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A Multiple Sclerosis–Associated Variant of CBLB Links Genetic Risk with Type I IFN Function

Klarissa Hanja Stürtner,* Uwe Borgmeyer,† Christian Schulze,‡ Ole Pless,§,1 and Roland Martin*,#,*1

Multiple sclerosis (MS) is a complex genetic disorder. Multiple genes and environmental factors, such as EBV infection, reduced vitamin D3 levels, and smoking, can contribute to the disease (1, 2). Genome-wide association studies (GWAS) have been highly successful in identifying an increasing number of common genetic variants and single nucleotide polymorphisms (SNPs) (3, 4), but knowledge regarding their functional contribution to MS pathogenesis remains limited, with the exception of MS risk-associated SNPs that lead to differential splicing of the IL-7 receptor and of TNFR1 (5-7). Furthermore, even less is known about mechanisms explaining the interaction and interplay of genetic and environmental factors on disease. A considerable fraction of the now more than 100 MS risk variants that have been identified by large GWAS studies (3, 4) are involved in T cell proliferation or setting their activation threshold, and hence indicate perturbations of T cell activation and function as an important aspect of MS pathogenesis. Among the known MS risk alleles, three are located near the Casitas B-lineage lymphoma proto-oncogene b gene (CBLB). The CBLB protein (CBL-B) is a key regulator of peripheral immune tolerance by limiting T cell activation and expansion and hence T cell–mediated autoimmunity through its ubiquitin E3-ligase activity. In this study, we show that CBL-B expression is reduced in CD4+ T cells from relapsing-remitting MS (RR-MS) patients during relapse. The MS risk-related single nucleotide polymorphism of CBLB rs12487066 is associated with diminished CBL-B expression levels and alters the effects of type I IFNs on human CD4+ T cell proliferation. Mechanistically, the CBLB rs12487066 risk allele mediates increased binding of the transcription factor C/EBPβ and reduced CBL-B expression in human CD4+ T cells. Our data suggest a role of the CBLB rs12487066 variant in the interactions of a genetic risk factor and IFN function during viral infections in MS. The Journal of Immunology, 2014, 193: 4439–4447.

Materials and Methods

Study population

All patients had MS as defined by the 2010 McDonald criteria and were recruited from the MS outpatient clinic of the Institute of Neuroimmunology and Multiple Sclerosis at the University Medical Center Hamburg-Eppendorf. All patients were without any immunomodulatory or immunosuppressive treatment for at least 3 mo before participating in our study. Blood samples during relapse were collected before the start of steroid treatment. Treatment response to IFN-β was defined according to published criteria (18); one relapse or at least one point of progression in the Expanded Disability Status Scale (EDSS) score both in combination with magnetic resonance imaging activity during 2 y of IFN-β treatment was used to define nonresponder status. “Intermediate responders” were
defined as patients suffering from a relapse within the first 3 mo of treatment or solely inflammatory magnetic resonance imaging activity without any relapse or EDSS progression. Healthy control donors (HDs) were recruited from coworkers at the University Medical Center Hamburg-Eppendorf. This study was approved by the local ethics committee (Ethik-Kommission der Ärztekammer Hamburg, No. 2758), and written informed consent was obtained from all study subjects.

Genotyping
We performed two-color TaqMan assays (Applied Biosystems) for the identification of homoduplex allele carriers using a FAM-labeled probe for the T allele (risk allele) and a VIC-labeled probe for the C allele for the rs12487066 and the rs9657904 SNP.

Linkage disequilibrium
Genotype data from the 1000 genomes project (19), European super population, for a genomic region comprising rs12487066, rs9657904, and rs2028597 plus 1 kb upstream and downstream were used to calculate linkage disequilibrium (HaploView 4.2).

Prediction of transcription factor binding sites
Match is a weight matrix–based tool for searching putative transcription factor binding sites (20). We used it for analyzing the DNA sequence starting 50 bp upstream and downstream of the CBLB SNP-containing region, focusing on the transcription factor binding prediction for sequence differences at the CBLB SNP base (C versus T) in the first instance. Only candidates binding to a sequence that included the CBLB SNP base were considered for further analysis. Identification of putatively binding transcription factors was based on their expression in CD4+ T cells, on high core and matrix match values provided by Match, and on differences in the match values for the different CBLB alleles.

