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Requirement of *bic/microRNA-155* for Normal Immune Function

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Abstract

MicroRNAs are a class of small RNAs that are increasingly being recognized as important regulators of gene expression. Although hundreds of microRNAs are present in the mammalian genome, genetic studies addressing their physiological roles are at an early stage. We have shown that mice deficient for *bic/microRNA-155* are immunodeficient and display increased lung airway remodeling. We demonstrate a requirement of *bic/microRNA-155* for the function of B and T lymphocytes and dendritic cells. Transcriptome analysis of *bic/microRNA-155*-deficient CD4⁺ T cells identified a wide spectrum of microRNA-155-regulated genes, including cytokines, chemokines, and transcription factors. Our work suggests that *bic/microRNA-155* plays a key role in the homeostasis and function of the immune system.

MicroRNAs (miRNAs) posttranscriptionally regulate gene expression by forming imperfect base pairing with sequences in the 3' untranslated region (3' UTR) of genes to prevent protein accumulation by repressing translation or by inducing mRNA degradation (1, 2). More than 500 miRNAs have been identified in mammals, although their functions are only now being elucidated (3). In the immune system, the enzyme responsible for regulatory RNA biogenesis, Dicer, is required for T cell function, which suggests regulatory roles for miRNAs in lymphocytes (4, 5). One miRNA, miR-155 (6), maps within, and is processed from, an exon of the noncoding RNA known as *bic* (7, 8), its primary miRNA precursor (9). *bic/miR-155* shows greatly increased expression in activated B and T cells (9–11), as well as in activated macrophages and dendritic cells (DCs) (12, 13). Overexpression of *bic/miR-155* has been reported in B cell lymphomas and solid tumors (14), and transgenic miR-155 mice have also been shown to develop B cell malignancies in vivo (15), indicating that the locus may also be linked to cancer.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/316/5824/608/DC1 Materials and Methods Figs. S1 to S8 Tables S1 to S4 References

To define the *in vivo* role of *bic/miR-155* (*bic*), we generated mutant alleles in embryonic stem cells (16) to obtain *bic*-deficient (*bic*^{m1/m1} and *bic*^{m2/m2}) mice (fig. S1, A and B). *bic*-deficient mice were viable and fertile but developed lung pathology with age. At 320 to 350 days, 56% (5 out of 9) of *bic*^{m1/m1} mice displayed significant remodeling of lung airways, with increased bronchiolar subepithelial collagen deposition and increased cell mass of sub-bronchiolar myofibroblasts (Fig. 1, B, D, and F), relative to age-matched control mice (*n* = 8) (Fig. 1, A, C, and E). A statistically significant increase in the ratio of collagen thickness/bronchiolar diameter and smooth muscle cell area/bronchiolar diameter could be measured in *bic*-deficient mice, compared with wild-type controls (Fig. 1, G and H). Increased airway remodeling in aged *bic*^{m1/m1} mice was accompanied by a significant increase in the numbers of leukocytes in bronchoalveolar lavage fluids (BAL) (Fig. 1, I) but not the lung interstitium. These changes are reminiscent of the lung fibrosis that often complicates systemic autoimmune processes with lung involvement (17, 18). We also noted that many *bic*^{m1/m1} mice developed enteric inflammation, a trait we have not investigated further. Thus, the phenotype we observed suggested that *bic/miR-155* may participate or play a role in regulating the homeostasis of the immune system.

The pathology observed in *bic*-deficient mice prompted us to examine the requirement of *bic/miR-155* in immunity. Although no gross defect in myeloid or lymphoid development in *bic*-deficient mice was observed (tables S1 and S2), protective immunity did appear to be impaired. Thus, after intravenous immunization with the live attenuated form of the enteric pathogen *Salmonella typhimurium* (*aroA* mutant strain), mice were assessed for their ability to resist oral challenge with virulent *S. typhimurium* bacteria (19, 20). Both unvaccinated *bic*^{m2/m2} and wild-type control mice (5 out of 5; *n* = 5) died within 7 days after infection (Fig. 2A). However, unlike their wild-type counterparts, *bic*^{m2/m2} mice were less readily protected by *aroA* vaccination, and the majority of mice (5 out of 6; *n* = 6) succumbed to challenge with the virulent strain by 33 days after infection (Fig. 2B). Thus, immunized *bic*-deficient mice, unlike wild-type mice, could not be protected by immunization to this pathogen.

