PNAS
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Supplementary Information for

Tyrosyl-tRNA synthetase stimulates thrombopoietin-independent hematopoiesis accelerating recovery from thrombocytopenia

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www.pnas.org/cgi/doi/10.1073/pnas.1807000115

Supplementary Information Text
SI Materials and Methods

Animal experiments. C57BL/6 wild type, IL-6$^{-/-}$ and MyD88$^{-/-}$ mice were obtained from The Jackson Laboratory. C-mpl (the human homologue of an oncogene from the mouse myeloproliferative leukemia virus) knockout mice were kindly provided by Warren S. Alexander (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). For injection studies, 6 to 8 week-old mice received 3-30 mg/kg purified recombinant YRS^{ACT}; blood cells were counted with the IDEXX ProCyte^{TM} (IDEXX Laboratories Inc., Westbrook, ME). To induce acute thrombocytopenia, 4 mg/kg of anti-GPIbα monoclonal antibody (5A7) was administered by retro-orbital injection.

Mouse BM cell culture. BM cells collected from mouse femurs and tibias were washed and resuspended at a concentration of $2.5 \cdot 10^7$ cells in 10 mL of Iscove modified Dulbecco medium (Life Technologies, Carlsbad, CA) supplemented with 15% fetal bovine serum (Life Technologies). Cells were cultured at 37°C in a humidified chamber with 5% CO_2 for 3 days and analyzed by flow cytometry. For cell enumeration, the instrument was calibrated using fluorescent microbeads (Bangs laboratories, USA).

Megakaryocyte ploidy analysis. *In vitro* cultured BM cells were harvested after 3 days of culture and culture supernatants were analyzed by ELISA to determine cytokine levels. Cells, both in suspension and adherent, were collected using 0.25% trypsin/EDTA and washed once before fixing in 1% parafolmaldehyde at 4°C for 2 hours. For analysis of BM cells freshly isolated from YRS^{ACT}-injected animals, BM cells were harvested from femurs and after centrifugation directly fixed in 1% parafolmaldehyde at 4°C for 2 hours. Cells were then washed and resuspended in PBS, incubated 10 minutes at room temperature with 1µg/mL Brilliant Violet 421^{TM} anti-mouse CD41 antibody (Biolegend, San Diego, CA) and 100

μg/mL propidium iodide (Sigma-Aldrich, St.Louis, MO). The volume of each sample was adjusted to 300μl and analyzed by flow cytometry using a LSRII Flow cytometer (Becton Dickinson, Franklin Lakes, NJ) or NovoCyte (ACEA Biosciences, San Diego, CA). Megakaryocytes were identified as $CD41^+$ cells and megakaryocyte number/ploidy was analyzed for 4 minutes.

Establishment and maintenance of a mouse hematopoietic progenitor cell line ($CD41^+$Lhx2). The retroviral vector expressing Lhx2 (pMY.FLAG.Lhx2; a gift of Dr. Kenji Kitajima, Tokyo Metropolitan Organization for Medical Research) was transfected into Plat-E cells using Lipofectamine LTX (Invitrogen) following manufacturer's instructions. $CD41^+$ cells - isolated from WT mouse BM cells using a PE-labeled anti-CD41 antibody (MWReg30) and anti-PE antibody-conjugated magnetic immunobeads (Miltenyi Biotec) - were transferred onto a retrovirus-preloaded RetroNectin-coated 12-well plate. After overnight culture with IMDM containing 15% FBS, 50 ng/mL mSCF, 20 ng/mL mTPO, 20 ng/mL mIL-6, the $CD41^+$ cells were seeded onto hTERT stromal cells (1).

Preparation of hPBMCs stimulated by YRS^{ACT}. Mononuclear cells were separated by density gradient centrifugation using Hitopaque1077 (Sigma-Aldrich) following manufacturer's instructions. For culture supernatant transfer experiment, hPBMCs seeded at $1.5 \cdot 10^6$ cells/mL were cultured in the presence of 200 nM YRS^{ACT} or control vehicle (PBS) for 2 days; culture supernatants were then collected for transfer to $CD41^+$Lhx2 cells in culture.