EMSA
Single-stranded oligonucleotides (Metabion, Martinsried, Germany) were annealed in 10 mM Tris-HCl (pH 7.5) and 60 mM NaCl and were stored at −20°C. Double-stranded oligonucleotides had 5’ overhangs of five nucleotides on both strands. For EMSAs, double-stranded oligonucleotides were labeled using Klenow polymerase (Roche Applied Science, Mannheim, Germany) with [α-32P]dATP (Hartmann Analytics, Braunschweig, Germany). Unincorporated nucleotides were removed with gel filtration on Sephadex G25 spin columns (Roche Applied Science), and 2 μl were used for EMSA. Labeled oligonucleotides were stored at 4°C in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 60 mM NaCl. Binding reactions were performed in a total volume of 1 μl consisting of 20 mM HEPES, pH 7.4, 80 mM NaCl, 20 mM KCl, 0.5 mM DTT, 0.1 μg Cot-1 DNA (Roche Applied Science), and 2 μl reticulocyte lysate. A 30-min preincubation was followed by the addition and binding of 1 μl of the labeled oligonucleotides for 30 min at room temperature. Complexes were resolved by denaturing PAGE in 0.5× TBE (45 mM Tris base, 45 mM boric acid, 0.5 mM EDTA) at 4°C at 20 V/cm for 4 h. The gels were dried and analyzed with the Fujix BAS 2000 bioimaging system using the Tina software (Raytest). The sequences used for EMSA were: CBLB-WT-F 5’-AACT-TAACGACCTAAAATGTACCGTACGTCGTG-3’; CBLB-WT-rev 5’-TGCTACAGCACCTTGTCAGTGAGA-3’; CBLB-mut-F 5’-AACT-TAACGACCTAAAATGTACCGTACGTCGTG-3’; and CBLB-mut-rev 5’-TGCTACAGCACCTTGTCAGTGAGA-TAACGACCTAAAATGTACCGTGCTG-3’.

Chromatin immunoprecipitation
Chromatin immunoprecipitations were performed as described previously (21) with minor modifications. PCR primers (BioMers) were chosen 100 bp upstream and downstream of the SNP. Sequences were as follows: for the rs12487066-F 5’-CTCCGCAAGTACAGGGATATT-3’; rs12487066-R 5’-CCTCTTATGGACTGTG-3’; and for the control rs9657904-F 5’-CTCTAACATTTACACAG-3’ and rs9657904-R 5’-ACCTCCATGTTGTCCTAT-3’.

Flow cytometric analysis of CBL-B expression
Measurements from whole blood samples of patients with RR-MS were performed using 250 μl EDTA blood. For surface marker analysis, cells were stained with mAbs and pooled with mAbs at room temperature, were lysed using lysis solution (BD Biosciences) for 10 min at room temperature. The following Abs were used: anti-C/EBP (APC, clone RPA-T4; eBioscience), anti-CD8 (mouse IgG1, clone R34.34; Beckmann Coulter); anti-CD5 (PE, clone 55E2; BD Biosciences), anti-CD19 (PE Cy7, clone 3D12; BD Biosciences). In vitro experiments, CD4+ T cells were washed in PBS and Fc-blocked with human IgG (Jackson ImmunoResearch) after 72 h in vivo incubation. Additional steps were performed in the dark. For CD4+, surface staining anti-CD4 (Phycoerythrin, clone M48; BD Biosciences) was performed 50% minimum for more than 30 min at room temperature. PBMCs and CD4+ T cells (from blood or in vitro activation, respectively) were fixed in 100 μl fixation buffer (eBioscience) for 20 min and then permeabilized in permeabilization buffer (PBS, 5% FCS, 2% BSA, 0.1% Triton X-100), containing a second Fc-block with human IgG (Jackson ImmunoResearch). For intracellular staining, either anti-human CBL-B (polyclonal rabbit IgG; Proteintech Group) at 0.005 μg/μl (1:50 diluted) or rabbit IgG as isotype control (Jackson ImmunoResearch) at 0.005 μg/μl was added for 35 min at room temperature. After washing, cells were stained with anti-rabbit IgG secondary Ab (Dianova, Hamburg, Germany) for 35 min at room temperature. All analyses were performed with an LSRII (BD Biosciences) flow cytometer. Data analysis was performed with BD FACSDiva for analyzing median fluorescence intensities (MedFI) for all data presented.

