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Overexpression of Methyl-CpG Binding Protein 2 Impairs T_H1 Responses

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Abstract

The DNA binding protein methyl-CpG binding protein 2 (MeCP2) critically influences neuronal and brain function by modulating gene expression, and children with overexpression of the *MECP2* gene exhibit postnatal neurological syndromes. We demonstrate that some children with *MECP2* duplication also display variable immunological abnormalities that include reductions in memory T and B cells and natural killer cells and immunoglobulin assay responses. Moreover, whereas mice with MeCP2 overexpression were unable to control infection with the intra-macrophage parasite *Leishmania major* and secrete interferon- γ (IFN- γ) from involved lymph nodes, they were able to control airway fungal infection by *Aspergillus niger* and mount protective T helper cell type 2 (T_H2)-dependent allergic responses. Relative to normal T cells, T_H cells from children and mice with *MECP2* duplication displayed similar impairments in IFN- γ secretion and T_H1 responses that were due to both MeCP2-dependent suppression of IFN- γ transcription and sequestration of the IFN- γ locus as assessed by chromatin immunoprecipitation assay. Thus,

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overexpressed MeCP2 aberrantly suppresses IFN- γ secretion from T_H cells, potentially leading to a partially immunodeficient state. Our findings establish a rational basis for identifying, treating, and preventing infectious complications potentially affecting children with *MECP2* duplication.

INTRODUCTION

Methyl-CpG binding protein 2 (MeCP2) is a pleiotropic DNA binding protein that preferentially binds to methylated CpGs and regulates gene expression (1). Duplication of the genomic region that contains the *MECP2* gene (Xq28) is linked to a syndrome characterized by multiple respiratory infections, hypertelorism, severe central nervous system (CNS) deterioration, and early death (2–4). The common core phenotype of these affected boys includes infantile hypotonia, mild dysmorphic features, severe to profound intellectual disability, poor or absent speech development, epilepsy, and progressive spasticity (3). The smallest region of genomic overlap that is associated with this phenotype includes the *MECP2* and interleukin-1 (IL-1) receptor–associated kinase 1 [*IRAK1*; Mendelian Inheritance in Man (MIM): 300283] genes (3, 5, 6). Mice subsequently engineered to overexpress human *MECP2* develop a similarly progressive neurological disease (7). The similar phenotypes of mice expressing twice the normal levels of MeCP2 and humans with increasing *MECP2* dosage from duplication to triplication (6, 8) indicate that *MECP2* duplication causes the neurological disorder that is now termed *MECP2* duplication syndrome (MIM: 300260).

Many children with *MECP2* duplication and triplication syndromes also experience severe, often lethal, pneumonias (3, 9, 10), suggesting that these individuals might have an immune deficit. A minority of these children exhibit decreased immunoglobulin A (IgA) levels and poor antibody responses to polysaccharide antigens (9). However, these uncommon abnormalities cannot explain the recurrent infections seen in most children with *MECP2* duplication (3). The *IRAK1* gene encodes a serine-threonine kinase that lies adjacent to the *MECP2* gene and is typically duplicated in *MECP2* duplication syndrome. IRAK1 is an intermediate signaling molecule in the Toll-like receptor (TLR) signaling pathway and is important for activation and regulation of innate and adaptive immunity (11–13). Aberrant expression of IRAK1 in *MECP2* duplication syndrome is therefore a potential cause of immune dysfunction.

Similar to its role in neuronal cell function as a regulator of chromatin structure and gene expression (1), MeCP2 may regulate gene expression in immune cells where it is also expressed (14). There is a precedent for the involvement of proteins with epigenetic regulatory function in immunological disease. For example, loss-of-function mutations in the DNA methyltransferase 3B gene (*DNMT3B*) (15) result in centromeric chromosomal instabilities that underlie about 50% of cases of the immunodeficiency, centromeric instability, and facial anomalies (ICF1) syndrome (MIM: 242860) (16).

To determine whether MeCP2 overexpression influences immune function, we conducted detailed studies of human subjects with *MECP2* duplication and studied mice in which a human *MECP2* transgene is overexpressed from its own promoter (MeCP2^{Tg3} mice). Our studies confirm that *MECP2* duplication is sufficient to confer immunological anomalies, which are manifested primarily by impaired secretion of the cytokine interferon- γ (IFN- γ) and reduced T helper cell type 1 (T_H1) responses. Our findings establish a rational basis for identifying, treating, and preventing infectious complications potentially affecting children with *MECP2* duplication.

