METHODS

Cell isolation and analysis by means of flow cytometry

Thymocytes and spleen and lymph node cells from *yy-1*$^{+/-}$ heterozygous mice at age 6 to 8 weeks and sex-matched littermate WT control animals were obtained by using standard protocols. Single-cell suspensions were analyzed by means of immunofluorescence and flow cytometry with saturating concentrations of isotype-matched controls and anti-CD4 (no. 553049), CD8 (no. 553031) and B220 (no. 553090, all from BD PharMingen) and NK1.1 (no. 11-5941-82; eBioscience, San Diego, Calf). To monitor TCR transgenic T cells from OTII mice, spleen cells were labeled with anti-CD4 antibodies together with anti-Vα2 (no. 553289) and anti-Vβ5 (no. 553190, BD Pharmingen).

Cytokine ELISA or cytometric bead array

ELISA kits were purchased from eBioscience (IFN-γ) and Biolegend (San Diego, Calif; IL-2 and IL-4) and used according to the manufacturer's specification. A Bio-Plex mouse cytokine assay (Bio-Rad, Hercules, Calif) was performed by using a 96-well filtration plate and 50-μL aliquots of premixed beads (Fig 2). Samples and controls were read with a Bio-Plex 200 suspension array system, and the data were analyzed with Bio-Plex Manager software with 5PL curve fitting.

ChIP assays

For more information, see Fig 1. Solubilized chromatin was prepared from CD4$^+$ splenic T cells (1×10^6 per antibody condition) isolated from WT and *yy-1*$^{+/-}$ heterozygous littermates by using an Acetyl H3 Histone ChIP assay kit (Upstate Biotechnologies, Lake Placid, NY), as previously described.[E1] After reversion of cross-links at 65°C for 4 hours, the immunoprecipitated DNA was extracted, and PCR was performed with the following primers specific for the murine IL-4 promoter: mIL4p263F sense, tctgaaaggccgattatggtg; antisense, taacaatgcaatgctggcaga.

T_H polarization

For more information, see Fig E4. Naive CD62L$^+$CD4$^+$ T cells were isolated from WT and *yy-1*$^{+/-}$ heterozygous splenocytes by means of positive (anti-CD62L) and negative (anti-CD4) immunomagnetic selection (Miltenyi Biotec). Cells (0.5×10^6 per well in 6-well plates precoated with 2 μg/mL anti-CD3 antibodies) were incubated with recombinant mIL-2 (50 ng/mL) under the following conditions: neutral (no other added cytokines or antibodies), T_H1 (5 ng/mL mIL-12 plus 3 μg/mL anti-mIL-4), or T_H2 (10 ng/mL mIL-4 plus 3 μg/mL anti-mIFN-γ). All recombinant murine cytokines were purchased from R&D Systems, and anti-cytokine antibodies were from Biolegend. After 7 days, cells were washed 3 times and restimulated with A23187 plus PMA for 18 hours, followed by cytokine secretion by means of ELISA.

PCR and RT-PCR

For more information, see Fig E1. Genomic DNA and total cellular RNA from splenocytes was obtained by using TRIzol reagent (Life Technologies, Grand Island, NY), respectively, as per the manufacturer's instructions. RNA samples were reverse transcribed, and gene-specific primers (Invitrogen, Carlsbad, Calif) were used to amplify selected regions of each gene. To verify that equal amounts of RNA were added in each RT-PCR, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Amplified PCR products were detected by using ethidium bromide gel electrophoresis. Primers used for PCR were as follows: neomycin forward, 5′ATGAACTGCAGGACGAGGCAGCG3′; neomycin reverse, 5′GGCGATAGAAGGCGATGCGCTG3′; YY-1 forward, 5′TCGCGCTGCAGCCGCTGGTGAC3′; YY-1 reverse, 5′CGCCACGGTGACCAGCGTCTGC3′; IL-4 forward, 5′CACTTGAGAGAGATCATCGGC3′; IL-4 reverse, 5′GAGTCTCTGCAGCTCCATGAG3′; GATA-3 forward, 5′GCCGAGGACATGGAGGTGA3′; GATA-3 reverse, 5′CTAACCCATGGCGGTGACCATG3′; GAPDH forward, 5′CAATTCAACGGCACAGTCAAG3′; GAPDH reverse, 5′CCTCACCCATTTGATGTTAGTG3′.

