

Mina, an *Il4* repressor, controls T helper type 2 bias

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T helper type 2 (T_H2) bias, which is the propensity of naive CD4⁺ T cells to differentiate into interleukin 4 (IL-4)-secreting T_H2 cells, is a genetic trait that affects susceptibility to infectious, autoimmune and allergic diseases. T_H2 bias correlates with the amount of IL-4 initially secreted by newly activated helper T cells that feeds back positively through the pathway of the IL-4 receptor and the transcription factors STAT6 and GATA-3 to drive T_H2 development. Here we identify Mina, a member of the jumonji C (JmjC) protein family, as a genetic determinant of T_H2 bias. Mina specifically bound to and repressed the *Il4* promoter. Mina overexpression in transgenic mice impaired *Il4* expression, whereas its knockdown in primary CD4⁺ T cells led to *Il4* derepression. Our findings collectively provide mechanistic insight into an *Il4*-regulatory pathway that controls helper T cell differentiation and genetic variation in T_H2 bias.

Naive CD4⁺ T cells are multipotent sentinels of the immune system, poised to respond to instructive signals from antigen-presenting cells by differentiating into distinct effector cell lineages. These include T helper type 1 (T_H1) and T_H2 cells, which are adapted differently for the control of intracellular and extracellular pathogens, respectively, in part through developmentally acquired potential for high expression of distinct cytokine genes¹. Dysregulated CD4⁺ T cell development can promote susceptibility to infectious, autoimmune and allergic diseases^{2–9}.

Interleukin 4 (IL-4; A001262), the canonical T_H2 effector cytokine, is also a critical developmental determinant that promotes T_H2 differentiation and inhibits T_H1 differentiation¹⁰. Recently activated helper T cells make small but functional amounts of IL-4 that induce positive feedback through the IL-4 receptor (A001263) and the transcription factors STAT6 (A002236) and GATA-3 (refs. 11,12) to promote the differentiation of T_H2 cells able to secrete copious amounts of IL-4 (refs. 10,13–18). Thus, regulation of autocrine IL-4 expression by activated helper T cells is a key control point in T helper cell lineage commitment. Nonetheless, the molecular mechanism underlying this regulation is incompletely understood.

T_H2 bias is a complex genetic trait characterized by variation in the propensity of naive helper T cells to differentiate into T_H2 (rather than T_H1) cells. T_H2 bias, measured experimentally as the amount of IL-4 produced by effector CD4⁺ T cells differentiated *in vitro* from naive helper T cells activated in 'neutral' conditions (that is, activated without the addition of exogenous cytokines, except IL-2, and cultured without cytokine-specific antibodies), varies over 50-fold from the high-producer phenotype of BALB/c mice to the

low-producer phenotype of B10.D2 mice and correlates with susceptibility to T_H2-dependent diseases such as bronchial asthma and leishmaniasis^{14–16,19–22}. Various cellular mechanisms have been suggested as the basis for T_H2 bias, including variation in the sensitivity to prostaglandin E₂-dependent inhibition of interferon- γ production²³, the timing of IL-12 receptor- β 2 downregulation^{24,25} and the capacity of activated helper T cells to produce autocrine IL-4 (refs. 14–16,20). Genetic approaches to delineate T_H2 bias have yielded many quantitative trait loci spread across mouse chromosomes 5, 12, 14 15, 16, 17, 18 and 19 (refs. 14,26–29) and a single discrete genetic locus on chromosome 11 (refs. 24,25). Several quantitative trait loci have been confirmed and isolated as discrete genetic loci by interval-specific congenic mapping^{14,26,28}. However, so far none of the underlying genetic determinants have been identified.

Here we combine classical genetic and transcriptional profiling analyses to identify Mina, a member of the jumonji C (JmjC) protein family, as a determinant of the T_H2 bias-regulatory locus *Dice1b* (called '*Dice1.2*' here), which was identified by its activity in the autocrine IL-4 pathway of activated helper T cells²⁸. We found that T_H2 bias and autocrine *Il4* expression correlated inversely with the Mina transcriptional rate, which in turn correlated with a *Mina* locus haplotype. Consistent with those findings, Mina bound to the *Il4* promoter, where it repressed *Il4* expression. We propose that a regulatory polymorphism that controls Mina expression in activated helper T cells determines the strength of Mina-dependent *Il4* repression and hence the degree of autocrine IL-4 production and, ultimately, the extent of T_H2 differentiation, thereby accounting at least in part for strain-specific differences in T_H2 bias.

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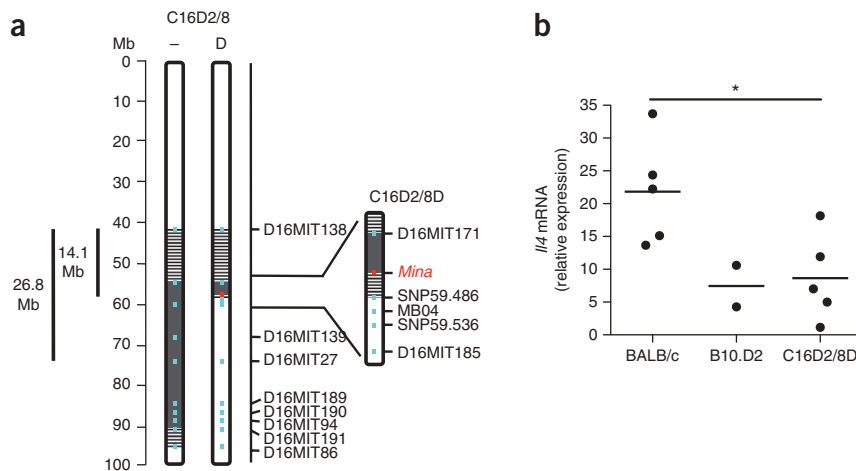


Figure 1 *Mina* is a *Dice1.2* candidate gene. (a) Location of congenic intervals in chromosome 16 of the C16D2/8 (–) and C16D2/8D (D) mouse strains, showing the chromosomal regions inherited from the B10.D2 strain (gray), the BALB/c strain (white) or ambiguous (striped); cyan dots indicate genotyped locations. Right margin, genotyping markers (red, *Mina*). Left margin, original (26.8 Mb) and newly identified (14.1 Mb) *Dice1.2* genetic intervals, respectively. (b) *Il4* expression in TCR crosslinking-activated CD4⁺ T cells from BALB/c, B10.D2 and C16D2/8D mice ($n = 2$ –5 per strain; results are presented in arbitrary units relative to the expression of *Hprt1* (encoding hypoxanthine guanine phosphoribosyl transferase). Each symbol represents an individual mouse; small horizontal lines indicate the mean. * $P = 0.0317$ (t -test). Data are representative of two experiments with similar results.

RESULTS

Mina is a *Dice1.2* candidate gene

Through the use of quantitative trait loci and interval-specific congenic mapping, an *Il4*-regulatory locus controlling T_H2 bias, *Dice1.2*, has been mapped to a 26.8-megabase (Mb) genomic interval in chromosome 16 (refs. 14,28; Fig. 1a). To resolve that interval further, we generated the subcongenic strain C16D2/8D, in which the *Dice1.2*-spanning genomic segment derived from B10.D2 mice (low T_H2 bias) that is present in the congenic strain C16D2/8 on the BALB/c background (high T_H2 bias)²⁸ is bisected to 14.1 Mb (D16MIT138–MB04; Fig. 1a). We primed splenic CD4⁺ T cells from BALB/c, B10.D2 and C16D2/8D mice for 16 h before analyzing *Il4* expression by quantitative RT-PCR. As expected, control BALB/c and B10.D2 cells showed phenotypes of high and low T_H2 bias, respectively (Fig. 1b). The production of *Il4* transcripts by C16D2/8D cells was similar to that of B10.D2 cells and was significantly less than that of BALB/c cells ($P = 0.0317$). This result indicates that *Dice1.2* is in the 14.1-Mb C16D2/8D congenic interval that spans from D16MIT138 to MB04 and contains 121 predicted or known genes³⁰. As 29 of these encode olfactory receptors, 92 remained as *Dice1.2* candidates.

The polymorphisms in *Dice1.2* that underlie the genetic variability in T_H2 bias may reside in coding or regulatory sequence. To test for the latter possibility, we investigated genes in the *Dice1.2* interval by transcriptional profiling with mRNA isolated from BALB/c and C16D2/8D CD4⁺ T cells stimulated by T cell antigen receptor (TCR) crosslinking for 16 h, a time at which differences in *Il4* expression were nearly maximal. The Affymetrix gene chip we used for initial screening contained 30 *Dice1.2* candidate genes. Of these, half were undetectable, and of the remainder, all but one had similar expression in BALB/c and C16D2/8D cells. The one exception was *Mina*, whose expression was two- to threefold higher in C16D2/8D cells than in BALB/c cells (Supplementary Fig. 1). Although expression profile analysis of the remaining genes in the *Dice1.2* interval might identify additional *Dice1.2* candidates, we explored here the possibility that *Mina* acts as a negative regulator of *Il4* expression and T_H2 bias.

Inverse correlation of IL-4 and *Mina*

First, to investigate whether the inverse correlation in the expression of *Mina* and *Il4* in BALB/c and B10.D2 cells could be generalized, we used quantitative RT-PCR to explore their expression in recently activated CD4⁺ T cells isolated from a panel of independent inbred mouse strains representing phenotypes of high and low T_H2 bias.

Whereas the kinetics of the transcriptional induction of *Mina* and *Il4* was similar across the strains tested, peak magnitudes were very dissimilar and segregated the strains into two discrete groups (Fig. 2a). In the C57BL/6, B10.D2 and C3H/HeN strains, which have low T_H2 bias, high *Mina* expression correlated with low *Il4* expression, whereas in the BALB/c, DBA/1J, DBA/2J and DBA/2N strains, which have high T_H2 bias, the converse was true (Fig. 2a). Thus, the inverse correlation in *Il4* and *Mina* expression could be generalized across strains with varying phenotypes of T_H2 bias.