RESULTS

Immunologic assessment of children with *MECP2* duplication syndrome suggests immune deficiency

Previous studies of children with *MECP2* duplication and triplication revealed a high incidence of recurrent infections, especially pneumonias (3, 8, 10). Infectious and inflammatory complications included life-threatening influenza B pneumonia with bacterial sepsis and oxygen-dependent chronic lung disease (Table 1) (8). To further address the apparent immunodeficiency in our own cohort of affected children with *MECP2* duplication, we reviewed the medical records of 10 affected children who are followed clinically at the Blue Bird Circle Rett Center at Texas Children's Hospital (TCH). Nine of our 10 patients with *MECP2* duplication experienced recurrent respiratory tract infections, including severe lower respiratory tract infections such as pneumonia (8 of 10) requiring hospitalization (Table 1). These findings confirm a high prevalence of especially pulmonary complications in children with *MeCP2* overexpression.

In keeping with previous observations (9), serum Ig levels including IgA, IgG, IgM, and IgE were normal with the exception of low IgA levels in two children with *MECP2* duplication (fig. S1 and table S1). Moreover, the production of specific IgG against diphtheria and tetanus toxoids and *Haemophilus influenzae* after boost vaccination was also abnormally attenuated in some, but not all, children (fig. S1 and table S1). In contrast, peripheral blood mononuclear cell (PBMC) responses to mitogenic stimulation were normal (fig. S4). Thus, patients from our local *MECP2* duplication syndrome cohort manifested objective evidence of immune anomalies, including mucosal inflammatory complications of the respiratory tract and variable Ig assay abnormalities.

To extend these findings, we prospectively collected and studied immune cells from 27 children with *MECP2* duplication syndrome having no signs or symptoms of acute disease. Total peripheral blood leukocyte counts of affected children were normal (Fig. 1A). However, when expressed as a percent of total leukocytes relative to established normal values, children with *MECP2* duplication manifested significantly reduced mature neutrophils and, reciprocally, increased immature neutrophils, which indicates concurrent infection or inflammation (Fig. 1, B and C). Peripheral blood eosinophils, basophils, and monocytes were also increased in number (Fig. 1, D to F), suggesting prominent activation of the innate immune system, although these abnormalities were not seen in children of all age groups.

More detailed immunophenotyping of lymphocytes from children with *MECP2* duplication by flow cytometry revealed an increase in total CD4⁺ T cells (CD4⁺CD45RA⁺) in peripheral blood, whereas total CD8⁺ T cells were normal (Fig. 1, G to I). Furthermore, reduced memory CD4⁺ T cells (CD45⁺CD4⁺CD45RO⁺) were accompanied by reciprocally increased naïve CD4⁺ T cells, together with reduced memory B cells (CD19⁺CD27⁺) and natural killer (NK) cells (CD56⁺), especially in younger affected children (Fig. 1, J to M). Total B cells were also reduced in older children (ages 10 to 18 years). These data are also presented, where available, as absolute leukocyte counts (fig. S2). Thus, although often subtle and inconsistently observed across children of different ages, these findings provide objective evidence of immune irregularities in affected boys, including reduced memory T and B cells and NK cells. Moreover, the increased numbers of innate immune cells in peripheral blood suggested the possibility of active infectious or inflammatory disease.

MeCP2 overexpression does not alter development of mouse immune cell lineages

To further delineate the possibility of immune dysfunction occurring as a result of *MECP2* duplication, we next turned to a mouse model in which human *MECP2*, and not other genes

or transcriptional elements, is overexpressed as a transgene (MeCP2^{Tg3} mice) (7, 17). As assessed by flow cytometry, the T cell lineage markers CD4 and CD8 were distributed normally among CD3⁺ T cells from both spleen and thymus of MeCP2^{Tg3} mice (Fig. 2A), indicating overall normal T cell development. The total numbers of lymphocytes in major subsets, including CD4⁺, CD8⁺, B cells (CD19⁺B220⁺) NK cells (NK1.1⁺), and memory/activated mouse T cells (CD44^{hi}CD62^{lo} CD4⁺), were also normal in MeCP2^{Tg3} mice (Fig. 2, B and C). Thus, *MECP2* overexpression per se does not influence development and homeostasis of major T cell subsets in mice.

MeCP2^{Tg3} mice are not able to control *Leishmania major* infection and have impaired T_H1 responses

To determine whether MeCP2 overexpression causes functional immunological deficits, we examined in transgenic mice how MeCP2 overexpression influences both T_H cell development and control of a variety of infections. Relative to otherwise genetically identical control mice, MeCP2^{Tg3} mice failed to control infection because of the intracellular protozoan parasite *Leishmania major* as assessed by progressive footpad swelling (Fig. 3A). Furthermore, IFN- γ -secreting lymph node cells from *L. major*-infected MeCP2^{Tg3} mice were significantly reduced (Fig. 3B). This finding was of particular interest because IFN- γ from T_H1 cells is required for control of *L. major* infection (18), suggesting that MeCP2 overexpression in T cells conferred a defect in T_H1 development. To test this possibility, we adoptively transferred syngeneic wild-type CD4⁺ T cells into MeCP2^{Tg3} mice and determined the susceptibility of these T cell chimeric mice to *L. major* infection. Indeed, immune reconstitution with wild-type T_H cells enhanced the ability of MeCP2^{Tg3} mice to mount lymph node IFN- γ responses and fully restored control of *L. major* infection as assessed by footpad swelling (Fig. 3, A and B).

