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Cutting Edge: A Critical Role for Gene Silencing in Preventing Excessive Type 1 Immunity¹

Anne S. Hutchins,* David Artis,[†] Brian D. Hendrich,^{2‡} Adrian P. Bird,[‡] Phillip Scott,[†] and Steven L. Reiner^{3*}

*Immunity often depends on proper cell fate choice by helper T lymphocytes. A naive cell, with minimal expression of IFN- γ and IL-4, must give rise to progeny expressing high levels of either one, but not both, of those cytokines to defend against protozoan and helminthic pathogens, respectively. In the present study, we show that inactivation of the Mbd2 gene, which links DNA methylation and repressed chromatin, results in enhanced resistance to the protozoan parasite *Leishmania major* but impaired immunity to the intestinal helminth *Trichuris muris*. Helper T cells from methyl CpG-binding domain protein-2-deficient mice exhibit exuberant patterns of cytokine expression despite appropriate silencing of genes encoding the lineage-specifying factors T-bet and GATA-3. These results suggest that gene silencing can facilitate the ability of a progenitor cell to give rise to appropriately differentiated daughter cells in vivo. These findings also point to novel pathways that could participate in genetic control of resistance to infection and autoimmunity. The Journal of Immunology, 2005, 175: 5606–5610.*

Lineage decisions occur frequently in development of multicelled organisms. The immune response to microbes also depends upon proper fate choice by Ag-specific helper T cells (1, 2). Successful clearance of protozoa, such as *Leishmania major*, requires Th1 cells that secrete large amounts of IFN- γ (3). Resistance to *L. major* is antagonized by Type 2 cytokines, including IL-4 and IL-10. Expulsion of intestinal helminths, such as *Trichuris muris*, requires Th2 cells that secrete IL-4 and IL-13 (4, 5). Resistance to *T. muris* is antagonized by IFN- γ produced by Th1 cells (6).

Proper maturation of Th1 and Th2 cells is regulated by the positive and negative actions of a variety of extrinsic factors, including Ags, cytokines, and other receptor-mediated signaling events (1, 2). The discovery of T-bet (7–9) and GATA-3 (10–13) as apparent master controllers of the Th1 and Th2 fate has

helped to establish the paradigm that distinct patterns of lineage-specific gene expression are sculpted by transcription factor regulatory networks (1, 2). However, mounting evidence suggests that covalent modification of histones and DNA, as well as the structural characteristics of chromosomes, may interact with conventional gene regulatory networks (14–16). Despite the attractiveness of chromatin-constrained models of gene expression and cell differentiation, there has been limited evidence that bona fide mammalian cell fate decisions are indeed controlled by epigenetic effects and gene silencing pathways.

Methyl CpG-binding domain protein-2 (MBD2)⁴ is a protein that links CpG methylation to repressive chromatin structure by recruiting complexes containing histone deacetylases and ATP-dependent nucleosome remodeling activities to methylated DNA (17). We previously offered in vitro evidence to suggest that MBD2-dependent gene silencing is necessary to facilitate temporally and spatially restricted patterns of gene expression (18). In the present study, we report the outcome of challenges of MBD2-deficient mice with two well-characterized parasitic pathogens that typically elicit polarized helper T cell responses. In the absence of efficient gene silencing, the cytokine gene expression of responding T cells is excessive, leading to profound changes in the susceptibility and resistance of *Mbd2* mutant mice to infectious diseases. These results support an epigenetic model of helper T cell differentiation and suggest that a matrix of gene silencing, not simply the choice of the correct *trans*-activator, might be an essential component of lineage specification.

Materials and Methods

Mice

Mbd2 mutant mice (B6.129-*Mbd2*^{tm1.Bird}) were generated as described previously (17). Experiments were performed with littermate cohorts from either C57BL/6 (B6) \times 129sv (129), N6 B6, or N10 BALB/c heterozygote matings. All animal work was in accord with guidelines of the University of Pennsylvania.

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⁴ Abbreviation used in this paper: MBD2, methyl CpG-binding domain protein-2; DN, dominant negative.

