Metalloproteases Regulate T Cell Proliferation and Effector Function via LAG-3.

Nianyu Li, Yao Wang, Karen Forbes, Kate M. Vignali, Bret S. Heale, Paul Saftig, Dieter Hartmann, Roy Black, John J. Rossi, Carl P. Blobel, Peter J. Dempsey, Creg J. Workman and Dario A.A. Vignali.

Supplemental Materials and Methods

sLAG-3 purification

For purification of sLAG-3, 1 x 10^9 LAG-3 transduced 3A9 cells were incubated with serum-free RPMI 1640 (Mediatech, Holly Hill, FL) for 2 days. Culture medium was harvested and applied to a C9B7W anti-LAG-3 mAb immunoaffinity column. sLAG-3 was eluted with 50 mM glycine, 150 mM NaCl, pH 2.5, and dialyzed against PBS overnight. Purity was confirmed by SDS-PAGE and Syproruby staining (PerkinElmer, Boston, MA). Protein concentration was determined by BCA protein determination kit (Pierce, Rockford, IL).

Bone marrow reconstitution and retroviral-mediated stem cell gene transfer

Bone marrow cells were harvested from ADAM17$^{+/+}$ and ADAM17$^{\Delta Zn/\Delta Zn}$ mice and lymphocytes (T and B cells) removed by AutoMACS (Miltenyi Biotech, Auburn CA). Irradiated (450 rads) B6.Rag1$^{-/-}$ mice were reconstituted with 4 x 10^6 purified bone marrow cells in 2% FBS/PBS with 2 U/ml heparin via the tail vein.

For retroviral-mediated stem cell gene transfer was performed as described (Szymczak *et al.*, 2004). Bone marrow was harvested from 8- to 10-wk-old donor mice 48 h after treatment with 150 mg/kg 5-fluoruracil (Pharmacia & UpJohn, Kalamazoo, MI). Bone marrow cells were cultured in complete DMEM with 20% FBS and the stem cells induced to proliferate with 20 ng/ml IL-3, 50 ng/ml IL-6, and 50 ng/ml stem cell factor (Biosource International, Camarillo, CA). Bone marrow cells were co-cultured for 48 h with the retroviral producer cell lines described earlier. Nonadherent, transduced bone marrow cells were collected, washed and 4 x 10^6 in PBS/2% FBS with 20 U/ml heparin injected via the tail vein into sublethally irradiated (900 rads) B6.Thy1.1$^+$ mice (B6.PL - The Jackson Laboratory, Bar Harbor, Maine). Mice were analyzed for T cell reconstitution and serum sLAG-3 concentration 8 wk post-transplant.

