Published in final edited form as:

Nat Immunol. 2009 August ; 10(8): 872–879. doi:10.1038/ni.1747.

***MINA*, an *IL-4* repressor controls T_H2 bias**

Mariko Okamoto^{1,*}, Melanie Van Stry^{4,*}, Linda Chung⁴, Madoka Koyanagi⁴, Xizhang Sun³, Yoshie Suzuki¹, Osamu Ohara², Hiroshi Kitamura², Atsushi Hijikata², Masato Kubo¹, and Mark Bix⁴

¹ Laboratory for Signal Network, Research Center for Allergy and Immunology, RIKEN Yokohama Institute, Suehiro-cho 1 7-22, Tsurumi, Yokohama, Kanagawa 230-0045, Japan

² Laboratory for Immunogenomics, Research Center for Allergy and Immunology, RIKEN Yokohama Institute, Suehiro-cho 1 7-22, Tsurumi, Yokohama, Kanagawa 230-0045, Japan

³ Department of Immunology, University of Washington, Seattle, WA 98195-7650

⁴ Department of Immunology, St. Jude Children's Research Hospital, 262 Danny Thomas Place St., Memphis, TN 38105 USA

Abstract

T helper-2 (T_H2)-bias, the propensity of naive $CD4^+$ T cells to differentiate into interleukin 4 (IL-4) secreting T_H2 cells, is a genetic trait impacting infectious, autoimmune and allergic disease susceptibility. T_H2 -bias correlates with the amount of IL-4 initially secreted by newly activated T_H2 cells that feeds back positively through the IL-4R-STAT6-GATA3 pathway to drive T_H2 development. Here, we identify *Mina*, a JmJC family member, 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, while its knockdown in primary $CD4^+$ T cells led to *Il4* derepression. Together, these findings provide mechanistic insight into an *Il4* regulatory pathway controlling T_H2 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 (T_H) 1 and T_H2 cells, differentially adapted for the control, respectively, of intra- and extracellular pathogens, in part via developmentally acquired potential for high expression of distinct cytokine genes¹. Dysregulated $CD4^+$ T cell development can promote susceptibility to infectious, autoimmune and allergic disease^{2–9}.

Interleukin 4 (IL-4) [<http://www.signaling-gateway.org/molecule/query?afcsid=A001262>], the canonical T_H2 effector cytokine, is also a critical developmental determinant, promoting

Correspondence should be addressed to M.B. (mark.bix@stjude.org.) or M.K. (raysolfc@rcai.riken.jp.), Mark Bix, Department of Immunology, St. Jude Children's Research Hospital, 262 Danny Thomas Place., Room E7064 Memphis, TN 38105 USA. Tel: (901) 595-5459, Fax: (901) 595-3107, Masato Kubo, Laboratory for Signal Network, Research Center for Allergy and Immunology (RCAI), RIKEN Yokohama Institute, Suehiro-cho 1 7-22, Tsurumi, Yokohama, Kanagawa 230-0045, Japan. Tel: 81 45-503-7047, Fax: 81 45-503-7046.

*Equal contribution

Accession codes

The microarray data are deposited in RCAI RefDIC (URL: <http://refdic.rcai.riken.jp/welcome.cgi>)⁵⁰ under the following accession codes: RMSPTB007001 and RMSPTB008001.

Author contributions

M.O. and M.V. did the experiments; L.C. did the *Mina* transcriptional analysis and supplemental figure 2; M.K. 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., M.Kubo and M.B. designed and conceptualized the research and analyzed the data; and M.B. prepared the manuscript.

T_H2 and inhibiting T_H1 differentiation¹⁰. Recently activated T_H cells make low but functional amounts of IL-4 that induce positive feedback through the IL-4 receptor (IL-4R) [http://www.signaling-gateway.org/molecule/query?afcsid=A001263] and transcription factors STAT6 [http://www.signaling-gateway.org/molecule/query?afcsid=A002236] and GATA3^{11, 12} to promote the differentiation of T_H2 cells possessing the capacity to secrete copious amounts of IL-4^{10, 13–18}. Thus, regulation of autocrine IL-4 expression by activated T_H 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 characterizing variation in the propensity of naive T_H cells to differentiate into T_H2 (as opposed to 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 T_H cells activated under ‘neutral’ conditions (no exogenous cytokines added, 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 2 (PGE₂)-dependent inhibition of interferon- γ (IFN- γ) production²³, the timing of IL-12R β 2 downregulation^{24, 25} and the capacity of activated T_H cells to produce autocrine IL-4 (refs. 14–16, 20). Genetic approaches to dissect T_H2-bias have yielded numerous quantitative trait loci (QTL) 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 QTLs have been confirmed and isolated as discrete genetic loci by interval specific congenic mapping^{14, 26, 28}. However, to date 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 (Jmj) family, as a determinant of the T_H2-bias regulatory locus *Dice1.2* that was identified through its activity in the autocrine IL-4 pathway of activated T_H cells²⁸. We found that T_H2-bias and autocrine *Il4* expression correlated inversely with *Mina* transcriptional rate that, in turn, correlated with *Mina* locus haplotype. Consistent with these findings, we show that *Mina* bound to the *Il4* promoter where it repressed *Il4* expression. We propose that a regulatory polymorphism controlling *Mina* expression in activated T_H 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.

