Loss of interleukin-10 activates innate immunity to eradicate adult T-cell leukemia-initiating cells

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Supplementary materials and methods

Cell lines, treatment and Short hairpin RNA (shRNA)

ATL-derived cell lines (HuT-102, MT-1) or HTLV-1-negative Jurkat malignant T-cell line, were maintained in RPMI medium supplemented with 10% fetal bovine serum and antibiotics. Cell lines were treated with arsenic (1 µM), IFNα (10^3 UI), PS-341 (10 nM) or their combinations for 24h.

In transduction experiments, cells were transduced with green fluorescent protein (GFP)-lentiviral vectors encoding scrambled (SCR) short hairpin RNA (shRNA) or shRNA against Tax (sh-Tax: CAGGCCTTATTTGGACATTTA). Lentiviruses were produced by transient transfections of HEK-293T cells. Infection of HuT-102, MT-1 and Jurkat cells was performed by spinoculation (3h at 1500 rpm and 32°C). GFP-positive transduced cells were sorted by flow cytometry (see below).

Flow cytometry and cell sorting

Antibody-based flow cytometry experiments were performed using a BD FACSAria cell sorter and analyzed by BD FACSDIVA™ software. Spleen cells were stained with anti CD25-PE (MACS) or CD25-FITC (BD Biosciences, 2µg/mL), anti CD11b+F4/80+ (macrophage marker), and anti CD335+ antibodies (NK marker) (BD Biosciences, 2ug/mL). Patient-derived cells were labeled with an anti-human CD25 FITC (BD Biosciences, 2µg/mL).

Sorting experiments were performed using a BD FACSAria cell sorter, using antibodies against murine CD25, murine CD335, murine CD11b, murine F4/80, GFP, or human CD25. *Tax* PCR using specific primers (Supplementary Table 1) confirmed that the sorted $CD25^+$ fraction

corresponds to *tax*-positive cells whereas the $CD25^-$ negative fraction corresponds to *tax*-negative cells.

Apoptosis assays

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to monitor apoptosis and was performed according to the recommendations of the manufacturer (Roche). Fluorescein-conjugated dUTP incorporated in nucleotide polymers was detected and quantified using flow cytometry.

Another apoptosis assay was performed using the annexin V–fluorescein isothiocyanate kit according to the recommendations of the manufacturer (BD Pharmingen). This assay measures phosphatidylserine exposure by flow cytometry analysis. Approximately 10000 cells per sample were acquired and analyzed using CellQuest software (Becton Dickinson).

Quantitative PCR

Total RNA was extracted using Trizol (Qiagen cat number 79306). Experiments were performed starting from 2µg of RNA in a total of 20µl. cDNA synthesis was performed using a Revert Aid First cDNA synthesis Kit (Thermo Scientific). Syber green qRT PCR was performed using the BIORAD CFX96 machine. Primers for IL-10, Rantes, IL-15 are listed in Supplementary Table 1. In qRT-PCR, individual reactions were prepared with 0.25 µM of each primer, 150 ng of cDNA and SYBR Green PCR Master Mix to a final volume of 10 µl. PCR reaction consisted of a DNA denaturation step at 95°C for 3min, followed by 40 cycles (denaturation at 95°C for 15 sec, annealing at 57°C for 60 sec, extension at 72°C for 30 sec). For each experiment, reactions were performed in duplicates and expression of individual genes was normalized to the housekeeping

gene Glyceraldehyde-3-Phosphate dehydrogenase GAPDH (Supplementary Table 1). The transcript expression level was calculated according to the Livak method.

Immunoblot analysis

One hundred µg of protein lysates were loaded onto a 12% SDS-polyacrylamide gel, subjected to electrophoresis, and transferred onto nitrocellulose membranes. Blots were incubated with specific antibodies against Tax (monoclonal antibody 168-A51, National Institutes of Health AIDS Research and Reference Reagent Program), p-STAT3 (4113S, cell signaling), STAT3 (9139S, cell signaling), actin (A2066, Sigma -Aldrish) and GAPDH (B2534M-HRP Abnova). Bands were visualized by autoradiography, following incubation with luminol chemiluminescent substrate (Bio-Rad, Cat# 170-5061).

Enzyme-linked immunosorbent assay (ELISA)

ELISA on murine cells was performed using Multi-Analyte ELISArray Kit (MEM-005A, Qiagen) according to the manufacturer's instructions. Following sonication, cell supernatants were spun for 10 min at 1000g, transferred to new Eppendorf tubes, and diluted using a specific cocktail of antigens including (IL-12, IFNγ, MIP-1α, MCP-1, and IL-10 among others) provided by the kit (Qiagen). ELISA on human cells was performed using Single Analyte ELISArray kit for human IL-10 (SEH00572A, Qiagen).