Isolation of $CD34^+$ cell from hPBSCs and differentiation into megakaryocytes. Peripheral blood stem cells were harvested by leukapheresis and cryopreserved as previously described (2). After thawing, the cells were cultured for 3-4 days in IMDM containing 15% FBS with 50 ng/mL SCF, 50 ng/mL TPO and 50 ng/mL Flt3 ligand; dead cells were removed

using Histopaque. $CD34^+$ cells were isolated using the MACS CD34 progenitor cell isolation kit (Miltenyi Biotec). Using 24 well plates, $1\text{-}2\cdot10^4$ $CD34^+$ cells were cultured with 1 mL of hPBMC supernatants pre-cultured for 2 days in the presence or absence of 200 nM YRS^{ACT}. On day 4, 1 mL of the supernatant was added to the cells, which were then analyzed on day 7.

***In vitro* generation of megakaryocytes from iPSCs.** Megakaryocyte differentiation was performed as previously reported with minor modifications (3). Small clumps of hiPSCs were transferred onto mitomycin-treated C3H10T1/2 cells and cultured for 14 days with hematopoietic cell differentiation cytokines including 20 ng/mL recombinant human VEGF. Culture medium was changed every 2-3 days and hematopoietic cells were generated via "sac-like" structures (iPS-sacs). On day14, $CD34^+$ cells were isolated by sorting and these cells were transferred onto mitomycin-treated C3H10T1/2 cells for further differentiation culture (at $1\cdot10^5$ cells/mL) in 1ml of medium containing 50 ng/mL human TPO, 50 ng/mL human SCF, and 25 U/mL heparin sodium in the presence or absence of 200 nM YRS^{ACT}. Fresh medium containing cytokines with or without YRS^{ACT} was added on day18 and cells were analyzed on day 23. For hPBMC culture supernatant transfer experiments, $CD34^+$ cells isolated on day 14 were seeded at $1\cdot10^5$ cells/mL and cultured with 1 mL of culture supernatant of hPBMCs exposed or not to 200 nM YRS^{ACT}. Supernatants were added on day 18 and cells analyzed on day 23.

Confocal analysis of BM cultured cells.

BM cells were cultured on 0.5% gelatin-coated coverslips and fixed with 2% paraformaldehyde at room temperature for 5 minutes. The slides were washed 2 times with PBS and then permeabilized with 0.2% Triton X-100 in PBS for 1 min at room temperature.

After incubation with 5% goat serum in PBS for 30 minutes, cells were stained with rabbit anti-F4/80 antibody (SP115), rat anti- Sca-1 antibody (E13), mouse anti-human GPIbα antibody (LJ1b1), followed by incubation with secondary antibodies of AlexaFluor647-labeled goat anti-rabbit IgG, AlexaFluor 488-labeled goat anti-rat IgG and AlexaFluor566-labeled goat anti-mouse IgG. Fluorescent signals were detected using laser scanning confocal microscope (Zeiss LSM 710, Zeiss, Oberkochen, Germany) and a 63x oil Plan Apo, 1.4na DIC.

Preparation of recombinant YRS^{ACT} protein. All plasmids were constructed utilizing standard site directed mutagenesis PCR using Pfu Ultra II polymerase (Agilent Technologies). The yeast SUMO gene with an N-terminal 6xHis tag was fused onto the N-terminus of each YRS construct in the pET28a vector (EMD Millipore)(4). Each construct was transformed into BL21-CodonPlus (DE3)-RIPL cells (Agilent Technologies) for protein expression. Six colonies were used to inoculate six different 3 L LB cultures overnight at 37°C for a total culture volume of 18 L. Once a 3 L culture reached an OD_{600} =0.6, it was put in an ice bath for 30 minutes and then induced at room temperature with 0.5 mM IPTG for 5 hours. From this point forward, all procedures were done on ice or at 4°C. Cells were collected by centrifugation and resuspended in 150 mL of binding buffer (10% glycerol, 20 mM Tris-HCl pH 7.9, 500 mM NaCl, 15 mM imidazole, 5 mM β-mercaptoethanol). The resuspended cells were lysed using a Microfluidics cell homogenizer and centrifuged at 30,000 g for 30 min. The lysate supernatant was poured over a column (12 mL resin bed volume) of Ni-NTA resin (Qiagen). The column was washed with 500 mL of wash buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 40 mM imidazole, 5 mM β-mercaptoethanol) and proteins were eluted with 30 mL of elution buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 350 mM imidazole, 5

mM β-mercaptoethanol). Following elution, the SUMO protease ULP1 was added to the elution to cleave off the 6xHis-SUMO tag. This protein mixture was buffer exchanged into PBS with 5 mM β-mercaptoethanol using a desalting column (GE Healthcare) and an ÄKTA Purifier. After desalting, proteins were concentrated and then separated using a Superdex 200 16/600 prep grade column (GE Healthcare) to fractionate out the SUMO tag, ULP1 protease, and aggregate proteins. Purified YRS protein was then poured over high capacity endotoxin removal resin (Pierce) and then passed through Acrodisc Mustang E membranes (Pall). Final endotoxin levels were tested using the Endosafe-PTS system (Charles River) and confirmed to be below 4.0 EU/mg. The buffer of purified protein was exchanged against PBS using a desalting column. Aliquots were frozen in liquid nitrogen and stored at -80°C until used.

