Supplemental Information:

Calcineurin/NFAT signaling inhibits myeloid hematopoiesis.

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Supplemental Figures:

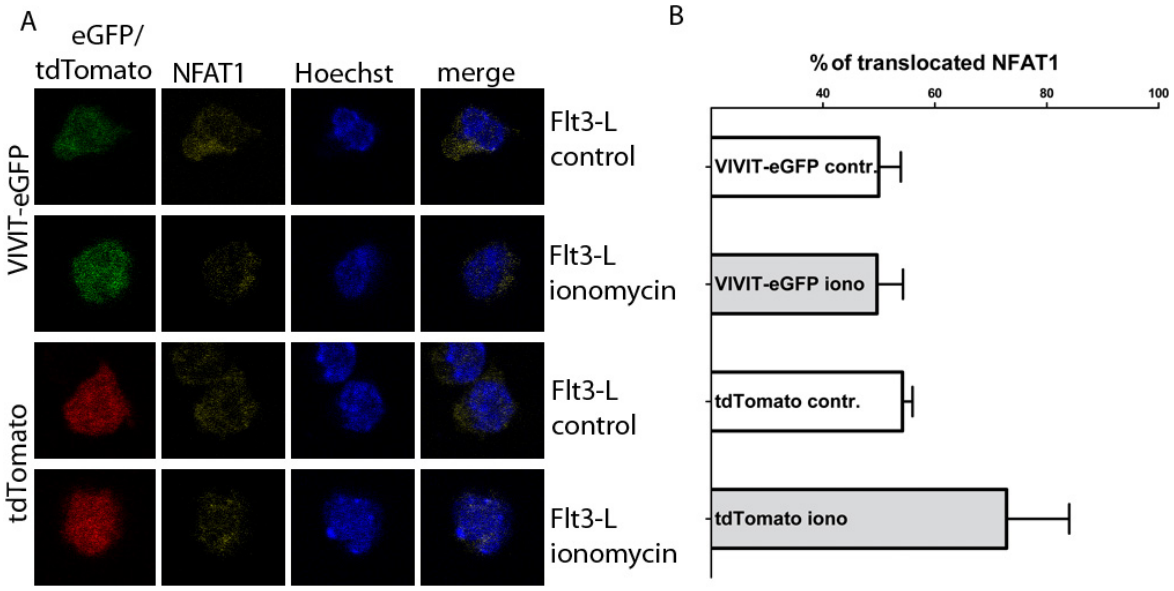


Figure S 1A, B: NFAT1 expression and translocation in HSC lines. Confocal images of VIVIT-eGFP and control-tdTomato expressing HSC lines stimulated for 48h with Flt3-L. NFAT translocation was induced by treatment with ionomycin for 2h (original magnification, x200). Intensity of NFAT signals from confocal images as quantified for Figure 1D and S1A using TissueQuest software and used to calculate the percentage of NFAT located in the nucleus