Samples were then loaded in the coated ELISA plaque, and were incubated for 2 hours. After 3 washes, the detection antibody was added and incubated for 2 hours. Then, Avidin-HRP was added for 30 min, and 4 washes were performed. Development solution was then added in dark and kept for 15 min, before addition of the stop solution. The optic density (O.D) was determined at 450 and 570 nm and calculated according to the standard values of a positive

control provided by the kit

β-Galactosidase histochemical staining

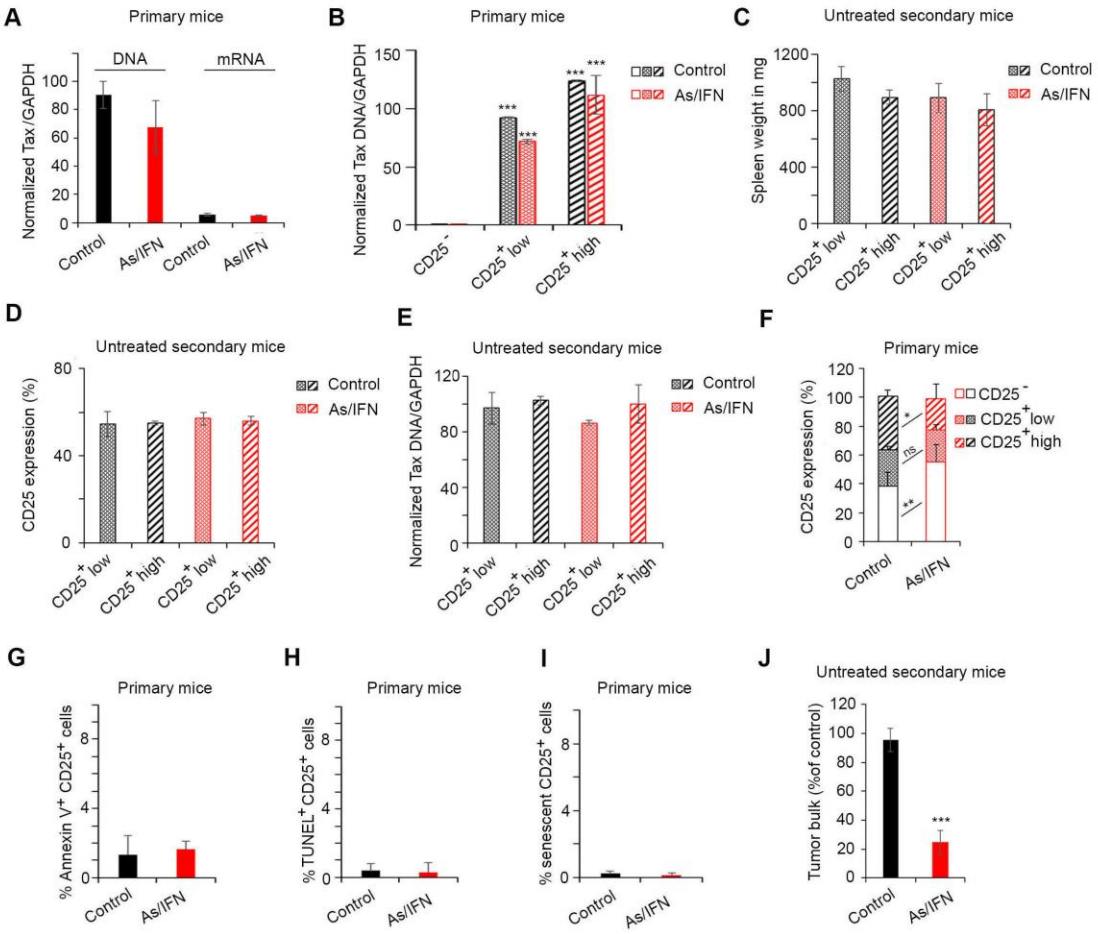
Cells were cultured overnight at 37% in 20% RPMI medium, washed twice with PBS and fixed with 4% paraformaldehyde for 30 minutes. The cells were then washed again with PBS and stained with a solution of 1 mg/mL 5-bromo-4-chloro-3-inolyl-b-galactosidase in dimethylformamide (20 mg/mL stock). Following overnight incubation at 37°C, the cells were washed twice with PBS, and the percentage of positively stained cells was determined after counting 10 random fields to reach 100 cells.

Statistical analysis

The t-test was performed to validate significance: *, ** and *** indicate *p* values ≤ 0.05; 0.01 and 0.001, respectively; *p*-values less than 0.05 were considered significant. Survival curves were calculated according to the methods of Kaplan and Meier.