Measurement of IL-6. Human PBMCs or THP-1 cells were seeded at $1 \cdot 10^6$ cells/mL in RPMI 1640 containing 10% FBS and treated with various concentration of YRS^{ACT} for 24 hours. After treatment, supernatants were collected and secreted IL-6 levels were measured by ELISA using Human IL-6 Quantikine ELISA Kit (R & D Systems). IL-6 levels in mouse BM culture supernatants were measured by ELISA using Mouse IL-6 Quantikine ELISA Kit (R & D Systems).

Immunoprecipitation and Western blotting. Recombinant YRS^{ACT} was incubated with purified His-tagged TLR2 or TLR4 (R & D Systems, 10 μg/mL, each) in 500 μl PBS containing 0.5% NP-40 for 1 hour at 4°C, followed by incubation with rabbit polyclonal antibody against human YRS or rabbit anti-IgG control (Cell Signaling) for 1 hour at 4 °C. Protein G-coated Sepharose beads were then added and incubated for 1 additional hour at 4 °C. After washing the beads thrice with 1ml of PBS-1% NP-40, gel-bound proteins were

eluted and subjected to Western blot analysis using rabbit anti-His antibodies (ICL Laboratory) to detect TLR2 and TLR4 or rabbit anti-YRS antibody.

Statistical analysis. Statistical analysis was performed with GraphPad Prism 7 (GraphPad Software. Inc., San Diego, CA). Decisions on the models used were based on the nature of the data, distributions, homogeneity of variance and other statistically validated criteria.

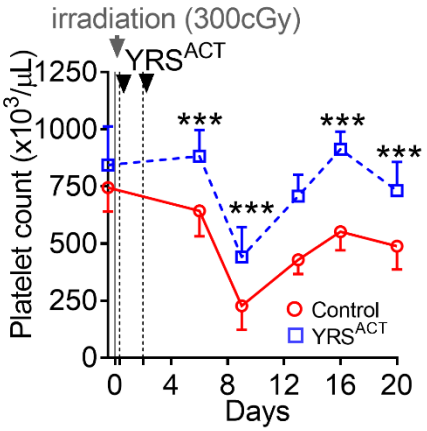

Fig. S1. YRS^{ACT} ameliorates irradiation-induced thrombocytopenia. YRS^{ACT} or PBS as control was administered to WT mice twice at 8 and 48 hours after 300cGy of irradiation (n = 4 in each group). Data are shown as mean ± 95% CI, ***$P < 0.001$ determined by two-way ANOVA with Sidak's multiple comparison test.