We conducted additional studies to understand whether MeCP2 overexpression influenced other T_H cell developmental programs, especially T_H2 responses that underlie allergic inflammation. In contrast to *L. major*-infected mice, MeCP2^{Tg3} mice infected intranasally with the fungus *Aspergillus niger* developed airway hyperresponsiveness as assessed by acetylcholine challenge, a canonical marker of airway obstruction in asthma; lung IL-4 responses, an index of T_H2 cell development and recruitment to lung; and allergic inflammation as assessed by airway eosinophil recruitment, which were either identical or enhanced relative to wild-type mice. Notably, however, IFN- γ responses were markedly diminished in MeCP2^{Tg3} mouse lungs after infection with *A. niger* (Fig. 3, C to E). MeCP2^{Tg3} mice further showed no impairment in controlling the resulting airway fungal infection as assessed by fungal cultures of lung homogenates and mounted normal lung IL-17 responses, indicative of normal T_H17 cell development, against *A. niger* (fig. S3). MeCP2^{Tg3} T_H cells were also capable of differentiating into T_H17 cells as efficiently as T cells from wild-type mice (fig. S3).

Together, these findings establish that MeCP2 overexpression in mice confers a selective defect in IFN- γ , but not IL-4 or IL-17A, production in diverse in vivo contexts but fails to resolve whether this reflects a global impairment in IFN- γ secretion or a lineage-specific defect in T_H1 responses. To address this, we compared the ability of CD8 and CD4 T cells from *MECP2* transgenic mice to differentiate into IFN- γ -secreting CD8⁺ cytotoxic T cells (Tc1) and T_H1 cells in vitro under lineage biasing conditions. Surprisingly, Tc1 differentiation was not affected by MeCP2 overexpression, but T_H1 development was markedly impaired (Fig. 3, F and G).

We further characterized the ability of MeCP2^{Tg3} mice to develop appropriate antibody responses. In mice, T_H cells function to assist B cell production of appropriate IgG antibodies, with T_H2 cells contributing to IgG1 responses and T_H1 cells, primarily through

IFN- γ , contributing to IgG2a responses (19). Immunization of mice with ovalbumin (OVA) revealed differential impairment of IgG responses, with no significant difference between wild-type and MeCP2^{Tg3} mice seen regarding IgG1 production, but significantly reduced IgG2a secretion was observed (Fig. 3H). Thus, MeCP2 overexpression confers a selective defect in IFN- γ secretion from T_H cells, leading to impaired IgG2a secretion in mice.

MECP2 duplication impairs human T_H1 cell differentiation

We further assessed T_H1 differentiation from naïve CD4 T cells from subjects with *MECP2* duplication. Similar to MeCP2^{Tg3} T cells, CD4⁺ T cells from affected subjects produced markedly less intracellular IFN- γ when cultured in vitro under either T_H0 or T_H1 biasing conditions and overall about one-third the total number of T_H1 cells as compared to normal subjects (Fig. 4, A and B). Notably, this was not due to impaired proliferation (Fig. 4, C and D, and fig. S4). Thus, similar to findings from MeCP2^{Tg3} mice, CD4⁺ T cells from children with *MECP2* duplication syndrome exhibit defective T_H1 responses.

TLR signaling is unaffected in mononuclear cells from children with *MECP2* duplication syndrome

Although the *IRAK1* gene is duplicated together with *MECP2* in *MECP2* duplication syndrome (5, 6), *IRAK1* is not duplicated in MeCP2^{Tg3} mice (7), providing an opportunity to test hypotheses regarding the role of this immunoregulatory serine/threonine kinase in altering immune function in *MECP2* duplication syndrome (20). We therefore determined the response of PBMCs from affected subjects to TLR ligand stimulation to uncover any potential perturbation in TLR signaling. We found no significant difference in inflammatory cytokine production [IL-1 β , IL-6, and TNF- α (tumor necrosis factor- α)] from these cells after stimulation with diverse TLR ligands (Fig. 5, A to C). Thus, independent of the aberrant expression of *IRAK1*, these findings support a primary role for MeCP2 in mediating the immune irregularities seen in *MECP2* duplication syndrome.

MECP2 overexpression suppresses T-bet–dependent IFN- γ transcription and sequesters the IFN- γ locus

We conducted additional experiments to determine the potential mechanism by which overexpression of MeCP2 impairs T_H1 responses. In addition to reduced IFN- γ protein production, both *MECP2* transgenic mouse T cells and T cells from study participants with *MECP2* duplication syndrome produced reduced levels of IFN- γ mRNA after stimulation under T_H1 biasing conditions, suggesting that MeCP2 suppressed *ifng* transcription (Fig. 6, A and B).