Infectious challenges

For protozoan challenge, mice were inoculated in the left hind footpad with either 2×10^6 or 2×10^7 stationary phase promastigote parasites of *L. major* (WHOM/IL/80/Friedlin). Footpad measurements, quantitative parasite cultures, Ag-specific restimulation, and ELISA were performed as described previously (19). For helminthic challenges, mice were infected orally with 150 *T. muris* eggs, as described previously (4–6). Parasite-specific Ab isotypes were measured as described previously (4–6). Cecum and adjacent colon were harvested to determine worm burdens at day 31 postinfection. One-centimeter segments of mid-cecum were removed and fixed for 24 h in 10% neutral buffered formalin. Paraffin-embedded tissue (5- μ m sections) were stained with H&E or Alcian blue-Periodic Acid Schiff (mucin stain) for detection of intestinal goblet cells.

In vitro differentiation of helper T cells

Splenocytes from naive mice were depleted of CD8⁺ cells and stimulated as described previously (18, 20). Briefly, 2×10^6 cells/ml were stimulated using soluble anti-CD3 mAb, anti-CD28 mAb, and rIL-2. Experimental Th1 conditions included mitogenic stimulation plus rIL-12 and anti-IL-4 mAb. Experimental Th2 conditions included mitogenic stimulation plus rIL-4 and anti-IL-12 mAb. CFSE labeling was performed as described previously (18, 21). Retroviral constructs encoding T-bet and dominant-negative (DN) T-bet were constructed and transduced as described previously (18, 20). Analysis of intracellular cytokines was performed as described previously (18, 21). Flow cytometric analyses in this article include only CD4⁺ events. Gates indicate specific staining of IFN- γ - or IL-4-positive cells as determined by fluorochrome-conjugated isotype control Abs. RT-PCR was performed using primers and probes described previously (18, 20). Northern blotting was performed as described previously (22).

Results and Discussion

MBD2 restricts IFN- γ induction during helper T cell differentiation in vivo

We previously showed that mice deficient in MBD2, a molecule proposed to link DNA methylation and repressed chromatin structure, have disordered helper T cell differentiation in vitro (18). MBD2-deficient helper T cells exhibit a higher frequency of IL-4- and IFN- γ -expressing cells under all polarizing conditions (18). Because we found ample evidence for ectopic expression of IL-4 in MBD2-deficient Th1 cells, we assessed the ability of MBD2-deficient mice to resist challenge with *L. major*, an intracellular protozoan parasite of macrophages. Successful eradication of *Leishmania* is contingent on development of IFN- γ -expressing helper T cells (3). In contrast, IL-4 plays a deleterious role by blocking the ability of IFN- γ to activate macrophages.

We challenged genetically resistant (B6 \times 129) littermate *Mbd2*^{+/+}, *Mbd2*^{+/-}, and *Mbd2*^{-/-} mice with hind footpad inoculation of a curable dose of 2×10^6 stationary phase promastigote parasites of *L. major*. Although genetically susceptible BALB/c mice are unable to control parasite replication using this inoculum, all genetically resistant mice, regardless of *Mbd2* status, were able to control the infection and resolve their footpad lesion (data not shown). When we challenged mice with a 10-fold higher dose of virulent parasites (2×10^7 promastigotes/footpad), we found that both BALB/c and wild-type B6 \times 129 (*Mbd2*^{+/+}) mice are unable to control parasite replication due to the overwhelming inoculum of parasites (Fig. 1A).