Knockdown of C/EBPβ in primary human CD4+ T cells
Small interfering RNA (siRNA) knockdown was performed according to the manufacturer’s instructions using Accell SMARTpool siRNA duplexes (Dharmacon, Lafayette, CO) targeting the 3'UTR and open reading frame of C/EBPβ and compared with a mixture of nontargeting control siRNAs. Briefly, 125,000 isolated human CD4+ T cells per well were resuspended in X-VIVO, seeded in 96-well plates, and incubated with 1 μM of the siRNA pool for C/EBPβ or the nontargeting pool. After administering the siRNA, CD4+ T cells were rested for 30 min at 37°C and were then stimulated with 75,000 anti-CD3, anti-CD2, anti-CD28–coated beads (Miltenyi Biotec) and EGF (100 ng/ml) for 72 h. As a control, siRNA with a nonsense/scrambled sequence from the Accell siRNA Control Kit was used. Knockdown efficiency was evaluated with real-time quantitative PCR (Supplemental Fig. 2A, 2B).

Real-time quantitative PCR
After 72 h of in vitro stimulation of CD4+ T cells, cells were washed once with PBS and resuspended in TRIZOL before snap-freezing in liquid nitrogen and storage at −80°C. RNA was extracted according to published protocols (22). RNA was purified from PBMCs of RR-MS patients and HDs. Real-time quantitative PCR was performed using an ABI real-time PCR 7900HT Fast System (Applied Biosystems). The TaqMan primer and probe set for C/EBPβ (Hs0072903_21), CBLB (Hs00180288_m1), and 18S (Hs00303631_g1) rRNA were purchased from Applied Biosystems and used according to the manufacturer’s instructions. 18S rRNA was used.
as endogenous control, and the relative gene expression was calculated with the ΔΔCT method using untreated CD4+ T cells as a calibrator.

Cytokine determination
IFN-β was measured in serum samples of HDs and patients with untreated RR-MS during relapse or remission using an IFN-β ELISA Kit (Catalogue No. KAC1201; Life Technologies) according to the manufacturer’s instructions. IL-2 was detected in culture supernatants of CD4+ T cells after 72 h of in vitro culture using an IL-2 ELISA set (Human ELISA MAX Deluxe; BioLegend) according to the manufacturer’s instructions.

Phosphorylation of PLC-γ1 by flow cytometry
PLC-γ1 expression and phosphorylation was measured using the Phosほう protocol (BD Biosciences) and the following Abs: anti-CDC4 (PacBlue, clone MT310; Dako/BIOZOL), anti-PLC-γ1 (PE, clone 10/PLC-γ1; BD Biosciences) and anti-PLC-γ1 pY783 (Alexa Fluor 647, clone 27/PLC; BD Biosciences). All analyses were performed on an LSRII (BD Biosciences) flow cytometer. Data analysis was performed with BD FACS Diva for analyzing MedFI and PLC-γ1 pY783 expression was normalized for PLC-γ1 expression [MedFI PLC-γ1 pY783/(MedFI PLC-γ1/medFI PLC-γ1 medium control)].

Proliferation
One hundred twenty-five thousand isolated human CD4+ T cells per well were resuspended in X-VIVO, seeded in 96-well plates, and stimulated for 72 h. After 72 h of in vitro stimulation of CD4+ T cells, cells were pulsed overnight with 1 μCi [methyl-3H]thymidine (Hartmann Analytica, Braunschweig, Germany) per well. Cells were harvested after 15 h of incubation, and incorporation of radioactivity was measured in a scintillation counter (Wallac 1450; PerkinElmer, Rodgau-Jürgensheim, Germany).

Statistical analysis
Statistical analyses were performed using Graph Pad Prism 5.0 Software (GraphPad Software, La Jolla, CA), conducting one-way ANOVA followed by Bonferroni multiple comparison test. Allele frequency was calculated using the Hardy-Weinberg equation and tested with the Pearson χ² test.

Results
When investigating its role in MS we observed lower CBL-B expression ex vivo in CD4+ T cells during relapses of RR-MS patients (Fig. 1A) but not in other lymphocyte subsets (Supplemental Fig. 1A–D). Further analysis of the CD4+ T cell subsets revealed that the decrease in CBL-B expression was primarily seen in naive CD4+ T cells (Supplemental Fig. 1E) and in CD4+ CD25+ T regulatory cells (Supplemental Fig. 1F).