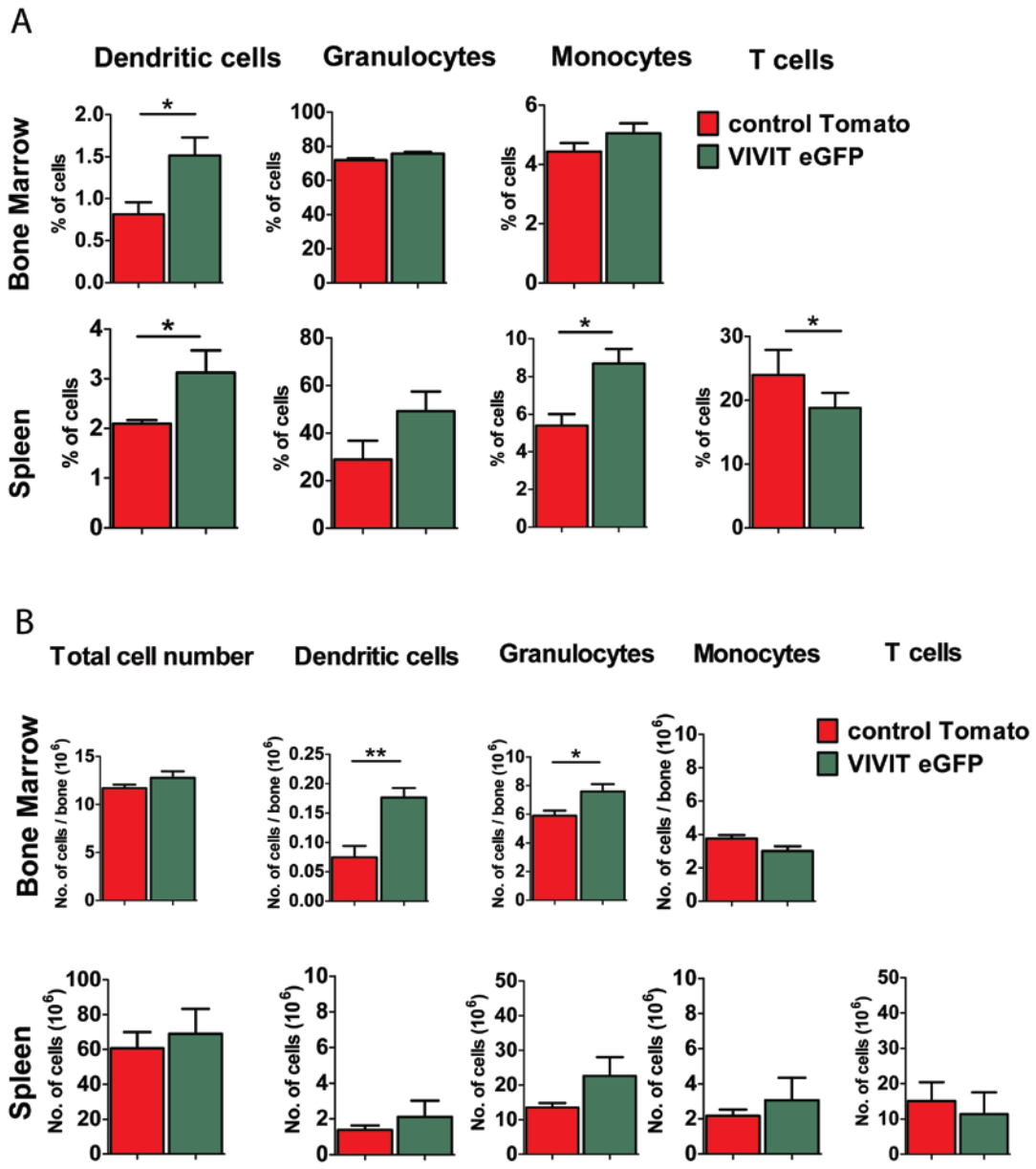


Figure S2A,B: Calcineurin/NFAT inhibition in HSC increase the numbers of cells in myeloid pool. Host mice were lethally irradiated and rescued by intravenous injection of HSC. HSC populations comprised cells expressing either VIVIT or a control plasmid with a fluorescent tag, mice were analyzed 8 weeks after engraftment. The percentage (S2A) or total numbers (S2B) of DC ($CD11c^{+}MHCII^{+}$), granulocytes ($CD11b^{+}Gr\text{-}1^{+}$), monocytes ($CD11b^{+}Gr1^{-}$) and T cells ($CD3^{+}$) in BM (upper panel) and spleen (lower panel) is shown.