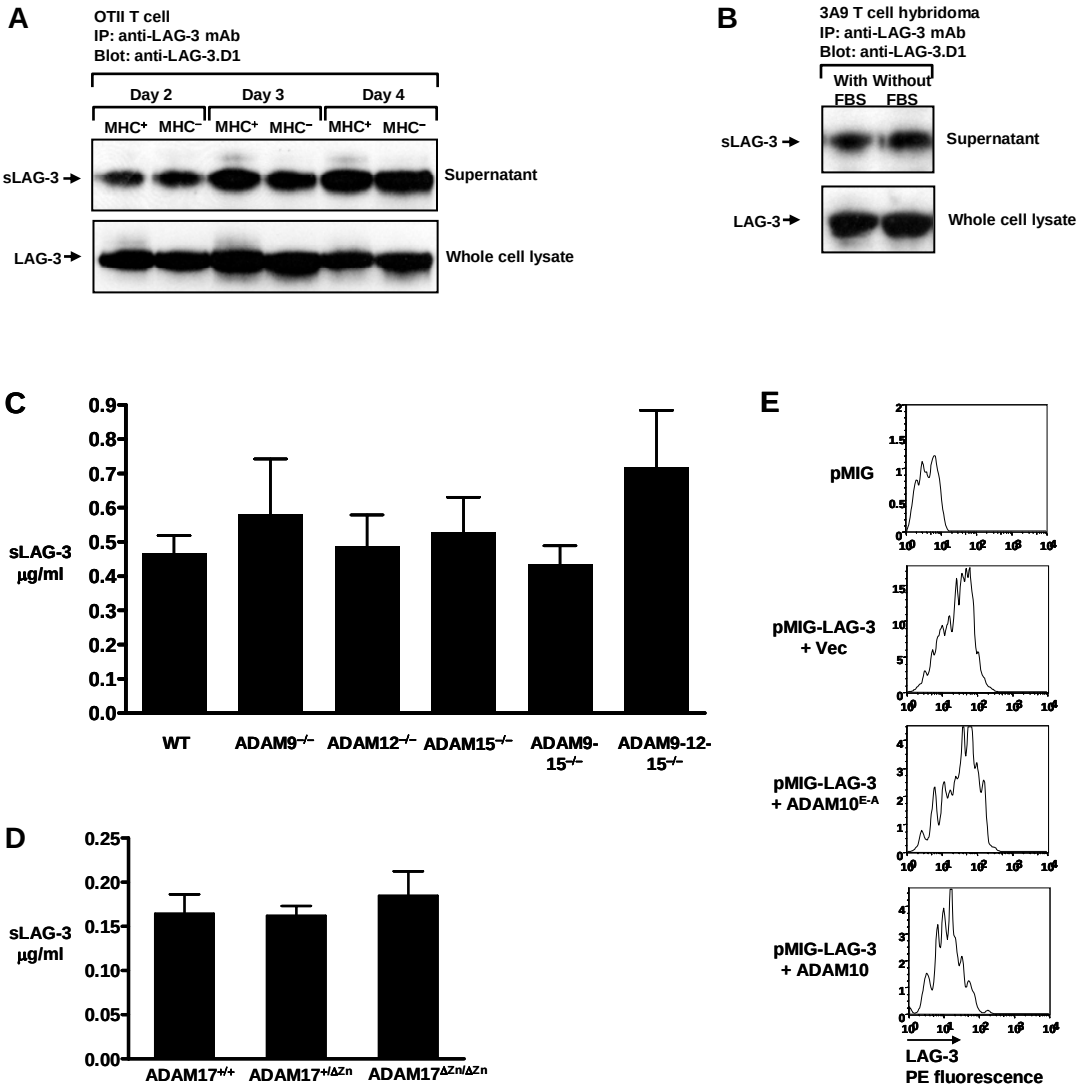
Construction of retroviral vector encoding shRNA targeting murine ADAM10.

Specific silencing of the endogenous murine ADAM10 was achieved using a retroviral shRNA expression vector. The target sequence was determined using a novel algorithm as previously described (Heale *et al.*, 2005). A murine U6-shRNA cassette was generated by PCR using the primers Hind-mU6.for as forward primer (5'-GCCAC CAAG CTTGATCCGACGCCG CCATCTCTAG-3') and AD10.RiN-1450 as reverse primer (5'-GCCACCAGATCTAAAAAA AGCAATCATCTTTGCACTGGTCACTGTAGCTACACAAA**CTACAGTGACCAGTGCAA AGATGATTGCT**AAACAAGGCTTTTCTCCAAGGGATATTTATAGTC-3') with a murine U6 promoter-containing plasmid (pLentilox) as template [ADAM10 1425-1453 target sequence in bold; annealing sequence underlined]. The PCR product was digested with BglII and HindIII and cloned into the modified MMLV retroviral vector pBan-GFP (kindly provided by David Wiest, Fox Chase Cancer Center). shRNA vector was verified by sequencing and virus generated as described below.