MeCP2 binds to methylated CpG islands of DNA and either activates or suppresses gene expression, and previous work indirectly implicated MeCP2 as a regulatory factor in *ifng* transcription (21). We first considered the possibility that MeCP2 overexpression could suppress expression of transcription factors required for IFN- γ production such as T-bet, the principal factor controlling IFN- γ production and secretion from T_H cells (22). However, intracellular staining of human T cells confirmed that T-bet expression is actually enhanced in the setting of *MECP2* duplication (Fig. 6C).

We next considered the possibility that overexpressed MeCP2 could directly suppress IFN- γ transcription. When expressed together with T-bet in the human T cell line Jurkat, MeCP2 markedly inhibited endogenous IFN- γ transcription (Fig. 6D). Transfection of a MeCP2-specific small interfering RNA (siRNA) into naïve CD4⁺ T cells from children with *MECP2* duplication syndrome both significantly reduced *MECP2* transcripts and partially restored IFN- γ mRNA levels, suggesting that overexpression of MeCP2 is directly linked to the suppression of IFN- γ transcription (Fig. 6E).

The IFN- γ promoter in naïve T cells is unmethylated (23), suggesting that methyl-CpG binding fails to explain this result. However, MeCP2 can also bind to nonmethylated DNA (24, 25) and affect large-scale chromatin reorganization independent of DNA modification as a major mechanism for gene suppression (26). To further explore this possibility, we performed chromatin immunoprecipitation (ChIP) assays using mouse T_H cells to determine the accessibility of the IFN- γ gene in the setting of MeCP2 overexpression. These studies demonstrated that, relative to wild-type cells, in the setting of MeCP2 overexpression, the IFN- γ locus is less likely to associate with acetylated histone protein 3 (Fig. 6, F and G). Thus, when overexpressed, MeCP2 renders the IFN- γ gene relatively inaccessible to transcriptional activation, most likely by sequestering the *IFNG* locus in association with histone proteins.

DISCUSSION

In addition to profound neurological disability, *MECP2* duplication syndrome is characterized by recurrent pneumonias and other inflammatory complications. We have combined studies of children with *MECP2* duplication and mice with a homologous alteration to determine whether overexpression of the *MECP2* gene causes immune dysfunction. Our findings confirm that when overexpressed in human and mouse T_H cells, *MECP2* selectively impairs IFN- γ secretion and T_H1 responses by acting as a transcriptional repressor and regulator of *ifng* locus accessibility. These findings indicate that children with duplication or triplication of the *MECP2* gene have immune impairments related to their inability to mount robust T_H1 responses, and suggest that the high frequency of pulmonary and other inflammatory complications affecting these children may be due to opportunistic pathogens.

A limitation of these studies is that although reduced T_H1 responses were observed equally in humans and mice with *MECP2* duplication, not all human immune abnormalities were reflected in the mouse. The markedly abnormal cell counts from peripheral blood of affected children that were not seen in the transgenic mice most likely represent differences in the environments encountered by humans and inbred research mice: Whereas humans constantly encounter pathogens from birth, specific pathogen-free (SPF) mice do not. We speculate that the relative lack of memory CD4⁺ T cells, balanced by reciprocal increases in naïve T cells, in children with *MECP2* duplication is potentially explained by their impaired T_H1 generation in response to diverse pathogens encountered in infancy and childhood. Chronic, unresolved infections due to lack of T_H1 responses in these children could also explain the marked immaturity and increased abundance of neutrophils, eosinophils, and monocytes (Fig. 1). Relative lack of T_H1 help could also explain the variable defects in antibody responses of some affected children (fig. S1). Thus, although confirmatory studies are needed, a single defect involving deficient IFN- γ responses involving only T_H cells potentially explains the diverse immunologic anomalies demonstrated in children as well as the discrepancies in observations between humans and mice with *MECP2* duplication.

Additionally, the mechanism by which MeCP2 controls gene expression in neurons and especially immune cells such as T cells remains incompletely understood. MeCP2 binds promiscuously and relatively homogeneously to chromosomal DNA, including methylated and nonmethylated regions, and may either transcriptionally suppress or enhance expression of neuronal genes (27). Little evidence exists to suggest that a major role of MeCP2 is to target individual genes to regulate their expression. Rather, MeCP2 appears to globally influence gene expression subtly and indirectly in a manner analogous to histone proteins by regulating chromosomal architecture (28).