Results

Mina is a *Dice1.2* candidate gene

Using QTL and interval specific congenic mapping, we previously mapped an *Il4* regulatory locus controlling T_H2-bias, *Dice1.2*, to a 26.8 Mb chromosome 16 genomic interval (Fig. 1a and 14, 28). To resolve this interval further, we generated sub-congenic strain C16D2/8D that bisects the *Dice1.2*-spanning B10.D2 (low T_H2-bias)-derived genomic segment contained in the BALB/c (high T_H2-bias) background congenic strain C16D2/8 (ref. 28) to 14.1 Mb (Fig. 1a, D16MIT138-MB04). Splenic CD4⁺ T cells from BALB/c, B10.D2 and C16D2/8D were primed for 16 h before analysis of *Il4* expression by quantitative reverse transcriptase PCR (RT-PCR). As expected, control BALB/c and B10.D2 cells exhibited, respectively, high and low T_H2-bias phenotypes (Fig. 1b). Production of *Il4* transcripts by C16D2/8D cells was similar to B10.D2 and significantly less than BALB/c ($P = 0.0317$). This result indicates that *Dice1.2* occurs within the 13.7 Mb C16D2/8D congenic interval that spans D16MIT138 to MB04 and contains 121 predicted or known genes³⁰. Twenty nine of these encode olfactory receptors, leaving 92 *Dice1.2* candidates.

The polymorphism(s) in *Dice1.2* underlying genetic variability in T_H2-bias may reside in coding or regulatory sequence. To test for the latter, we interrogated genes in the *Dice1.2* interval by transcriptional profiling using mRNA isolated from BALB/c and C16D2/8D CD4⁺ T cells stimulated by T cell receptor (TCR) cross-linking for 16 h, a time point when differential *Il4* expression was nearly maximal. The Affymetrix gene chip used for initial screening contained 30 *Dice1.2* candidate genes. Of these, half were undetectable and of the remainder, all but one displayed similar expression in BALB/c and C16D2/8D cells. The one exception was *Mina* which exhibited two-to-three-fold higher expression in C16D2/8D than in BALB/c cells (Supplementary Fig. 1 online). Although expression profile analysis of the remaining genes in the *Dice1.2* interval could reveal additional *Dice1.2* candidates, we explored here the possibility that *Mina* acts as a negative regulator of *Il4* gene expression and T_H2-bias.

Inverse correlation in IL-4 and *Mina* levels

First, to investigate whether the inverse correlation in *Mina* and *Il4* expression observed in BALB/c and B10.D2 was generalizable, 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 high and low T_H2-bias phenotypes. Whereas the kinetics of *Mina* and *Il4* transcriptional induction was similar across the tested strains, peak magnitudes were markedly dissimilar, segregating the tested strains into two discrete groups (Fig. 2a). In low T_H2-bias C57BL/6, B10.D2 and C3H/HeN strains, high *Mina* expression correlated with low *Il4* expression; while in high T_H2-bias BALB/c, DBA/1J, DBA/2J and DBA/2N strains, the converse was true (Fig. 2a). Thus, the inverse correlation in *Il4* and *Mina* expression was generalizable across strains with varying T_H2-bias phenotypes.

From the previous analysis of splenic CD4-enriched T cells, it is not clear whether the inverse correlation in *Il4* and *Mina* expression occurs in naïve progenitor T_H or effector T_H cells. To investigate this question, we compared cytokine gene expression in highly purified naïve T_H cells from BALB/c and C57BL/6. Quantitative RT-PCR analysis of naïve T_H cells (99% CD4⁺CD62L^{hi}CD44^{lo}; less than 0.2 % natural killer T cell contamination) stimulated for 24 h with plate-bound antibodies to TCR and CD28 revealed an inverse correlation in *Mina* and *Il4* expression, similar to that observed in CD4-enriched cells (Fig. 2b, top). Next, we compared restimulated memory effector cells generated from C57BL/6 T_H cells primed under 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 naïve T_H cells from C57BL/6 and BALB/c, T_H1 and T_H2 cells displayed similar kinetics and magnitude for *Mina* expression. Thus, the disparate *Il4* expression potential of T_H1 and T_H2 cells is not inversely correlated with *Mina* expression. By contrast, natural variation in the capacity of naïve T_H 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 between *Il4* and *Mina* expression, either negative regulation of *Il4* by *Mina* or negative regulation of *Mina* by IL-4 signaling. To distinguish these, we compared *Mina* mRNA abundance from 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). Similar results were obtained with highly purified naïve T_H cells (Supplementary Fig. 2a online). Given the ability of IL-12 to promote and inhibit T_H1 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, addition of IL-12 did not

enhance the low *Mina* expression of BALB/c naive T_H cells (Supplementary Fig. 2b). Together, these results indicate that neither the IL-4 nor the IL-12 signaling pathways regulate *Mina* expression, providing support for the idea that *Mina* acts as a negative regulator of *Il4* expression in naive T_H cells.