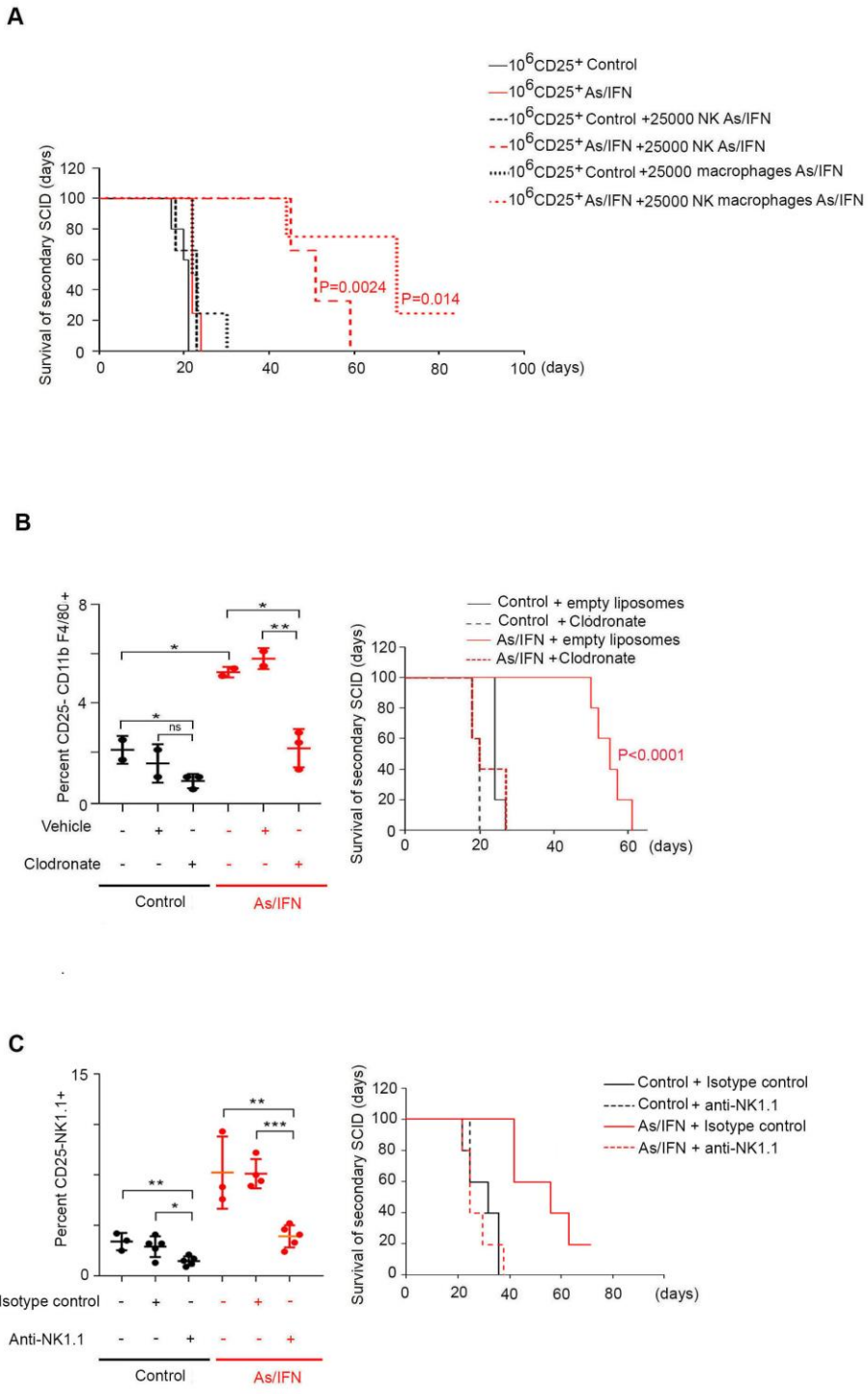
Overall survival is defined as time from injection of ATL cells to death. Mice that are still alive are censored at the time they were last known to be alive. Analyses were performed using Excel® software. The *p* value was obtained by log-rank statistical analysis.