From the analysis reported above of splenic T cell samples enriched for CD4⁺ cells, it was not clear whether the inverse correlation in the expression of *Il4* and *Mina* occurred in naive progenitor helper T cells or effector helper T cells. To investigate this question, we compared the expression of genes encoding cytokines in highly purified naive helper T cells from BALB/c and C57BL/6 mice. Quantitative RT-PCR analysis of naive helper T cells (99% CD4⁺CD62L^{hi}CD44^{lo}; less than 0.2% contamination by natural killer T cells) stimulated for 24 h with plate-bound antibody to TCR (anti-TCR) and anti-CD28 demonstrated an inverse correlation in the expression of *Mina* and *Il4*, similar to that in the cell samples enriched for CD4⁺ cells (Fig. 2b, top). Next we compared restimulated memory effector cells generated from C57BL/6 helper T cells primed in T_H1 and T_H2 conditions. As expected, *Il4* expression was low and high in T_H1- and T_H2-primed cells, respectively (Fig. 2b, bottom). However, in contrast to naive helper T cells from C57BL/6 and BALB/c mice, T_H1 and T_H2 cells had similar kinetics and magnitude of *Mina* expression. Thus, the disparate *Il4* expression potential of T_H1 and T_H2 cells is not inversely correlated with *Mina* expression. In contrast, natural variation in the capacity of naive helper T cells to express *Il4* is inversely correlated with *Mina* expression.

Mina acts 'upstream' of IL-4 and IL-12

Two possibilities could account for the inverse correlation in the expression of *Il4* and *Mina*: negative regulation of *Il4* by *Mina*, or negative regulation of *Mina* by IL-4 signaling. To distinguish between these possibilities, we assessed the abundance of *Mina* mRNA in activated B10.D2 CD4⁺ T cells in the presence and absence of exogenously added IL-4. The addition of IL-4 to B10.D2 CD4⁺ T cells did not diminish its 'high-*Mina*' phenotype. Conversely, IL-4 neutralization with an IL-4-specific antibody did not enhance the 'low-*Mina*' phenotype of BALB/c CD4⁺ T cells (Fig. 2c). We obtained similar results with highly purified naive helper T cells (Supplementary Fig. 2a). Given the ability of IL-12 to promote and

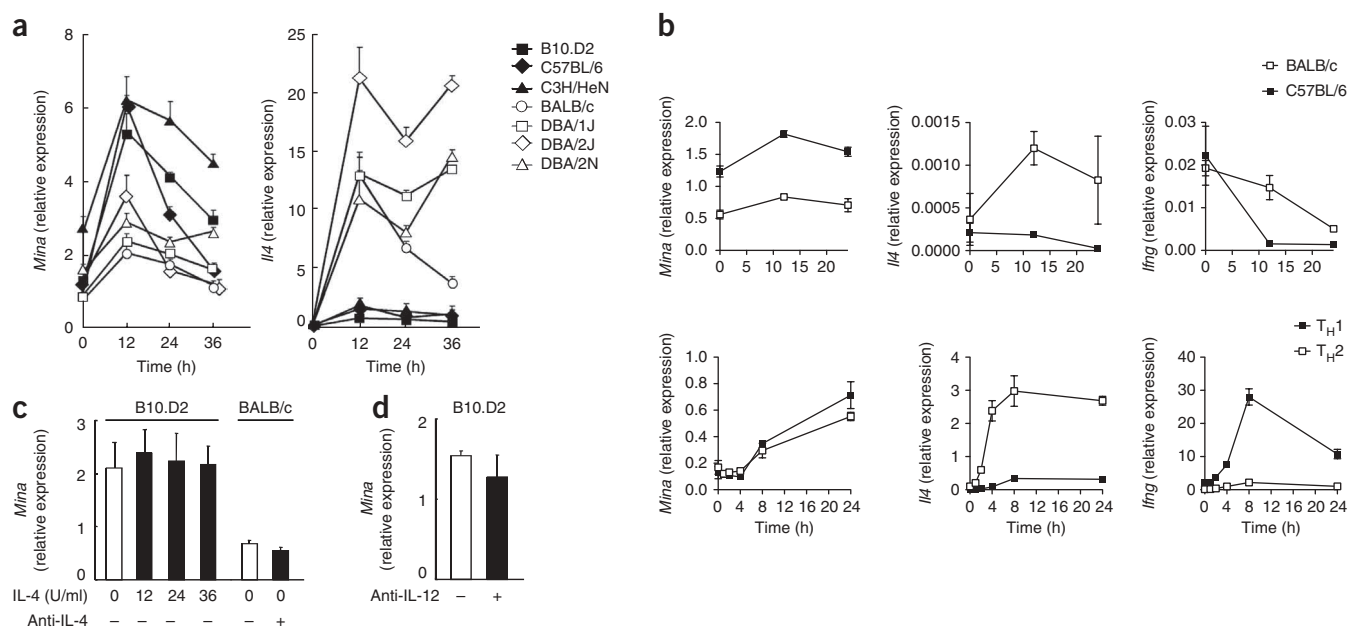


Figure 2 Inverse correlation of *Mina* and *Il4* expression in naive helper T cells. **(a)** Time course of the expression of *Mina* mRNA (left) and *Il4* mRNA (right) in TCR crosslinking-activated CD4⁺ T cells isolated from strains with low T_H2 bias (B10.D2, C57BL/6 and C3H/HeN; filled symbols) and high T_H2 bias (BALB/c, DBA/1J, DBA/2J and DBA/2N; open symbols); results are presented in arbitrary units relative to the expression of *Actb* (encoding β -actin). Data are from three independent experiments (mean and s.e.m.). **(b)** Time course of the expression of *Mina*, *Il4* and *Ifng* mRNA in BALB/c or C57BL/6 naive helper T cells activated by TCR crosslinking (top row) or from T_H1- or T_H2-primed C57BL/6 naive helper T cells (bottom); results are presented in arbitrary units relative to *Hprt1* expression. Data are representative of two independent experiments (error bars, s.e.m. of triplicate PCR). **(c)** *Mina* expression in CD4⁺ T cells stimulated for 24 h with TCR crosslinking in the presence of 'graded' doses of IL-4 (B10.D2) or anti-IL-4 (BALB/c); results are presented in arbitrary units relative to *Actb* expression. Data are from three independent experiments (mean and s.e.m.). **(d)** *Mina* expression in B10.D2 CD4⁺ T cells stimulated for 24 h in the presence of anti-IL-12; results are presented (in arbitrary units) relative to *Actb* expression. Data are from three independent experiments (mean and s.e.m.).

inhibit T_H1 development and T_H2 development, respectively, it was also possible that *Mina* expression was regulated by IL-12. However, neutralization of IL-12 with an IL-12-specific antibody did not diminish the high *Mina* expression of B10.D2 cells (Fig. 2d). Furthermore, the addition of IL-12 did not enhance the low *Mina* expression of BALB/c naive helper T cells (Supplementary Fig. 2b). Together these results indicate that neither the IL-4 signaling pathway nor the

IL-12 signaling pathway regulates *Mina* expression, which provides support for the idea that *Mina* acts as a negative regulator of *Il4* expression in naive helper T cells.

Mina is transcriptionally regulated

We next sought to determine whether differences in *Mina* transcript abundance led to differences in *Mina* protein abundance. We activated

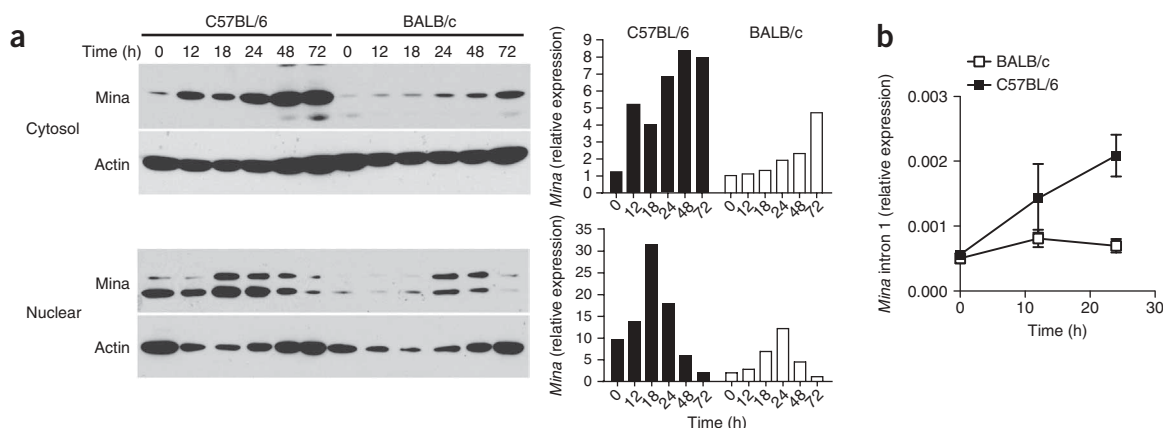


Figure 3 *Mina* is transcriptionally regulated. **(a)** Immunoblot analysis of *Mina* and actin in cytosolic and nuclear extracts of BALB/c and C57BL/6 naive helper T cells activated for various times (above lanes) with phorbol 12-myristate 13-acetate (PMA) and ionomycin. The top band in the nuclear *Mina* immunoblot is nonspecific. Right, quantification of *Mina* expression, presented relative to actin expression. Data are representative of two independent experiments. **(b)** Time course of the expression of *Mina* pre-mRNA in naive helper T cells isolated from pools of BALB/c mice ($n = 5$) and C57BL/6 mice ($n = 20$) and activated by TCR crosslinking, measured by quantitative PCR with primers targeting sequences from *Mina* intron 1 and presented (in arbitrary units) relative to *Hprt1* expression. Data are representative of two independent experiments (error bars, s.e.m. of triplicate PCR).