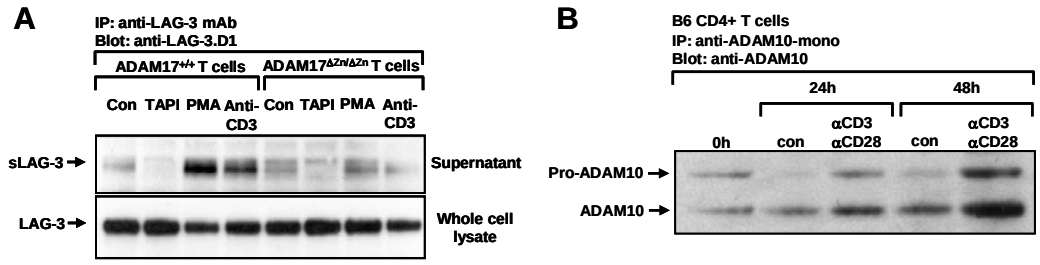
References

Heale BS, Soifer HS, Bowers C, and Rossi JJ (2005). siRNA target site secondary structure predictions using local stable substructures. *Nucleic Acids Res,* **33,** e30.

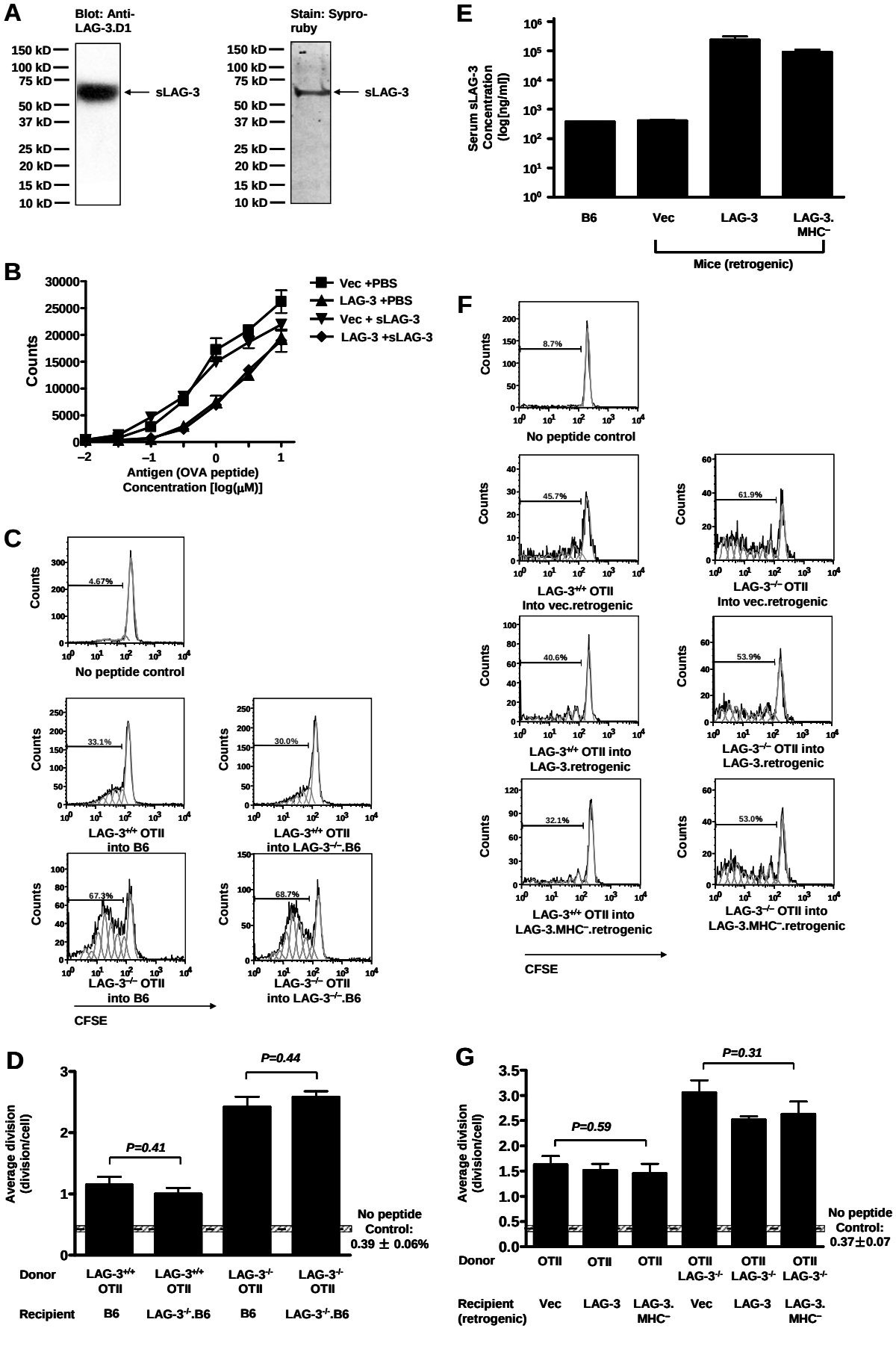
Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, and Vignali DA (2004). Correction of multi-gene deficiency *in vivo* using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nat Biotechnol*, **22,** 589-594.