Supplementary Figure 1

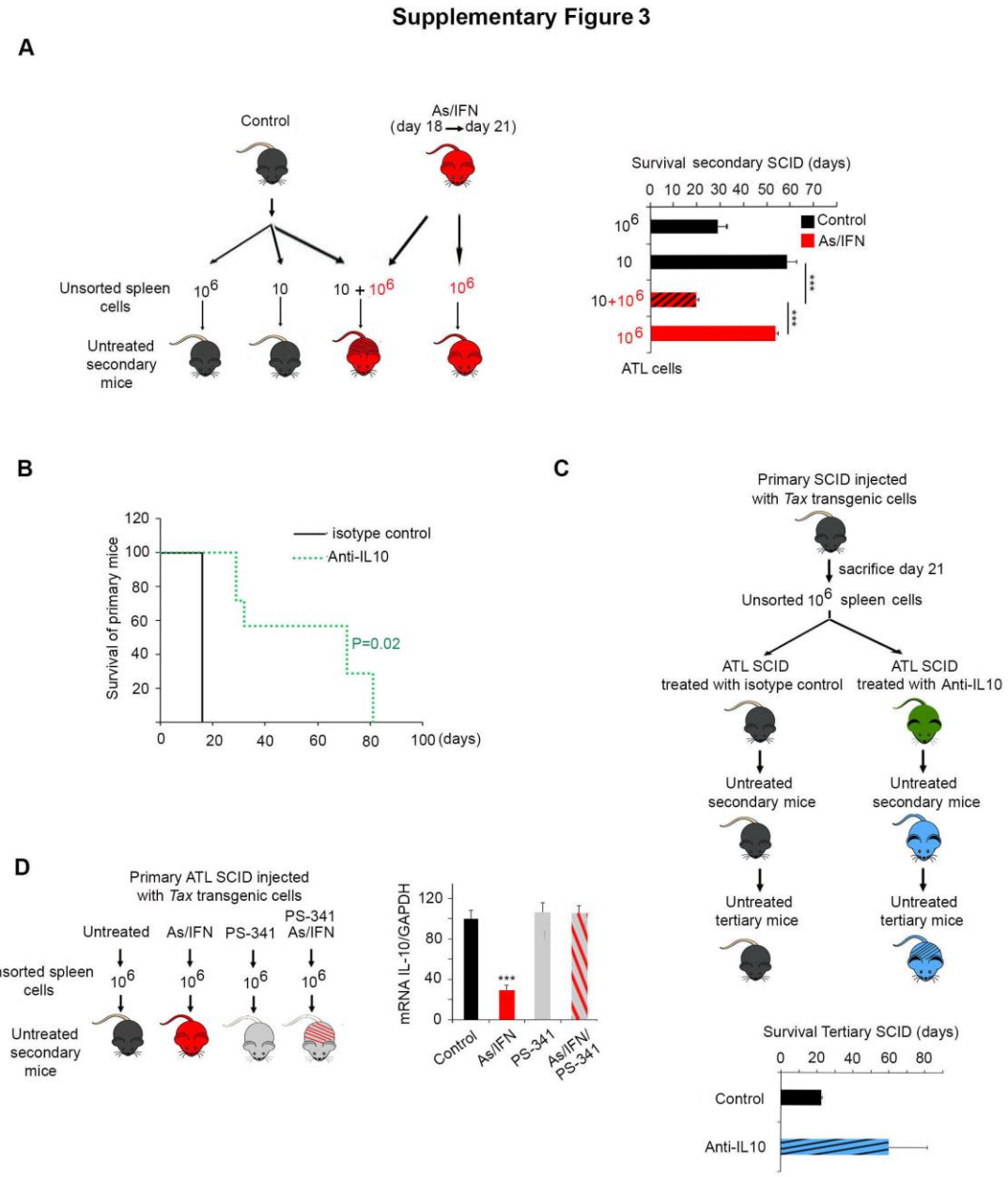


Supplementary Figure 1. A. Quantitative PCR for *tax* DNA and Tax mRNA levels in spleen-derived cells from untreated (black) or AS/IFNα-treated primary ATL mice (red) (n=3/condition). **B**. Quantitative PCR for *tax* DNA levels in spleen-derived cells from ATL SCID mice following sorting of $CD25^{-}$ (white histogram), $CD25^{+low}$ (crossed histograms), or $CD25^{+high}$ cells (dashed histograms) from untreated (control; black) or AS/IFNα-treated primary mice (red) (n=3/condition). **C.** Spleen weight of secondary SCID recipients injected with 10^6 sorted $CD25^{+low}$ (crossed histograms) or $CD25^{+high}$ (dashed histograms) cells derived from untreated (control; black) or AS/IFNα-treated primary ATL mice (red) (n=4/condition). **D.** Flow cytometry showing percent of murine CD25 expression in spleen-derived cells from untreated secondary SCID mice injected with 10^6 sorted $CD25^{+low}$ (crossed histograms) or $CD25^{+high}$ (dashed histograms) cells derived from untreated (control; black) or AS/IFNα-treated primary ATL mice (red) (n=4/condition). **E.** Quantitative PCR for *tax* DNA levels in spleen-derived cells from untreated secondary SCID mice injected with one million sorted $CD25^{+low}$ (crossed histograms) or $CD25^{+high}$ (dashed histograms) cells derived from untreated (control; black) or AS/IFNα-treated primary ATL mice (red) (n=4/condition). **F.** Spleen-derived cells from untreated (control) or AS/IFNα-treated primary ATL SCID mice were analyzed by flow cytometry for murine CD25 expression. Percent $CD25^{-}$ (white histogram), $CD25^{+low}$ (crossed histograms), or $CD25^{+high}$ (dashed histograms) cells from untreated (black) or AS/IFNα-treated primary mice (red) are shown as indicated (n=6/condition). **G.** Percent Annexin V positivity in sorted $CD25^{+}$ spleen-derived cells from untreated ATL SCID mice (control; black histograms) or AS/IFNα-treated primary mice (red histograms) (n=3/condition). **H.** Percent TUNEL positivity in sorted $CD25^{+}$ spleen-derived cells from untreated ATL SCID mice (control; black histograms) or AS/IFNα-treated primary mice (red histograms) (n=3/condition). **I.** Beta galactosidase assay and quantification of senescent cells in sorted $CD25^{+}$ spleen-derived cells from untreated ATL SCID mice (control; black histograms) or AS/IFNα-treated primary mice (red histograms) (n=3/condition). **J.** Tumor bulk (spleen weight x percentage of *tax* DNA in spleen cells) in untreated secondary mice injected with 10^6 spleen cells derived from either untreated (black) or AS/IFNα-treated (red) primary ATL mice (n=5/condition) and sacrificed at week 3. The t-test was performed to validate significance. *, ** and *** indicate *p* values ≤ 0.05; 0.01 and 0.001, respectively. *p*-values less than 0.05 were considered significant.