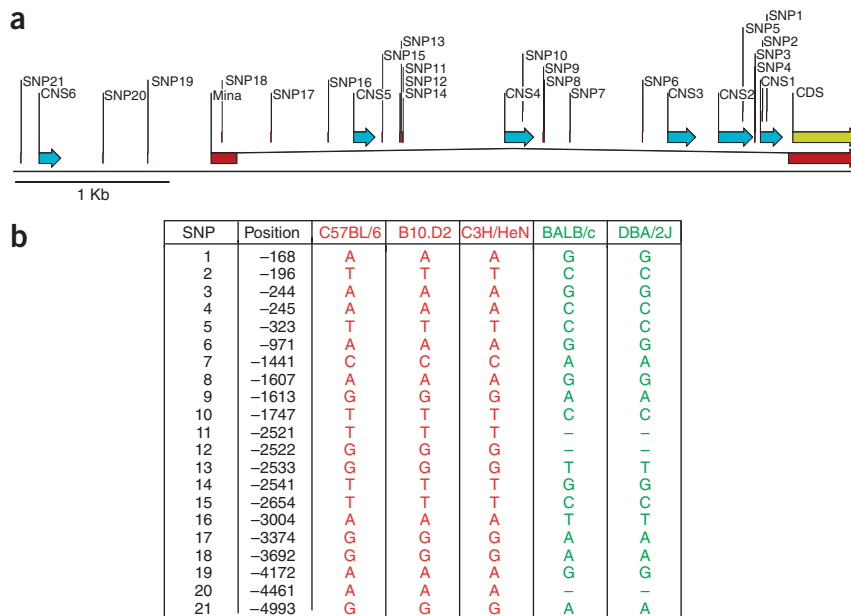


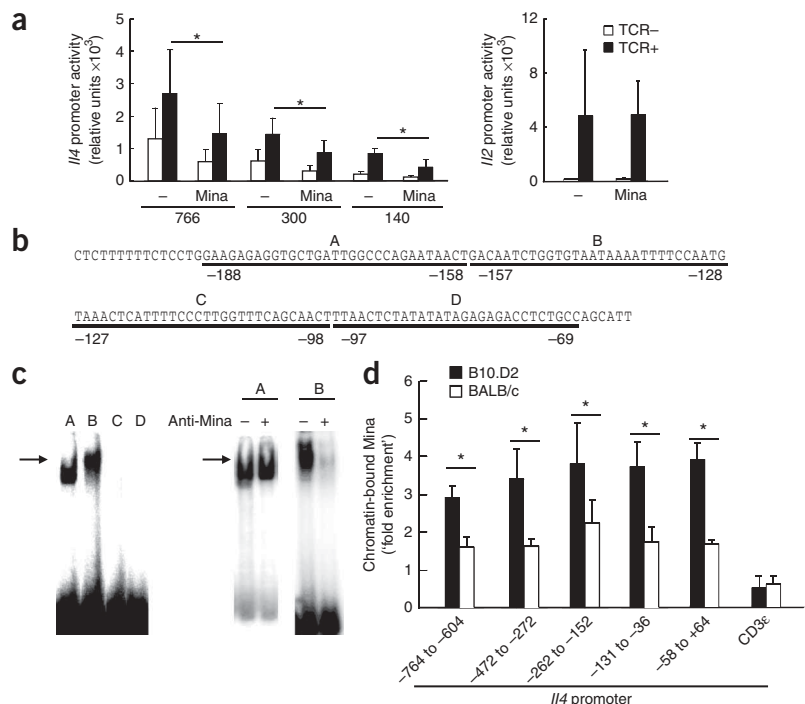
Figure 4 *Mina* haplotype can be used to predict T_H2 -bias phenotype. **(a)** Promoter-proximal end of the *Mina* genomic locus, showing the 21 SNPs identified across this interval, along with exons (red), the coding region (yellow) and conserved noncoding sequences (CNS1–CNS6; cyan). **(b)** Position (relative to the translational start site in exon 2) and allelic identity of each SNP for three strains with low T_H2 bias (red) and two strains with high T_H2 bias (green; – indicates a gap in the sequence).

purified BALB/c and C57BL/6 naive helper T cells over a 72-hour time course with plate-bound anti-TCR and anti-CD28 and analyzed the resulting nuclear and cytoplasmic extracts by immunoblot with a *Mina*-specific antibody (**Fig. 3a**). Whereas cytosolic *Mina*

(C57BL/6, B10.D2/OsN and C3H/HeN). We identified 21 single-nucleotide polymorphisms (SNPs) that precisely defined two haplotypes (**Fig. 4**) that correlated with the phenotypes of T_H2 bias and *Mina* expression (**Fig. 2**). Thus, *Mina* haplotypes are a useful genetic

Figure 5 *Mina* can bind to and repress transcription from the *I/I4* promoter.

(a) Transcriptional response of the *I/I4* promoter (left) and *I/I2* promoter (right) to *Mina*, assessed in cells transduced with a *Mina* or empty (–) expression vector and a luciferase reporter driven by the *I/I4* promoter (promoter deletion mutant length below (in bp): 766, positions –830 to –64; 300, positions –364 to –64; 140, positions –204 to –64 (all positions relative to the translational start site)) or *I/I2* promoter and then left unstimulated (TCR–) or stimulated for 12 h with anti-TCR (TCR+). * $P < 0.05$ (t -test). Data are from three independent experiments (average and s.e.m.). **(b)** Nucleotide sequence of the proximal *I/I4* promoter, showing regions used as oligonucleotide probes (A, B, C and D; underlined) in EMSA. **(c)** EMSA of nuclear extracts of B10.D2 CD4⁺ T cells stimulated for 24 h by TCR crosslinking and incubated with end-labeled probes A–D in the presence (right; probes A and B only) or absence (left) of 2 μ g anti-*Mina*. Arrows indicate the location of the nucleoprotein complex containing *Mina*. Data are representative of three independent experiments. **(d)** ChIP analysis of chromatin-bound *Mina* at five sites along the *I/I4* promoter (horizontal axis; positions relative to translational start site) and at the locus encoding CD3 ϵ (negative control) in BALB/c and B10.D2 CD4⁺ T cells stimulated for 24 h by TCR crosslinking; results are presented as immunoprecipitated relative to input. * $P < 0.05$ (t -test). Data are from five independent experiments (mean and s.e.m.).



increased with time, nuclear *Mina* appeared transiently, peaking around 24 h in both BALB/c cells and C57BL/6 cells. In both subcellular compartments, *Mina* protein amounts were consistently higher (about two- to fivefold) in C57BL/6 cells than in BALB/c cells, which demonstrated tight linkage between the abundance of *Mina* protein and that of *Mina* mRNA. To determine whether *Mina* was regulated transcriptionally, we exploited the fact that the abundance of pre-mRNA correlates with transcriptional rate³¹. Measurement of *Mina* pre-mRNA abundance showed higher expression in newly activated C57BL/6 helper T cells than in newly activated BALB/c helper T cells, which correlated with the differences in *Mina* mRNA abundance (**Fig. 3b**). These results collectively indicate that *Mina* protein abundance is transcriptionally regulated.

To explore whether regulatory polymorphisms could account for the differences in *Mina* transcription in strains with high and low T_H2 bias, we sequenced the 5' end of the *Mina* genomic locus (including intron 1 and 1,304 base pairs (bp) of the promoter) from strains with high T_H2 bias (BALB/c and DBA/2J) and those with low T_H2 bias

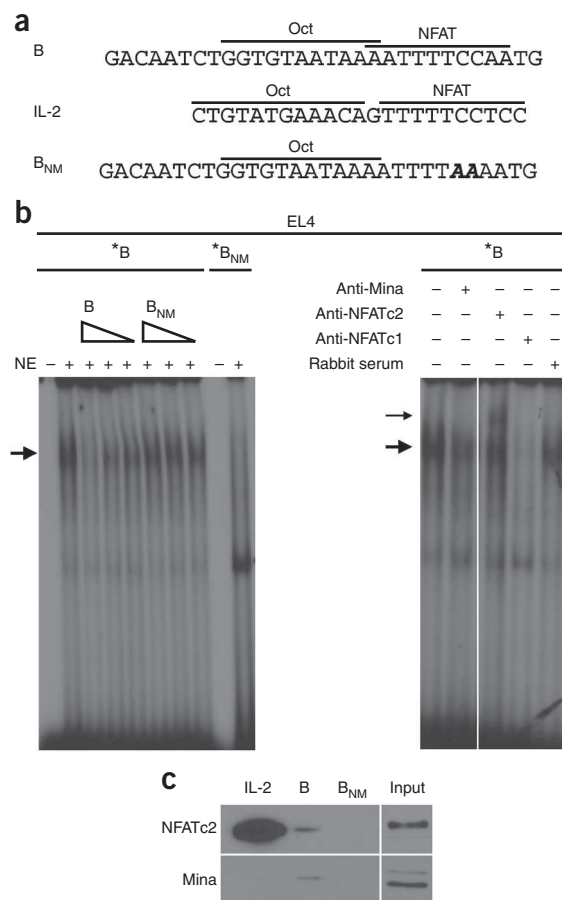


Figure 6 Recruitment of Mina to the *Il4* promoter requires NFAT.

(a) Oligonucleotide probes from the *Il4* promoter (B and B_{NM}) and *Il2* promoter (IL-2) used in the EMSA and DNA-precipitation experiments in b,c; above, NFAT- and Oct-binding sites. Probe B_{NM} is identical to probe B except for a dinucleotide mutation in the NFAT-binding site (bold italics). (b) EMSA of nuclear extracts of EL4 cells induced for 4 h with PMA-ionomycin and incubated with end-labeled probe B (*B) or probe B_{NM} (*B_{NM}) in the presence or absence of 100, 33 or 11 ng (wedges) of unlabeled probe B or probe B_{NM} (left) or with end-labeled probe B and 3 μg anti-Mina, 2 μg anti-NFATc1, 2 μg anti-NFATc2 or rabbit serum (right). NE-, absence of nuclear extract; NE+, presence of nuclear extract. Thick and thin arrows indicate locations of the Mina complex and the anti-NFAT 'supershifted' complex, respectively. Data are representative of three independent experiments. (c) Blots of precipitation products formed by the combination of biotin-labeled probes (IL-2, B and B_{NM}) with nuclear extracts from EL4 cells induced for 4 h with PMA and ionomycin (Input), followed by isolation of complexes of DNA and protein (NFATc2 and Mina) with streptavidin-agarose beads. Data are representative of two independent experiments.