Supplementary Figure S1 (A) MHC class II ligation does not induce LAG-3 cleavage. OTII TCR transgenic T cells were purified by MACS and activated with 2 µM anti-CD3 / 2 µM anti-CD28 in the presence of irradiated splenocytes from either MHC+ or MHC– Rag1–/– mice as antigen presenting cells. After the days indicated, supernatants were collected and cells lysed. Both were immunoprecipitated with the anti-LAG-3 mAb, eluted proteins separated by SDS-PAGE and blotted with anti-LAG-3.D1 antisera. **(B)** Fetal bovine serum has no effect on sLAG-3 production. T cell hybrids (1 x 106) were cultured in either normal culture medium (RPMI-1640 with 10% FBS) or serum free medium. Supernatants were collected and cells lysed after 12 h. Both were immunoprecipitated with the anti-LAG-3 mAb, eluted proteins separated by SDS-PAGE and blotted with anti-LAG-3.D1 antisera. (**C-D)** The concentration serum sLAG-3 in the mouse strains indicated was determined by ELISA. Data are presented as the mean of three mice ± SE. **(E)** ADAM10$^{-/-}$ MEF cells were either transfected with pMIG alone or cotransfected with LAG-3 in pMIC (LAG-3) plus an IRES-GFP vector pIRES (Vec), a dominant negative bovine ADAM10 cDNA in pIRES (ADAM10^{E-A}), or wild type bovine ADAM10 in pIRES as described in **Figure 2D**. Surface LAG-3 expression of GFP positive cells was measured by FACS.



Supplementary Figure S2 (A) TCR signaling induces ADAM17-mediated LAG-3 cleavage. Irradiated B6.Rag1−/− mice were reconstituted with bone marrow from either ADAM17+/+ or ADAM17ΔZn/ΔZn mice. CD4+ T cells were isolated by MACS and stimulated with 10 μg/ml of SEB in the presence of irradiated antigen presenting cells for 4 days. Activated T cells were then treated as indicated [as above plus 2 μg/ml anti-CD3 (2C11), anti-CD3 and TAPI, or anti-CD3 and 50 μg/ml Genistein] for 1 h. **(B)** TCR signaling induces ADAM10 expression in T cells. CD4+ T cells from C57/BL6 mice were isolated by MACS and stimulated with 2 μg/ml anti-CD3 (2C11) 10 μg/ml for 0, 24, 48 hours. ADAM10 expression was detected by western blot.