Supplementary Figure 2



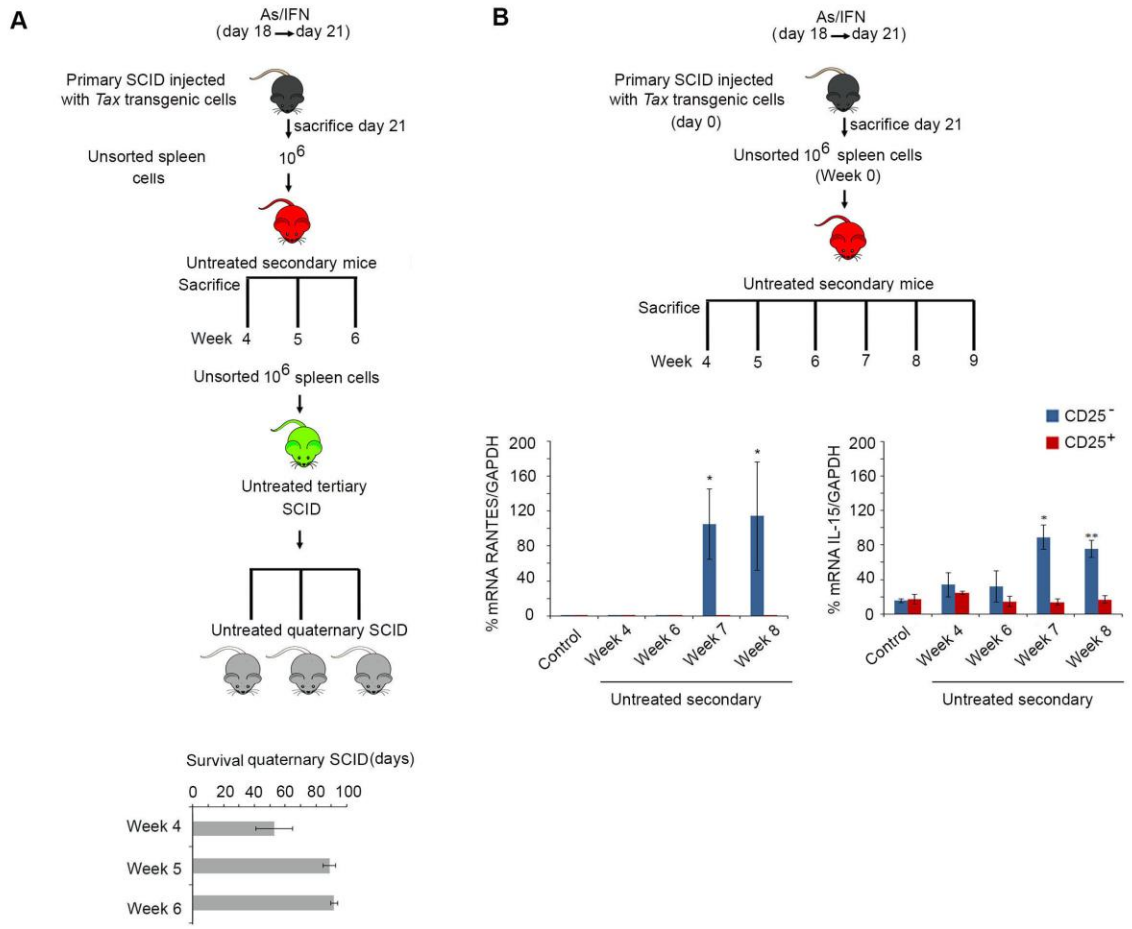
Supplementary Figure 2. A. Primary mice injected with 10^6 ATL cells were treated with AS/IFNα as described in Fig. 1A then sacrificed. Spleen-derived cells were sorted using the murine CD25 and/or the CD335, CD11b and F4/80 markers. Kaplan–Meier curves represent survival of untreated secondary SCID mice injected with one million $CD25^+$ cells from untreated (control; n=5, black line) or AS/IFNα-treated primary mice (n=5, red line); survival of untreated secondary SCID mice injected with one million $CD25^+$ cells from untreated primary mice and 25,000 $CD25^-CD335^+$ cells from AS/IFNα-treated primary mice (n=3, dashed black lines); survival of untreated secondary SCID mice injected with one million $CD25^+$ cells from AS/IFNα-treated primary mice and 25,000 $CD25^-CD335^+$ cells from AS/IFNα-treated primary mice (n=3, dashed red lines); survival of untreated secondary SCID mice injected with one million $CD25^+$ cells from untreated primary mice and 25,000 $CD25^-CD11b^+F4/80^+$ cells from AS/IFNα-treated primary mice (n=4, spotted black lines); and survival of untreated secondary SCID mice injected with one million $CD25^+$ cells from AS/IFN-treated primary mice and 25,000 $CD25^-CD11b^+F4/80^+$ cells from AS/IFNα-treated primary mice (n=4, spotted red lines). **B.** Secondary SCID mice injected with 10^6 spleen cells from untreated (control) or AS/IFNα-treated primary SCID mice were treated with Clodronate or empty liposomes as indicated. Macrophage depletion was verified by flow cytometry using $CD25^-CD11b^+F4/80^+$ markers (n=2 or n=3, Left graph). Kaplan–Meier survival curves of secondary SCID mice injected with 10^6 spleen-derived cells from untreated (black) or AS/IFNα-treated primary mice (red) and treated with empty liposomes (lines) or Clodronate (dashed lines) (n=5, right graph). **C.** Secondary SCID mice injected with 10^6 spleen cells from untreated (control) or AS/IFNα-treated primary ATL SCID mice were treated with anti-NK1.1 or mouse IgG isotype control as indicated. NK depletion was verified by flow cytometry using $CD25^-NK1.1^+$ markers. Kaplan–Meier survival curves of secondary SCID mice injected with 10^6 spleen-derived cells from untreated (black) or AS/IFNα-treated primary ATL mice (red) and treated with mouse IgG isotype control (lines) or anti-NK1.1 (dashed lines) (n=5/condition).