Unexpectedly, we found that both *Mbd2*^{+/-} and *Mbd2*^{-/-} mice are robustly resistant to the normally incurable challenge (Fig. 1A). Identical results were obtained using littermate *Mbd2*^{+/+}, *Mbd2*^{+/-}, and *Mbd2*^{-/-} mice that were generated from the intercross of sixth generation B6 *Mbd2*^{+/-} mice (data not shown). MBD2-deficient mice (heterozygote and homozygote) controlled the local replication of the parasite in their footpad lesions, as assessed by quantitative culture (Fig. 1B),

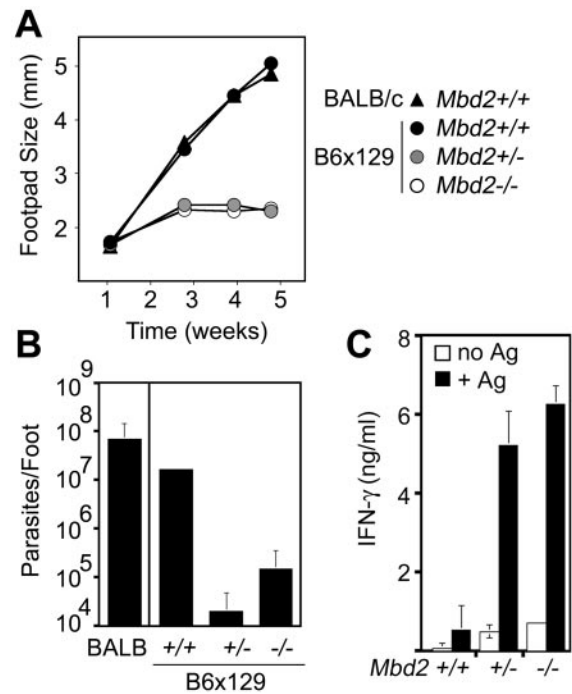


FIGURE 1. *Mbd2*^{+/-} and *Mbd2*^{-/-} mice are hyperresistant to *L. major* infection. B6 \times 129 *Mbd2*^{+/+}, *Mbd2*^{+/-}, *Mbd2*^{-/-}, and BALB/c mice were infected in the hind footpad with 2×10^7 stationary phase promastigote parasites of *L. major*. **A**, Disease progression was monitored by growth of footpad lesions. Results represent mean footpad diameter of two infected animals per group (<15% variance in measurements). Similar results were obtained in three separate experiments, using littermates from B6 or B6 \times 129 background. Parasite control was achieved in both ^{+/-} and ^{-/-} mice, regardless of background. **B**, Parasite burdens in hind feet were determined by presence of motile organisms in serial dilution cultures 5 wk postinfection. Data represent mean \pm SD of triplicate determinations of representative animal of each genotype. **C**, Parasite-specific IFN- γ secretion was measured by ELISA from cell suspensions prepared from draining popliteal lymph nodes of 5-wk infected mice that had been stimulated with (■) or without (□) *Leishmania* Ags (100 μ g/ml) for 48 h. Data represent mean \pm SD of triplicate determinations of representative animal of each genotype. Results are representative of three experiments.

and exhibited less dissemination of the parasite to visceral organs (data not shown). Consistent with their increased resistance to infection, cells recovered from the draining lymph nodes of *Mbd2*^{+/-} and *Mbd2*^{-/-} mice secreted more IFN- γ than cells from *Mbd2*^{+/+} mice when restimulated in vitro with soluble *Leishmania* Ags (Fig. 1C). Hyperresistance of B6 \times 129 and B6 *Mbd2*^{-/-} mice was completely abrogated by treatment with neutralizing anti-IFN- γ Abs, resulting in an explosive footpad lesion of greater severity than infected wild-type mice (data not shown). Thus, hemizygous or homozygous loss of *Mbd2* results in an exuberant Th1 response and enhances host resistance against an intracellular protozoan parasite. MBD2 has not been reported to be expressed in a monoallelic fashion, suggesting that minimally dysfunctional alleles of *Mbd2* might exert significant immunological phenotypes. However, the *Mbd2* gene is 44 cM from the centromere of mouse chromosome 18, which is some distance from a putative *L. major* resistance locus that is situated between 16 and 24 cM from the centromere (23) and distinct from the chromosomal locations of the other known resistance loci (24–27).