Mina is transcriptionally regulated

We next asked whether differential *Mina* transcript abundance led to differential protein abundance. Purified BALB/c and C57BL/6 naive T_H cells were activated over a 72-h time course with plate-bound antibodies to the TCR and CD28 and the resulting nuclear and cytoplasmic extracts were analyzed by immunoblot using a *Mina*-specific antibody (Fig. 3a). Whereas cytosolic *Mina* increased with time, nuclear *Mina* appeared transiently, peaking around 24 h in both BALB/c and C57BL/6. In both subcellular compartments *Mina* protein amounts were consistently higher (~2–5 fold) in C57BL/6 than in BALB/c, demonstrating tight linkage between *Mina* protein and mRNA abundance. To determine whether *Mina* was regulated transcriptionally, we exploited the fact that pre-mRNA abundance correlates with transcriptional rate³¹. Measurement of *Mina* pre-mRNA abundance revealed higher expression in C57BL/6 than in BALB/c newly activated T_H cells, correlating with differential *Mina* mRNA abundance, (Fig. 3b). Taken together, these results indicate that *Mina* protein abundance is transcriptionally regulated.

To explore whether regulatory polymorphisms could account for differential *Mina* transcription between high and low T_H2-biased strains, we sequenced the 5' end of the *Mina* genomic locus (including intron 1 and 1304 bp of the promoter) from high (BALB/c and DBA/2J) and low (C57BL/6, B10.D2/OsnJ, C3H/HeN) T_H2-bias phenotype strains. We identified 21 SNPs that defined precisely two haplotypes (Fig. 4), correlating with T_H2-bias and *Mina* expression phenotypes (Fig. 2). Thus, *Mina* haplotypes are a useful genetic marker for T_H2-bias and may contain regulatory polymorphism(s) responsible for differential *Mina* expression across strains with distinct T_H2-bias phenotypes.

Mina specifically binds and represses the *Il4* promoter

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

To further refine the *Mina* responsive region, we performed electrophoretic mobility shift assays (EMSA). Nuclear extracts isolated from TCR-stimulated B10.D2 CD4⁺ T cells were tested against a series of end-labeled DNA probes spanning the 140 bp *Il4* promoter (Fig. 5b). Nucleoprotein complexes were formed with probes A and B but not C or D (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 probe B region that extends from -157 to -128 bp.

To determine whether *Mina* binds to the *Il4* promoter *in vivo*, we performed anti-*Mina* 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 -157 to -128 region, we observed *Mina* enrichment in the *Il4* promoter but not in the CD3

epsilon promoter (Fig. 5d). Furthermore, the magnitude of binding activity at the *Il4* promoter was consistently higher from B10.D2 as compared to BALB/c cells, correlating with the relative expression of Mina in these two strains (Fig. 2e). A broad ChIP survey of the entire T_H2 locus in 24 h activated B10.D2 CD4⁺ T cells also detected a strong peak of Mina binding at the *Il4* promoter (Supplementary Fig. 3). Taken together, these data support a model in which Mina is recruited to the proximal *Il4* promoter where it exerts a concentration-dependent transcriptional repressive effect.

Mina recruitment 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 previously described binding sites for the transcription factors NFAT

[<http://www.signaling-gateway.org/molecule/query?afcsid=A001640>; <http://www.signaling-gateway.org/molecule/query?afcsid=A000024>] and Oct^{33, 34}, raising the possibility of their involvement in recruiting Mina to the *Il4* promoter (Fig. 6a). To test this, we used wild-type and mutant probe B to perform EMSA analysis with nuclear extracts from the Mina-expressing T cell thymoma line EL4 (ref. 35). 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 online). This complex was sensitive to disruption by a Mina-specific antibody, indicating that the probe B complex formed with EL4 extract (like that formed with B10.D2 CD4⁺ T cell extract) contained Mina (Fig. 6b). Whereas a wild-type NFAT consensus sequence could compete with probe B for complex formation, a wild-type Oct consensus sequence could not (data not shown), suggesting that the Mina complex contains NFAT but not Oct. Furthermore, a probe B mutant incapable of binding Oct was still able to form the Mina complex (data not shown). Thus, Oct is neither a constituent of, or required for Mina recruitment to the *Il4* promoter.

Formation of higher-order complexes using antibodies specific for NFATc1 and NFATc2 confirmed the presence of both NFAT proteins in the probe B-Mina complex (Fig. 6b and data not shown). A mutant probe B (B_{NM}), unable to bind NFAT³⁴, could not form the Mina complex and failed to compete probe B for complex formation (Fig. 6b), suggesting a requirement of NFAT for Mina recruitment. To confirm this notion, we performed DNA 'pull-down' assays with biotinylated probes B and B_{NM}. Whereas both Mina and NFAT (c1 and c2) could be coprecipitated with the biotinylated wild-type probe B, B_{NM} failed to coprecipitate with either NFAT protein or Mina (Fig. 6c and data not shown). Interestingly, a biotinylated NFAT-binding sequence from the *Il2* promoter failed to coprecipitate Mina despite binding both NFATc1 and NFATc2 (Fig. 6c and data not shown), indicating that NFATc1 and NFATc2 were insufficient for Mina recruitment. Finally, nuclear extracts from 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). Taken together, these data suggest that NFAT is necessary, but not sufficient, to recruit Mina into the *Il4* promoter-specific nucleoprotein complex.