Supplementary Figure 3. A. Survival of untreated secondary SCID mice injected with 10^6 or 10 cells from spleens of untreated primary mice (control; n=3, black histograms), 10^6 cells from primary ATL mice treated with AS/IFNα for 3 days (n=3, red histogram), or 10 cells from untreated primary mice together with 10^6 cells from primary ATL mice treated with AS/IFNα for 3 days (n=3, red-black dashed histogram). Histograms represent data using untreated mice injected with cells from one original *tax* transgenic and treated mice injected with cells from another original *tax* transgenic. **B.** Kaplan–Meier survival curves of primary SCID mice injected with 10^6 spleen-

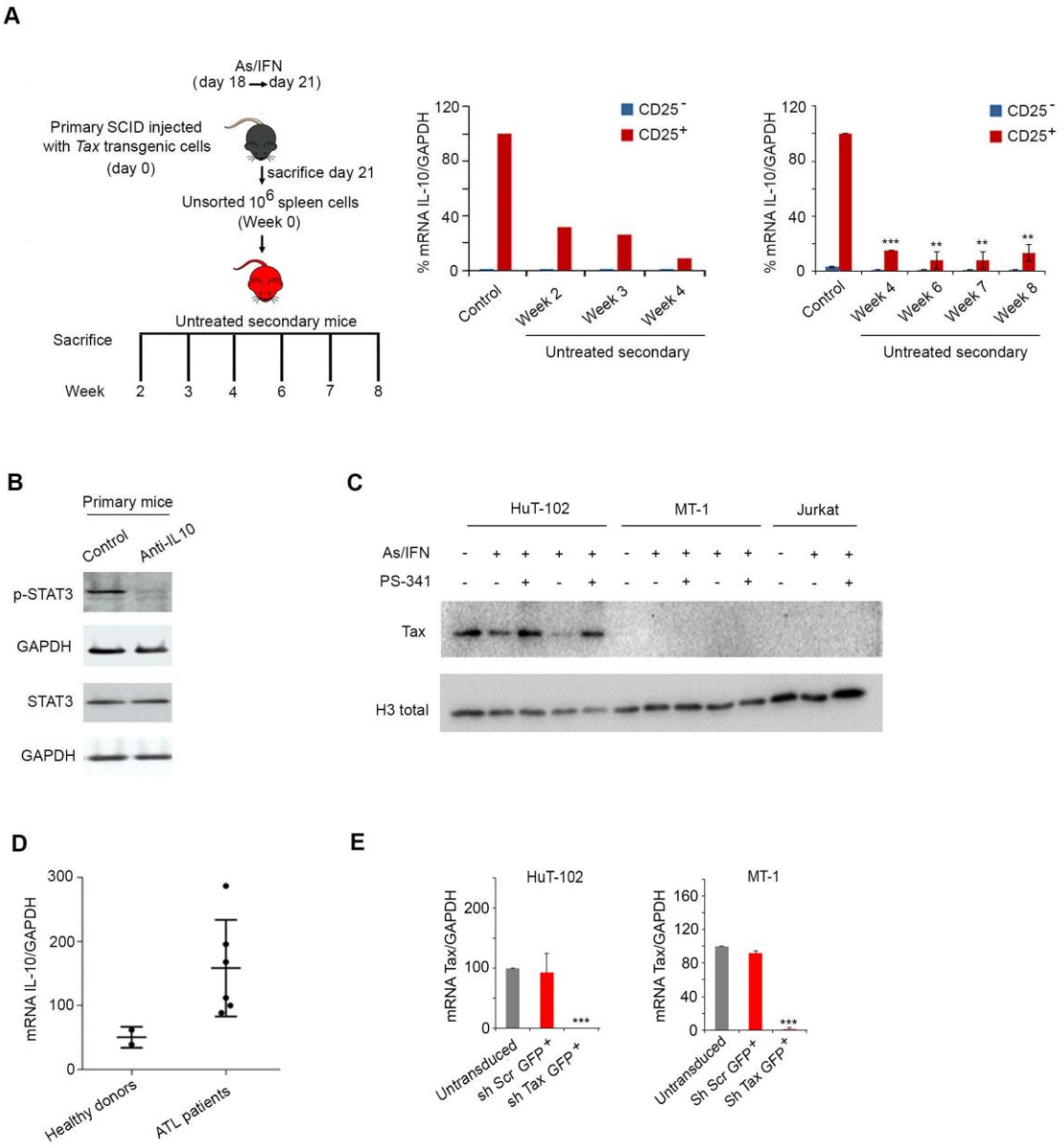
derived cells and treated with anti-IL-10 antibodies (green dashed line) or control isotype (n=7, black line) (see experimental design in Figure 2G). **C.** Secondary SCID recipients were injected with 10^6 spleen-derived cells derived from primary SCID mice treated with anti-IL-10 antibodies or isotype control. Survival of tertiary recipient SCID mice injected with 10^6 spleen-derived cells derived these secondary untreated mice (n=6/condition). **D.** Quantitative RT-PCR for IL-10 mRNA levels in spleen-derived cells of SCID mice injected with 10^6 spleen cells and left untreated (black histogram, n=2), or treated with AS/IFNα for 3 days (red histogram, n=4), the proteasome inhibitor PS-341 for one week (gray histogram, n=2), or the combination of AS, IFNα and PS341 (gray/red dashed histograms, n= 2). The t-test was performed to validate significance. *, ** and *** indicate p values ≤ 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