Mutation of *Mbd2* partially counteracts genetic susceptibility to *L. major*

We also challenged genetically susceptible BALB/c *Mbd2*^{+/+}, *Mbd2*^{+/-}, and *Mbd2*^{-/-} mice with the lower dose of 2×10^6 stationary phase promastigote parasites of *L. major*. The inability of the BALB/c strain to control *L. major*, as well as its predisposition to enhanced Th2 differentiation in vitro, is a complex genetic trait, involving multiple, independently segregating loci (3, 23–27). We found that loss of both *Mbd2* alleles on the BALB/c background confers substantial protection against *L. major* with regard to footpad lesion size (Fig. 2A) and control of parasite dissemination to the spleen (Fig. 2B). In contrast to the situation in genetically resistant backgrounds, we found that BALB/c *Mbd2*^{+/-} mice are substantially more susceptible than BALB/c *Mbd2*^{-/-} mice.

Cells recovered from the draining lymph nodes of infected mice were restimulated in vitro with soluble *Leishmania* Ags and analyzed at the single-cell level for expression of the signature cytokines of Th1 and Th2 cells, IFN- γ and IL-4, respectively (Fig. 2C). In genetically resistant B6 mice, we detected primarily single-positive, IFN- γ -expressing, Ag-specific Th1-like cells. In susceptible BALB/c *Mbd2*^{+/+} mice, we detected significantly more Ag-specific, single-positive, IL-4-expressing, Th2-like cells. In BALB/c *Mbd2*^{-/-} mice, we found an increase in IFN- γ -expressing, Ag-specific, Th1-like cells. Therefore, mutation of *Mbd2* can partially ameliorate the genetic susceptibility of BALB/c mice to *L. major*.

MBD2 is required for immunity to *T. muris*

A well-characterized parasitic infestation that elicits a polarized Th2 response in many strains of mice is infection with the gut-

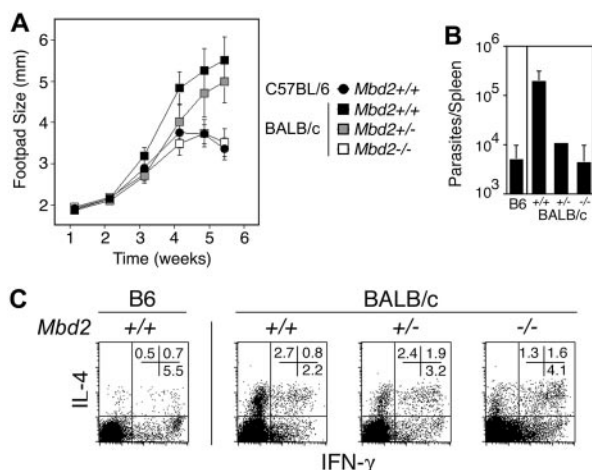


FIGURE 2. Mutation of *Mbd2* partially counteracts genetic susceptibility to *L. major*. BALB/c *Mbd2*^{+/+}, *Mbd2*^{+/-}, *Mbd2*^{-/-}, and B6 mice were infected in the hind footpad with 2×10^6 *L. major*. **A**, Disease progression was monitored by growth of footpad lesions. Results represent mean footpad diameter \pm SD of three infected animals per group. Similar results were obtained in three experiments. Although BALB/c *Mbd2*^{-/-} mice were always more resistant than MBD2-proficient littermates, there was variability in the degree to which BALB/c *Mbd2*^{-/-} were protected compared with B6 mice (data not shown). **B**, Quantitative parasite burdens of spleens were determined by serial dilution cultures 6 wk postinfection. Data represent mean \pm SD of triplicate determinations of representative animal of each genotype. **C**, Parasite-specific cytokine expression in draining popliteal lymph node cells of 6-wk infected mice stimulated with *Leishmania* Ags for 72 h. Intracellular accumulation of IFN- γ and IL-4 among CD4⁺ T cells was assessed using flow cytometry. Percentage of positive cells is indicated. Results are representative of three experiments.

dwelling helminth *T. muris*. Immunity to *T. muris* is dependent on Th2 cells that secrete IL-4 and IL-13 (4, 5), while resistance to infection is antagonized by IFN- γ (4, 6). Wild-type BALB/c mice and B6 \times 129 *Mbd2*^{+/+}, *Mbd2*^{+/-}, and *Mbd2*^{-/-} littermate mice were orally infected with embryonated *Trichuris* eggs to assess their ability to expel adult worms. *Mbd2*^{-/-} mice had an increase in the number of parasitic worms recovered from their ceca compared with *Mbd2*^{+/+} and *Mbd2*^{+/-} mice at day 15 (data not shown) and day 31 (Fig. 3A) postinfection. At later time points, all genotypes with the exception of *Mbd2*^{-/-} mice cleared the parasites. Thus, deficiency in *Mbd2* results in chronic parasitic infestation with the helminth *T. muris*.

