Supplementary Methods

Trans-splicer Expression Cassettes

The construction of the 3' *trans*-splicers was based on a strategy to correct CD40L-knockout (KO) mice (C57Bl/6 background, Jackson Laboratories, Bar Harbor, MA). The wild type murine CD40L gene has 5 exons (Figure 1A). The CD40L-KO mouse is missing exons 3 and 4 (panel B). This mouse *cis*-splices the CD40L gene to create a mutant exon 1-2-5 mRNA transcript (panel C). The *trans*-splicer constructs included (5' to 3'): a hybridization domain, spacer, branch point, polypyrimidine tract and splice acceptor domains followed by exons 2-5 and a polyA/stop-signal (panel D). Through *trans*-splicing, this design should create a normal CD40L transcript that includes exons 1-5.

All plasmids for *in vitro* experiments were based on a backbone derived from the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA). In preliminary studies (see below), four hybridization domains were tested (see Figure 1D, labeled a-d; the *trans*-splicer construct shown uses hybridization domain b as an example). Hybridization domains were all derived from *Mus musculus* chromosome X genomic contig (GenBank accession # NT_039706.2/MmX_39746_32); hybridization domain “a” included nt 2615895 to 2616206; domain “b” nt 2616246 to 2616557; domain “c” nt 2616555 to 2616866; domain “d” nt 2617015 to 2617326. The spacer was comprised of a *de novo* sequence (5'-GCGGCCGCCCGAATAAGTGATTGATTGAGTTT-3'), that is non-homologous to any murine or human sequence and contains stop codons in all three frames. This was followed by the artificial branch point and polypyrimidine tract and splice acceptor sequence 5'-TACTAACTGATATCTCTTCTTTTTTTTTTCCGGAAAACAG-3' as described previously[1]. Exons 2-5 were cloned from the murine CD40L cDNA[2]. The polyA/stop signal included bovine growth hormone polyadenylation signal and transcription termination sequence, derived from the pcDNA3.1(+) vector backbone. All of the *trans*-splicer expression

cassettes were driven by the cytomegalovirus (CMV) immediate/early promoter/enhancer, also derived from the pcDNA3.1(+) vector backbone.

Optimization of *Trans*-splicer Hybridization Domains

Preliminary studies demonstrated that the optimal size of the hybridization domain for maximal efficiency of *trans*-splicing was 312 nt. To determine the optimal hybridization location within the target intron for maximal efficiency of *trans*-splicing, *in vitro* studies were carried out to determine the most efficient construct. A mutant CD40L gene target was constructed in pcDNA3.1(+) with the following elements 5' to 3' in the expression cassette: CMV promoter/enhancer followed by the murine CD40L exon 1 and intron 1 followed by the coding sequences of exons 2 and 3, a hemagglutinin (HA) tag sequence[3] and a polyA/stop signal. Location of the optimal *trans*-splicer hybridization domain was determined by transfecting the target and various *trans*-splicer plasmids (including hybridization domains a, b, c or d) into HeLa cells and quantifying intact CD40L transcripts using RT-PCR. The day before transfection, $1.2x10^5$ HeLa cells were plated on 12-well plates and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and streptomycin (100 μg/ml) for 24 hr, 37°C. Cells were transfected with the target and *trans*-splicer expression plasmids using Lipofectamine Plus Reagent (Invitrogen) according to the manufacturers protocol. In a typical co-transfection reaction, 0.2 μg target plasmid and 0.2 μg *trans*-splicer plasmids were transfected into cells. For controls, 0.2 μg target plasmid and 0.2 μg null plasmid (pNull; a vector identical to *trans*-splicer plasmids without transgene) or 0.2 μg pCD40L plasmid and 0.2 μg pNull were used. Thirty-six hr after transfection, the plates were rinsed with phosphate buffered saline, pH 7.4 (PBS) and total RNA was isolated using TRIzol (Invitrogen). Reverse transcriptase reaction was carried out (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen) and oligo(dT). PCR was done using a PLATINUM Taq DNA Polymerase kit (Invitrogen). To

detect the *in vitro cis*-spliced product primers 1F and 1R were used. To detect the *in vitro trans*-spliced product, primers 2F and 2R were used (see Figure 1). Primers sequences for *in vitro* analysis were as follows; primer 1F (5'-TGATAGAAACATACAGCCAACCTT-3', exon 1), primer 2F (5'-CTGTGCTTTTTGCTGTGTATCTTC-3', exon 1); primer 1R (5'-CTAGCGTAATCCGGAACATCGTA-3', HA-tag specific) and 2R (5'-GACTTGAGTGTAGACATAATAGAG-3', exon 5). PCR amplification (25-40 cycles) was carried out at 94 °C, 0.5 min, annealing at 58 °C for 0.5 min, extension at 72 °C, 1 min followed by final extension at 72 °C for 5 min. The reaction products were analyzed by agarose gel electrophoresis. To assess the fidelity of the *trans*-splicing, the RT-PCR products with primers 2F and 2R were electrophoresed and purified with a gel extraction kit (QIAquick gel extraction kit; Qiagen, Valencia, CA), and the products sequenced.