In contrast, our findings suggest that, when overexpressed, MeCP2 interacts specifically with T-bet to inhibit *ifng* transcription. The Sin3A-HDAC (histone deacetylase) complex is displaced by T-bet at the *ifng* locus, which, although possibly only a correlative observation, may be important for the induction of *ifng* transcription and proper T_H1 differentiation (29). Sin3A-HDAC is recruited by MeCP2 to target gene loci to suppress gene transcription, strongly supporting the importance of the Sin3A-HDAC/MeCP2 complex in the regulation of IFN- γ production (30). Although speculative, it is possible that increased binding of MeCP2 to T-bet in the setting of *MECP2* duplication may be sufficient to preclude the full displacement of Sin3A-HDAC at the *IFNG* promoter, leading to DNA hypoacetylation and condensation on histone proteins.

Several human immunodeficiency syndromes are marked by deficiency in IFN- γ activity and involve genetic mutations in the IFN- γ receptor, signal transducer and activator of transcription 1 (STAT1), and the IL-12 receptor β 1 (IL-12R β 1) chain. Patients with these mutations exhibit profound immunodeficiency marked by susceptibility to lethal invasive infections due to intracellular pathogens such as bacteria and viruses (31–34). Pneumonia, often due to mycobacteria, is a consistent clinical finding in these patients, but infections due to viruses and other bacteria have also been described (31, 32, 34). These findings in patients with unrelated genetic anomalies that underlie parallel immune irregularities further suggest that the pneumonias seen in children with *MECP2* duplication may be infectious in nature and caused by immune dysfunction, specifically impaired IFN- γ , and not solely by neurological impairments.

Notably, however, IFN- γ secretion and T_H1 responses are not entirely ablated in the setting of MeCP2 overexpression (Fig. 4), perhaps reflecting subtle differences in the intranuclear levels of MeCP2 in immune cells in the setting of gene duplication. Defective IFN- γ responses were further limited to T_H cells, although we cannot exclude the possibility that MeCP2 duplication confers an IFN- γ defect in NK cells. Together, these issues may explain the variable immune phenotypes observed in our cohort with *MECP2* duplication and the possibly attenuated infectious disease course of children with *MECP2* duplication syndrome as compared to the more severe global IFN- γ deficiencies (such as IL-12R β 1 deficiency) in which disseminated infections are often seen (35). IFN- γ further serves as a growth and differentiation factor for NK cells (36). This suggests that the mild deficiency in NK cells observed in our study subjects with MeCP2 overexpression could be due to the relative lack of the trophic effects of IFN- γ , although the reduction in NK cells could also be related to the depleting effects of recurrent infections.

In summary, we have shown that in addition to neurological phenotypes, duplication of the *MECP2* gene causes immune dysfunction due in part to the suppression of IFN- γ production from T_H cells. Affected children may be susceptible to infections caused by a wide variety of intracellular pathogens that are capable of causing pneumonia and other infections and possibly contributing to CNS disease. Identifying specific infectious agents that contribute to pneumonia and possibly neurological decline in children with *MECP2* duplication will help to clarify the extent and severity of immune dysfunction.

MATERIALS AND METHODS

Human subjects and sample collection

All experimental procedures were approved by the Institutional Review Board of Baylor College of Medicine and TCH. Participants received a diagnosis of *MECP2* duplication syndrome according to standard molecular criteria. Blood samples were collected from 27 consecutive individuals with a diagnosis of *MECP2* duplication syndrome confirmed by review of medical records and clinical genetic testing results and from 26 age-matched

control subjects who assembled at Baylor College of Medicine for the 1st International *MECP2* Duplication Syndrome Family Conference in May 2011. Unrelated controls were recruited through the TCH phlebotomy laboratory and were prospectively recruited at the time of a clinical blood draw. Subjects were excluded if they had a clinical diagnosis suggesting the possibility of immune deficiency (such as recurrent infections, organ transplant, primary immunodeficiency, and autoimmune disease), if they were taking immunomodulating therapies, or if they had signs/symptoms of active infection at the time of the blood draw.

Mice

Fvb/NJ mice were purchased from Jackson Laboratories and used as controls for mice with duplication of the *MECP2* gene (FVB/N MeCP^{Tg3}) (7). MeCP^{Tg3} mice contain a 99-kb human PAC (P1 artificial chromosome) clone that contains all exons of the *MECP2* gene and no other transcriptional units or exons. All experiments included male mice between the ages of 4 and 6 weeks. Mice were bred under SPF conditions, and all animal protocols were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Antibodies and reagents

Except where otherwise indicated, all antibodies and cytokines were purchased from BD Biosciences. Anti-acetyl-histone H3 antibody was purchased from Millipore. Vector pcDNA3.1-*MECP2A*(E2)-CTAP containing a full-length human *MECP2A* complementary DNA (cDNA) with CTAP tag was generated by first subcloning human *MECP2A*(E2) cDNA from vector MECP2-pBi-EGFP (37) into pMK33-CTAP (GenBank accession no. AY727854) and then subcloning the *MECP2A*(E2)-CTAP fusion into pcDNA3.1 vector (Invitrogen). Vector expressing human *TBX21* was purchased from GeneCopoeia.