The enhanced susceptibility of *Mbd2*^{-/-} mice is not likely due to an inability to mount a Th2 response against *T. muris*. Indeed, the serum levels of parasite-specific, Th2-associated IgG1 Ab were equivalent or higher in *Mbd2* mutant mice compared with *Mbd2*^{+/+} control mice (Fig. 3B). However, *Mbd2*^{+/-} and *Mbd2*^{-/-} mice exhibited higher levels of parasite-specific, IFN- γ -associated IgG2a Ab (Fig. 3B). *Mbd2*^{-/-} mice also developed intestinal pathology consistent with excessive IFN- γ production (6). The protective response of *Mbd2*^{+/+} mice was accompanied by Th2 cytokine-dependent goblet cell responses and mucin secretion (Fig. 3C). By contrast, *Mbd2*^{-/-} mice exhibited severe colonic inflammation, lymphatic dilation, edema, mucosal thickening, and numerous

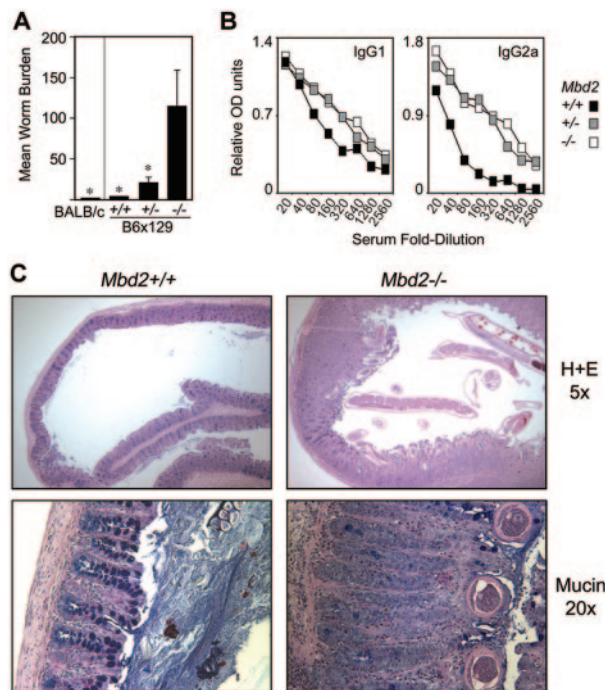


FIGURE 3. MBD2 is required for expulsion of *T. muris* infection and prevention of Th1-associated colonic pathology. B6 \times 129 *Mbd2*^{+/+}, *Mbd2*^{+/-}, *Mbd2*^{-/-}, and BALB/c mice were infected with 150 embryonated *T. muris* eggs. **A**, Cecal worm burdens were assessed in three mice per group at day 31 postinfection. Results are expressed as the mean \pm SD. Significant differences between marked bars and values of *Mbd2*^{-/-} mice are indicated. **B**, Parasite-specific serum Ab titers were performed by ELISA with *T. muris* Ag-coated dishes and anti-isotype detection Abs. **C**, Representative histology of colonic tissue of *Mbd2*^{+/+} and *Mbd2*^{-/-} mice at day 31 postinfection. Note that MBD2-deficient mice have persistent intraluminal parasites and develop Th1-associated, colitis-like destructive intestinal pathology following *T. muris* infection. Results are representative of three separate experiments.

intraluminal parasites (Fig. 3C; 5 \times). Extensive transmural inflammatory infiltrate in the intestinal mucosa of infected *Mbd2*^{-/-} mice was associated with marked crypt hyperplasia and loss of goblet cells and mucin responses (Fig. 3C; 20 \times). The pathology of infected *Mbd2*^{-/-} mice is similar to Th1 cytokine-associated intestinal inflammatory responses reported in models of inflammatory bowel disease (6). Despite the systemic evidence for a mixed Th1/Th2 response in MBD2-deficient mice, the net effect on target intestinal tissue was an aborted Th2 response, with Th1-associated pathology and impaired immunity.