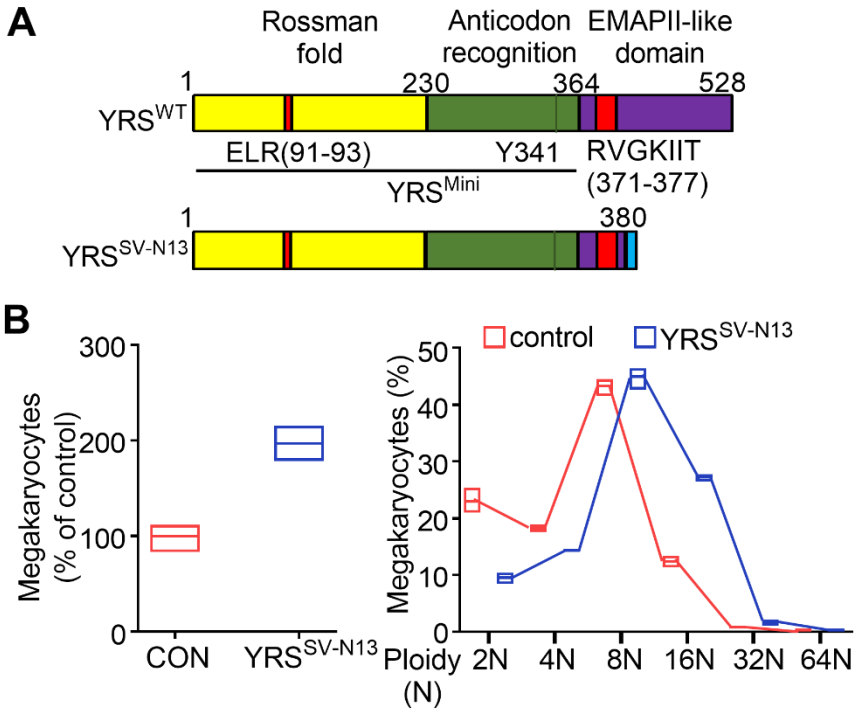

Fig. S2. Human YRS splicing variant that lacks C-terminal domain has thrombopoietic activity similar to YRS^{ACT}. (A) $YRS^{SV\text{-}N13}$ (also referred as AS10) is one of the previously reported YRS splicing variants that skip exon 11, leading to a frameshift and premature stop codon. (B) Pooled BM cells from three WT mice were cultured with added $YRS^{SV\text{-}N13}$ (500 nM) or PBS (CON) for 3 days and analyzed for MK number and ploidy distribution by flow cytometry. Technical triplicates of each condition are shown as min to max floating bars with mean. In the right panel, color-coded lines join the mean values of each ploidy distribution.

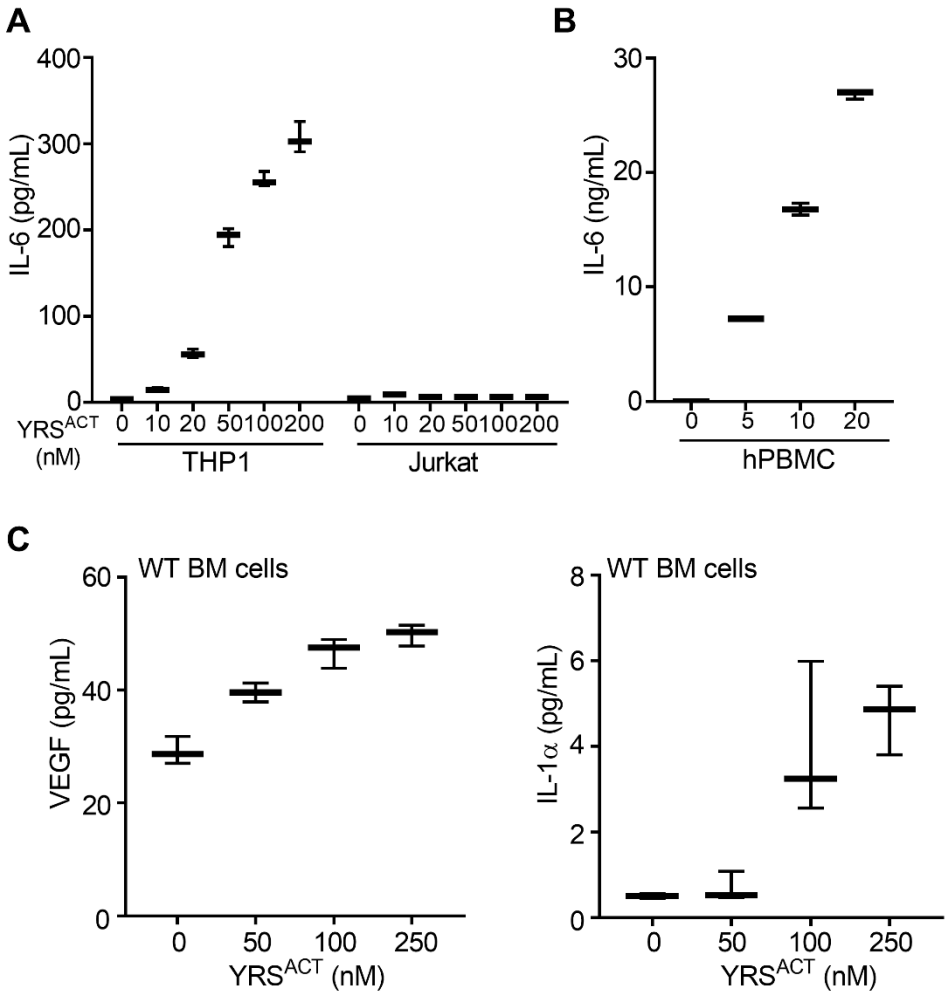

Fig. S3. YRS^{ACT} dose-dependent effect on cytokine secretion by monocytic cells. (A) THP1 and Jurkat cells and (B) hPBMCs were treated with different doses of YRS^{ACT}, after which IL-6 secreted into supernatants was measured by ELISA. (C) WT mouse BM cells were treated with different doses of YRS^{ACT} and secreted VEGF (left) and IL-1α (right) were measured by ELISA in culture supernatants. Results are shown as 25^{th}-75^{th} percentile bars with median and min to max whiskers.

