1^+$ and $NK1.1^-$ Swiss mice as visualized by flow cytometry on blood samples using anti-NK1.1 and anti-NKG2A,C,E antibodies. (*Lower*) Serum viral loads of mice after injection of WT (●) or immunosuppression-negative DM (□ and ■) F-MLV as measured in untreated Swiss mice (● and ■) or in $NK.1.1^-$ Swiss mice injected with the anti-NK1.1 antibody (□; *Materials and Methods*). Each symbol corresponds to the mean ± SD of more than three mice. PE, phycoerythrin.

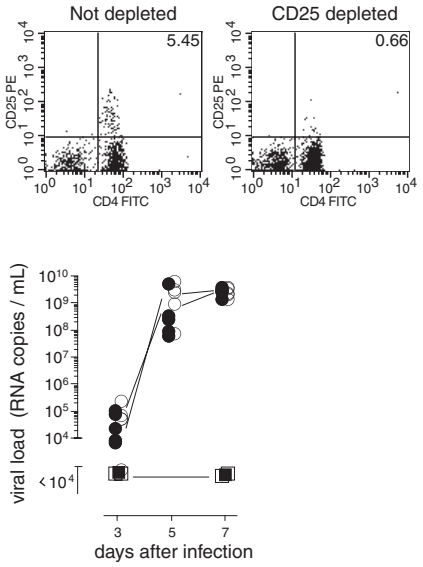

Fig. S3. Natural Treg cells do not affect F-MLV viremia. Swiss mice were injected with 100 μg of anti-CD25 IgG on day −1 (*Materials and Methods*) and were infected at day 0 with 4×10^7 copies of WT or DM F-MLV. (*Upper*) Cell depletion was checked by flow cytometry on blood samples 2 days postinfection. Numbers in the dot plot corners indicate the percentage of labeled cells in the corresponding quadrant. (*Lower*) Serum viral loads after injection of WT (○ and ●) or DM (□ and ■) F-MLV was detected in untreated (● and ■) or CD25-depleted (○ and □) Swiss mice. Each circle corresponds to one individual mouse, with the lines connecting the geometric means, and results are representative of three independent experiments. PE, phycoerythrin.

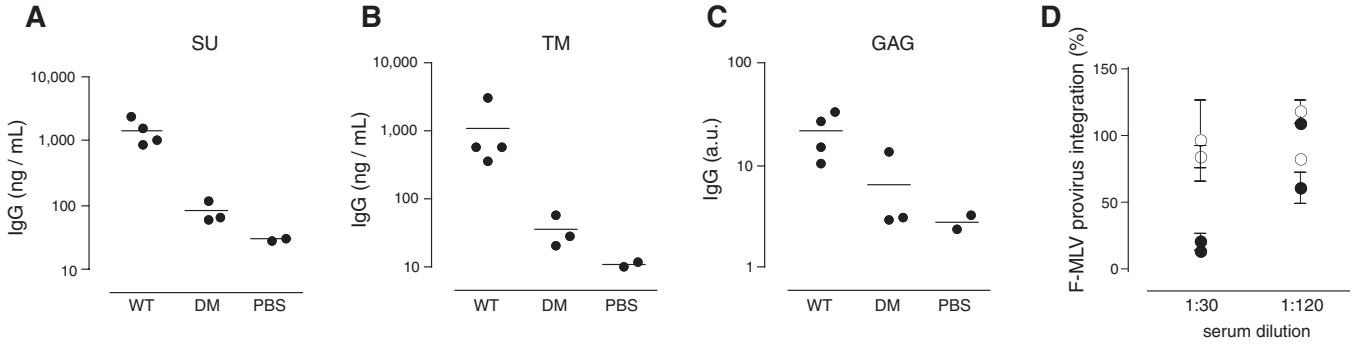

Fig. S4. Analysis of antiviral antibody responses to WT and DM F-MLV [and control (PBS)]. Swiss mice were i.v. inoculated as in Fig. 2 with 2×10^7 copies of WT or DM F-MLV, and serum was harvested 8 weeks postinfection. (*A–C*) Serially diluted serum was used to detect surface (SU)- (*A*), TM- (*B*), and Gag- (*C*) specific IgG by ELISA. Each symbol represents one individual mouse with the bars corresponding to the means, and results are representative of three independent experiments. (*D*) Assay for F-MLV neutralizing antibodies in the collected sera. Neutralizing antibodies were tested for their ability to inhibit infection of NIH/3T3 target cells by F-MLV. Results for serum of WT (●) or DM (○) F-MLV-infected mice are expressed as percentage of integrated proviral copies relative to controls with serum from mock-infected (with PBS) mice. Each symbol corresponds to the mean of triplicates ± SD for each individual mouse.