To estimate the efficiency of *trans*-splicing *in vitro*, real-time quantitative RT-PCR, TaqMan PCR (Applied Biosystems, Forster City, CA) was performed using primers, 4F, included (5'-TCTTCATAGAAGATTGGATAAGGTCG-3', exon 1 and 2) and 5R, (5'-GGATGCTGCATTACTGTTGGGC-3', exon 4) were used with a 6-carboxyfluorescein labeled probe (5'-6FAM- AGGATCCTCAAATTGCAGCACACGTTGTTA-TAMRA-3', exon 4). Ribosomal RNA was used as internal control. RT was done using random primer and Reverse Transcriptase Reagent (Applied Biosystems, Foster City, CA). PCR amplification was performed for 40 cycles, with each cycle at 95°C for 15 sec and 60°C for 1 min, in triplicate using the ABI Prism 7700 SDS (Applied Biosystems). The threshold cycle values were averaged and data were used to determine the relative amounts of *trans*-spliced products mRNA using comparative CT methods. As a control, mRNA harvested from cells were transfected with a full length murine CD40L cDNA driven by a CMV promoter/enhancer. To determine the cellular level of both *trans*-spliced and *cis*-spliced product, primers 5F, (5'-GGACTTCCAGCGAGCATGAA-3', exon 1) and 6R (5'-

TCCTCTTCGACCTTATCCAATCTT-3', exon 1 and 2) were used with a 6FAM-labeled probe (5'-6FAM-CACAGCAAAAAGCACAGATCCAATCATTTG-TAMRA-3', exon 1). RT-PCR and subsequent analyses were done as described above and relative amounts of *trans*- and *cis*-spliced products determined using controls as described above. The percentage of *trans*-splicing of total RNA splicing was calculated as 100 x (*trans*-spliced products relative to control) / (*trans*- + *cis*-spliced products relative to control).

In Vitro Assessment of CD40L Protein Produced by _Trans_-splicing

To assess the expression of *trans*-spliced CD40L protein, transfected HeLa cells were stained with a phycoerythrin (PE)-conjugated monoclonal antibody specific for CD40L (clone MR1, BD Pharmingen, San Diego, CA) and isotype control (BD Pharmingen, anti-trinitrophenol, clone A19-4). Stained cells were examined by flow cytometry (FACSCalibur; Becton-Dickinson, San Jose, CA); a minimum of 10,000 events were collected and analyzed. The percentage of *trans*-splicing of control was calculated as [(mean fluorescence intensity (MFI) of *trans*-splicer + target) - (MFI pNull x100)]/[(MFI pCD40L) - (MFI pNull)].

Analysis of Function of _Trans_-spliced CD40L _In Vitro_

To assess the effect of *trans*-spliced CD40L on B cell proliferation *in vitro*, splenic B cells were negatively selected from spleen of wild type C57Bl/6 mice using anti-CD4 microbeads, anti-CD8 microbeads, and anti-CD11c microbeads (Militenyi Biotech, Auburn,CA) and stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) according to the manufacturer's instructions. The purified B cells averaged 90% B220-positive and < 1% CD4 and CD8 positive. Briefly, splenic B cells were resuspended in 4 ml PBS and stained with 2.5 μM CFSE and incubated 5 min, 23 °C. FBS (4 ml) was added to quench unbound CFSE, the mixture incubated 1 min, 23 °C and then washed twice with PBS. Splenic B cells (10^5) were cultured in 96-well culture plates with irradiated (7.7 Gy) HeLa cells previously transfected with plasmids in RPMI 1640 medium

(10% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 55 μM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate) supplemented with recombinant mouse IL-4 (rmIL-4) and IL-5 (both 10 ng/ml; both from R & D Systems, Minneapolis, Minnesota). Mixed cells were stained with alophycocyanin-labeled anti-B220 mAb (clone RA3-6B2, BD Pharmingen) and B cell proliferation was assessed by flow cytometry.