MBD2 limits IFN- γ expression but is not required to repress T-bet expression

Despite the disorganized phenotype of cytokine expression in the absence of MBD2 in vitro (18) and in vivo, we found that mutant cells were capable of restricting expression of the forbidden *trans*-activators. As assessed by Northern blotting, both *Mbd2*^{+/+} and *Mbd2*^{-/-} cells cultured in vitro can induce T-bet and repress GATA-3 in Th1 conditions (Fig. 4A). Similarly, both *Mbd2*^{+/+} and *Mbd2*^{-/-} cells are able to induce GATA-3 and repress T-bet in Th2 conditions. The possible induction of substantial amounts of ectopic T-bet in developing Th2 cells from *Mbd2* mutant mice was further excluded using real-time PCR (Fig. 4B).

Taken together, these results suggest that developing *Mbd2*^{-/-} Th2 cells can misexpress "T-bet-less" IFN- γ . Such findings parallel our previous suggestion that developing *Mbd2*^{-/-} Th1 cells misexpress "GATA-less" IL-4 (18). The competitive relationship between T-bet and MBD2 could be further visualized by genetic manipulation of each factor. Provision of exogenous T-bet, using retroviral transduction, induces higher levels of IFN- γ in the absence than in the presence of *Mbd2* (Fig. 4C). Additionally, antagonism of T-bet with a DN factor prevents IFN- γ induction with lesser efficiency in the absence of *Mbd2* (Fig. 4D). Moreover, preliminary analysis of compound mutant mice also indicates that loss of *Mbd2* can partially rescue the Th1 defect caused by loss of *T-bet* (A. S. Hutchins and S. L. Reiner, unpublished results). Thus, T-bet and MBD2 are in a genetic competition to activate and repress IFN- γ , respectively. This competition may also extend to the STAT4 pathway (28) because the absence of MBD2 also reduces the requirement for IL-12 to induce IFN- γ expression. We have not yet been able to detect MBD2 binding to the *Ifng* gene body or promoter, although it was detected at the *Il4* locus (18). This could indicate that MBD2 binding to the *Ifng* gene is ephemeral, that it occurs at a more distant *cis*-acting sequence, or that derepression of a *trans*-acting factor other than T-bet might account for the apparent "T-bet-less" IFN- γ expression. Whether the *Ifng* gene is a direct or indirect target, one role of MBD2 seems to be to limit IFN- γ expression in vivo.

Conclusions

We now show that the loss of MBD2, a component of DNA methylation-mediated gene silencing, perturbs the normal restraints on effector cytokine expression in maturing helper T cells in vivo. Using small molecule inhibitors of maintenance methylation or histone deacetylases (21, 29), as well as gene inactivation of *Mbd2* (18) or the maintenance methyltransferase *Dnmt1* (30, 31), it was suggested previously that helper T cell differentiation was constrained by DNA methylation-mediated