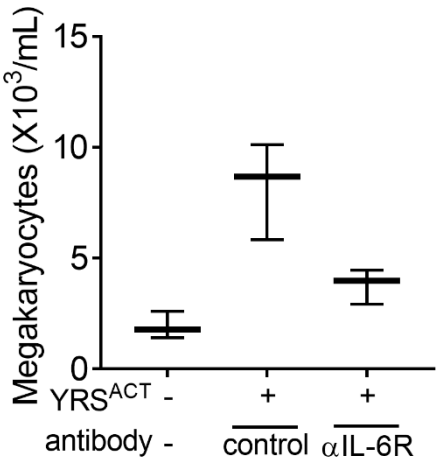

Fig. S4. Role of IL-6 in YRS^{ACT}-induced megakaryocyte expansion. WT mouse BM cells were treated with 100 nM YRS^{ACT} in the presence of IL-6 receptor blocking antibody or control IgG. Cells were harvested after 3 days of culture and analyzed by flow cytometry. The results of technical triplicates are shown for each experiment as 25^{th}-75^{th} percentile bars with median and min to max whiskers.

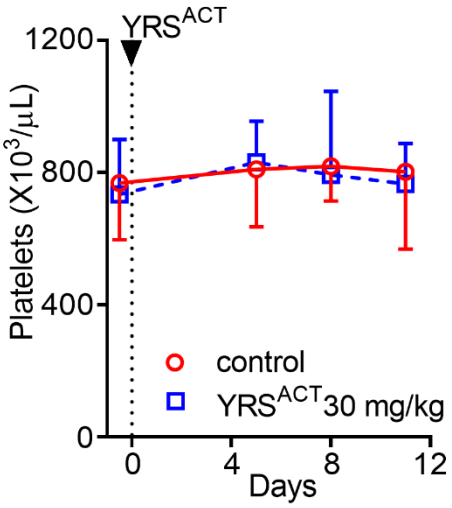

Fig. S5. YRS^{ACT} does not enhance platelet production in MyD88$^{-/-}$ mice. MyD88$^{-/-}$ mice (n = 4) were given a single intravenous dose of YRS^{ACT} (30 mg/kg) and platelet counts were monitored for 12 days after the injection. Data are shown as mean ± 95% CI.