Mina is necessary and sufficient to constrain IL-4 expression

To test directly whether Mina can repress *Il4* transcription in CD4⁺ T cells, we generated 3 independent BDF1XB6 mouse lines expressing a *Mina* transgene driven by a lymphocyte-specific proximal *Lck* promoter-E_μ enhancer³⁶. We analyzed mice after 3 backcrosses to BALB/c, at which point they displayed normal T cell development and activation marker expression (data not shown). Using quantitative RT-PCR to compare *Mina* expression in CD4⁺ T cells, we found that all three transgenic lines displayed high basal *Mina* expression that diminished over a 48 h activation time course to lower expression comparable to or higher than that exhibited at the 'peak of expression' by their respective wild-type littermate

controls (Fig. 7). To determine the effect of transient enforced elevation of Mina abundance on cytokine gene expression, we examined the transcriptional induction of *Il2*, *Il4* and *Ifng*. In comparison to wild-type littermate controls expression of *Il4*, but not *Il2* and *Ifng*, was markedly impaired in all three Mina transgenic lines (Fig. 7). Thus, transient enforced elevation of Mina in CD4⁺ T cells specifically impairs expression of *Il4* but not *Il2* or *Ifng*.

To test whether Mina-dependent repression is required to maintain normal expression of *Il4*, we used anti-sense morpholino technology to knockdown Mina protein in primary CD4⁺ T cells. C57BL/6 CD4⁺ T cells pre-treated for 16 h with *Mina*-morpholino (MinaMO) expressed half the amount of Mina protein as mock or control-morpholino (CoMO) pre-treated cells 24 h after activation (Fig. 8). By contrast, MinaMO pre-treated cells expressed ~2-fold higher amounts of *Il4* (but not *Ifng*) mRNA than mock or CoMO pre-treated cells (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*-T_H2-bias regulatory locus *Dice1.2*. Our data suggest that Mina is a necessary and sufficient dose-dependent *Il4*-specific repressor in naive T_H cells, affecting the magnitude of the early autocrine IL-4 burst required to program T_H2 development. Furthermore, our results show how natural variation in *Mina* transcription (likely 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 (2OG)-dependent dioxygenase enzymes. The catalytic dioxygenase core comprises three iron- and two 2OG-binding residues, of which 4 are conserved in Mina³⁷. 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), was shown recently to possess 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 HIF39⁴⁰. Despite imperfect conservation of its core catalytic residues, Mina is a functional Fe(II)-2OG-dependent dioxygenase enzyme (personal communication, Christopher Schofield). Thus, an attractive hypothesis is that Mina acts as an *Il4* repressor by demethylating histones at key positive regulatory elements such as the 3' HSV site⁴¹, reported to be required for Notch-mediated activation of autocrine IL-4 expression in naive T_H cells^{42, 43}. Preliminary results suggest that H3K4me3 abundance is similar at HSV, HSVa, HSIV and HSS3 between naive CD4⁺ T cells from BALB/c and C57BL/6, inconsistent with a histone demethylating role for Mina. Another attractive hypothesis is that Mina catalyzes post-translational changes that modulate key *Il4* transcription factors. In this regard, it is tantalizing 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 indicate that while Oct is excluded from the Mina complex, an NFAT-dependent interaction is required for Mina recruitment to the *Il4* promoter. However, though necessary, NFAT is not sufficient for Mina recruitment to the *Il4* complex as it does not associate with an *Il2* promoter fragment capable of recruiting both NFATc1 and NFATc2. This result implies the existence of specificity-determining sequence elements in addition to those required for NFAT

recruitment. Identification of these elements may assist identification of factors required with NFAT to recruit *Mina* to the *Il4* promoter.

Mina was first identified as a Myc-induced nuclear antigen of 53 kDa, found to be overexpressed and associated with poor prognosis in a variety of human cancer cells^{45–48}. Studies using ChIP and inducible Myc-ERTam showed that *Mina* is a direct Myc target in human promyelocytic leukaemia and human glioblastoma cells⁴⁸. Comparison of the *Mina* promoter sequence between high and low T_H2-biased strains failed to reveal a polymorphism in a presumptive Myc binding site (data not shown), suggesting that differential Myc activity may not be responsible for differential *Mina* expression in high and low T_H2-biased strains. Furthermore, Myc expression in activated naive T_H cells is similar in high and low T_H2-biased strains and its acute deletion does not impair *Mina* expression in naive T_H cells (M. Koyanagi, unpublished data). Thus, in naive T_H cells *Mina* may not be a *bona fide* Myc target.

21 *Mina* promoter and intron-1 SNPs define two distinct haplotypes that correlate with *Mina* expression phenotype. The polymorphism(s) underlying differential *Mina* expression is likely to 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 used to identify gene regulatory elements as regions of so-called conserved non-coding sequence (CNS49). We have identified 6 CNSs in the 5 kb region of the *Mina* locus containing the 21 SNPs. Four of the 21 haplotype-defining SNPs (SNP-1, 2, 5 and 10), occur in 3 CNSs (CNS-4, 2 and 1) and are thus candidates for the functional regulatory polymorphism underlying differential *Mina* expression that distinguishes high and low T_H2-biased strains. Identification of *Mina* regulatory polymorphism(s) will provide candidates for the causative *Dice1.2* genetic lesion and a foothold to dissect critical upstream pathways.