To determine whether Mina binds to the *Il4* promoter *in vivo*, we used anti-Mina for chromatin immunoprecipitation (ChIP) analysis of BALB/c and B10.D2 CD4⁺ T cells that had been activated for 24 h. Consistent with the binding activity detected by EMSA in the region from position -157 to position -128, we found Mina enrichment in the *Il4* promoter but not in the promoter of the gene encoding CD3ε (Fig. 5d). Furthermore, the magnitude of binding activity at the *Il4* promoter was consistently higher in B10.D2 cells than in BALB/c cells, which correlated with the relative expression of Mina in these two strains (Fig. 2e). A broad ChIP survey of the entire T_H2 locus in B10.D2 CD4⁺ T cells activated for 24 h also detected a strong peak of Mina binding at the *Il4* promoter (Supplementary Fig. 3). These data collectively support a model in which Mina is recruited to the proximal *Il4* promoter, where it exerts a concentration-dependent transcription-repressive effect.

Recruitment of Mina to the *Il4* promoter requires NFAT

As Mina lacks a predicted DNA-binding domain, it is likely that its recruitment to the *Il4* promoter is indirect. Probe B contains published binding sites for the transcription factors NFAT (NFATc1 (A001640) and NFATc2 (A000024)) and Oct^{33,34}, which raises the possibility of their involvement in recruiting Mina to the *Il4* promoter (Fig. 6a). To test this possibility, we used wild-type probe B and a mutant probe B (B_{NM}) unable to bind NFAT³⁴ for EMSA with nuclear extracts from the T cell thymoma line EL4, which expresses Mina³⁵. Nuclear extracts from activated but not resting EL4 cells (and C57BL/6 CD4⁺ T cells) formed a nucleoprotein complex with wild-type probe B (Supplementary Fig. 4). This complex was sensitive to disruption by a Mina-specific antibody (Fig. 6b), which indicated that the probe B complex formed with EL4 extracts, like that formed with B10.D2 CD4⁺ T cell extracts, contained Mina. Although a wild-type NFAT consensus sequence was able to compete with probe B for complex formation, a wild-type Oct consensus sequence did not (data not shown), which suggests that the Mina complex contains NFAT but not Oct. Furthermore, a probe B mutant unable to bind Oct was still able to form the Mina complex (data not shown). Thus, Oct is neither a constituent of the Mina-probe B complex nor required for recruitment of Mina to the *Il4* promoter.

The formation of higher-order complexes with anti-NFATc1 and anti-NFATc2 confirmed the presence of both NFAT proteins in the probe B-Mina complex (Fig. 6b and data not shown). The mutant probe B_{NM} that is unable to bind NFAT³⁴ did not form the Mina complex and failed to compete with probe B for complex formation

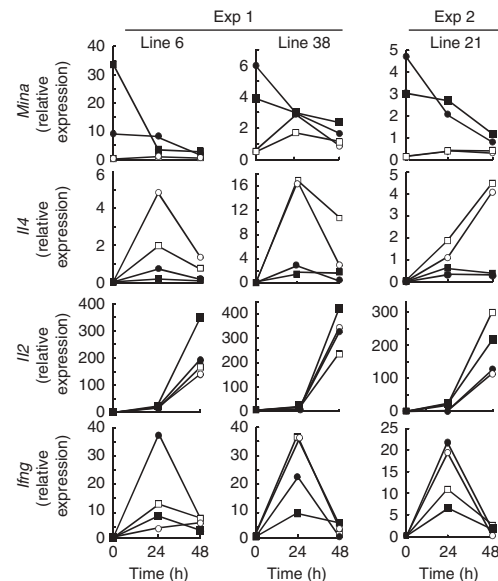
marker for T_H2 bias and may contain regulatory polymorphisms responsible for differences in *Mina* expression across strains with distinct phenotypes of T_H2 bias.

Mina specifically binds and represses the *Il4* promoter

To investigate whether Mina can repress transcription from the *Il4* promoter, we did transient reporter assays, cotransfecting 68-41 mouse T cell hybridoma cells with a Mina or empty expression vector and a luciferase reporter driven by either an *Il4* promoter or an *Il2* promoter³². Mina inhibited luciferase activity driven by the *Il4* promoter but not activity driven by the *Il2* promoter (Fig. 5a). To define the Mina-responsive region in the 766-bp *Il4* promoter, we used the transient reporter assay to study a series of *Il4* promoter deletion mutants. Neither truncation of the *Il4* promoter to 300 bp nor truncation to 140 bp was sufficient to eliminate sensitivity to Mina-dependent repression (Fig. 5a). Thus, a target site for the repressive activity of Mina is in the first 140 bp of the *Il4* promoter.

To further refine the Mina responsive region, we used electrophoretic mobility-shift assay (EMSA). We tested nuclear extracts isolated from TCR-stimulated B10.D2 CD4⁺ T cells against a series of end-labeled DNA probes spanning the first 140 bp of the *Il4* promoter (Fig. 5b). Nucleoprotein complexes were formed with probe A (positions -188 to -158) and probe B (positions -157 to -128) but not probe C (positions -127 to -98) or probe D (positions -97 to -69; Fig. 5c); however, only the probe B complex was sensitive to disruption by a Mina-specific antibody. Thus, Mina can associate with the *Il4* promoter over the 30-bp region of probe B that extends from position -157 to position -128.

Figure 7 A transient enforced increase in *Mina* impairs *Il4* expression in CD4⁺ T cells. Quantitative real-time RT-PCR analysis of the expression of *Mina*, *Il4*, *Il2* and *Ifng* in CD4⁺ T cells isolated from three independent lines from *Mina*-transgenic mice (lines 6, 38 and 21; filled symbols; $n = 2$ mice each) and their corresponding littermate controls (open symbols; $n = 2$ mice each), then stimulated for various times (horizontal axis) with anti-TCR and anti-CD28. Results are presented in arbitrary units relative to *Actb* expression; each line represents an individual mouse. Data are from two independent experiments (Exp 1 and 2).



(Fig. 6b), which suggests a requirement for NFAT in *Mina* recruitment. To confirm that idea, we did DNA precipitation assays with biotinylated probes B and B_{NM}. Whereas both *Mina* and NFAT (NFATc1 and NFATc2) could be precipitated together with the biotinylated wild-type probe B, probe B_{NM} failed to precipitate together with either NFAT protein or *Mina* (Fig. 6c and data not shown). Notably, a biotinylated NFAT-binding sequence from the *Il2* promoter failed to precipitate *Mina* despite binding both NFATc1 and NFATc2 (Fig. 6c and data not shown), which indicated that NFATc1 and NFATc2 were insufficient for *Mina* recruitment. Finally, nuclear extracts of cells treated with the calcineurin inhibitor cyclosporin A, to prevent NFAT dephosphorylation and nuclear translocation, failed to form the *Mina* nucleoprotein complex (Supplementary Fig. 4). These data collectively suggest that NFAT is necessary but not sufficient for recruitment of *Mina* to the *Il4* promoter-specific nucleoprotein complex.

Mina is necessary and sufficient to constrain IL-4 expression

To directly test whether *Mina* can repress *Il4* transcription in CD4⁺ T cells, we generated three independent BDF1 × C57BL/6 mouse lines expressing a *Mina* transgene driven by a lymphocyte-specific proximal promoter–E_μ enhancer of the gene encoding the kinase *Lck*³⁶. We analyzed mice after three backcrosses to BALB/c mice, at which point they showed normal T cell development and expression of activation markers (data not shown). Using quantitative RT-PCR to compare *Mina* expression in CD4⁺ T cells, we found that all three transgenic lines had high basal *Mina* expression that diminished over a 48-h activation time course to lower expression similar to or higher than the peak of expression by their respective wild-type littermate controls (Fig. 7). To determine the effect of a transient enforced increase in *Mina* abundance on cytokine gene expression, we examined the transcriptional induction of *Il2* and *Il4* and of the gene encoding interferon-γ (*Ifng*). Expression of *Il4* was considerably impaired in all

three *Mina*-transgenic lines relative to its expression in wild-type littermate controls, but expression of *Il2* and *Ifng* was not (Fig. 7). Thus, a transient enforced increase in *Mina* in CD4⁺ T cells specifically impairs expression of *Il4* but not of *Il2* or *Ifng*.

To test whether *Mina*-dependent repression is required to maintain normal expression of *Il4*, we used antisense morpholino technology to ‘knock down’ *Mina* protein in primary CD4⁺ T cells. At 24 h after activation, C57BL/6 CD4⁺ T cells pretreated for 16 h with *Mina*-morpholino expressed half the amount of *Mina* protein expressed by cells given mock pretreatment or pretreatment with control-morpholino (Fig. 8). In contrast, cells pretreated with *Mina*-morpholino expressed about twofold more *Il4* mRNA (but not more *Ifng* mRNA) than did cells given mock pretreatment or pretreatment with control-morpholino (Fig. 8). Thus, the quantitative potential of CD4⁺ T cells to express *Il4* is normally constrained by tight *Mina*-dependent negative regulation.

DISCUSSION

We have identified *Mina* as a genetic determinant of the *Il4*- and T_H2 bias-regulatory locus *Dice1.2*. Our data suggest that *Mina* is a necessary and sufficient dose-dependent *Il4*-specific repressor in naive helper T cells that affects the magnitude of the early autocrine IL-4 burst required for the ‘programming’ of T_H2 development. Furthermore, our results have shown how natural variation in *Mina* transcription (probably due to regulatory SNPs at the *Mina* locus) contributes to natural variation in T_H2 bias.