To assess the ability of *trans*-spliced CD40L in HeLa cells to initiate immunoglobulin class-switching in B cells, 10^5 splenic B cells from wild type C57Bl/6 mice were cultured with irradiated transfected HeLa cells in 96-well culture plates in RPMI 1640 medium supplemented with rmIL-4 (10 ng/ml). Six days later, the conditioned medium was harvested and the concentration of IgE determined using an enzyme-linked immunosorbent assay (ELISA) using anti-mouse IgE mAbs (BD PharMingen, clone R35-72 as capture, and biotinylated clone R35-118 mAb as detection) and avidin-horseradish peroxidase, according to the manufacturer (BD PharMingen). Mouse IgE (anti-DNP, Sigma, Saint Louis, MO, clone SPE-7) was used as a standard.

To assess the effect of *trans*-spliced CD40L on activation of macrophages, 10^5 RAW 264.7 macrophage-like cells (TIB-71, ATCC, Manassas, VA) were cultured with $5x10^4$ irradiated HeLa cells transfected with plasmid in 96-well culture plates in DMEM as described above. Six, 24, and 48 hr after co-culture, conditioned media were harvested and the concentration of TNF-_ determined by ELISA (R&D Systems).

To assess the production of peptides by *trans*-splicer mRNA, we performed *in vitro* transcription/translation analysis using ^{35}S methionine and Linked in vitro SP6/T7-Transcription/Translation Kit-radioactive (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions.

Lentivirus *Trans*-splicer Vectors

Based on the result that hybridization domain b was the most efficient at promoting *trans*-splicing (see Results), the lentivirus vector LVtsCD40L2-5 was generated using the pLVtsCD40L2-5 expression plasmid, containing the same expression cassette used in the b hybridization domain plasmid (see above). As a control, lentivirus (LVeGFP) vector was prepared using the identical expression plasmid[4] (pTK113) but expressing enhanced green fluorescent protein[4] (eGFP). The lentivirus vectors were generated by standard transient three-plasmid transfection using a vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyping plasmid[5] (5 μg), _NRF packaging plasmid[4] (10 μg), and the CD40L *trans*-splicer or eGFP expression plasmids (15 μg) into subconfluent 293T cells (ATCC, CRL-11268) using calcium phosphate precipitation[6]. Lentivirus vector particles were collected from conditioned medium at 60 hr post-transfection and concentrated by two-rounds of ultracentrifugation and resuspended in serum free, StemPro34 SFM medium (Invitrogen).

***Ex vivo* Lentivirus-mediated Transfer of the Expression Cassette to Bone Marrow Cells**

Animal experiments were performed with the authorization of the institutional review board and Animal Care and Use Committee of Weill Medical College of Cornell University. Bone marrow cells from C57Bl/6 CD40L wild type or CD40L-KO mice were harvested four days after administration of 5-fluorouracil (5-FU; 150 mg/kg; Sigma) to enrich the stem cell population[7]. The medium used for lentiviral transduction contained factors previously reported to enhance transduction efficiency of stem cells, including mouse stem cell factor (SCF, 100 ng/ml, R&D Systems), human thrombopoietin[8] (TPO, 100 ng/ml, R&D Systems), mouse IL-3 and mouse IL-6[9] (both 20 ng/ml, both R&D Systems). After addition of 4 μg/ml polybrene (Sigma), the medium containing LVtsCD40L2-5 or LVeGFP (MOI 30) was centrifuged with cells at 600 x g for 45 min, followed by a 24 hr incubation. Transduced bone marrow cells ($2x10^5$) were transplanted intravenously into lethally irradiated (8.5 Gy) CD40L-KO mice. Ten wk after transplantation, bone marrow cells, peripheral blood cells and spleens were

harvested, and genomic DNA and total RNA were isolated using DNeasy Tissue Kit (Qiagen) or TRIzol (Invitrogen), respectively. As a transduction control, peripheral blood cells were isolated from LVeGFP-treated CD40L-KO mice. CD40L or eGFP-positive CD4+CD8+ (T cells), B220+ (B cells), or CD11b+ Gr-1+ (monocytes/granulocytes) peripheral blood cells were examined using flow cytometry to analyze expression at the protein level using alophycocyanin-labeled monoclonal antibodies specific for CD4 (clone RM4-5, BD Pharmingen), CD8 (clone 53-6.7, BD Pharmingen), CD11b (clone M1/70, BD Pharmingen) and Gr-1 (clone RB6-8C5, BD Pharmingen).