Although *Mina* can explain the *Il4*–T_H2-bias regulatory activity of *Dice1.2*, it remains possible that other genes in the minimal *Dice1.2* interval contribute to its activity. Furthermore, *Dice1.2* is one of two (the other being *Dice1.1*) *Il4*–T_H2-bias regulatory loci originally identified on chromosome 16 (ref. 28). Thus, the identification of the molecular basis of *Dice1.1* is likely to add further mechanistic insight into the complex regulation of *Il4* and T_H2-bias. Interestingly, *Dice1.2* (but not *Dice1.1*) is tightly linked genetically to a host response modifying locus (*LmrA*) for the protozoan parasite *Leishmania major*, responsible for leishmaniasis disease²⁸. As T_H2-bias plays a critical role in determining leishmania disease susceptibility, it is possible that *Mina* is *LmrA*. In this regard, it is perhaps relevant that among hematopoietic cell types *Mina* is expressed in CD4⁺ T cells and dendritic cells, the latter cell type recently implicated, too, as a contributor to the differential T_H2-bias phenotypes of BALB/c and B10.D2. A dual T_H2-bias regulatory role of *Mina* (via separate activities in naive T_H and dendritic cells) could explain the linkage of *Dice1.2*, but not *Dice1.1*, to *LmrA*. Investigation of the role of *Mina* in dendritic cells may thus provide further insight into the mechanism of T_H2-bias.

Together, our data reveal a novel IL-4 repressive pathway in which *Mina* plays a key role regulating the amount of IL-4 produced by naive T_H 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, the downstream targets and the repressive mechanism of *Mina* and the extent to which the *Mina* pathway may be conserved and manipulated in humans.

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 purchased from Jackson Lab, Taconic Farms, CLEA and Charles River Laboratory.

Reagents and antibodies

Anti-TCR β was purified from hybridoma H57.597. Anti-CD28 was purified, respectively, from hybridomas PV-1 and 37N51.1, kind gifts, respectively from R. Abe (Science University of Tokyo) and J. Allison (Memorial Sloan Kettering Cancer). 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 (gift from G. Trinchieri, NIH). Anti-Mina antibody was purchased from (Zymed, clone M532, ref. 46-47). Anti- NFATc2 (NFAT1/NFATp; Tamar Laboratory Supplies; clone 25A10.D6.D2), anti-NFATc1 (NFAT2; Santa Cruz, sc-13033), anti-Oct1 (Santa Cruz, sc-232), anti-Actin (Santa Cruz sc-1616), anti-rabbit secondary antibody (Santa Cruz), anti-goat HRP secondary (Santa Cruz), anti-H3K4me3 (Upstate 07-030) and isotype control IgG (Abcam AB46540-1) were purchased. Human recombinant IL-2 was used at 20 U/ml. Mouse recombinant IL-4 was purchased from PeproTech.

Expression profiling

CD4⁺ T cells isolated from spleen and lymph nodes were stimulated with plate-bound anti-TCR β mAb (1 μ g/ml) and anti-CD28 mAb (10 μ g/ml). After 24 h, total RNA was extracted (Tri reagent, Sigma-Aldrich) and purified on RNeasy mini-columns (Qiagen). cRNA were synthesized from the isolated RNA according to Affymetrix protocols. Biotinylated cRNAs were hybridized to Affymetrix GeneChips (mouse Genome 430 version 2, Affymetrix). Microarray data were globally normalized using GeneSpring software (Agilent Technologies).

Quantitative RT-PCR

cDNA was synthesized from total cellular RNA using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR analysis was performed on MX4000 (Stratagene) and 7500 (Applied Biosystems) real-time PCR systems. See Supplementary Table 1 online for primer sequences. mRNA expression was normalized to either *Actb* or *Hprt1*, as indicated in Figure legends.

Congenic mouse strains and phenotypic screening of Th2-bias

Generation of congenic strain C16D2/8 was described previously²⁸. C16D2/8D was selected from the backcross progeny of [C16D2/8 $\times$ BALB/c]. The congenic interval in C16D2/8D was bred to homozygosity by intercrossing and characterized by genotyping with the markers shown in Fig. 1a and Supplementary Table 1. Splenic CD4⁺ T cells, isolated by MACS (CD4⁺ T cell Isolation kit, Miltenyi), were stimulated with plate-bound anti-TCR β (1 μ g/ml) and anti-CD28 (10 μ g/ml) antibodies for 16 h prior to harvesting RNA (Tri reagent, Sigma-Aldrich) and determining *Il4* and *Hprt1* expression by reverse transcriptase real time PCR, as described^{41, 51}. In some experiments, splenic CD4⁺ T cells were isolated by iMag (CD4 T cell Isolation kit, Sigma) and stimulated with plate-bound anti-TCR (1 μ g/ml) and soluble anti-CD28 (PV-1, 10 μ g/ml) antibodies for 24 h prior to harvesting RNA.