Cells and cultures

PBMCs were isolated with standard Ficoll-Hypaque procedure as previously described (38). CD4⁺ T cells were further purified by positive selection (Miltenyi) and cultured in RPMI 1640 with L-glutamine, penicillin-streptomycin, and 10% fetal bovine serum. Cells were activated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-2 (T_H0 condition) or in the presence of recombinant human IL-12 (10 ng/ml) and antibodies to IL-4 (10 µg/ml) to induce T_H1 differentiation. Naïve mouse CD4⁺ T cells were purified by positive immunomagnetic selection (Miltenyi) from splenocytes. Naïve CD4⁺ T cells were cultured in plates coated with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) in supplementary IL-2 (neutral conditions) or also with recombinant murine IL-12 and anti-IL-4 mAb (10 µg/ml) (T_H1 conditions) or recombinant murine IL-6, IL-1β, and transforming growth factor-β (T_H17 conditions) for 3 to 7 days.

Flow cytometry

Human and mouse cells were stained with the indicated antibodies, and flow cytometry data were collected with a BD LSRII (BD Biosciences) and analyzed by FlowJo software (Tree Star). For intracellular cytokine and protein detection, cells were restimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of monensin (BD Biosciences) for 5 hours at 37°C in 5% CO₂. Cells were then fixed and stained with Cytofix/Cytoperm kit (BD Biosciences). Dilution of the fluorescent cytoplasmic stain CFSE (Invitrogen) was used to detect proliferating cells according to the manufacturer's instructions.

TLR stimulation assay

Cytokine secretion in response to TLR ligand stimulation was assessed from peripheral blood by ARUP Laboratories.

Leishmania infection

L. major strain (MRHO/SU/59/P/LV39) was maintained and cultured as described (39, 40). Mice were infected with 2×10^6 stationary-phase *L. major* promastigotes in the hind footpads. MeCP2^{Tg3} mice were intraperitoneally transferred with 10^7 CD4⁺ T cells 24 hours before infection. Footpad thickness was monitored weekly over 80 days after infection with a micrometer. Cytokine-secreting popliteal lymph node cells were detected by ELISpot assay (41).

Analysis of allergic lung disease

Mice were intranasally challenged with 400×10^3 conidia of *A. niger* every other day for 2 weeks. Airway responsiveness to acetylcholine challenge, BAL fluid collection, and enumeration of total lung IL-4- and IFN- γ -secreting cells by ELISpot were performed as previously described (42–44).

Allergen sensitization

Mice were sensitized with OVA precipitated in alum (OVA/alum) once a week for 3 weeks, and OVA-specific IgG isotype production was determined by ELISA as previously described (41, 42).

Cell transfection

Jurkat T cells were transfected with the Amaxa Jurkat Nucleofector Kit (Lonza) according to the manufacturer's instructions. Cells were then incubated for 48 hours in 37°C with 5% CO₂.

MECP2 siRNA knockdown

Silencer Select siRNA targeting *MECP2* gene was purchased from Life Technologies predesigned siRNA sequences GCUUCCCCGAUUAACUGAAAtt (sense) and UUCAGUUAUUCGGGAAGCtt (antisense). Silencer Select Negative Control siRNA was used as negative control. CD4⁺ T cells from patients were nucleofected with either control or *MECP2*-specific siRNA according to the manufacturer's protocol. Cells were used for functional studies 24 hours after nucleofection.

mRNA isolation and qPCR

Cell pellets were lysed with TRIzol (Invitrogen), and mRNA was extracted for qPCR according to the manufacturer's directions. Probes for *Ifng* (Hs00989291_m1) and *Mecp2* (Hs00172845_m1) were purchased from Applied Biosystems. Gene expression was normalized to 18S ribosomal RNA (Hs99999901_s1) expression.

ChIP assay

ChIP experiments were performed with Pierce Agarose ChIP Kit (Thermo Fisher Scientific) according to the manufacturer's protocols. In brief, for each analysis, 2×10^6 differentiated TH1 cells were cross-linked with 1% formaldehyde at room temperature (RT) for 10 min, and then the reaction was stopped with 0.2 M glycine for 5 min at RT. Chromatin was digested with micrococcal nuclease (8 U/ μ l), and sheared chromatin was immunoprecipitated with anti-acetyl-histone H3 antibody or rabbit IgG together with agarose beads overnight at 4°C. DNA was thereafter purified from the mixture, and a 252–

base pair region of the IFN- γ promoter was amplified (forward primer, 5'-CGTAATCCCGAGGAGCCTTC-3'; reverse primer, 5'-CTTTCAATGACTGTGCCGTGG-3') by PCR.