Supplementary Figure 4



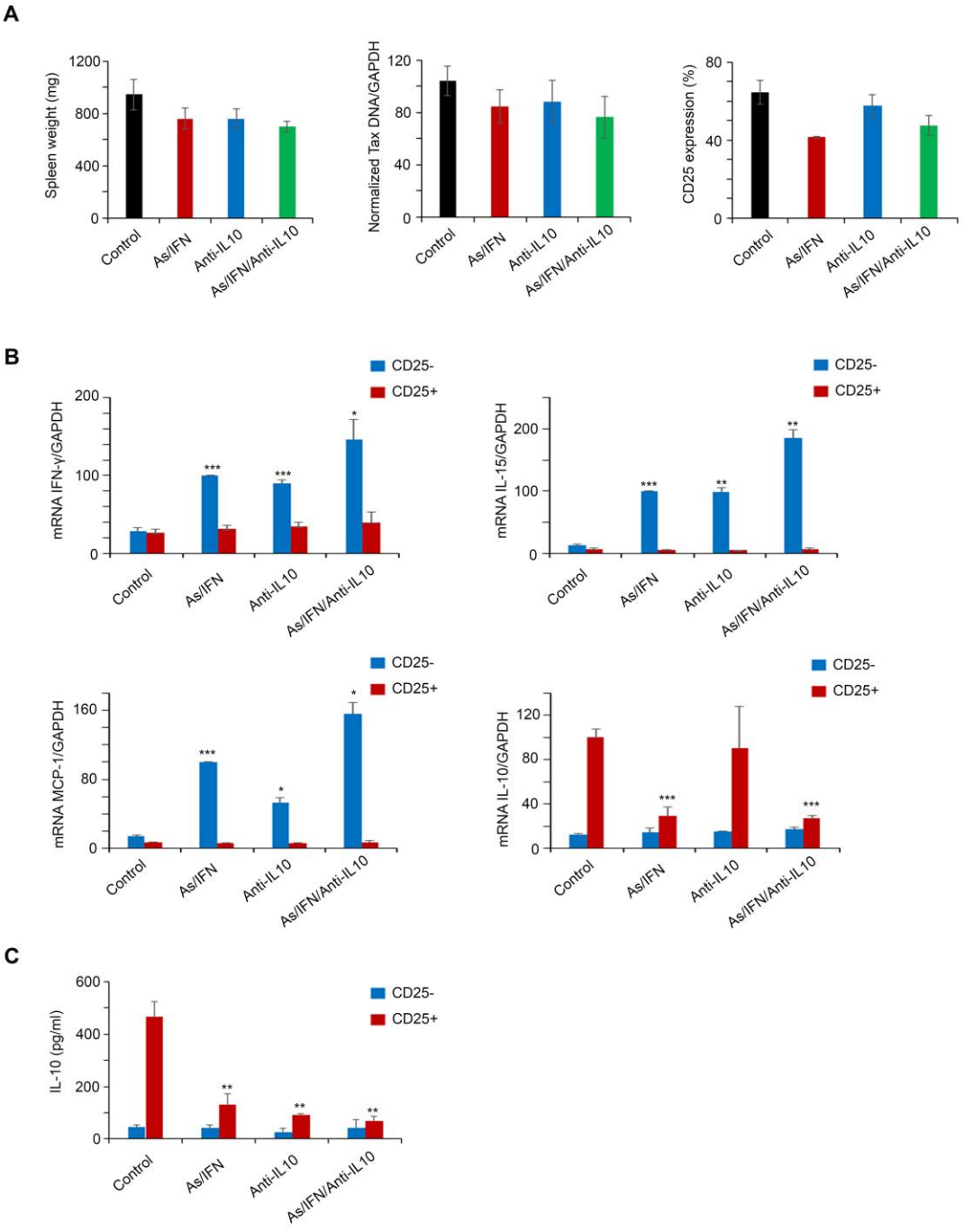
Supplemental Figure 4. A. Primary ATL SCID mice were treated with AS and IFNα for 3 days then sacrificed. One million spleen-derived cells were injected into secondary SCID mice that were left untreated and sacrificed on weekly basis (Week 4 to Week 9). One million spleen cells derived from weekly sacrificed untreated secondary mice were injected into tertiary SCID mice (n=10 per week) that were left untreated. One million spleen cells from tertiary mice who died of ATL were injected into quaternary SCID mice (n=5 per week). The weeks refer to the time of sacrifice of the untreated secondary mice. Histograms represent the survival of untreated quaternary SCID mice. **B.** Primary mice were treated with AS and IFNα for 3 days as described above. One million spleen-derived cells were injected into secondary SCID mice that were left untreated and sacrificed on weekly basis (Week 4 to Week 8) (n=3 per condition). Quantitative RT-PCR for RANTES and IL-15 mRNA levels in homogenized spleen-derived sorted $CD25^+$ (red histograms) and $CD25^-$ cells (blue histograms) from weekly (Week 4-Week 8) sacrificed untreated secondary mice injected with 10^6 spleen cells from AS/IFNα treated primary ATL mice.