Immunoblots

Naive T_H cells were stimulated with phorbol myristate acetate (PMA) and ionomycin. Nuclear and cytoplasmic protein were isolated from 15×10^6 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce, 78833). 15 µg of nuclear and cytoplasmic protein were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes and reacted with anti-Mina and anti-Actin antibody. After reaction with HRP conjugated anti rabbit secondary antibody, blots were visualized by chemiluminescence substrate (Roche). Mina expression was normalized with Actin expression.

Transient reporter assay

Strain B10.D2 *Mina* cDNA was inserted into pCMV10 (Sigma-Aldrich) to generate pCMV FLAG-Mina expression construct. 68-41 T cell hybridoma cells were cotransfected with plasmids encoding Firefly luciferase driven by the *Il4* or *Il2* promoters (pIL-4 Luc and pGL-2), pCMV10 or pCMV FLAG-Mina and a pancreatic alkaline phosphatase (PAP) expression plasmid (pSVPAP, included to control for transfection efficiency). 40 h post-transfection, cells were stimulated for 12 h with plate-bound anti-TCR mAb and total cell lysates were prepared. Luciferase activity measured with a Microplate luminometer (Promega) was normalized to PAP activity.

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-TCRβ (10 µg/ml) and anti-CD28 (20 µg/ml), in the presence or absence of cyclosporin A (1 µg/ml, Calbiochem52). Nuclear extracts were prepared with the NE-PER extraction kit according to manufacturers directions (Pierce). Protein concentrations were determined using the Bradford Assay (Pierce) with bovine serum albumin as a standard. Oligonucleotides were annealed in annealing buffer (100 mM Tris pH 7.5, 10 mM EDTA, 2 M NaCl, 50 mM MgCl₂) by heating to 95 °C followed by slow cooling to 22°C. For gel shifts, annealed oligonucleotides were end-labeled with ATP-γ³²P and T4 polynucleotide kinase (Promega). Nuclear extracts (10 µg) or bovine serum albumin (BSA), ³²P-labeled oligonucleotide (1×10^4 c.p.m.), and poly(dI:dC) (125 ng, Sigma) were incubated together at 22°C for 30 min in 20 µl binding buffer (10 mM Tris pH 7.5, 60 mM KCl, 2 mM MgCl₂, 0.15 mM dithiothreitol). For supershifts, antibody (2–3 µg) was included in the reaction. Binding reactions were resolved in 1 X TAE 4% polyacrylamide gels.

DNA pull-down

Pull-down assays were performed as described previously⁵³. Briefly, streptavidin-agarose beads (300 µl, Pierce) were pre-absorbed with BSA (500 µl, 1 mg/ml), polydI:C (50 µg, Sigma), and sheared salmon sperm DNA (50 µg), then washed three times and resuspended in EMSA binding buffer (300 µl). Annealed 5' biotin-labeled oligonucleotides (1 µg) were incubated for 30 min at 22°C with nuclear extract (300 µg) from 4 h PMA/IONO-stimulated EL4 cells in EMSA binding buffer with polydI:C (10 µg). 30 µl of pre-absorbed beads were added to the oligonucleotide–protein mixture and incubated at 4 °C for 4 h. DNA-protein-streptavidin-agarose complexes were washed three times with EMSA binding buffer, eluted in Laemmli buffer, resolved on 12% SDS-PAGE gels, transferred to nitrocellulose membranes and blotted with anti-Mina (1 µg/ml) and anti-NFATc2 (1:5000 dilution).

ChIP

ChIP was performed using both the equivalent mass and equivalent volume methods. Briefly for the equivalent mass method (described previously⁵⁴), DNA recovered from an aliquot of sheared chromatin was used as the “input” sample. The remaining chromatin was pre-

cleared with protein A- and protein G-agarose (Upstate, cat. 16–156 and 16–266) and then incubated with anti-Mina antibody (2 µg/ml) overnight at 4 °C. DNA recovered after immunoprecipitation (IP) was isolated using a PCR purification kit (QIAGEN) and quantified using picogreen fluorescence (Molecular Probes). Equivalent mass of IP 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 sec, 61 °C for 1 min and 72 °C for 40 sec. Data are presented as the ratio of IP to input C_T values.

For the equivalent volume method (described previously⁵⁵), chromatin was prepared as described above and then incubated with anti-Mina or control IgG (10 µg/ml) overnight at 4 °C. Input DNA and DNA recovered after immunoprecipitation (IP) were isolated using a PCR purification kit (QIAGEN). 10 µl of IP or 10 µl of a 1:20 diluted input DNA was analyzed by real time PCR as described above for RT-PCR with the following modifications. Taq polymerase was from Sigma and cycling conditions were: 94 °C for 15 min followed by 45 cycles of 94 °C for 20 sec, 61 °C for 1 min and 72 °C for 40 sec. Data are presented as the ratio of IP to input C_T values.