Protective immunity requires the function of T and B lymphocytes. Therefore, we next examined the *in vivo* B and T cell responses of *bic*-deficient mice immunized with the T-dependent antigen, tetanus toxin fragment C protein (TetC). Immunized *bic*^{m1/m1} mice produced significantly reduced amounts of immunoglobulin M (IgM) and switched antigen-specific antibodies (Fig. 2C), indicative of impaired B cell responses. For examination of T cell function, splenocytes from mice immunized with TetC were restimulated *in vitro*, and the levels of interleukin (IL)-2 and interferon (IFN)- γ cytokines were measured. As expected, splenocytes from wild-type mice immunized with TetC produced significantly increased levels of IL-2 and IFN- γ relative to naive mice (Fig. 2D). In contrast, *bic*^{m1/m1} immunized mice failed to produce significant levels of these cytokines (Fig. 2D). Thus, B and T cell responses were diminished in *bic*-deficient mice, possibly contributing to their impaired enteric immunity.

To understand the nature of defective immune responses *in vivo*, we explored the possibility of an intrinsic requirement for *bic/miR-155* in B cells and T cells. Dendritic cell (DC) function was also tested, because these cells act as professional antigen presenting cells (APCs) with the ability to influence T cell activation and differentiation. Production of IgG1 by lipopolysaccharide (LPS)- and IL-4-stimulated *bic*^{m2/m2} B cells was significantly reduced (Fig. 2E), although this defect did not appear to correspond with abnormal proliferation (fig. S2). After encountering antigen, DCs increase their immunostimulatory capacity (21) through a process that is mimicked *in vitro* by stimulation with LPS. After treatment with LPS *bic*^{m2/m2}, bone marrow-derived DCs expressed levels of major histocompatibility complex-II and costimulatory molecules similar to those seen on

identically treated matured wild-type DCs (fig. S3, A and B), which indicates that *bic/miR-155* is not required for maturation. Nevertheless, *bic*^{m2/m2} DCs failed to efficiently activate T cells, consistent with defective antigen presentation or costimulatory function (Fig. 2F). Collectively, these results suggest that the effects of *bic/miR-155* may operate in part on T cells through its influence on DC function.

To establish whether there is also an intrinsic requirement for *bic/miR-155* in T cell function, the response of receptor-stimulated naïve *bic*^{m2/m2} CD4⁺ T cells was tested. Despite normal proliferation, uncommitted *bic*^{m2/m2} CD4⁺ cells showed a significant reduction of the T helper (Th)–1 cytokine, IFN- γ , after stimulation with antibodies to CD3 and CD28 (fig. S4). A reduction by a factor of 5 in the number of IFN- γ -producing cells was also observed after restimulation of *bic*^{m2/m2} CD4⁺ T cells cultured under conditions designed not to polarize Th responses (Fig. 3A); and was accompanied by a doubling in the number of IL-4 single-producing cells (Fig. 3A). In light of the expression of *bic/miR-155* in both Th1 and Th2 cell lineages (fig. S5, A and B), we next examined the phenotype of *bic*^{m2/m2} CD4⁺ T cells after culture in conditions that promote Th1 or Th2 cell differentiation. The levels of IFN- γ , as well as the number of *bic*^{m2/m2} Th1 cells secreting cytokine, were similar to controls, which indicates that *bic/miR-155* is not required for Th1 differentiation (Fig. 3, A and B). However, phenotypic alterations were observed as *bic*^{m2/m2} Th1 cells produced elevated levels of CCL-5 (Fig. 3B and table S3). By contrast, increased commitment to the Th2 pathway was evident in *bic*^{m2/m2} Th2 cell cultures as higher numbers of IL-4–producing cells were observed (Fig. 3A). In support of this result, enhanced levels of the Th2 cytokines IL-4, IL-5, and IL-10 were generated by *bic*^{m2/m2} cells after culture in Th2 polarizing conditions (Fig. 3C). Taken together, these data demonstrate that *bic*-deficient CD4⁺ T cells are intrinsically biased toward Th2 differentiation. Moreover, Th1 cells may have altered function despite normal production of IFN- γ .