Mina belongs to the Jmj protein family whose hallmark JmjC domain is homologous to the family of Fe(II)- and 2-oxoglutarate-dependent dioxygenase enzymes. The catalytic dioxygenase core comprises three iron-binding residues and two 2-oxoglutarate-binding residues, of which four are conserved in *Mina*³⁷. Dioxygenases can catalyze a variety of post-translational modifications on substrate proteins, including demethylation and hydroxylation. For example, JMJD6, a member of the JmjC-only subfamily (to which *Mina* belongs), has been shown to have histone arginine-demethylase activity³⁸, and FIH (also a member of the JmjC-only subfamily) is an asparagine hydroxylase, responsible for inactivating the transcriptional activation domain of hypoxia-inducing factor^{39,40}. Despite imperfect conservation of its core catalytic residues, *Mina* is a

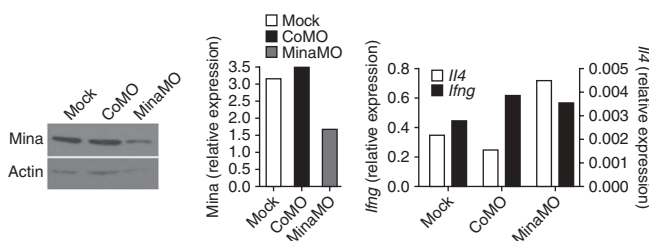


Figure 8 *Il4* expression in CD4⁺ T cells is constrained by *Mina*-dependent repression. Left, immunoblot analysis of the expression of *Mina* and actin in total cellular extracts from CD4⁺ T cells (4×10^6 to 6×10^6) given 16 h of mock pretreatment or pretreatment with control morpholino (CoMO) or *Mina*-morpholino (*Mina*MO) and then activated for 24 h with plate-bound anti-TCR and anti-CD28. Middle, quantification of *Mina* expression in the blot at left, presented relative to actin expression. Right, expression of *Il4* and *Ifng* in the cells at left; results are presented (in arbitrary units) relative to *Hprt1* expression. Data are representative of two independent experiments (mean of duplicate PCR).

functional Fe(II)–2-oxoglutarate-dependent dioxygenase enzyme (C. Schofield, personal communication). Thus, a likely hypothesis is that *Mina* acts as an *Il4* repressor by demethylating histones at key positive regulatory elements such as the 3' DNase I-hypersensitive site V (ref. 41), reported to be required for Notch-mediated activation of autocrine expression of IL-4 in naive helper T cells^{42,43}. Preliminary results suggest that abundance of histone 3 trimethylated on lysine 4 is similar at DNase I-hypersensitivity sites HSV, HSVa, HSIV and HSS3 in naive CD4⁺ T cells from BALB/c mice and C57BL/6 mice, inconsistent with a histone-demethylating function for *Mina*. Another likely hypothesis is that *Mina* catalyzes post-translational changes that modulate key *Il4* transcription factors. In this context, it is notable that arginine methylation augments the ability of the transcription factor NIP45 to promote *Il4* transcription⁴⁴. Finally, it is possible that the *Il4*-repressive effect of *Mina* may be mediated by a mechanism independent of its dioxygenase activity.

Mina, which lacks an obvious DNA-binding domain, associates with a region of the *Il4* promoter that contains binding sites for NFAT and Oct. Our results have indicated that whereas Oct is excluded from the *Mina* complex, an NFAT-dependent interaction is required for recruitment of *Mina* to the *Il4* promoter. However, although it is necessary, NFAT is not sufficient for recruitment of *Mina* to the *Il4* complex, as it did not associate with an *Il2* promoter fragment able to recruit both NFATc1 and NFATc2. This result indicates the existence of specificity-determining sequence elements in addition to those required for NFAT recruitment. Identification of these elements may assist in the identification of factors required along with NFAT to recruit *Mina* to the *Il4* promoter.

Mina was first identified as a nuclear antigen of 53 kilodaltons induced by the myelocytomatosis oncogene product *Myc* and was found to be overexpressed and associated with poor prognosis in a variety of human cancer cells^{45–48}. Studies using ChIP and tamoxifen-inducible fusion protein *Myc*-ERTam have shown that *Mina* is a direct target of *Myc* in human promyelocytic leukemia and human glioblastoma cells⁴⁸. Comparison of the *Mina* promoter sequence in strains with high or low T_H2 bias failed to demonstrate polymorphism in a presumptive *Myc*-binding site (data not shown), which suggests that differences in *Myc* activity may not be responsible for the differences in *Mina* expression in strains with high or low T_H2 bias. Furthermore, *Myc* expression in activated naive helper T cells is similar in strains with high or low T_H2 bias, and its acute deletion does not impair *Mina* expression in naive helper T cells (M. Koyanagi, unpublished data). Thus, in naive helper T cells, *Mina* may not be a true *Myc* target.

There are 21 SNPs of the *Mina* promoter and intron 1 that define two distinct haplotypes that correlate with *Mina* expression phenotype. The polymorphisms that underlie the differences in *Mina* expression probably reside in a key regulatory element whose function is modulated by one or several of the 21 (or perhaps additional) haplotype-defining SNPs. Phylogenetic sequence conservation has been used to identify gene-regulatory elements as regions of 'conserved noncoding sequence'⁴⁹. We have identified six conserved noncoding sequences in the 5-kilobase region of the *Mina* locus containing the 21 SNPs. Four of the twenty-one haplotype-defining SNPs (1, 2, 5 and 10) are in three conserved noncoding sequences (4, 2 and 1) and are thus candidates for the functional regulatory polymorphisms that underlie the differences in *Mina* expression that distinguish strains with high or low T_H2 bias. The identification of *Mina*-regulatory polymorphisms will provide candidates for the causative *Dice1.2* genetic lesion and a foothold for delineation of the critical 'upstream' pathways.

Although differences in *Mina* expression can explain the *Il4*- and T_H2 bias-regulatory activity of *Dice1.2*, it remains possible that other genes in the minimal *Dice1.2* interval contribute to the T_H2 bias-regulatory activity of *Dice1.2*. Furthermore, *Dice1.2* is one of two *Il4*- and T_H2 bias-regulatory loci originally identified on chromosome 16 (the other being *Dice1a*, called '*Dice1.1*' here)²⁸. Thus, identification of the molecular basis of the action of *Dice1.1* will probably add further mechanistic insight into the complex regulation of *Il4* and T_H2 bias. Notably, *Dice1.2* (but not *Dice1.1*) is tightly linked genetically to a host response-modifying locus (*LmrA*) for the protozoan parasite *Leishmania major* that is responsible for leishmaniasis²⁸. As T_H2 bias is critical in determining susceptibility to leishmaniasis, it is possible that *Mina* is *LmrA*. In this context, it is perhaps relevant that among hematopoietic cell types, *Mina* is expressed in CD4⁺ T cells and dendritic cells; the latter cell type has also been suggested to contribute to the different T_H2-bias phenotypes of BALB/c and B10.D2 mice. A dual T_H2 bias-regulatory function for *Mina* (with separate activities in naive helper T cells and dendritic cells) could explain the linkage of *Dice1.2* but not *Dice1.1* to *LmrA*. Investigation of the function of *Mina* in dendritic cells may thus provide further insight into the mechanism of T_H2 bias.

Our data have identified a previously unknown IL-4-repressive pathway in which *Mina* serves a key function in regulating the amount of IL-4 produced by naive helper T cells, thereby controlling the extent of T_H2 differentiation and T_H2 bias. Natural genetic variation in this pathway contributes to the variation in T_H2 bias characteristic of distinct inbred mouse strains. It will be important to determine the 'upstream' regulators, 'downstream' targets and repressive mechanism of *Mina* and the extent to which the *Mina* pathway may be conserved and manipulated in humans.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Accession codes. UCSD-Nature Signaling Gateway (<http://www.signaling-gateway.org>): A001262, A001263, A002236, A001640 and A000024; Reference Database of Immune Cells of the Research Center for Allergy and Immunology⁵⁰: microarray data, RMSPTB007001 and RMSPTB008001.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

M.O. and M.V.S. did the experiments; L.C. did the *Mina* transcriptional analysis and **Figures 2b** and **3b** and **Supplementary Figure 2**; M.Ka. did the *Mina* immunoblots; X.S. analyzed the C16D2/8D mice; Y.S. did the transgenic experiments; Y.S. and L.C. maintained the mouse colonies; O.O., H.K. and A.H.

did the expression profiling; M.O., M.V.S., M.Ku and M.B. designed and conceptualized the research and analyzed the data; and M.B. prepared the manuscript.

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ONLINE METHODS

Mice. Animals were bred and maintained in specific pathogen-free conditions in accordance with the guidelines of the Institutional Animal Care and Use Committees of St. Jude Children's Research Hospital and the RIKEN Institute. BALB/c, C57BL/6, C3H/HeN, B10.D2, DBA/2J, DBA/1J and DBA/2N mice were from Jackson Laboratory, Taconic Farms, CLEA and Charles River Laboratories.

Reagents and antibodies. Anti-TCR β was purified from hybridoma H57.597. Anti-CD28 was purified from hybridomas PV-1 and 37N51.1 (gifts from R. Abe and J. Allison, respectively). Anti-IL-4 was used as purified protein or 10% culture supernatant from hybridoma 11B11. Anti-IL-12 was used as purified protein or 10% culture supernatant from hybridoma C17.8 (a gift from G. Trinchieri). Other antibodies were as follows: anti-Mina (M532; 40–9500; Zymed)^{46,47}; anti-NFATc2 (NFAT1-NFATp; 25A10.D6.D2; Tamar Laboratory Supplies), anti-NFATc1 (NFAT2; sc-13033; Santa Cruz), anti-Oct1 (sc-232; Santa Cruz), anti-actin (sc-1616; Santa Cruz), anti-rabbit secondary antibody (SC-2305; Santa Cruz), horseradish peroxidase-conjugated anti-goat secondary antibody (SC-2020; Santa Cruz) and isotype control immunoglobulin G (AB46540-1; Abcam). Human recombinant IL-2 was used at a concentration of 20 U/ml. Mouse recombinant IL-4 was from PeproTech.

Expression profiling. CD4⁺ T cells isolated from spleen and lymph nodes were stimulated with plate-bound monoclonal anti-TCR β (1 μ g/ml; H57.597) and monoclonal anti-CD28 (10 μ g/ml; 37N51.1). After 24 h, total RNA was extracted (Tri reagent, Sigma-Aldrich) and was purified on RNeasy minicolumns (Qiagen), then cRNA was synthesized according to Affymetrix protocols. Biotinylated cRNA was hybridized to an Affymetrix GeneChip (Mouse Genome 430 2.0 Array; Affymetrix). Microarray data were globally normalized with GeneSpring software (Agilent Technologies).