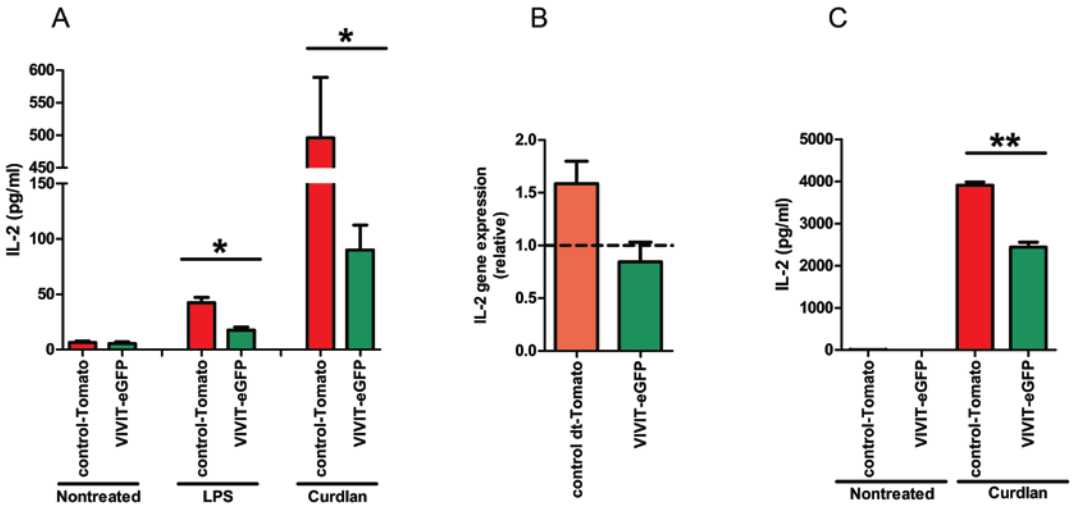


Figure S3: **Expression of VIVIT peptide inhibits IL-2 up-regulation induced in DCs by LPS or curdlan**. **(A)** IL-2 protein concentration and **(B)** mRNA levels were determined 24h (protein) and 4h (mRNA) after LPS or curdlan stimulation of splenic $CD11c^+$ cells. $CD11c^+$ cells were isolated from spleens 8 weeks after mouse reconstitution with VIVIT-eGFP or control-tdTomato HSC. **(C)** IL-2 protein expression determined 24 h after curdlan stimulation of DCs derived from VIVIT-eGFP and tdTomato expressing HSC lines in Flt3-L supplemented medium.