To understand how *bic/miR-155* regulates Th2 commitment and to gain a more global insight into the extent of deregulation in Th1 cells, we analyzed gene expression in *bic*^{m2/m2} Th1 or Th2 cells using microarray analysis. In addition, because the 5' region of miRNAs (referred to as the “seed” region) is believed to be crucial for target mRNA recognition (1, 2), we searched the 3' UTRs of significantly up-regulated genes in microarrays for the presence of seed matches specific for miR-155. In *bic*-deficient Th1 cells, we identified 46 of 53 up-regulated transcripts as potential miR-155 targets (table S3 and fig. S6). In *bic*-deficient Th2 cells, 53 out of 99 up-regulated transcripts were predicted targets (table S4 and fig. S7). To confirm these genes as likely targets of miR-155, we then searched the 3' UTRs for seed matches specific for all of the known mouse miRNAs in the miRbase public database (3). miR-155 seed sequences were significantly overrepresented over all other tested mouse miRNAs, indicating a significant probability that these genes are direct targets of miR-155 (Fig. 4, A and B). This computational data strongly suggests that miR-155 represses a wide assortment of genes in CD4⁺ T cells and lends support for the hypothesis that miRNA targets are generally abundant in mammals (22).

A wide spectrum of miR-155 target genes with diverse molecular roles, such as T cell costimulation (e.g., *Tnfrsf9*), chemotaxis (e.g., *Ccl-5*), and signaling (e.g., *Ikbke*), were identified. Among these, we noted that the transcription factor *c-Maf* contains phylogenetically conserved miR-155 seed matches in the 3' UTR (fig. S8). *c-Maf* is a potent transactivator of the *IL-4* promoter, and ectopically expressed *c-Maf* is sufficient to cause increased IL-4, IL-5 and IL-10 production by Th2 cells (23–25). In concordance with the microarray results, a significant induction of *c-Maf* mRNA was detected in *bic*^{m2/m2} Th2 cells, and the levels of *c-Maf* protein were correspondingly increased (Fig. 4, C and D). By contrast, levels of *Gata3* transcript, which does not contain a miR-155 seed, were not elevated (Fig. 4C). Increased expression of *c-Maf* may thus contribute, at least in part, to the

increased Th2 cytokine production phenotype observed in *bic*^{m2/m2} Th2 cells. To further confirm whether *c-Maf* is a direct target of miR-155, we cloned its 3' UTR into a luciferase reporter plasmid. The wild-type *c-Maf* reporter exhibited significant miR-155-dependent repression relative to the reporter with a mutant seed sequence, which indicates that this is a direct target for miR-155 (Fig. 4E). We conclude from these experiments that *bic/miR-155* modulates levels of *c-Maf* in CD4⁺ T cells and this is likely to contribute to the attenuation of Th2 cell responses in vivo.

Our data demonstrate that mice carrying a null mutation in the *bic/miR-155* gene display altered immune responses. Thus, along with an increase in airways remodeling suggestive of altered homeostasis, we observed that *bic/miR-155* regulates the function of both lymphocytes and DCs, leading to an overall diminution of immune responses. The identification of multiple novel potential targets of miR-155 supports the view that *bic/miR-155* is a core regulator of gene expression in multiple cell types, with a “targetome” optimized to modulate the immune response. Interestingly, *bic*-deficient mice share some of the cellular features observed in CD4-*Cre/Dicer*^{FL} mice, including defects in CD4⁺ T cell cytokine production and immune homeostasis (3, 4). It will now be important to define the patho-physiology of *bic*-deficient lymphocytes and further test the role of miR-155-dependent repression of *c-Maf* on immune responses in vivo. The strength of the *bic/miR-155* mutant phenotype more generally suggests critical roles for miRNAs in vivo, with potentially severe loss-of-function phenotypes directly relevant to human disease. In this regard, it is intriguing that the human *BIC/miR-155* gene maps to an asthma, pollen sensitivity, and atopic dermatitis susceptibility region on chromosome 21q21 (26–28). Given the severe phenotypes noted in these mice, *BIC/miR-155* should be investigated as a potential immune disease locus in humans.

Acknowledgments

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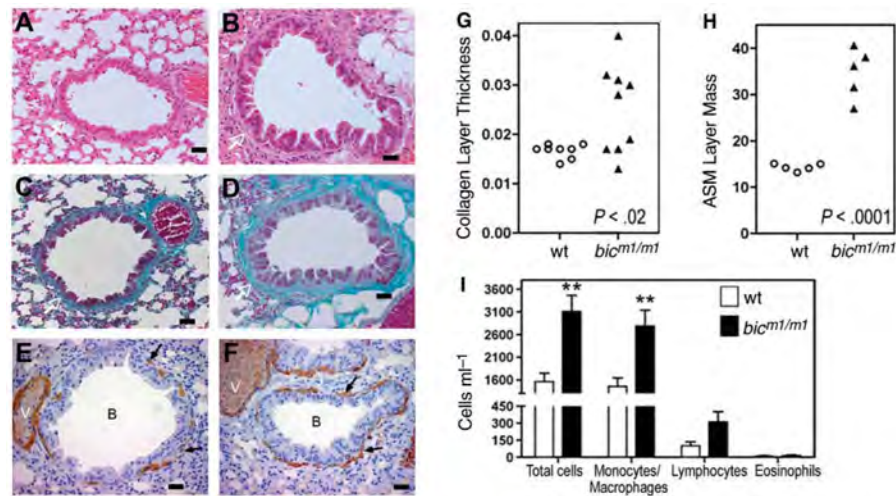