Quantitative RT-PCR. First, cDNA was synthesized from total cellular RNA with SuperScript III reverse transcriptase (Invitrogen). Then, MX4000 (Stratagene) and 7500 (Applied Biosystems) real-time PCR systems were used for quantitative RT-PCR analysis (primer sequences, **Supplementary Table 1**), and mRNA expression was normalized to the expression of *Actb* or *Hprt1*.

Congenic mouse strains and phenotypic screening of T μ 2 bias. The generation of congenic strain C16D2/8 has been described²⁸. C16D2/8D mice were selected from the backcross progeny of C16D2/8 $\times$ BALB/c crosses. The congenic interval in C16D2/8D was bred to homozygosity by intercrossing and was characterized by genotyping (markers, **Fig. 1a** and **Supplementary Table 1**). Splenic CD4⁺ T cells isolated by magnetic cell sorting (CD4⁺ T cell Isolation kit; Miltenyi) were stimulated for 16 h with plate-bound anti-TCR β (1 μ g/ml; H57.597) and anti-CD28 (10 μ g/ml; 37N51.1), then RNA was collected (Tri reagent; Sigma-Aldrich) and expression of *Il4* and *Hprt1* was analyzed by real-time RT-PCR as described^{41,51}. In some experiments, splenic CD4⁺ T cells were isolated with the IMag magnetic cell separation system (BD) and were stimulated for 24 h with plate-bound anti-TCR (1 μ g/ml; H57.597) and soluble anti-CD28 (PV-1; 10 μ g/ml) before collection of RNA.

Immunoblot analysis. Naive helper T cells were stimulated with PMA and ionomycin. Nuclear and cytoplasmic proteins were isolated from 15×10^6 cells with the NE-PER nuclear and cytoplasmic extraction reagent (78833; Pierce). Nuclear and cytoplasmic proteins (15 μ g each) were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes and hybridized to anti-Mina and anti-actin. After reaction with horseradish peroxidase-conjugated anti-rabbit secondary antibody (SC-2305; Santa Cruz), blots were visualized with chemiluminescence substrate (Roche). Mina expression is presented relative to actin expression.

Transient reporter assay. *Mina* cDNA from strain B10.D2 was inserted into pCMV10 (Sigma-Aldrich) to generate the pCMV-FLAG-Mina expression construct. The 68–41 T cell hybridoma cells were cotransfected with plasmids encoding firefly luciferase driven by the *Il4* or *Il2* promoter (pIL-4-Luc and pGL-2), pCMV10 or pCMV-FLAG-Mina and a pancreatic alkaline phosphatase expression plasmid (pSPVAP; included to control for transfection efficiency). At 40 h after transfection, cells were stimulated for 12 h with plate-bound

monoclonal anti-TCR (H57.597) and total cell lysates were prepared. Luciferase activity measured with a Microplate luminometer (Promega) is presented relative to the activity of pancreatic alkaline phosphatase.

EMSA. EL4 cells or CD4⁺ T cells (enriched from B10.D2 or C57BL/6 spleen and lymph nodes) were stimulated with either PMA (Sigma) and ionomycin (Sigma) or plate-bound anti-TCR β (10 μ g/ml; H57.597) and anti-CD28 (20 μ g/ml; 37N51.1) in the presence or absence of cyclosporin A (1 μ g/ml; Calbiochem⁵²). Nuclear extracts were prepared with the NE-PER extraction kit according to the manufacturer's directions (Pierce). Protein concentrations were measured by the Bradford assay (Pierce) with BSA as a standard. Oligonucleotides were annealed in annealing buffer (100 mM Tris, pH 7.5, 10 mM EDTA, 2 M NaCl and 50 mM MgCl₂) by heating to 95 °C followed by slow cooling to 22 °C. For gel shifts, annealed oligonucleotides were end-labeled with [γ -³²P]ATP and T4 polynucleotide kinase (Promega). Nuclear extracts (10 μ g) or BSA, ³²P-labeled oligonucleotide (1 $\times$ 10⁴ c.p.m.) and poly(dI:dC) (125 ng; Sigma) were incubated together for 30 min at 22 °C in 20 μ l binding buffer (10 mM Tris, pH 7.5, 60 mM KCl, 2 mM MgCl₂ and 0.15 mM dithiothreitol). For 'supershifting', 2–3 μ g antibody was included in the reaction. Binding reactions were resolved by electrophoresis through 1 $\times$ TAE–4% polyacrylamide gels.

DNA precipitation. Precipitation assays were done as described⁵³. Streptavidin-agarose beads (300 μ l, Pierce) were preabsorbed with BSA (500 μ l; 1 mg/ml), poly(dI:dC) (50 μ g; Sigma) and sheared salmon sperm DNA (50 μ g), then were washed three times and were resuspended in EMSA binding buffer (300 μ l). Annealed 5' biotin-labeled oligonucleotides (1 μ g) were incubated for 30 min at 22 °C with nuclear extracts (300 μ g) from EL4 cells stimulated for 4 h PMA and ionomycin, in EMSA binding buffer along with poly(dI:dC) (10 μ g). Then, preabsorbed beads (30 μ l) were added to the oligonucleotide-protein mixture, followed by incubation for 4 h at 4 °C. DNA-protein-streptavidin-agarose complexes were washed three times with EMSA binding buffer, eluted in Laemmli buffer, resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-Mina (1 μ g/ml) and anti-NFATc2 (1:5000 dilution; 25A10.D6.D1; Tamar Laboratory Supplies).

ChIP. Equivalent-mass and equivalent-volume methods were used for ChIP. For the equivalent-mass method⁵⁴, DNA recovered from an aliquot of sheared chromatin was used as the 'input' sample. The remaining chromatin was precleared with protein A and protein G agarose (16–156 and 16–266; Upstate) and then was incubated overnight at 4 °C with anti-Mina (2 μ g/ml, 40–9500; Zymed). DNA recovered after immunoprecipitation was isolated with a PCR purification kit (QIAGEN) and was quantified by PicoGreen assay (Molecular Probes). An equivalent mass of immunoprecipitated and input DNA was analyzed by real-time PCR as described above for RT-PCR with the following modifications: Taq polymerase was from Qiagen (Hot Start), and cycling conditions were 94 °C for 15 min, followed by 45 cycles of 94 °C for 20 s, 61 °C for 1 min and 72 °C for 40 s. Data are presented as the ratio of the change-in-threshold (C_T) value for immunoprecipitated DNA to that of input DNA.

For the equivalent-volume method⁵⁵, chromatin prepared as described above was incubated overnight at 4 °C with anti-Mina or control immunoglobulin G (10 μ g/ml; ab4640-1; Abcam). Input DNA and DNA recovered after immunoprecipitation were isolated with a PCR purification kit (QIAGEN). Then, 10 μ l of immunoprecipitated DNA or 10 μ l of a 1:20 dilution of input DNA was analyzed by real-time PCR as described above for RT-PCR with the following modifications: Taq polymerase was from Sigma, and the cycling conditions were 94 °C for 15 min, followed by 45 cycles of 94 °C for 20 s, 61 °C for 1 min and 72 °C for 40 s. Data are presented as the ratio of the change-in-threshold value for immunoprecipitated DNA to that of input DNA.

Mina-transgenic mice. For the establishment of *Mina*-transgenic mice, B10.D2 *Mina* cDNA was cloned downstream of an element composed of the proximal promoter and E μ enhancer of the gene encoding Lck in the p1026X expression vector⁵⁶. PCR of genomic DNA obtained from tail tissue identified ten independent BDF1 $\times$ C57BL/6 transgenic founders. Transgene copy number was determined by Southern blot analysis. Three founders containing over ten copies of the transgene were selected and were backcrossed for three generations to the

BALB/c strain before further analysis. Protein expression in splenic CD4⁺ T cells was confirmed by immunoblot analysis with anti-Mina (40–9500; Zymed).

Antisense morpholino-mediated knockdown. CD4⁺ T cells were enriched from C57BL/6 spleen and lymph nodes by complement-mediated lysis with antibody to heat-stable antigen (anti-CD24; J11d), anti-major histocompatibility complex class II (BP107) and anti-CD8 (53.67.2) and then were incubated overnight with Endo-Porter reagent⁵⁷ (6 µl/ml) and fluorescein isothiocyanate-labeled standard control or Mina-morpholino (10 µM; Gene-Tools; **Supplementary Table 1**) in the presence of IL-7 (3 ng/ml). The transfection efficiency was over 80% as determined by flow cytometry. CD4⁺ T cells were then activated for 24 h with plate-bound anti-TCR (H57.597) and anti-CD28 (37N51.1) in the presence of IL-2 (25 U/ml) before immunoblot analysis of the knockdown of Mina and actin and quantitative RT-PCR analysis of the expression of *Il4* and *Ifng*.

Gene annotation. Gene annotation in the D16MIT138–MB04 interval was retrieved from the Mouse Genome Database (Mouse Genome Informatics, The Jackson Laboratory; May, 2009)³⁰.