Data and statistical analyses

Aggregate data are presented as means $\pm$ SEM. Prism software was used for statistical analyses. Except for airway physiology data, which were log-transformed to achieve a normal distribution suitable for analysis with the two-tailed Student's *t* test, significant differences between paired samples were analyzed with the Mann-Whitney *U* test. Comparisons of immune phenotyping data in which subject data are compared to a clinically established normal range (95th percentile) were performed with the R program binominal distribution. A *P* value of less than 0.05 was considered significant for all analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Children with *MECP2* duplication syndrome have reduced numbers of memory T and B cells and NK cells. (A to F) Peripheral blood from 27 children with *MECP2* duplication syndrome was analyzed for total leukocytes (A) and the leukocyte subgroups (B) mature neutrophils, (C) immature neutrophils, (D) eosinophils, (E) basophils, and (F) monocytes, expressed as percentages of total leukocytes. (G to M) Lymphocyte subgroups are indicated as the proportion of CD45⁺ cells that were (G) B cells (CD45⁺CD19⁺), (H) CD4⁺ T cells (CD45⁺CD3⁺CD4⁺), (I) CD8⁺ T cells (CD45⁺CD3⁺CD8⁺), (J) memory CD4⁺ T cells (CD45⁺CD4⁺CD45RO⁺), (K) naïve CD4⁺ T cells (CD45⁺CD4⁺CD45RA⁺), (L) memory B cells (CD45⁺CD19⁺CD27⁺), and (M) NK cells (CD45⁺CD56⁺) as determined by flow cytometry. Age-based normal ranges (TCH) for each parameter are indicated by gray shading. $n = 27$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to normal ranges. Statistics was performed with the R program binominal distribution.

Fig. 2.

MeCP2 overexpression does not alter immune system development and homeostasis in mice. Splenocytes and thymocytes from wild-type (WT) and MeCP2 overexpression (MeCP2^{Tg3}) mice were analyzed for the frequency of major lymphocyte subsets and memory T cells by flow cytometry by comparing the expression of the lineage markers CD4, CD8, CD19, and NK1.1. (A) Representative analysis of CD4 and CD8 expression on splenocytes (top panel) and thymocytes (bottom panel). (B) Absolute numbers of splenic CD4 and CD8 T cells, B cells, and NK cells ($n = 4$ per group). (C) Representative analysis of the activation and memory CD4⁺ T cell markers CD44 and CD62L from splenocytes. (D) Absolute number of CD44^{hi}CD62L^{lo} CD4⁺ splenocytes ($n = 5$ per group).

Fig. 3.

Impaired T_H1 , but normal T_H2 , responses in $MeCP2^{Tg3}$ mice. (A and B) Impaired control of *L. major* infection and reduced IFN- γ responses in $MeCP2^{Tg3}$ mice. WT ($n = 7$) Fvb and syngeneic *MECP2* transgenic mice ($MeCP2^{Tg3}$, $n = 6$) were infected in footpads with stationary-phase *L. major* promastigotes. Footpad thickness was measured over 80 days (A), and total popliteal lymph node cells spontaneously secreting IFN- γ and IL-4 were quantified by enzyme-linked immunospot (ELISpot) (B). A third group of $MeCP2^{Tg3}$ mice received intraperitoneally naïve WT $CD4^+$ T cells 24 hours before infection ($MeCP2^{Tg3} + CD4$, $n = 6$). The footpad thickness data were analyzed with Student's *t* test for each time point. Statistics refer to WT versus $MeCP2^{Tg3}$. The ELISpot data were analyzed with Mann-Whitney *U* test. (C to E) Normal T_H2 -dependent allergic responses in $MeCP2^{Tg3}$ mice. (C) Airway responsiveness to acetylcholine provocation was assessed in WT and $MeCP2^{Tg3}$ mice challenged intranasally over 2 weeks with either phosphate-buffered saline (PBS) or the conidia of *A. niger*. (D) Total IL-4- and IFN- γ -secreting cells from whole lung of the same mice as determined by ELISpot assay. (E) Total numbers of eosinophils (Eos), monocytes (Mono), neutrophils (Neutro), and lymphocytes (Lym) in bronchoalveolar lavage (BAL) fluid of the same mice. $n = 3$ in PBS WT; $n = 4$ in PBS $MeCP2^{Tg3}$; $n = 7$ in both fungus WT and fungus $MeCP2^{Tg3}$ group. Data were analyzed with Student's *t* test. (F and G) *MeCP2* overexpression impairs T_H1 development in vitro. Naïve splenic $CD4^+$ and $CD8^+$ T cells were activated under neutral (T_H0) or T_H1 -differentiating conditions as indicated. Flow cytometry was used to determine intracytoplasmic production of IL-4 and IFN- γ . The aggregate percent of IFN- γ -secreting cells deriving from naïve $CD4^+$ T cells using this protocol is shown ($n = 4$ per group; $**P < 0.01$, Student's *t* test). (H) Impaired IFN- γ -dependent IgG isotype production in $MeCP2^{Tg3}$ mice. WT and $MeCP2^{Tg3}$ mice were immunized with OVA/alum weekly for 3 weeks, after which serum levels of OVA-specific IgG1 and IgG2a were determined by enzyme-linked immunosorbent assay (ELISA).