Western blotting

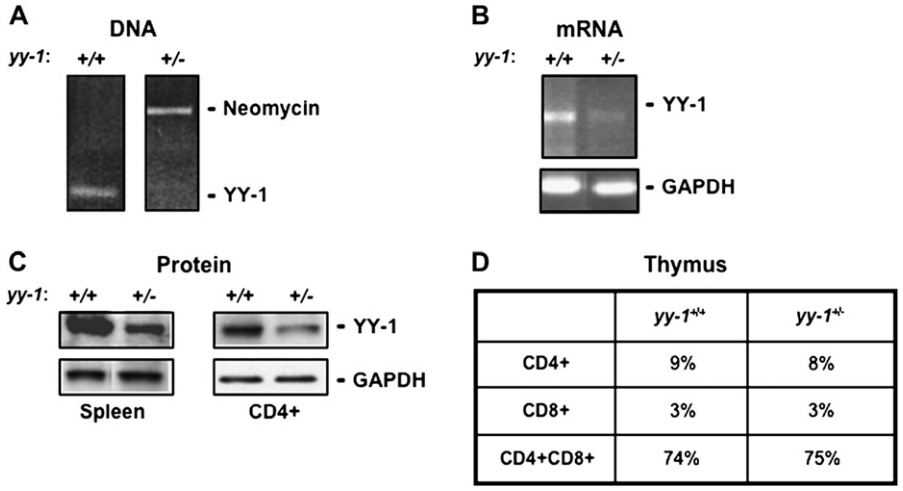
For more information, see Fig E1. Whole-cell lysates (30 μg per lane) were separated on 4% to 20% SDS-polyacrylamide gels, electroblotted onto nitrocellulose membranes (Hybond-C extra; Amersham Pharmacia Biotech, Little Chalfont, Bucks, United Kingdom), blocked for 1 hour at room temperature in blocking buffer (1× PBS, 0.1% Tween 20, and 5% [wt/vol] Carnation non-fat dry milk), and washed 3 times for 10 minutes each in washing buffer PBST (PBS and 0.1% Tween 20). Blots were incubated with a 1:1000 dilution of primary antibody in PBST buffer overnight at 4°C with anti-YY-1 (H-10, Santa Cruz Biotechnology) or anti-GAPDH antibodies (AB8246, Abcam, Cambridge, Mass) followed by washing in PBST buffer at room temperature. The secondary antibody, (anti-mouse IgG-horseradish peroxidase, NA931V, Amersham Pharmacia Biotech, and anti-goat IgG horseradish peroxidase, R&D Systems) were diluted 1:10,000 in PBST buffer, incubated for 30 minutes at room temperature, and then washed 3 times in PBST buffer. Secondary antibody–horseradish peroxidase was detected by means of chemiluminescence (Western Blotting Luminol Reagent sc-2048, Santa Cruz Biotechnology).

Quantitative RT-PCR targeted array

For more information, see Fig E2. CD4$^+$ T cells from 6- to 9-week-old WT and *yy-1*$^{+/-}$ mice (n = 3 each) were incubated with immobilized anti-CD3 on magnetic beads (2 μg/mL) and soluble anti-CD28 (2 μg/mL) for 48 hours, and total RNA was isolated with RNA Easy kits (Qiagen, Valencia, Calif). RT-PCR was performed with a FirstStrand cDNA Synthesis Kit (SuperArray, Gaithersburg, Md), and quantitative gene expression was analyzed with a 96-well RT-PCR array (Mouse $T_H1/T_H2/T_H3$ Pathway Array, SuperArray) and an IQ5 thermocycler (Bio-Rad). Relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method, in which Ct indicates the fractional cycle number at which the fluorescent signal reaches the detection threshold. The ΔΔ method uses the normalized ΔCt value of each sample calculated by using a total of 5 endogenous control genes (18S rRNA, HPRT1, RPL13A, GAPDH, and ACTB). Values are presented as an average fold change of $2^{-(\text{average } \Delta\Delta Ct)}$ for genes in CD4$^+$ T cells from *yy-1*$^{+/-}$ versus WT mice.