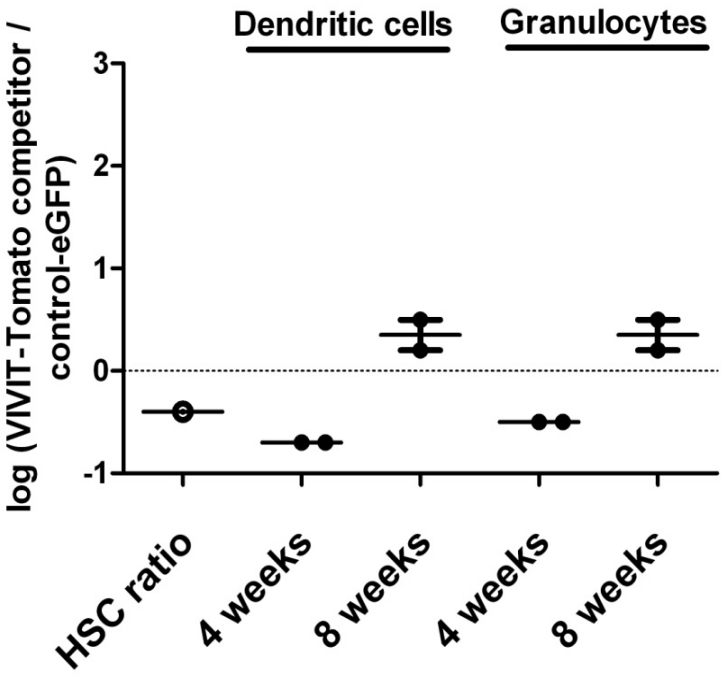
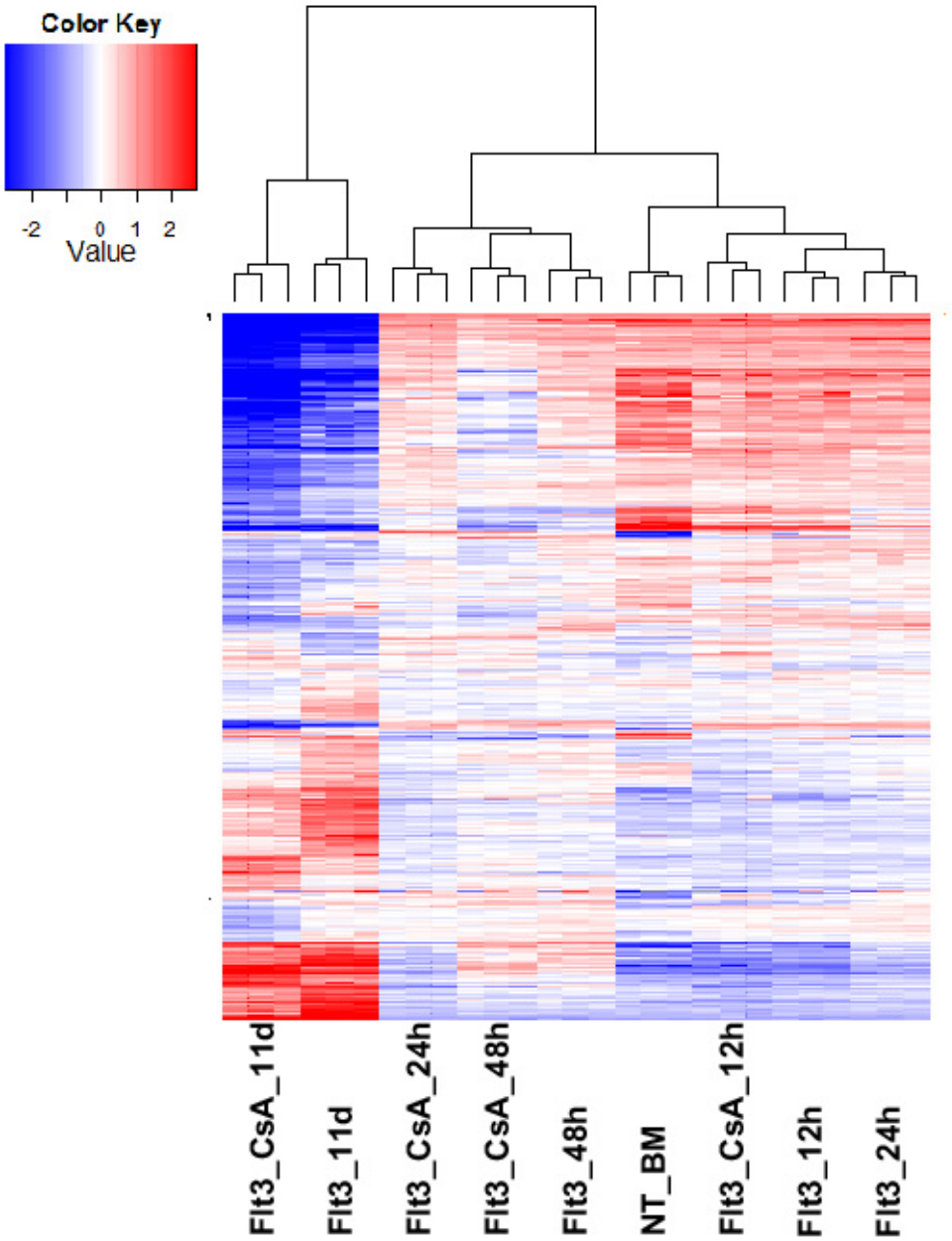


Figure S4: Preferential growth advantage of myeloid cells impaired in calcineurin/NFAT signaling. Flow cytometric analysis of DCs ($CD11c^{+}MHCII^{+}$**)** and granulocytes ($CD11b^{+}Gr\text{-}1^{+}$) in the bone marrow of mice reconstituted with VIVIT-tdTomato and control eGFP expressing HSC lines. Lethally irradiated mice were reconstituted with the mixture of HSC in the competitive ratio of 30% VIVIT-tdTomato to 70% of control eGFP HSC lines. Bone marrow cells were analyzed 4 and 8 weeks after the transplantation. Data are representative of two independent experiments.