Supplementary Figure 5



Supplementary Figure 5 A. Quantitative RT-PCR for IL-10 mRNA levels in spleen-derived sorted $CD25^+$ (Red histograms) and $CD25^-$ cells (Blue histograms) from weekly (Week 2-4) sacrificed untreated secondary mice injected with 10^6 spleen cells from AS/IFNα treated primary mice (Each condition represents pooled cells from 3 mice) (middle) and from weekly (Week 4-Week 8) sacrificed untreated secondary mice injected with 10^6 spleen cells from AS/IFNα-treated primary mice (right). Results show a selective production of IL-10 by the $CD25^+$ ATL cells and a sharp decrease of IL-10 transcripts in untreated secondary mice. The t-test was performed to validate significance. *, ** and *** indicate p values ≤ 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant. **B.** Western blot for STAT3 protein levels in cells from primary ATL SCID mice treated with anti-IL-10 antibodies. **C.** Western blot analysis of Tax in ATL-derived (HuT-102 and MT-1) or HTLV-1 negative (Jurkat) cell lines after treatment with AS/IFNα alone or together with the proteasome inhibitor PS-341 for 24h. **D.** Transcript levels of hIL-10 in freshly isolated peripheral blood mononuclear cells from two healthy donors and six patients with ATL. **E.** Quantitative RT-PCR for Tax mRNA levels in ATL-derived cell lines (HuT-102 and MT-1). Histograms correspond to un-transduced (Grey), sorted GFP^+ ShScr (Red) or ShTax (red) transduced cells as indicated. Results show a selective downregulation of hIL-10 transcripts in ATL-derived cell lines upon knock down of Tax (2 independent experiments). The t-test was performed to validate significance. *, ** and *** indicate *p* values ≤ 0.05; 0.01 and 0.001, respectively. *p*-values less than 0.05 were considered significant.

Supplementary Figure 6



Supplementary Figure 6. **A.** Spleen weight (left panel) of primary ATL SCID mice left untreated (Black histograms) or treated with AS/IFNα (Red histograms), an anti-IL-10 antibody (Blue histogram), or the triple combination AS/IFNα/anti-IL-10 antibody (Green histogram) (n=5 per condition). Quantitative PCR for *tax* DNA levels in spleen-derived cells (middle panel) from primary ATL SCID mice left untreated (Black histograms) or treated with AS/IFNα (Red histograms), an anti-IL-10 antibody (Blue histogram), or the triple combination AS/IFNα/anti-IL-10 antibody (Green histogram) (n=3 per condition). Flow cytometry showing murine CD25 expression (right panel) in spleen-derived cells from primary ATL SCID mice left untreated (Black histograms) or treated with AS/IFNα (Red histograms), an anti-IL-10 antibody (Blue histogram), or the triple combination AS/IFNα/anti-IL-10 antibody (Green histogram) (n=3 per condition). **B.** Quantitative RT-PCR for IFN-γ, IL-15, MCP-1 and IL-10 mRNA levels in sorted $CD25^+$ (Red histograms) or $CD25^-$ (Blue histograms) spleen-derived cells from primary ATL SCID mice untreated or treated with AS/IFNα, an anti-IL-10 antibody, or the triple combination AS/IFNα/anti-IL-10 antibody (n=3 per condition). **C.** IL-10 levels determined by ELISA in the supernatant of homogenized spleen-derived $CD25^+$ and $CD25^-$ sorted cells from primary ATL SCID mice untreated or treated with AS/IFNα, an anti-IL-10 antibody, or the triple combination AS/IFNα/anti-IL-10 antibody (n=3 per condition). The t-test was performed to validate significance. *, ** and *** indicate p values ≤ 0.05; 0.01 and 0.001, respectively. *p*-values less than 0.05 were considered significant.