($n = 6$ per group; $*P < 0.05$, Students' t test). Data are averages $\pm$ SEM. $*P < 0.05$, $**P < 0.01$.

Fig. 4.

CD4⁺ T cells from PBMCs of children with *MECP2* duplication syndrome exhibit impaired T_H1 differentiation and dysregulated proliferation in vitro. **(A)** Representative flow cytometric analyses of intracytoplasmic IFN- γ production (*y* axis) and cell proliferation as assessed by dilution of the cytoplasmic dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (*x* axis) of stimulated CD4⁺ T cells from a normal (control) volunteer and one with *MECP2* duplication (MeCP2). **(B)** Aggregate analysis of the percent of IFN- γ -secreting cells under T_H1 differentiation conditions comparing control and *MECP2*-overexpressing T cells from 10 independent experiments. Statistics was performed with paired *t* test. Bar represents mean. **(C and D)** Representative histograms depicting proliferation of IFN- γ -secreting and nonsecreting T cells as assessed by intracellular CFSE dilution under the indicated conditions.

Fig. 5.

TLR signaling is unaffected in children with *MECP2* duplication syndrome. Production of the indicated cytokines by PBMCs from children with *MECP2* duplication syndrome (MECP2 dupl) after stimulation with the indicated TLR ligands was determined by multiplex analysis (control, $n = 18$; *MECP2* duplication, $n = 9$). (A) IL-1 β . (B) TNF- α . (C) IL-6.

Fig. 6.

MeCP2 overexpression suppresses T-bet-dependent IFN- γ transcription. (**A** and **B**) IFN- γ mRNA level is regulated at the transcriptional level in T_H1 cells from both MeCP2^{Tg3} mice and children with *MECP2* duplication. (**A**) CD4⁺ splenocytes from WT and MeCP2^{Tg3} mice were cultured as in Fig. 4, and IFN- γ mRNA was quantified by real-time quantitative polymerase chain reaction (qPCR) ($n = 4$ per group). (**B**) CD4⁺ T cells isolated from PBMCs of children with *MECP2* duplication and controls were cultured under T_H1-polarizing conditions for 3 days, and IFN- γ mRNA levels were quantified by real-time qPCR ($n = 4$ per group). (**C**) Intracellular T-bet was quantified by flow cytometry comparing T_H1 cells from a control individual and one with *MECP2* duplication. Accumulated data from six independent subjects in each group are shown. Statistics was performed with paired t test. Bar represents mean. (**D**) Endogenous IFN- γ transcription is suppressed when T-bet is overexpressed with MeCP2. Jurkat T cells were transfected with vectors expressing either *TBET* or *TBET* and *MECP2*, and the effect on IFN- γ mRNA expression was determined by real-time qPCR (* $P < 0.05$, $n = 3$ per group). (**E**) Impaired IFN- γ secretion is partially rescued by *MECP2* siRNA knockdown in CD4⁺ T cells. CD4⁺ T cells from children with *MECP2* duplication were transfected with *MECP2*-specific and irrelevant (control) siRNA, and CD4⁺ T cells from control individuals received control siRNA. IFN- γ and MeCP2 transcripts were then quantified by real-time qPCR ($n = 3$ per group). (**F** and **G**) The IFN- γ locus is sequestered in MeCP2^{Tg3} T cells. Naïve CD4⁺ T cells from WT and MeCP2^{Tg3} mice were cultured under T_H1 biasing conditions. After 3 days, chromatin regions linked to acetylated histone protein 3 (H3Ac) were immunoprecipitated with a specific antibody, and the relative abundance of the IFN- γ promoter was assessed by qPCR. (**G**) The ratio of anti-H3Ac-associated PCR product to input-associated product from three independent experiments was determined by densitometric quantitation of the PCR bands. All statistical comparison were performed with Student's t test (* $P < 0.05$).

Table 1

Infectious disease history summary of children with MeCP2 overexpression.

	Infection-related hospitalizations	Pneumonia	Other infectious and inflammatory disorders
<i>MECP2</i> duplication	8/10	8/10	Fungemia (1/10)
<i>MECP2</i> triplication *	3/3	3/3	Acute respiratory distress syndrome and methicillin-resistant <i>Staphylococcus aureus</i> sepsis complicating influenza B (1/2) Oxygen-dependent chronic lung disease (3/3)

* Previously reported (8). Data are for three of five children surviving to hospital discharge.