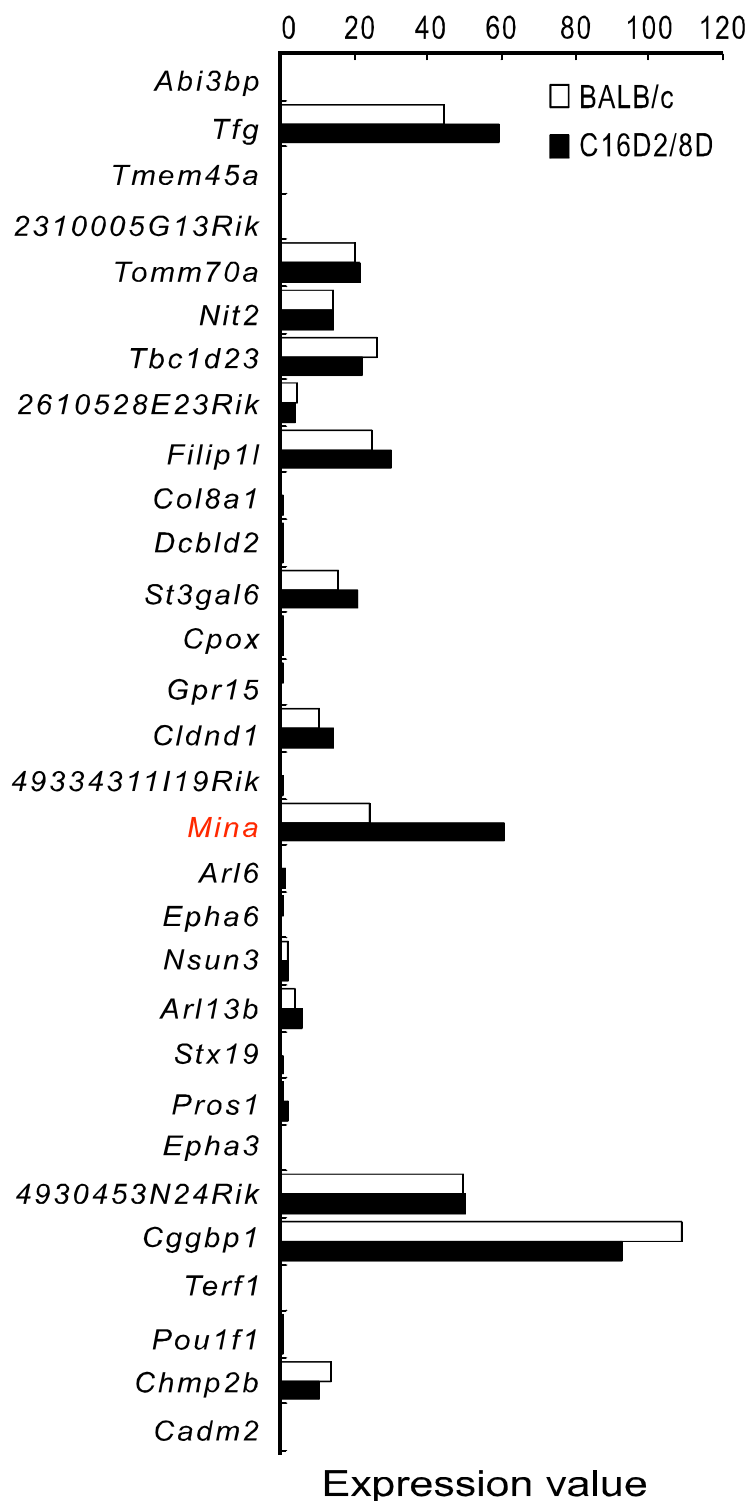
Statistical analysis. Prism software was used for nonparametric *t*-tests.

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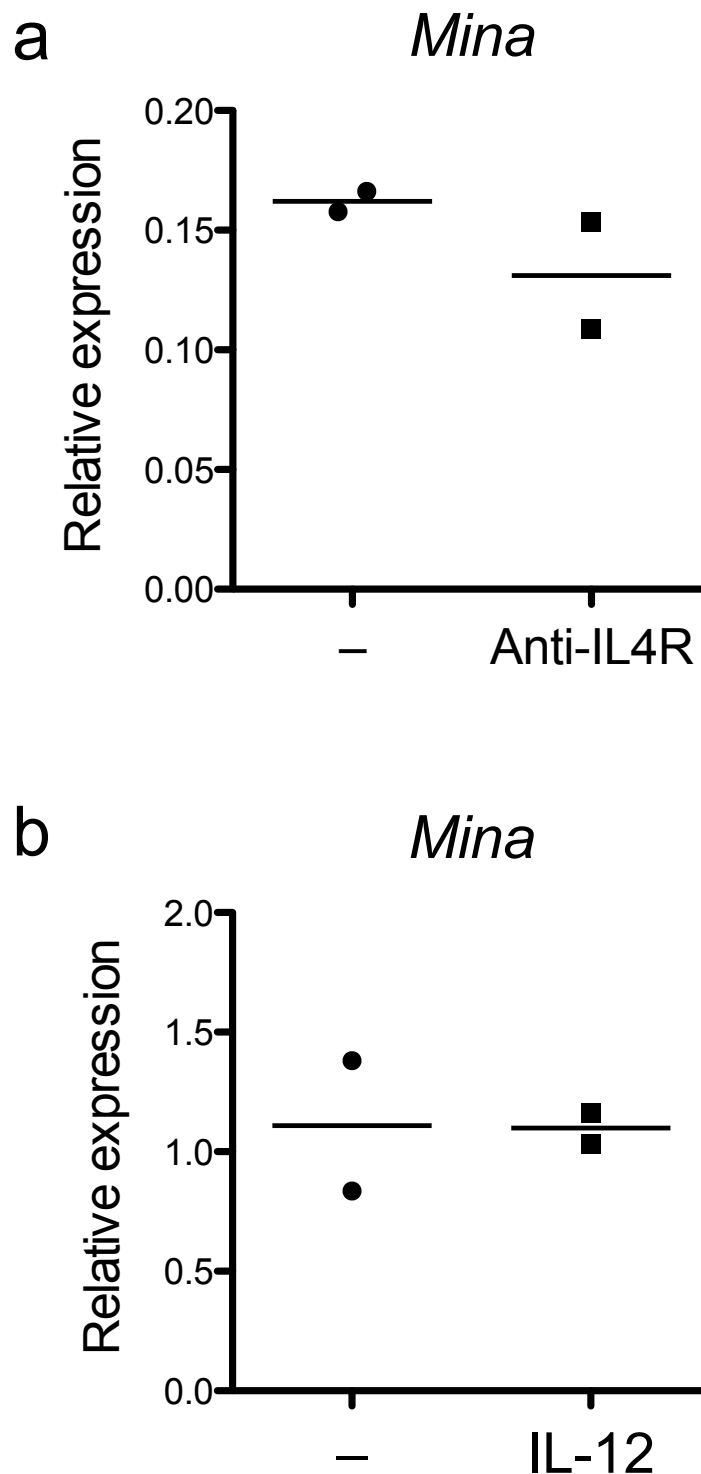
Supplementary Information