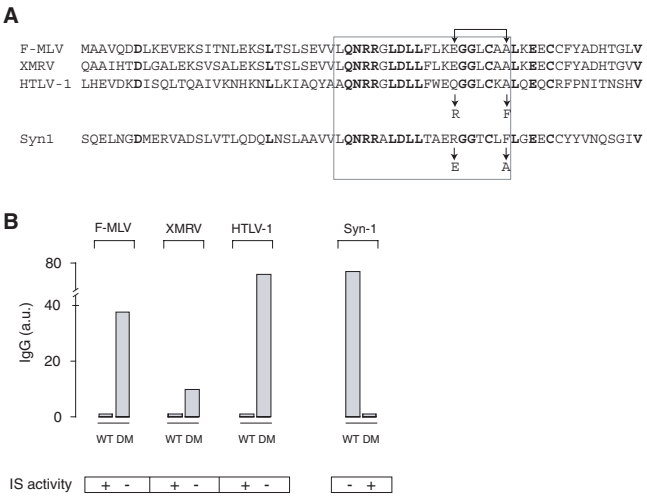

Fig. S5. Functional immunosuppressive domains in human retrovirus Envs. (*A*) Alignment of the F-MLV, XMRV, and HTLV-1 retrovirus and of the syncytin-1 (Syn-1) ectodomains and identification of the two residues controlling their IS activity. (*B*) (*Upper*) Differential antibody responses induced by WT and DM recombinant ectodomains. Recombinant proteins corresponding to the 64 amino acids (*A*) of the WT or DM TM ectodomain for F-MLV, XMRV, and Syn-1 and to the 84 amino acids of the WT or DM TM ectodomain fused with the MBP protein for HTLV-1 were i.v. injected three times with a 1-week interval into Swiss mice (50 μg per injection). One week after the last injection, mice were blood-sampled and IgG levels were determined by ELISA using plates coated with the appropriate WT ectodomain protein for F-MLV, XMRV, and Syn-1 or with MBP-LacZ for HTLV-1. IgG levels are expressed relative to that of the WT ectodomain set as unity for retroviruses (and to that of the DM ectodomain for syncytin-1). Results are representative of 2-5 independent experiments. Similar results were observed when ELISA plates were coated with the DM ectodomain proteins from F-MLV, XMRV, and Syn-1. a.u, arbitrary units. (*Lower*) IS activity (+ or –) of the corresponding ectodomains as measured by the MCA205 tumor rejection assay in Fig. 1C. MCA205 tumor cells were transduced with pDFG expression vectors encoding either the WT or DM version of secreted F-MLV, XMRV, HTLV-1, and Syn-1 ectodomains. The tumor rejection assay was performed as described in *Materials and Methods*.

Table S1. Primer list

	5′ – 3′ Sequence
1	ATACACTCGAGtCCGGaccaactagaaccATGGCGTGTTCAACGCTCCC
2	ATACATACGCGTTTATCATGGCTCGTATTCTAGTGGTTTTAGCT
3	CAACCTTACCAACCCTGATAAAACTCAAGA
4	CAGTCCTCCTCTTTTTAGGAACAACAGGTCTAGGC
5	TGTGCTGCCCTAAAAGAAGAATGTTGTT
6	GGACTAAAGCCTGGACTACTGAGATCCTG
7	CAGTCCTCCTTCTTTTAGGAACAACAGGT
8	TGTGCTTTCCTAAAAGAAGAATGTTGTTTCTAT
9	ATACATCCATGGCTGCCGTACAAGATGATCTCA
10	ATACATCTCGAGATCTCTTACTAGGCCTGTATGGTCAGC
S1	AGGATTGTTTAACAGATCCCCCT
S2	GCGACGCGTATGTATGTCGACTTATCATGGCTCGTATTCTAGTGGTTTTA
S3	ATACATACGCGTATAAAAGATTTTATTTAGTTTCCAGAAAAAGG
S4	CGCGGCTACAATTAATACATAACCTTA
S5	ATACATTTAATTAACTCTCGAGGTTAACGAATTCCG
S6	ATACATACGCGTCTTACTTGTACAGCTCGTCCATGC
S7	ACATGGCCCAGCCGGCCATGGCTGCCGTACAAGATGATCTC
S8	GTATACGCGTTTATACTAGGCCTGTATGGTCAGC
S9	ACATGGCCCAGCCGGCCCTCCAGGCAGCCATAC
S10	GTATACGCGTTTATACGCCAGTGTGGTCC
S11	ATACATCCATGGGGCTCCAGGCAGCCATAC
S12	ATGTATCTCGAGATCTCTTACTACGCCAGTG
S13	CTGTTCCTAAAAAGAGGAGGATTATGTGCTTTCCTAAAAGAAGAATGC
S14	GCATTCTTCTTTTAGGAAAGCACATAATCCTCCTCTTTTTAGGAACAG
S15	CCCCTTTTCCTTGTCACCTGTTCC
S16	TCCTCCTCGCTCCCAGAACAGGAGATCAAGGCCTCGTCTG
S17	CGAGGGGAGGTGTCGTAGCTGA
S18	TTATGCAAATTTTTACAAGAACAGTGCTGTTTTCTGA
S19	ACATGGCCCAGCCGGCCCTCCTACATGAGGTG
S20	GTATACGCGTTTAATGGGAATTGGTAATATTC
S21	CTGAAATCACCCCGGACAAA
S22	ATACATGGCTGCAGCATTAGTCTGCGCGTCTTTCAGG
S23	ATACATGCTGCAGCCATGTCCCTCGCCTCAGGAA
S24	ATACATAAGCTTTTAATTCTCAAGGGGGGGTCTTTC