A
Blot: Anti-LAG-3.D1
Stain: Sypro-ruby
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
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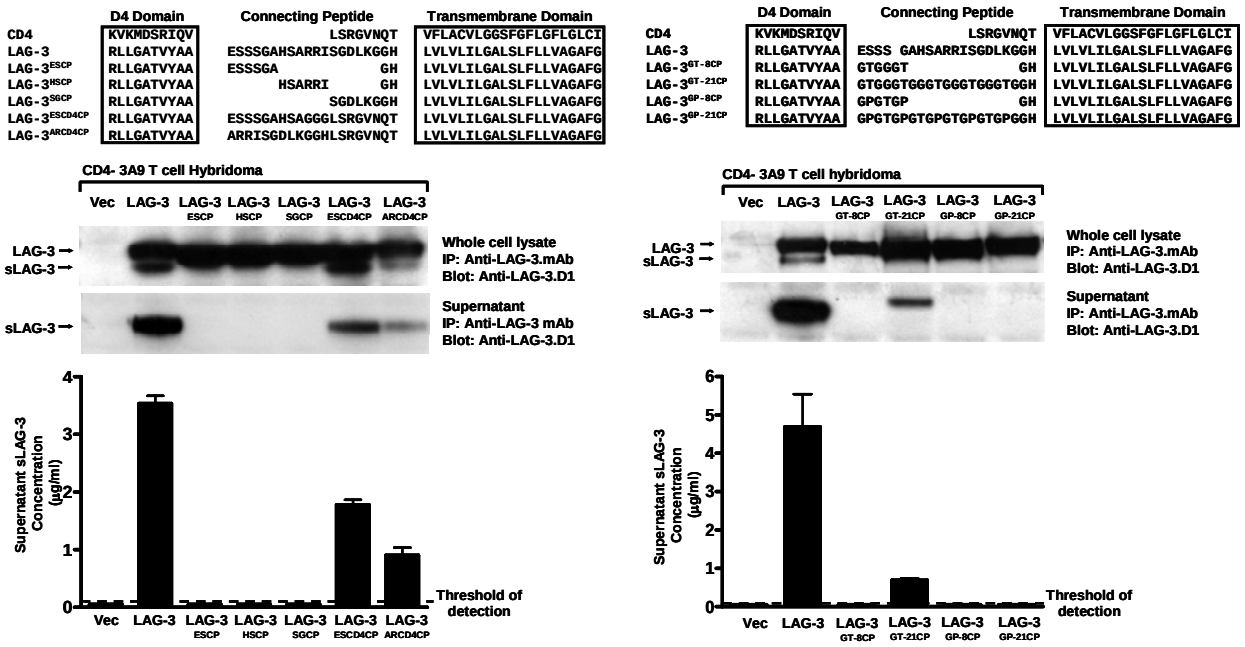
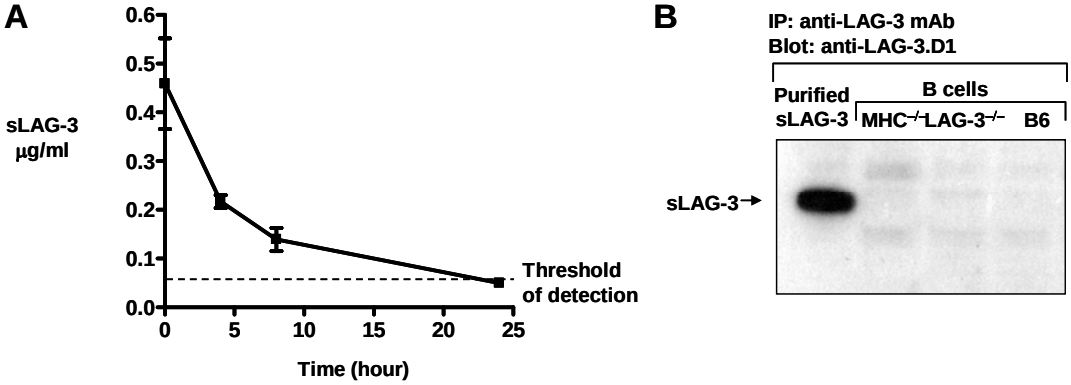
15 kD
10 kD
sLAG-3
B
Counts
Antigen (OVA peptide) Concentration [log(μM)]
Vec +PBS
LAG-3 +PBS
Vec + sLAG-3
LAG-3 +sLAG-3
C
No peptide control
LAG-3+/+ OTII into B6
LAG-3+/+ OTII into LAG-3−/−.B6
LAG-3−/− OTII into B6
LAG-3−/− OTII into LAG-3−/−.B6
CFSE
D
Average division (division/cell)
P=0.41
P=0.44
No peptide Control: 0.39 ± 0.06%
Donor
Recipient
LAG-3+/+ OTII
LAG-3+/+ OTII
LAG-3-/- OTII
LAG-3-/- OTII
B6
LAG-3-/-.B6
B6
LAG-3-/-.B6
E
Serum sLAG-3 Concentration (log[ng/ml])
B6
Vec
LAG-3
LAG-3. MHC−
Mice (retrogenic)
F
No peptide control
LAG-3+/+ OTII Into vec.retrogenic
LAG-3−/− OTII Into vec.retrogenic
LAG-3+/+ OTII into LAG-3.retrogenic
LAG-3−/− OTII into LAG-3.retrogenic
LAG-3+/+ OTII into LAG-3.MHC−.retrogenic
LAG-3−/− OTII into LAG-3.MHC−.retrogenic
CFSE
G
Average division (division/cell)
P=0.59
P=0.31
No peptide Control: 0.37±0.07
Donor
Recipient (retrogenic)
OTII
OTII
OTII
OTII LAG-3-/-
OTII LAG-3-/-
OTII LAG-3-/-
Vec
LAG-3
LAG-3. MHC−
Vec
LAG-3
LAG-3. MHC−