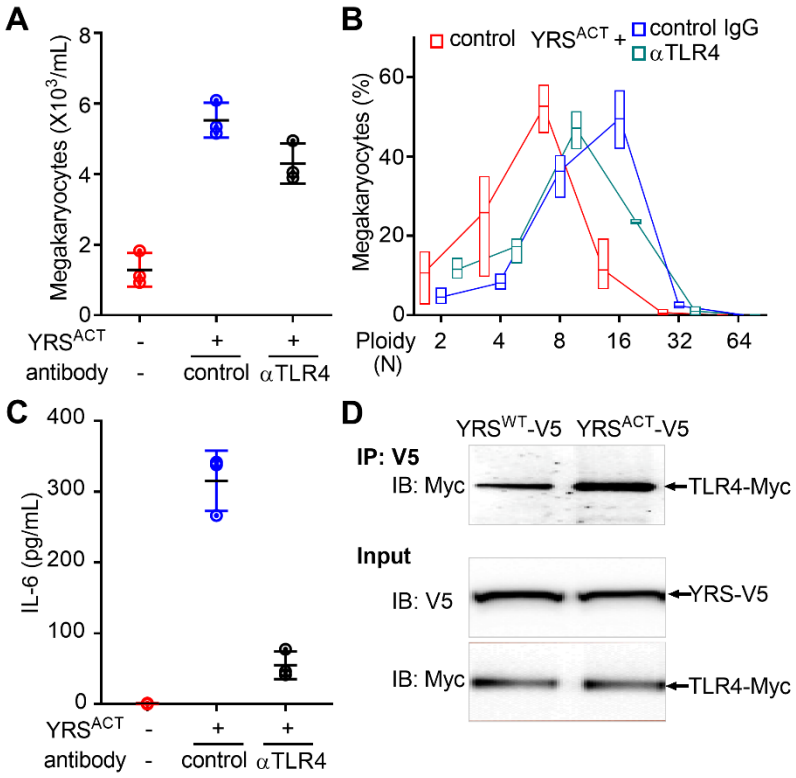

Fig. S6. Effect of YRS^{ACT} is mediated not only by TLR2 but also by TLR4. (A,B) BM cells from $TLR2^{-/-}$ mice were treated with 100 nM YRS^{ACT} in the presence of an anti-TLR4 blocking antibody or control IgG. Cells were harvested after 3 days of culture and analyzed for MK count and ploidy distribution. (C) Culture supernatants were collected for cytokine measurement. The results of technical triplicates are shown as dot plot with mean ± S.D (A,C) or min to max floating bars with mean (B); in the latter panel, color-coded lines join the mean values of each ploidy distribution. (D) V5-tagged YRS^{WT} or YRS^{ACT} and c-Myc-tagged TLR4 were co-transfected into HEK293 cells. Cell lysates were immunoprecipitated with anti-V5 antibody and immunoblotted with antibodies against c-Myc (top panel). Immunoblotting of total cell lysate is shown as input in the lower panels.

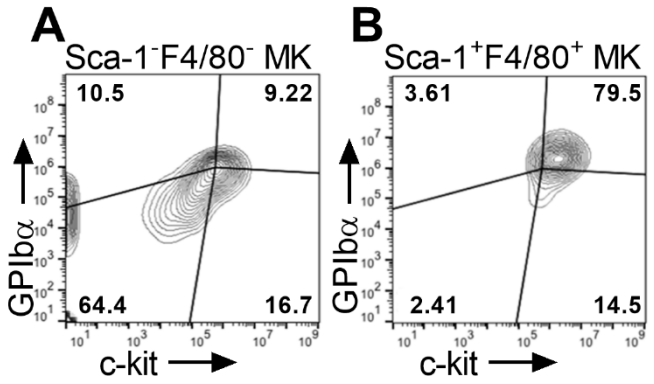

Fig S7. Sca-1^{+}F4/80^{+} MKs express-kit as well as late stage mature MK marker, GPIbα. Sca-1^{+}F4/80^{+} and Sca-1^{-}F4/80^{-} MKs (4.21% and 77.9%, respectively, see Figure 3D) identified in the BM of WT mice were analyzed for the expression of c-kit and GPIbα.

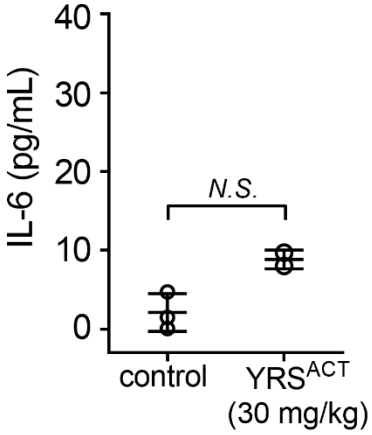

Figure S8. Effect of YRS^{ACT} administration on plasma IL-6 levels. WT mice received an intravenous injection of YRS^{ACT} (30 mg/kg; n = 2) or PBS control (n = 3). After 3 days, plasma samples were collected for IL-6 measurement by ELISA. Data are shown as dot plot with mean ± S.D; *P* value calculated by Mann-Whitney two-tailed unpaired t test.

Cytokines/Chemokines	Fold
Monocyte Chemotactic Protein-5	73.7
Interleukin-6	54.1
Interferon gamma	>19.3
Monocyte Chemotactic Protein-3	17.1
Monocyte Chemotactic Protein-1	12.0
Granulocyte-Macrophage Colony-Stimulating Factor	>9.1
Interleukin-10	>5.9
Interleukin-1 alpha	4.4
Macropahge Colony-Stimulating Factor	3.4
Factor VII	2.7
Vascular Endothelial Growth Factor A	2.1

Supplementary Table 1. YRS^{ACT} induces production of cytokines/chemokines by mouse BM cells cultured *in vitro*. Supernatants of WT mouse BM cells cultured in the presence or absence of YRS^{ACT} were analyzed for changes in expression of 59 different proteins by RodentMap array analysis (Myriad RBM). Data are presented as n-fold increase.

References

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