Fig. 1.

Mice deficient for *bic/miR-155* show increased lung airway remodeling (A to F) Histological examination of sections of lung bronchioles from control wild-type (A, C, and E) and *bic*^{m1/m1} mice (B, D, and F). Scale bar, 100 μ m. (A and B) Haematoxylin and eosin stain; (C and D) Masson Trichrome stain; (E and F) Immunohistochemical staining for smooth muscle actin. Collagen layer (white arrows), lung myofibroblasts (black arrows), bronchioles (B), and blood vessels (V) are indicated. (G) Quantitation of peribronchiolar collagen thickness or (H) airways smooth muscle cell (ASM) mass in *bic*^{m1/m1} mice compared with that of wild-type mice. (G) $P < 0.02$ or (H) $P < 0.0001$, in comparison with wild-type group, Student's two-tailed t test. Open circles, control mice; filled triangles, *bic*^{m1/m1} mice. Notably, *bic*^{m1/m1} mice with increased collagen layer thickness also had increased ASM mass. (I) Total and differential cell counts in BAL from the indicated mice. Data are the mean + SE from seven *bic*-deficient mice and six control mice. ** $P < 0.01$ in comparison with wild-type group, Student's two-tailed t test.

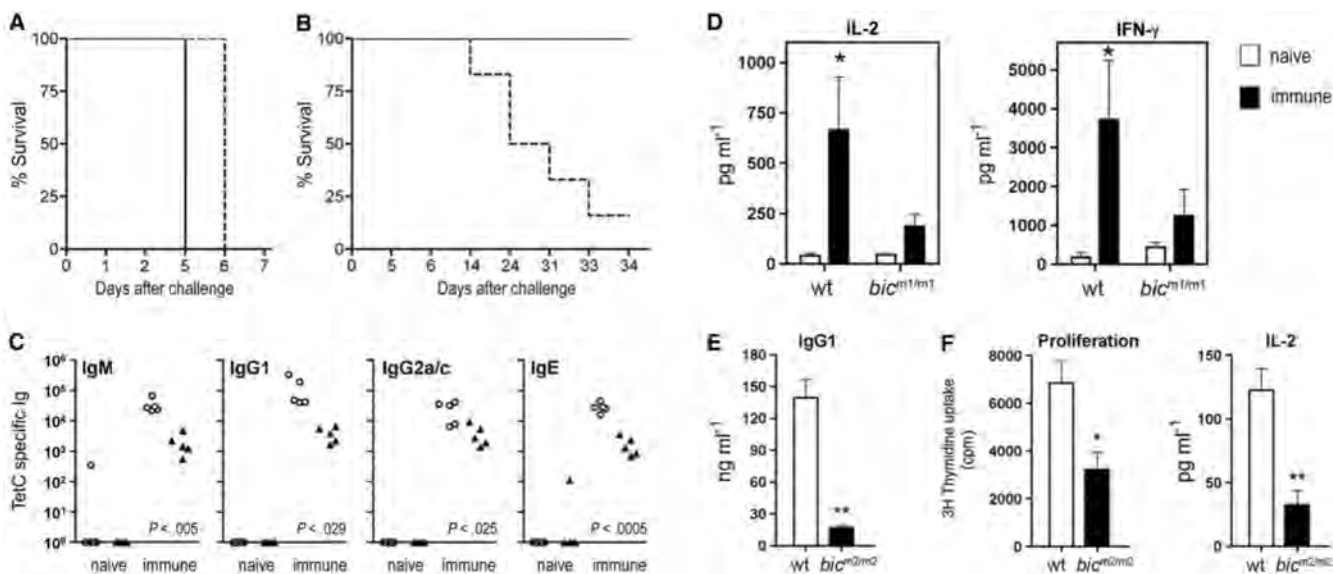
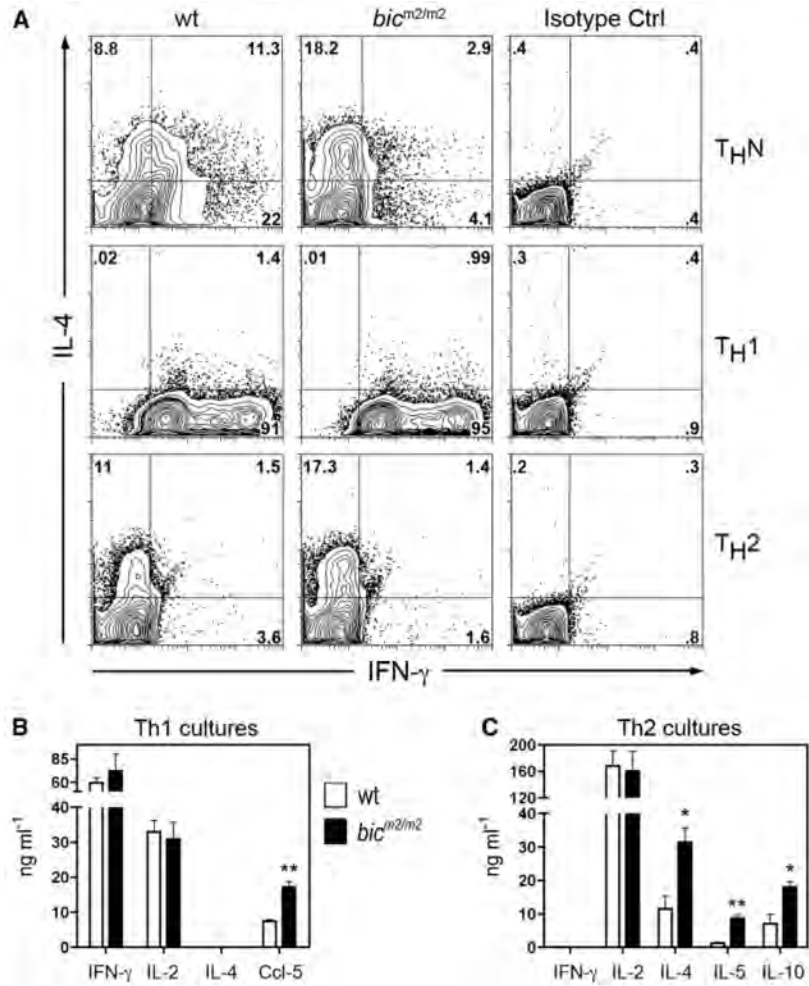