Supplementary Table 1. Primers and probes

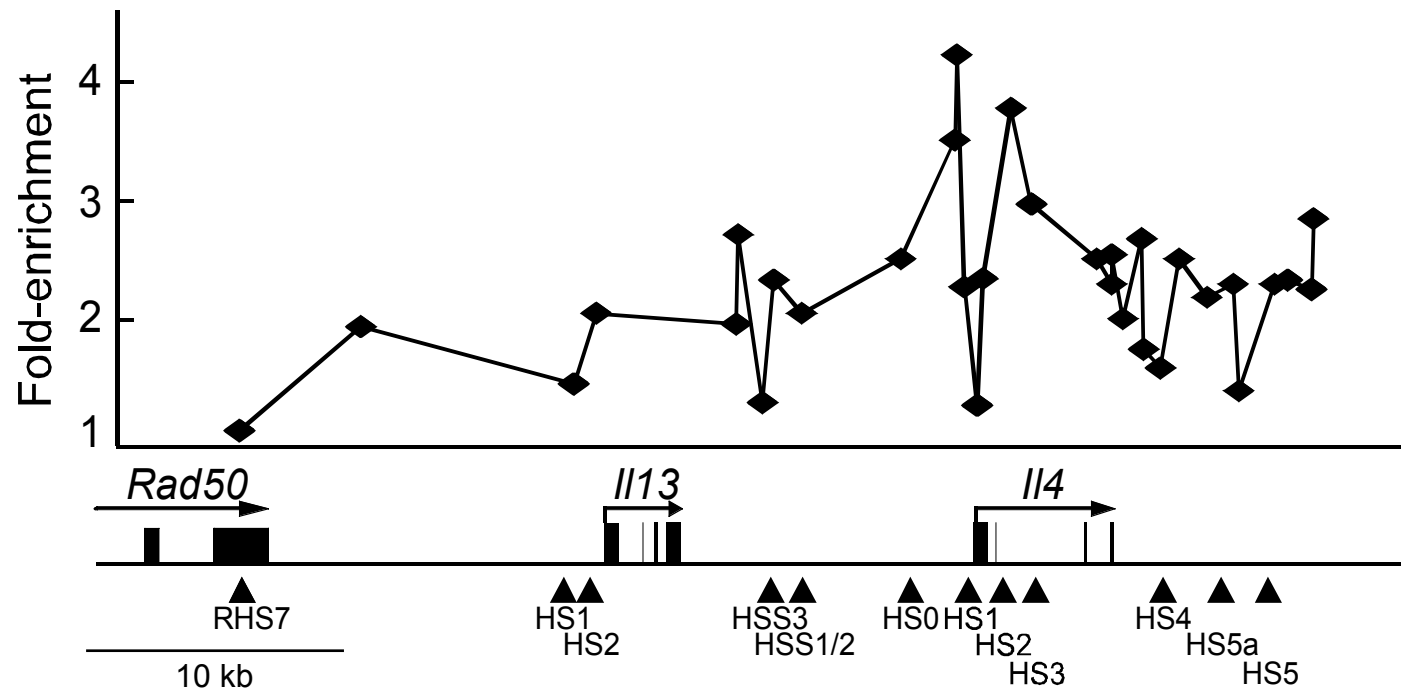
		Forward 5' to 3'	Reverse 5' to 3'
Mouse genotyping	SNP59.486 MB04 SNP59.536	ACTCATCACTAGGTGAGCGACACT AATATCTTTGGGCTTCCTACTGC TAACAGCAAGCCACTCTTCGTCCT	AGGCGTGTATGTGTCAGCCTTTCT ITCAATTGCTTTCATTTGGATCT TTGCCACCATGTCTTTGCTCTGTG
ChIP	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 -764/-604 -472/-272 -262/-152 -131/-36 -58/+64 CD3e	TCCTGTGAATCCTTCATGTCAAGC CAATCTTGTTCCTCCAGTAG GTCCTCTTATCGACCCCATC GTCTATATCCCTCCCACTCGT TCAAGCCTGATGACCTATGC GAATGCCAGTGTCTTACAGT GCAAAAGAACTCTCAGCAAGGCC CGAGGCCTCATTATCTTCAT TGCATGTACTTTGGTAGGGTC TCCAGCCTCTGCCTTATC CAAAGAGAGACCCGGTCTCC AGGTAAGACCCAGAGTCAGCTTTC CACTTGAGAGAGATCATCGG GCCTTGCTTGATACGGTATCT AGGCCTCTTCTGGTCACCTAACA CCCAGCGTTTACATGAGC CCCCACTTGGTTATTTATGAC GCATTTAGCCTACCGTGGC GAGAAGAACAGAGGCATGGCACTAC TGGCACTACACCTACCTTCCAT CCCAGGCTATTCCCAGTGTG CATCTGACACCATTTCTCGA CTGTGAGTACTTCGTGCAG AAGTAAGTCGTGAGGCTTGT GTGCGCACGTTGAAGTATGTGA GGCAGCTACTGCAGGAAGT ATGCCAACCTGAAGAACTAAC TCAGAGAGATGCAGCCTTTGACTAA GTGGTGCACACAGTGTAGGC CTGCCCATTCTTAATTAC TCACATGAGCCTTTGCAAGACA CAGGCCACACTCACAATAGAAGATG CAAAGAGAGACCCGGTCTCC AGGTAAGACCCAGAGTCAGCTTTC GTCTGAAAGGCCGATTATGG CTCCTGGAAGAGAGGTGCTG CATTTTCCTTGGTTTCAGC GGCCCGGATTTCTCAGTTA	AAGTGTCAATCATCGCTGGAGC AAGATGGAATCATGCCAGT AAAGGCTTGGGGAAACAC GGGCAGGTGAGTATCAGTCTA AGGGTCTCCAGAAGATTAGCC CATGCCAGTTGAGATTGT CCGTCCCTCTCGCTTCAGT GGAGATGACGAGGTTTGCTA AGAGTGGCACCTGAGACTTAG CTGGAGCAAAAGAATAGACCAT GGAGGTTCTGGGCTAGGTTG TCAGCGTAGGGTTGCCACTG CCACCTCTCTAGCAACTCAG GCCTGTAGGGACCATACGA GTCCCTCCCACTACAAATGGA GGGTGTGAATAAGCCATATTG CCCCACCACAATATTAGG AAGCTGCTATGTGAGTGCCG GTATGTATGATGCGTATGTGG ATGCGTATGTGGAGGTGAGAACAC CAGTCTCCCTGCCCTGTCTTA CATAGAGCATCTTCTGACGG AGAGTGCCAAGTCACTGTTAC AGGCATTATCCATGAGGTAC ACCAACCGCCAGCTTTATTGAC CGCCTAAGCACAATTCATT CAGATCAGACGCTCTCCACACT ACAGCCTGTTCTCTCAGCATT TGCTGTTACCGACAGGAAACA AGCCAGAACATCAGGGTTAG GGCTGCGGATGACTGATCAG TGAGACTTCCACGTGAGGATAAA GGAGGTTCTGGGCTAGGTTGAA CAGCGTAGGGTTGCCACTG TTTTCTGTGAAATCAGACC GTTGCTGAAACCAAGGGAAA CAATAGCTCTGTGCCGTCAG GCCATAGTAGGATGAAGGAG
Expression	<i>Mina</i> <i>Il4</i> <i>Il2</i> <i>Ifng</i> <i>Hprt</i> <i>Bactin</i> <i>Mina</i> <i>Intron 1</i>	CAGTAGGGCCAGATAAGAATCCAT CTTATCGATGAATCCAGGCATCG ATGTACAGCATGCAGCTCGCATC GGATGCATTATGAGTATTGC GAGGGTAGGCCTATGGCT ACTATTGGCAACGAGCGGTTT GTACTGGTGCTGATTGCAGCCAAA	CATGTGCATCTGCCTCACATT CATCGGCATTTTGAACGAACGAGGTCA GGCTTGTTGTTGAGATGATGCTTTGAC CCTTTCCGCTTCCTGAGG GTTGGATACAGGCCAGACTTTGTTG GGATGCCACAGGATTCCATACC TACTGAGTGCAGTCTGTGCGTTCA
EMSA and Pull-down	B B _{NM} IL2	Oligo GACAATCTGGTGTAAATAAAATTTTCCAATG GACAATCTGGTGTAAATAAAATTTTAAAATG CTGTATGAAACAGTTTTTCTCTCC	
Morpholino	Control Mina	CCTCTTACCTCAGTTACAATTTATA CTGTGGGCTGCACCTTCTTTGGCAT	



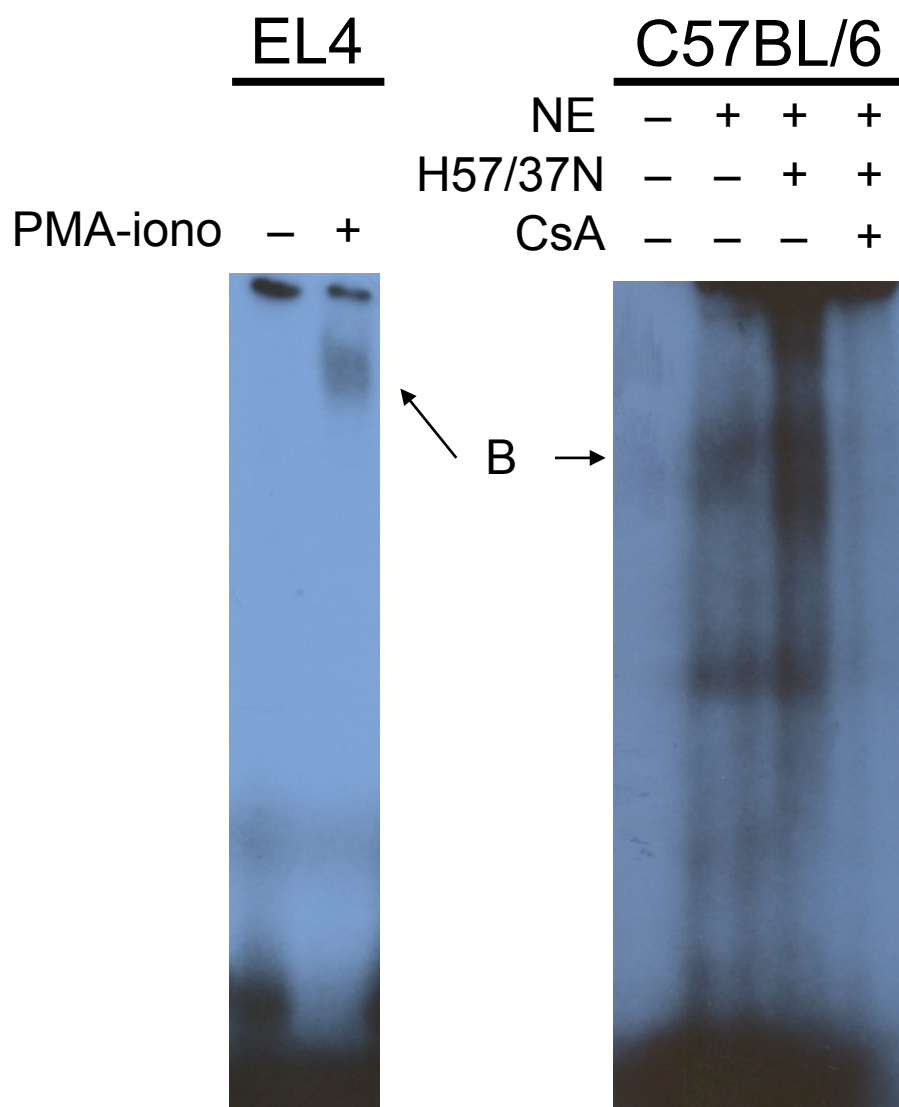
Supplementary Figure 1. *Mina* is a differentially expressed gene in the *Dice1.2* genetic interval. Comparative expression profiling of genes in the *Dice1.2* genetic interval. Shown for 30 of 81 *Dice1.2* candidate genes represented on Affymetrix expression profiling chips are expression values of mRNA isolated from BALB/c and C16D2/8D CD4⁺ T helper cells activated for 16 h by TCR cross-linking. This experiment was performed once. Similar results were obtained in a comparison of mRNA isolated from BALB/c and B10.D2 CD4⁺ T helper cells activated for 16 h by TCR cross-linking on duplicate Affymetrix chips.



Supplementary Figure 2. *Mina* acts upstream of IL-4 and IL-12 in naive T_H cells. *Mina* expression (*Hprt1*-normalized, arbitrary units) as measured by quantitative RT-PCR from BALB/c naive T_H cells stimulated with TCR cross-linking for 72 h **(a)** in the presence or absence of anti-IL4 receptor (M1, 25 µg/ml); **(b)** for 12 h in the presence or absence of IL-12 (100 ng/ml). Data are from 2 independent experiments. Each symbol represents an individual pool of mice (at least 4 mice/pool) and the mean is indicated by a horizontal line.



Supplementary Figure 3. ChIP survey of Mina binding at the TH2 locus. Top, ChIP plot of Mina fold-enrichment across the TH2 locus in B10.D2 CD4⁺ T cells that had been activated for 24 h. Bottom, schematic representing the *Il4* locus with exons indicated as tall rectangles, transcriptional orientation with horizontal arrows. And the location of DNaseI hypersensitive sites by triangles. This experiment was performed once.



Supplementary Figure 4. Formation of the Mina-probe B nucleoprotein complex requires cellular activation and is cyclosporin A sensitive. EMSAs were performed with radiolabeled probe B incubated with nuclear extracts (NE) isolated from EL4 thymoma cells (left) or C57BL/6 CD4⁺ T cells (right) following mock activation or treatment with TCR–CD28 crosslinking (H57/37N, 16 h) or PMA and ionomycin (PMA-iono, 4 h), where indicated in the presence of 1 μ g/ml cyclosporin A (CsA). Arrows indicate the location of the Mina-containing probe B nucleoprotein complex. Data are representative of 3 independent experiments.