A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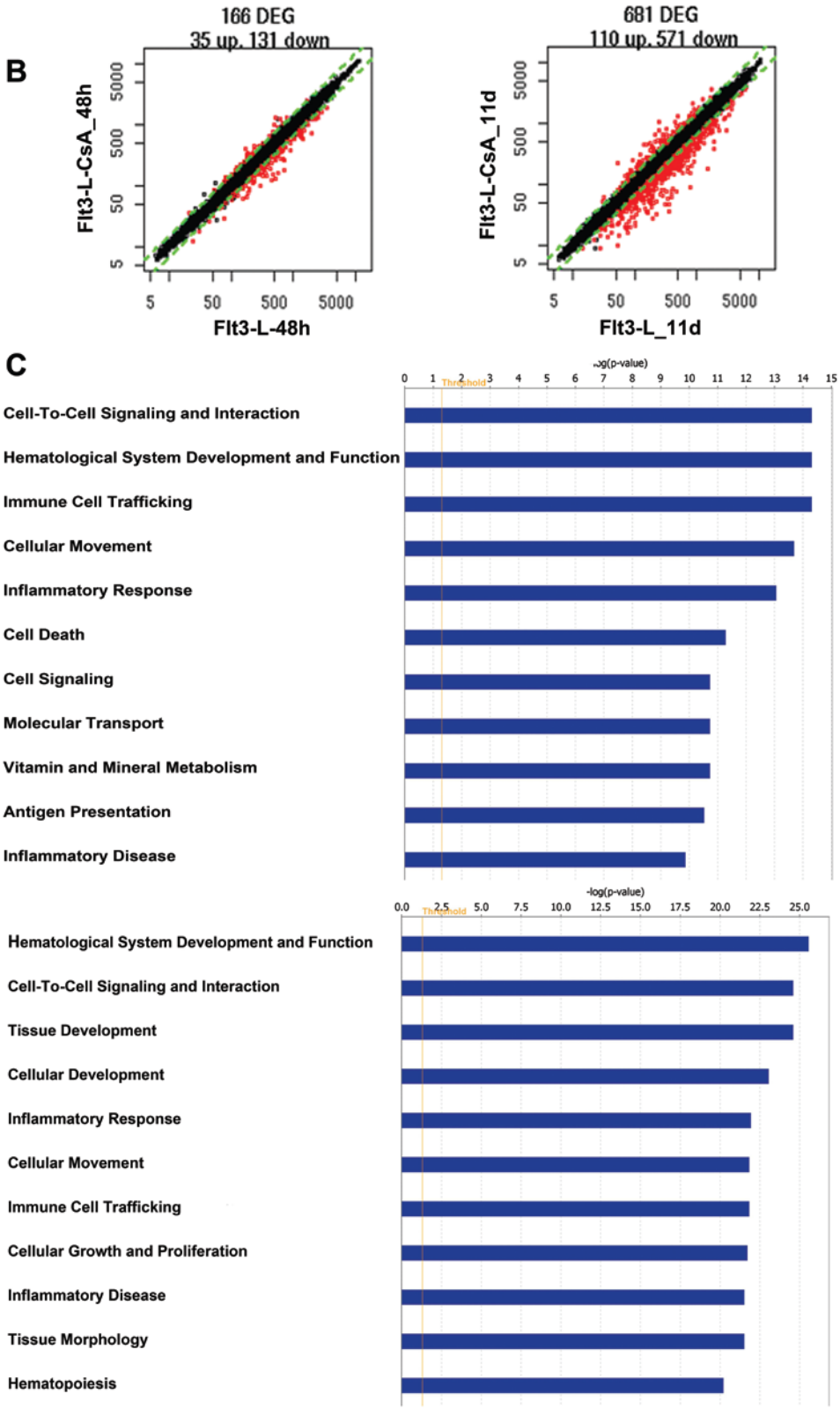
B
166 DEG
35 up, 131 down
Flt3-L-CsA_48h
Flt3-L-48h
5
50
500
5000
681 DEG
110 up, 571 down
Flt3-L-CsA_11d
Flt3-L_11d
C
-log(p-value)
Threshold
Cell-To-Cell Signaling and Interaction
Hematological System Development and Function
Immune Cell Trafficking
Cellular Movement
Inflammatory Response
Cell Death
Cell Signaling
Molecular Transport
Vitamin and Mineral Metabolism
Antigen Presentation
Inflammatory Disease
-log(p-value)
Threshold
Hematological System Development and Function
Cell-To-Cell Signaling and Interaction
Tissue Development
Cellular Development
Inflammatory Response
Cellular Movement
Immune Cell Trafficking
Cellular Growth and Proliferation
Inflammatory Disease
Tissue Morphology
Hematopoiesis