To assess the distribution and expression of LVtsCD40L2-5, PCR and RT-PCR were used, respectively. To detect LVtsCD40L2-5 *in vivo*, primers 3F and 2R were used. To detect the *cis- and trans*-spliced products of LVtsCD40L2-5 *in vivo* primers 1F and 3R were used, and to detect the *in vivo trans*-spliced product of LVtsCD40L2-5 only, primers 2F and 4R were used (see Figure 1). Primers sequences for *in vivo* analysis were as follows: primer 3F (5'-CCGAATAAGTGATTGATTGAGTTTTACT-3', *trans*-splicer vector specific); primer 3R (5'-TCACAGTTCAGCAAGGATAAAGAT-3', exon 2); and primer 4R (5'-TGCATTACTGTTGGCTTCGCTTAC-3', exon 4). Sequence of primers 1F, 2F, 1R and 2R and PCR and RT-PCR reactions were performed as described above for *in vitro* analysis. For RT-PCR analysis, primers 2F and 4R were used to demonstrate CD40L mRNA derived from *trans*-splicing only, while primers 1F and 3R were used to demonstrate both *trans*- and *cis*-spliced CD40L mRNAs. RNA from wild type mouse was used as a positive control with primers 2F and 4R because it contains exon 4 (the binding site of the downstream primer) in the CD40L gene, whereas the CD40L-KO mice do not have exon 4, and the only potential primer binding site derives from the *trans*-splicer pre-mRNA. Determination of efficiency of *trans*-splicing in vivo was performed using the same primers and procedures as described above.

Secondary Transplantation

Secondary transplantations were performed to confirm transduction of primitive hematopoietic cells and to look for corrective effects that could be passaged to a second generation. As described for primary transplantation, 4 months after the primary transplant, bone marrow cells from CD40L-KO primary transplant recipient mice were harvested 4 days after administration of 150 mg/kg 5-FU. Transduced bone marrow cells ($2x10^5$) were transplanted intravenously into lethally irradiated CD40L-KO mice (8.5 Gy). Six wk after secondary transplantation, peripheral blood cells were harvested and genomic DNA and total RNA were isolated using DNeasy Tissue Kit (Qiagen) or TRIzol (Invitrogen), respectively, and analyzed as described above. Studies to assess the corrective effects of the secondary transplanted cells are described below.

Real-Time Quantitative PCR and RT-PCR *In Vivo*

To assess the copy number of the *trans*-splicer gene in cells, we performed quantitative real-time PCR using primers 6F (5'-GAAAACAGCTTTGAAATGCAAAGA-3', exon 3), primer 7R (5'- TTCTTGGCCCACTGTAGAACG -3', exons 4 and 5) and probe (5'-FAM-CTCAAATTGCAGCACACGTTGTAAGCGA-TAMRA-3', exon 4). A standard curve was generated using a serial dilution of *trans*-splicer plasmid DNA ranging from 4.5×10^7 to 4.5 copies. No template controls with water instead of template DNA were included in each experiment. All standards, samples and controls were run in triplicate. To assess the efficiency of *trans*-splicing of total splicing *in vivo*, we performed quantitative RT-PCR as described above.

Restoration of Immunity Against a Thymus-dependent Antigen

To determine whether *trans*-spliced CD40L contributed to the restoration of a normal immune response to a thymus-dependent antigen, mice were immunized with 50 μg of

keyhole limpet hemocyanin (KLH, Sigma), suspended in 100 μl PBS and mixed with 100 μl complete Freund's adjuvant and injected intraperitoneally. Booster immunization were done at 2 and 4 wk after the initial inoculation, and sera and spleens were collected on day 34. To assess the titer of KLH-specific IgM and IgG1, KLH-specific ELISAs were carried out with 96-well plates coated with KLH (1 μg/well), using rabbit anti-mouse IgM or IgG1 polyclonal antibody as primary antibody (both from Bio-Rad, Hercules, CA) with goat anti-rabbit IgG (H+L)-HRP conjugate as secondary (Bio-Rad). The reaction was developed with horseradish peroxidase substrate (Bio-Rad, #172-1064) and absorbance measured at 415 nm. End-point titers were determined as the reciprocal of the dilution at or below a fixed absorbance value of 0.1; negative results were given a titer of the lowest dilution[10].

Assessment of Regulated Expression of CD40L

To assess the regulated expression of CD40L in CD4+ T cells, splenic CD4+ T cells were harvested from C57Bl/6 wild type mice, CD40L-KO mice and *trans*-splicer treated CD40L-KO mice 8 wk after initial transplantation using a mouse T Cell CD4 subset column kit (R&D systems). CD4+ T cells (10^6 cells) cultured in 6-well culture plates previously coated with anti-CD3e antibody (10 μg/well, BD Pharmingen, Clone #145-2C11) in RPMI 1640 medium as described above, were analyzed by flow cytometry. As a marker of early T cell activation, fluoresecin isothiocyanate (FITC)-labeled anti-CD69 antibody (BD Pharmingen, clone H1.2F3) was used. Splenic CD4+ T cells from secondary transplant recipients were stimulated and analyzed in an identical manner 4 months after first transplantation.

Supplemental Methods References

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