***Mina* transgenic mice**

To establish *Mina* transgenic mice, a B10.D2 *Mina* cDNA was cloned downstream of the *Lck* proximal promoter and E_μ enhancer in the p1026X expression vector⁵⁶. PCR of genomic tail DNA identified ten independent BDF1XB6 transgenic founders. Transgene copy number was determined by Southern blot analysis. Three founders harboring over 10 copies of the transgene were selected and backcrossed for three generations to BALB/c prior to further analysis. Protein expression in splenic CD4⁺ T cells was confirmed by immunoblot analysis with anti-Mina.

Anti-sense morpholino mediated knock-down

CD4 T cells (enriched from C57BL/6 spleen and lymph nodes using complement mediated lysis with anti-HAS (J11d), anti-classII (BP107), anti-CD8 (53.67.2) were incubated overnight with endoproteinase reagent⁵⁷ (6 µl/ml) and FITC-labeled standard control or *Mina* morpholino (Supplementary Table 1) (10 µM, Gene-Tools) in the presence of IL-7 (3 ng/ml). Transfection efficiency was greater than 80% as determined by FACs. CD4⁺ T cells were then activated with plate-bound H57/37N in the presence of IL-2 (25 U/ml) for 24 h prior to immunoblot analysis for knock-down of *Mina* and Actin and quantitative RT-PCR expression analysis 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, Bar Harbor, Maine. World Wide Web (URL: <http://www.informatics.jax.org>); May, 2009)³⁰.

Statistical analysis

Prism software was used for non-parametric *t*-tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank A. Matsuno, M. Nakamura, M. Natsume, J. Epler, Y. Zhang, N. Li, S. Brown and R. Cross for technical help; J. Partridge for discussions; D. Green, H. Beere, and J. Kang (U. Mass. Medical School) for comments on the manuscript. Supported by the Cancer Research Institute (M.B.), the Burroughs Wellcome Fund (M.B.), American Lebanese Syrian Associated Charities (M.B.), MEXT of Japan (M.Ku.), the RIKEN RCAI International Collaboration Award Program (M.B. and M.Ku.), a Grant-in-Aid for Scientific Research (B) (M.K.), a Grant-in-Aid for Scientific Research on Priority Areas of the Ministry of Education, Culture, Sports, Science, and Technology (Japan) (M.Ku.), the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (M.Ku.), and the National Institutes of Health (M.B., AI048636).

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Figure 1.

Mina is a *Dice1.2* candidate gene. **(a)** Location of chromosome 16 congenic intervals in C16D2/8 and C16D2/8D. Shown for each strain are the chromosomal regions inherited from B10.D2 (grey), BALB/c (white) or undetermined (striped). Genotyped locations are indicated with cyan dots. Genotyping markers are indicated on the right axis, with *Mina* in red. Long and short vertical bars indicate the original and new *Dice1.2* genetic intervals, respectively. **(b)** Relative *Il4* expression (*Hprt1*-normalized, arbitrary units) in TCR-activated CD4⁺ T cells from BALB/c (BALB), B10.D2 (D2) and C16D2/8D mice ($n = 2-5$; each symbol represents an individual mouse and the mean is indicated by a horizontal line).

* Statistical significance was determined using a *t*-test; $P = 0.0317$. Data are representative of 2 experiments with similar results.

Figure 2.

Inverse correlation in *Mina* and *Il4* expression in naïve T_H cells. (a) *Mina* (left) and *Il4* (right) mRNA expression (*Actb*-normalized, arbitrary units) time course from TCR cross-linking-activated $CD4^+$ T cells isolated from low (B10.D2, C57BL/6, and C3H/HeN; filled symbols) and high (BALB/c, DBA/1J, DBA/2J, and DBA/2N; open symbols) T_H2 -biased strains. Data are the mean of three independent experiments and error bars indicate SEM. (b) *Mina*, *Il4* and *Ifng* mRNA expression (*Hprt1*-normalized, arbitrary units) time course from (top) TCR cross-linking-activated BALB/c (open symbol) and C57BL/6 (closed symbol) naïve T_H cells or from (bottom) T_H1 - (closed symbol) and T_H2 - (open symbol) primed C57BL/6 naïve T_H cells. Data are representative of two independent experiments

and error bars indicate SEM for triplicate PCR measurements. **(c)** *Mina* expression (*Actb*-normalized, arbitrary units) in CD4⁺ T cells stimulated with TCR cross-linking for 24 h in the presence of graded doses of IL-4 (B10.D2) or anti-IL-4 (BALB/c). Data are the mean of three independent experiments and error bars indicate SEM. **(d)** *Mina* expression (*Actb*-normalized, arbitrary units) in CD4⁺ T cells from B10.D2 mice stimulated for 24 h in the presence of anti-IL12. Data are the mean of three independent experiments and error bars indicate SEM.

Figure 3.

Mina is transcriptionally regulated. **(a)** Mina and Actin immunoblots from cytosolic and nuclear extracts of BALB/c and C57BL/6 naïve T_H cells activated by PMA-ionomycin over the indicated time course. The upper band in the nuclear Mina immunoblot is non-specific. Actin-normalized data are plotted to the right of each set of blots. Data are representative of two independent experiments. **(b)** *Mina* pre-mRNA expression (*Hprt1*-normalized, arbitrary units) time course from TCR-crosslinking activated naïve T_H cells isolated from pools of BALB/c (open squares, n = 5) and C57BL/6 (closed squares, n = 20) mice. Expression was measured by quantitative PCR using primers targeting sequences from *Mina* intron 1. Data are representative of 2 independent experiments and error bars indicate SEM for triplicate PCR measurements.