Figure S5: Analysis of differentially expressed genes in Flt3-L cultures of BM cells treated with NFAT inhibitor Cyclosporin A. (A) Clustering of gene expression during BM-DCs differentiation in Flt3-L supplemented medium in the presence or absence of CsA. (B, C) Analysis of differentially expressed genes. **(B)** Number of differentially expressed genes in Flt3-L supplemented bone marrow cultures treated with CsA versus controls (48h left and 11 days right). **(C)** Function analysis of differentially expressed genes 48 h (upper panel) and 11days (lower panel) using Ingenuity Pathway Analysis platform. Graphs display most significant cellular functions form 166 (upper panel) and 681 (lower panel) differentially expressed genes. Data represent 3 biological replicates for each culture condition.

Supplemental methods:

Array hybridization and analysis

Total RNA was extracted following the double extraction protocol: RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (Trizol Invitrogen) followed by a Qiagen RNeasy clean-up procedure. RNA purity was assessed by spectrophotometer (Nanodrop): 260/280 and 260/230 ratios were evaluated. If the 260/280 and 260/230 ratios are < of 1.8, RNA samples are ethanol precipitated. Total RNA integrity was assessed by Agilent Bioanalyzer and the RNA Integrity Number (RIN) was calculated and RIN greater than 9, were used for microarray analysis. ssDNA was prepared, fragmented and labelled according to the Affymetrix protocol. Fragmented ssDNAs were hybridized to the standard arrays for 17h at 45°C; the arrays were then washed and stained using the fluidics station and then scanned using GeneChip Scanner 3000. The images were analyzed using Command Console and comparison analyses were carried out according to the instructions provided by Affymetrix.
Microarray data handling was mainly done with an R package AMDA (Pelizzola et al, 2006) and Bioconductor 2.6 (Gentleman et al, 2004). The Robust Multichip Average (RMA) (Irizarry et al, 2003) method was employed to calculate probe set intensity. To filter out noisy data before the selection of differentially expressed genes a filter based on Interquartile Range (IQR) was applied. All the probe sets whose IQR value is lower than 0.15 were eliminated from the data set. The identification of differentially expressed genes (DEGs) was addressed using linear modeling and empirical Bayes methods (Smyth, 2004) together with Benjamini-Hochberg false discovery rate correction of the p value. The comparison between CsA treated and control Flt3-L-derived DCs was performed selecting DEGs with a threshold p value of 0.05 and fold change of 1.5. The list of differentially expressed genes and proteins was input into the Ingenuity Pathway Analysis (IPA) platform for the identification of biological processes as well as canonical pathways comparing CsA treated and control Flt3-L derived DCs. All microarray data files are available for free download at the Gene Expression Omnibus (GEO accession number: GSE29094; http://www.ncbi.nlm.nih.gov/geo).