Supplementary Figure S3. sLAG-3 does not affect T cell activation. **(A)** Immunoaffinity purified sLAG-3 was run on an SDS-PAGE gel and analyzed by western blot analysis using anti-LAG-3.D1 antisera (left panel) and by protein gel staining with sypro-ruby followed by visualization under UV (right panel). A single band of the predicted molecular weight was observed. **(B)** LAG-$3^{-/-}$ OTII TCR transgenic T cells were MACS purified, activated and transduced with either empty pMIG vector (Vec) or LAG-3.pMIG (LAG-3) retrovirus. Cells were then stimulated with APCs plus $OVA_{326-339}$, at the concentrations indicated, in the presence or absence of 1μg/ml purified sLAG-3 [~2-fold more sLAG-3 than detected following T cell stimulation in vitro and ~5-fold more than found in sera (Li et al., 2004)]. Cultures were pulsed with [^{3}H]thymidine during the last 8 h of a 48 h assay. Data are representative of 3 independent experiments. **(C, D)** LAG-$3^{+/+}$ and LAG-$3^{-/-}$ $Thy1.1^+$ OTII T cells were MACS purified, labeled with CFSE, adoptively transferred into $Thy1.2^+$ B6 or LAG-$3^{-/-}$ mice and stimulated in vivo 24 h later with (no peptide control) or without $OVA_{326-339}$ (50μg IP). Spleens were removed 6 d later and the percentage of dividing cells determined by gating on $CFSE^{negative-low}$/$Thy1.1^+$/$CD4^+$ T cells. **(C)** Representative CFSE histograms are shown with individual divisions displayed using FlowJo. **(D)** The mean ± SE of 3 independent experiments (5 mice per group) is shown with the hatched horizontal bar representing the mean ± SE of the no peptide controls. P values show that there were no significant differences between the groups indicated as determined by the Student's t-test. **(E-G)** $Thy1.1^+$ B6.PL bone marrow cells transduced with pMIG (Vec), LAG-3 or a non-MHC class II binding mutant (LAG-3.MHC^- [R72E/Y73F/R99A]) were used to reconstitute irradiated B6.PL mice. **(E)** The serum sLAG-3 concentration in LAG-3 or LAG-3.MHC^- bone marrow reconstituted mice was ~1000-fold higher than in unmanipulated mice as determined by ELISA, 8 weeks post-transplant. Data are the mean ± SE of 8-9 mice per group. **(F, G)** These mice were used as recipients for LAG-$3^{+/+}$ or LAG-$3^{-/-}$ $Thy1.2^+$ OTII T cells that were MACS purified and labeled with CFSE prior to adoptive transfer. OTII T cells were then stimulated in vivo 24 h later with (no peptide control) or without $OVA_{326-339}$ (50μg IP). Spleens were removed 6 days later and the percentage of dividing cells determined by gating on $CFSE^{negative-low}$/$Thy1.2^+$/$CD4^+$ T cells. **(C)** Representative CFSE histograms are shown with individual divisions displayed using FlowJo. **(G)** The mean ± SE of 3 independent experiments (8-9 mice per group) is shown with the hatched horizontal bar representing the mean ± SE of the no peptide controls. P values show that there were no significant differences between the groups indicated as determined by the Student's t-test.