Fig. 2.

Defective adaptive immunity by *bic*-deficient mice. (A) Survival curve for mice ($n = 5$ in each group) infected orally with 1×10^8 colony-forming units (CFU)–virulent *S. typhimurium* strain SL344. As expected for mice of this genetic background, all failed to survive challenge. (B) Survival of mice ($n = 6$ in each group) infected intravenously with 1×10^4 CFU of *S. typhimurium* aroA strain followed by oral challenge with *S. typhimurium* SL344 6 weeks after prime. In contrast with control mice, *bic*^{m2/m2} mice demonstrate reduced survival after challenge. (A and B) Line, control C57BL/6J (wild-type) mice; dashed line, N5 C57BL/6J backcross *bic*^{m2/m2} mice. (C) TetC-specific Ig levels from control mice (open circles) or *bic*-deficient mice (filled triangles) immunized with TetC at days 1 and 21 and analyzed 13 days after secondary immunization. *P* values denote significant differences; Student's two-tailed *t* test. (D) Production of IL-2 and IFN- γ by splenocytes isolated from wild-type or *bic*^{m1/m1}-naïve mice (open bars) or immunized with TetC as in (C) (closed bars) and cultured for 48 hours in the presence of TetC. Data are the mean \pm SE from four mice. **P* < 0.05 versus naïve mice; Student's two-tailed *t* test. (E) Reduced IgG1 production by *bic*^{m2/m2} B cells cultured in the presence of LPS and IL-4 for 4 days. Data are the mean \pm SE from 3 mice. ***P* < 0.01 versus wild-type; Student's two-tailed *t* test. (F) Significantly reduced proliferation and IL-2 production by ovalbumin T cell receptor transgenic (OT-II) cells cultured with LPS-matured, bone marrow–derived, *bic*-deficient DCs in the presence of cognate (2.5 μ M) ovalbumin protein. Cell proliferation was determined by [³H]-thymidine incorporation at 72 hours. IL-2 was measured from supernatants by enzyme-linked immunosorbent assay (ELISA) at 48 hours. Data are the mean \pm SE from five mice of each genotype. **P* < 0.05 versus wild-type; Student's two-tailed *t* test.