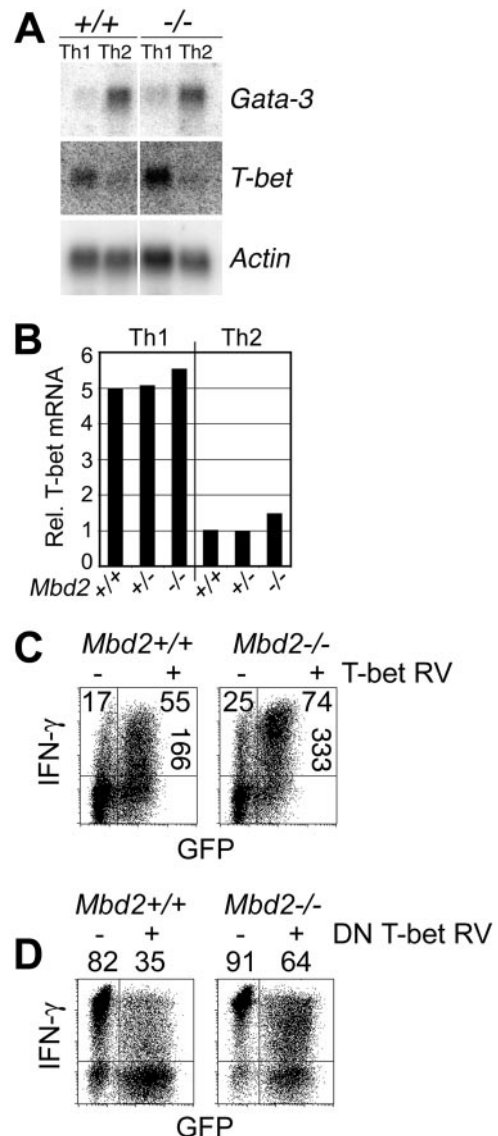


FIGURE 4. MBD2 and T-bet inversely regulate IFN- γ expression. *A*, Northern analysis of Gata-3, T-bet, and β -actin mRNA in *Mbd2*^{+/+} and *Mbd2*^{-/-} CD4⁺ T cells stimulated in Th1 and Th2 conditions for 5 days. *B*, Real-time RT-PCR analysis of T-bet mRNA from *Mbd2*^{+/+}, *Mbd2*^{+/-}, and *Mbd2*^{-/-} CD4⁺ T cells stimulated in Th1 and Th2 conditions for 5 days. T-bet levels expressed relative to hypoxanthine phosphoribosyltransferase levels. Values represent mean of triplicate determinations (variance < 20%). *C*, *Mbd2*^{+/+} and *Mbd2*^{-/-} cells were cultured in Th2 conditions to repress endogenous T-bet, and retrovirus encoding both T-bet and GFP was introduced after 24 h. Expression of GFP and IFN- γ (among CD4⁺ cells) was assessed 3 days later. Frequency of IFN- γ -expressing cells on each side of the plot is indicated horizontally. y -axis mean fluorescence intensity of T-bet-transduced IFN- γ ⁺ cells is indicated vertically. *D*, DN T-bet was introduced into developing Th1 cells of ^{+/+} and ^{-/-} mice after 24 h. After another 3 days, expression of GFP and IFN- γ was assessed. Frequency of IFN- γ ⁺ cells on each side of the plot is indicated. All results are representative of at least two separate experiments.

silencing. Prior studies emphasized the misexpression of "GATA-less" IL-4 in Th1 cells and CD8⁺ T cells in vitro (18, 30). Unexpectedly, we found that the dominant derangement of MBD2-deficient mice is an excessive IFN- γ -mediated response against both protozoan and helminthic invaders. This is unlikely the result of defective counterregulation because we found that MBD2-deficient cells also overexpress the regulatory

cytokine IL-10 and that *Mbd2* mutant mice have normal numbers of FoxP3⁺CD25⁺CD4⁺ regulatory T cells (A. S. Hutchins and S. L. Reiner, unpublished results). Even highly enriched naive T cells from MBD2-deficient mice cultured in vitro in the absence of regulatory T cells and APCs exhibit excessive effector cytokine expression compared with MBD2-proficient cells (A. S. Hutchins and S. L. Reiner, unpublished results), suggesting a T cell-autonomous component to the phenotypes observed in vivo.

These findings provide formal in vivo evidence that gene silencing organizes signaling and transcription factor networks important for the maturation of cytokine expression in helper T cells. The unexpectedly significant protection against *L. major* afforded by inactivation of only one allele of *Mbd2* also raises a novel possibility regarding the genetics of disease resistance to infectious and autoimmune processes. Future determination of candidate loci might rightly include gene silencing pathways, in addition to traditional regulators of subset-specific cytokines.

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Disclosures

The authors have no financial conflict of interest.

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