Supplementary Figure S4 The cleavage of LAG-3 depends on the length and composition of its connecting peptide. A series of LAG-3 mutants that contained 8 or 20 amino acid CPs of differing composition were generated and expressed in a LAG-3^{-}/CD4^{-} 3A9 T cell hybridoma by retroviral transduction. Top panel details the amino acid sequences of the CP mutants used. Letters in the boxes indicate the amino acids of the domains. Uniformed expression of the LAG-3 mutants was ensured by FACS and confirmed by western blot. Cells were sorted for LAG-3/GFP equivalence and placed in culture (1 x 10^6 in 1ml) for 1 h. Supernatants were then collected and cells lysed. Constitutive shedding by unstimulated cells was assessed by detection of sLAG-3 using western blot and ELISA. For the middle panel, both were immunoprecipitated with the anti-LAG-3 mAb, eluted proteins separated by SDS-PAGE and blotted with anti-LAG-3.D1 antibody. Cells expressing the empty pMIG vector (Vec) or wild type LAG-3 (LAG-3) were included as controls. In the lower panel, sLAG-3 concentration in the supernatant was determined by ELISA. LAG-3 concentration was calculated using a standard curve generated with affinity purified sLAG-3. Data are presented as the mean of 3 separate experiments. Three sets of LAG-3 mutants were examined. LAG-3^{ESCP}, LAG-3^{HSCP} and LAG-3^{SGCP} possessed different 8 amino acid portions of the LAG-3 CP. All were resistant to cleavage as indicated by the lack of detectable sLAG-3 by western blot and ELISA. This also correlated with the absence of cell surface-associated sLAG-3 that dimerizes with intact LAG-3 molecules (lower band of the upper gel). Second, LAG-3$^{ES\text{-}CD4CP}$ and LAG-3$^{AR\text{-}CD4CP}$ possessed extensions of the CD4 CP by insertion of different LAG-3 CP residues. Both were cleaved but at significantly reduced efficiency. Third, four LAG-3 mutants were generated with synthetic CPs containing either 'GTGG' or 'GPGT' repeats to further assess cleavage promiscuity. Two short and one long CP LAG-3 mutant (LAG-3$^{GT\text{-}8CP}$, LAG-3$^{GP\text{-}8CP}$ & LAG-3$^{GP\text{-}21CP}$) were not cleaved. One of the LAG-3 mutants with a long flexible CP, LAG-3$^{GT\text{-}21CP}$, was cleaved but at a significantly reduced rate compared with wild type LAG-3 (6 fold reduction by ELISA).



Supplementary Figure S5 sLAG-3 is rapidly excreted and/or degraded in *vivo*. **(A)** sLAG-3 (5 μg/mice) was injected into LAG-3$^{-/-}$ mice and serum sLAG-3 concentration before or after injection measured by ELISA. Normal B6 serum concentration ~0.1 μg/ml. **(B)** B cells from MHC class II deficient mice, LAG-3$^{-/-}$ mice, or B6 mice were MACS purified. B cells (2 x 10^7) were lysed and then immunoprecipitated with the anti-LAG-3 mAb, eluted proteins separated by SDS-PAGE and blotted with anti-LAG-3.D1 antisera. Purified sLAG-3 (0.2 μg) was used as control.