**Fig. 3.**

Increased Th2 polarization and amplified Th2 cytokine production by *bic*-deficient CD4⁺ T cells. CD4⁺CD62L⁺ cells of indicated genotypes were cultured under (A, middle panel, and B) Th1 conditions, (A, lower panel, and C) Th2 in vitro differentiation conditions, or (A, upper panel) nonpolarizing (ThN) conditions and restimulated with immobilized antibody to CD3 (10 μg/ml) and soluble 2 μg/ml antibody to CD28 on day 6. (A) Intracellular cytometric analysis for IFN-γ and IL-4 production (16). The panel shows a representative result of three mice of each genotype analyzed in the same experiment. Data are representative of two independent experiments ($n = 3$ per genotype). Numbers in each quadrant are percentages of cells of indicated phenotype. (B and C) Cytokine levels were assayed by ELISA 21 hours after restimulation of cells cultured under (B) Th1 or (C) Th2 polarizing conditions. Data are the mean + SE from three individual mice. * $P < 0.05$ or ** $P < 0.01$ versus wild-type; Student's two-tailed t test.

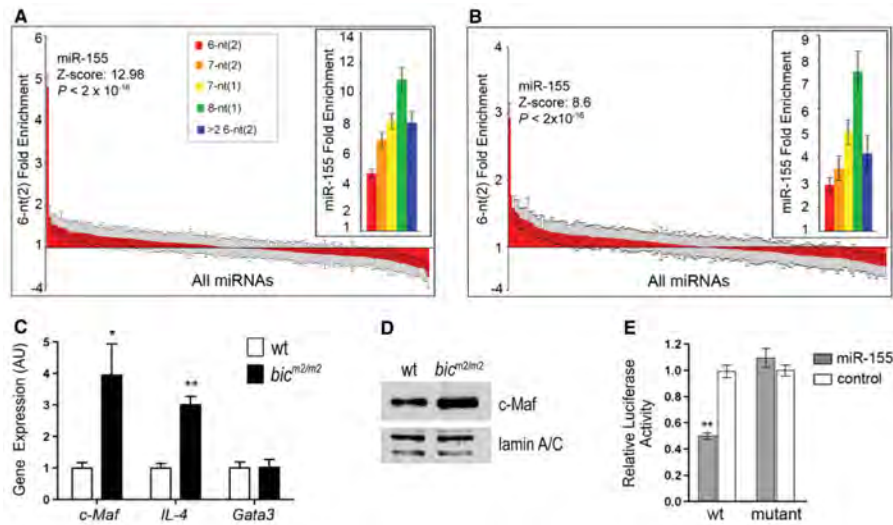


Fig. 4. miR-155 pattern sequences are enriched in the Th1 and Th2 cell up-regulated genes, and *c-Maf* is a bona fide target of miR-155. (A and B) Fold enrichment of 5' miRNA pattern sequences of the indicated types contained in the 3' UTRs of the (A) Th1 or (B) Th2 cDNA microarray significantly up-regulated gene sets. The standard deviation, *Z* score, and *P* value were calculated by sampling 1000 random sets of 53 (for Th1 set) or 99 (for Th2 set) genes from the mouse genome (16). Data are fold enrichment \pm SD. (C) Quantitative PCR analysis for *Gata3*, *c-Maf*, and *IL-4* transcript levels from Th2 cells re-stimulated with antibodies to CD3 and CD28. Data are the mean \pm SE from three mice. * $P < 0.05$ versus wild-type; Student's two-tailed *t* test. (D) c-MAF protein levels were assessed by Western blot of nuclear extracts of Th2 cells isolated from the indicated genotypes. Expression of lamin A/C was used as loading control. (E) miR-155-dependent repression of *c-Maf* reporter in vitro. A luciferase (Rluc) reporter was used to validate *c-Maf* as a direct target of miR-155. Wild-type (wt) or mutant plasmids (mut) were cotransfected with the indicated duplex miRNA for miR-155 (open bars) or control Cel-miR-64 (filled bars) into HeLa S3 cells. Data are mean \pm SE from three experiments. ** $P < 0.0001$ in comparison with wild-type plasmid treated with nonspecific RNA duplex, Cel-miR-64; Student's two-tailed *t* test.