REFERENCE

E1. Valapour M, Guo J, Schroeder JT, Keen J, Cianferoni A, Casolaro V, et al. Histone deacetylation inhibits IL4 gene expression in T cells. J Allergy Clin Immunol 2002;109:238-45.



	yy-1$^{+/+}$	*yy-1*$^{+/-}$
CD4+	9%	8%
CD8+	3%	3%
CD4+CD8+	74%	75%

FIG E1. Characterization of *yy-1*$^{+/+}$ WT and *yy-1*$^{+/-}$ heterozygous mice. **A**, Genomic DNA was isolated from WT and heterozygous mice and analyzed by using PCR with primers amplifying the neomycin cassette or endogenous *yy-1* gene (see the Methods section in the Online Repository). Results are from 1 mouse representative of more than 20, indicating reduced abundance of the *yy-1* gene in heterozygous mice. **B**, Total mRNA was extracted from resting splenocytes and analyzed by means of RT-PCR with primers amplifying YY-1 or GAPDH cDNAs. **C**, YY-1 expression was analyzed by means of Western blotting in whole-cell lysates from splenocytes or purified spleen $CD4^+$ T cells, with anti-GAPDH antibodies as lane-loading controls. Results in panels Fig E1, *B* and *C*, are from 1 pair of age- and sex-matched WT or heterozygous littermates and are representative of 6. **D**, Thymocytes from 6-week-old WT or heterozygous mice were analyzed by means of flow cytometry with antibodies directed against CD4 or CD8 alone or in combination. The table shows that the average number of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ cells was not significantly different in 3 mice per genotype. The size and total cellularity of the thymus was also similar (data not shown).

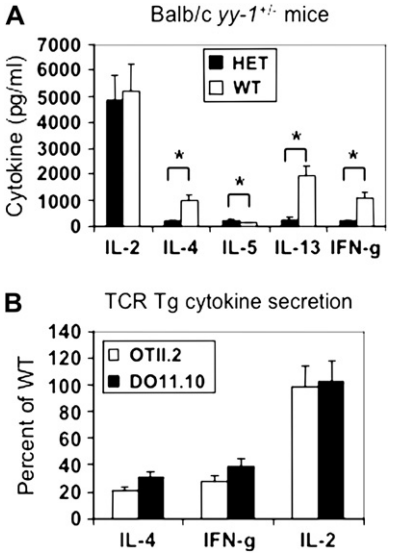


FIG E2. Reduced effector cytokine secretion in *yy-1*$^{+/-}$ heterozygous mice on different genetic backgrounds. **A**, CD4^{+} T cells from heterozygous *(HET) yy-1*$^{+/-}$ mice on the BALB/c background *(solid bars)* or littermate WT control animals *(open bars)* were stimulated with anti-CD3 plus anti-CD28 antibodies for 72 hours, and cell-free supernatants were analyzed by using the multiplex cytokine bead array for the indicated cytokines. *$P < .05$ based on genotype. **B**, TCR transgenic mice on the C57BL/6 (OTII.2) or BALB/c (DO11.10) backgrounds were generated that were either WT or heterozygous at the *yy-1* locus by means of selective breeding. Spleen CD4^{+} cells were obtained from the respective strains and incubated with OVA peptide–loaded syngeneic APCs (see the Methods section) for 48 hours, and cytokine secretion was measured by means of ELISA. Results are expressed as the relative secretion of the indicated cytokines from HET TCR transgenic T cells compared with WT cells and are the means ± SEMs of 3 per group.