Figure 4. *Mina* haplotype predicts T_H2 -bias phenotype. **(a)** Map of the promoter- proximal end of the *Mina* genomic locus. Indicated are the 21 SNPs identified across this interval, along with exons (red), coding region (yellow) and conserved non-coding sequences (CNS; cyan). **(b)** Position of each SNP (relative to the translational start site in exon2) and its allelic identity for 3 low (red) and 2 high (green) T_H2 -bias strains.

Figure 5.

Mina can bind to and repress transcription from the *Il4* promoter. **(a)** Identification of a Mina-responsive region in the *Il4* promoter. The transcriptional response to Mina of the *Il4* promoter (left; varying in length from 766 to 140 bp, indicated below the graph) and the *Il2* promoter (right) is shown. Reporter activity was measured from transductants stimulated (open bars) or not (filled bars) with anti-TCR for 12 h. Data are the average of three independent experiments and error bars indicate SEM (* $P < 0.05$; t test). **(b)** Nucleotide sequence of the proximal *Il4* promoter with regions (indicated as horizontal lines) used as oligonucleotide probes (A, B, C, and D) in electrophoretic mobility shift assays (EMSAs). **(c)** Mina interacts with the proximal *Il4* promoter in EMSAs. Nuclear extracts from 24 h TCR-stimulated B10.D2 CD4⁺ T cells were incubated with end-labeled probes A–D in the absence (left) or presence (right, probes A and B only) of 2 μ g anti-Mina. Arrows indicate the location of the Mina-containing nucleoprotein complex. Data are representative of 3 independent experiments. **(d)** Differential binding of Mina53 to the *Il4* promoter. ChIP plot depicting fold-enrichment for chromatin-bound Mina53 at five sites (numbered relative to the transcriptional start site) across the *Il4* promoter and at the *Cd3e* locus (as a negative

control) in 24 h TCR-stimulated CD4⁺ T cells from strains BALB/c (open bars) and B10.D2 (filled bars). Data are the mean of five independent experiments and error bars indicate SEM (* $P < 0.05$, t test).

Figure 6.

Mina recruitment to the *Il4* promoter requires NFAT. **(a)** Nucleotide sequence of oligonucleotide probes from the *Il4* (B and B^{NM}) and *Il2* (IL2) promoters used in EMSA and DNA pull down experiments in **b** and **c**. NFAT and Oct binding sites are labeled. Probe B^{NM} is identical to probe B except for a dinucleotide mutation in the NFAT-binding site, indicated in bold italicized type. **(b)** The Mina nucleoprotein complex with Probe B requires NFATc1 and NFATc2 binding. Left, nuclear extracts from 4 h PMA-ionomycin induced EL4 cells were incubated with end-labeled probes B and B_{NM} in the presence or absence of 100, 33, or 11 ng of 'cold' Probe B or B_{NM}. Right, nuclear extracts from 4 h PMA-ionomycin induced EL4 cells were incubated with end-labeled Probe B and rabbit serum, 3

μg anti-Mina, 2 μg anti-NFATc1 or 2 μg of anti-NFATc2. Thick and thin arrows indicate the locations of the Mina complex and the anti-NFAT 'supershifted' complex, respectively. Data are representative of 3 independent experiments. (c) NFATc2 and Mina protein blots of 'pull-down' products formed by combining nuclear extracts (input) from 4 h PMA-ionomycin-induced EL4 cells with biotin-labeled probes (IL2, B and B_{NM}). DNA-protein complexes were isolated using streptavidin-agarose beads. Data are representative of 2 independent experiments.

Figure 7.

Transient enforced *Mina* elevation impairs *Il4* expression in CD4⁺ T cells. CD4⁺ T cells isolated from three independent *Mina* transgenic lines (Lines #6, #38 and #21; closed symbols, 2 mice each) and their corresponding littermate controls (open symbols, 2 mice each) were stimulated with antibodies to the TCR and CD28. RNA harvested at the indicated times post-stimulation was analyzed for *Mina*, *Il4*, *Il2* and *Ifng* by quantitative real-time RT-PCR. Data, expressed as relative expression (*Actb* normalized, arbitrary units), are from two independent experiments. Each trace represents an individual mouse.

Figure 8.

Il4 expression in CD4⁺ T cells is constrained by Mina-dependent repression. (left) Protein immunoblots of Mina and Actin expression in total cellular extracts from $4-6 \times 10^6$ CD4⁺ T cells activated for 24 h following 16 h mock, CoMO or MinaMO pre-treatment. (middle) Quantification of Mina expression from the protein blot. (right) Relative *Il4* and *Ifng* expression (*Hprt1*-normalized, arbitrary units) in the same cells shown in the previous two panels. Shown is the mean of duplicate PCR reactions. Data are representative of 2 independent experiments.