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J et al (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome biology 5**:** R80

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP (2003) Summaries of Affymetrix GeneChip probe level data. Nucleic acids research 31**:** e15

Pelizzola M, Pavelka N, Foti M, Ricciardi-Castagnoli P (2006) AMDA: an R package for the automated microarray data analysis. BMC bioinformatics 7**:** 335

Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Statistical applications in genetics and molecular biology 3**:** Article3

Immunofluorescent labeling

Cells were cultured for 48h in Flt3-L supplemented medium, followed by stimulation with ionomycin (500 ng/ml) for 2h. Cells were fixed for 20min at room temperature in 4% paraformaldehyde, collected, washed twice in PBS, permeabilized in 0.5% Triton X100 for 2 minutes at room temperature, washed and then blocked for 1h with 3% BSA. Cells were incubated for 1h (37°C) with anti-NFAT1 antibody (Thermo Scientific) diluted to 10 µg/ml in the blocking solution, washed 3 times in PBS and incubated for 1h (37°C) with the secondary antibody (AlexaFluor 633 goat anti-mouse IgG, Molecular Probes) at 1 µg/ml, and Hoechst at 2 µg/ml. Cells were mounted in FluorSave (Calbiochem). Cellular localization of NFAT1 was analyzed using an Olympus FV1000 confocal microscope. Intensity of NFAT signals from confocal images were quantified using TissueQuest software (TissuGnostics, Austria) and used to calculate the percentage of NFAT located in the nucleus.

qPCR primers

Gene	Primer	Sequence (5'- 3')
NFATc1	Forward	TGCAAGCCAAATTCCCTGGTGG
	Reverse	GGGGTCGGGAGGCATGGTGA
NFATc2	Forward	CTGGTCTACGGGGGCCAGCA
	Reverse	GGCAGGGACTGGGTGGTAGG
IL-2	Forward	CCCAGGATGCTCACCTTC
	Reverse	CAACAGTTACTCTGATATTGCTGA
cdk4	Forward	TTGTGCAGGTAGGAGTGCTG
	Reverse	TGCCAGAGATGGAGGAGTCT
ccna2	Forward	GTGGTGATTCAAAACTGCCA
	Reverse	AGAGTGTGAAGATGCCCTGG
ccnb2	Forward	CAGAGAAAGCTTGGCAGAGG
	Reverse	TGAAACCAGTGCAGATGGAG
E2f4	Forward	AGCTCATGCACTCTCTCGTG
	Reverse	TACCTCCTTTGAGCCCATCA
GAPDH	Forward	TCGTCCCGTAGACAAAATGG
	Reverse	TTGAGGTCAATGAAGGGGTC
p21	Forward	ATCACCAGGATTGGACATGG
	Reverse	CGGTGTCAGAGTCTAGGGGA
NFia	TaqMan	Mm00447981_m1
Etv1	TaqMan	Mm00514804_m1
Nupr1	TaqMan	Mm00498104_m1
Atf5	TaqMan	Mm00459515_m

Flow cytometry antibodies

Antibody	Conjugated	Clone	Company
CD3e	Biotin, APC	145-2C11	BD Biosciences
CD4	Pacific Blue	RM4-5	Biolegend
CD8a	APC-Cy7	53-6.7	Biolegend
CD11c	APC, eFluor450	N418	ebioscience
CD11b	Biotin, APC-eFluor780, FITC	M1/70	ebioscience
CD45R (B220)	Biotin, PE-Cy7	RA3-6B2	ebioscience
CD19	Biotin, PE	eBio1D3	ebioscience
I-A/I-E	APC-eFluor780, Alexa Fluor700	M5/114.15.2	ebioscience
Gr-1	Biotin, AlexaFluor700, Pacific Blue	RB6-8C5	Biolegend
F4/80	APC-Cy7, PE-Cy7	CI:A3-1	Biolegend
NK1.1	Biotin	PK136	BD Biosciences
TER119	Biotin	TER-119	ebioscience
CD127 (IL-7Ra)	Biotin	A7R34	ebioscience
CD135	APC	A2F10	ebioscience
Ly-6A/E (Sca-1)	Pacific Blue	E13-161.7	Biolegend
CD117 (c-kit)	PE-Cy7	2B8	Biolegend
Siglec-H	PE	eBio440c	eBioscience
PDCA-1	Pe-Cy7	eBio927	eBioscience