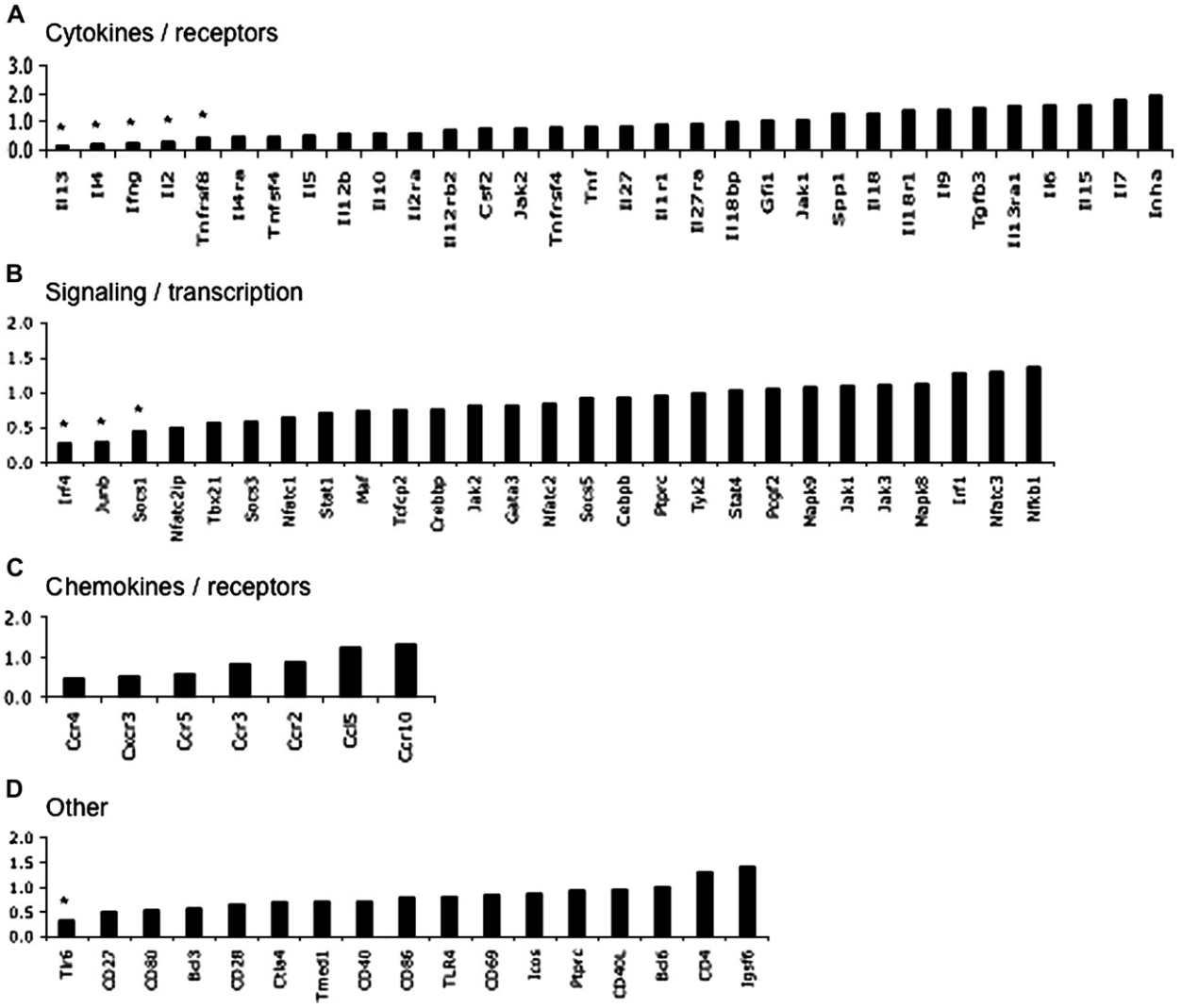


FIG E3. Analysis of gene expression in *yy-1*$^{+/-}$ T cells by using quantitative RT-PCR arrays. Spleen CD4^{+} T cells from C57BL/6 WT and *yy-1*$^{+/-}$ heterozygous mice (n = 3 each) were stimulated for 48 hours with anti-CD3 plus anti-CD28 antibodies, and total mRNA was extracted (see the Methods section in the Online Repository). Quantitative gene expression was analyzed by using a 96-well RT-PCR array (Mouse $T_H1/T_H2/T_H3$ pathway array, SuperArray) according to the manufacturer's instructions. Relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method normalized to the expression of 5 endogenous control genes (18S rRNA, HPRT1, RPL13A, GAPDH, and ACTB), which did not differ significantly based on genotype (data not shown). Values are presented as an average fold change of $2^{-(\text{average } \Delta\Delta Ct)}$ for genes in CD4^{+} T cells from *yy-1*$^{+/-}$ versus WT mice grouped according to functional category as indicated **(A-D)**. *$P < .05$.

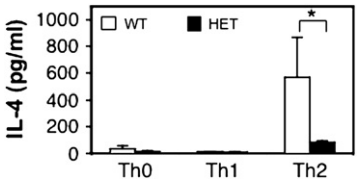


FIG E4. Partial deficiency of *yy-1* compromises T_H2 differentiation. Spleen $CD4^+$ T cells from C57BL/6 WT and *yy-1*$^{+/-}$ heterozygous mice (n = 3 each) were differentiated under neutral (T_H0), T_H1, or T_H2 conditions as indicated, and IL-4 expression was measured by means of ELISA after cell restimulation (see the Methods section in the Online Repository). *yy-1*$^{+/-}$ T cells under neutral conditions produced less IL-4, although this result did not reach statistical significance. $*P < .05$, showing reduced IL-4 secretion under T_H2 conditions.