Next, we examined whether the MS risk-associated CBLB SNPs are involved in the immune alterations in MS. To date, three SNPs have been associated with MS risk: rs12487066 was identified in the first GWAS study for MS (8) and was reproduced in a family case-control study (23), rs9657904 was identified in the Sardinian population (9), and rs2028597 was identified in the second major GWAS study for MS (3). Using data from the 1000 Genomes Project (19), we examined the linkage disequilibrium (LD) of these three reported SNPs. There is no indication for linkage disequilibrium between rs12487066 and rs9657904/rs2028597, supporting the idea that rs12487066 is independent of these two alleles (Fig. 2A). Because at the time of our study the rs12487066 was the only reproduced MS-associated SNP and because the populations in which the rs12487066 was identified were more similar to our Northern German cohort, we focused on the rs12487066 that seems to confer MS risk independently of the other two SNPs.

Because we observed a decrease in CBL-B expression in CD4+ T cells during relapse, probably caused by the active phase of the disease, we only considered patients during remission for the analysis of CBL-B expression concerning the corresponding genotype. MS patients carrying the risk allele (T) of the first described CBLB-attributed SNP rs12487066 showed significantly lower CBL-B expression in CD4+ T cells than patients lacking this allele (Fig. 1B). There was no difference in CBL-B expression between heterozygous and homozygous allelic state in MS patients, indicating a dominant effect of the T allele and a lack of a gene dosing effect. Surprisingly, when we applied the genotyping to healthy rs12487066 allele carriers, we were not able to reproduce the difference in CBL-B expression between T and non-T allele carriers (Fig. 1C). Consequently, we hypothesized that the rs12487066-associated differences in CBL-B expression are likely to be related to the disease state—that is, MS, and that some as yet unknown factors are responsible. To identify potential mediators, we examined the genomic location of the CBLB risk SNP rs12487066 in more detail. As shown in Fig. 2A, rs12487066 is located ~320 kb upstream of the CBLB coding region and surrounded by regulatory and epigenetic modification sites (methylation and acetylation) specifically reported for CD4+ T cells according to the Ensembl database.

Using an unbiased bioinformatics analysis that applied the weight matrix–based tool Match to predict transcription factor binding to the risk versus non-risk allele SNP sequence (20) gave

FIGURE 1. CBL-B expression is diminished in CD4+ T cells of RR-MS patients during relapse and in CD4+ cells of RR-MS patients carrying the rs12487066 risk allele (T). (A) CBL-B expression is significantly diminished in MS patients during relapse (RR-MS/R*) as measured by flow cytometry in comparison with HDs and MS patients during remission (RR-MS, n = 15). HD n = 15; RR-MS n = 15; RR-MS/R* n = 10. One-way ANOVA, p = 0.0122. (B) CBLB rs12487066 risk allele carriers (T) show a reduced CBL-B expression in CD4+ T cells in comparison with homozygote non-risk allele carriers (CC) in RR-MS patients. CC n = 8; CT n = 14; TT n = 15. One-way ANOVA, p = 0.02. (C) CBL-B expression is not altered in CD4+ T cells from HDs of CBLB rs12487066 risk allele carriers. CC n = 7; CT n = 14; TT n = 13. One-way ANOVA, p = 0.9721. All values are presented as MedFI normalized to the isotype control (MedFI/Isotype control × 100). n.s., not significant.
as the top-scoring candidate transcription factors C/EBPβ (C/EBPβ) and Growth factor independent protein-1 (Gfi-1; data not shown). Interestingly, binding was predicted only for the C/EBPβ binding site if it contained the risk (T) allele of rs12487066 (Fig. 2B), whereas the prediction for rs9657904 and rs2028597 showed no differential binding for C/EBPβ, C/EBPβ and Gfi-1.
were subsequently tested for differential binding to risk and non-risk allele sequences of the rs12487066 in EMSAs and, consistent with the aforementioned prediction, C/EBPβ shifts oligonucleotides containing the risk allele (T) significantly better than the non-risk oligonucleotide (Fig. 2C). Gfi-1 showed no binding to either oligonucleotide.

C/EBPβ is involved in inflammation, immune regulation, and T cell biology (24). In particular, C/EBPβ has been shown to be upregulated significantly in MS tissue samples in a study examining gene expression in MS brain tissue (25). Furthermore, it has been associated with viral infections, among which is EBV (26), the strongest environmental risk factor for MS (1). IFN-β is produced by a broad range of cells in response to viral infection; it limits T cell proliferation and cytokine production, is used as a first-line drug to treat MS, and is known to induce C/EBPβ (27). We therefore considered IFN-β and treatment with this cytokine as a meaningful model for the inflammatory host response causing C/EBPβ induction in vitro. We were able to confirm and expand previous observations by showing increased expression of the full-length C/EBPβ isoform LAP (Fig. 2F) upon stimulation of naive and activated CD4+ cells with IFN-β. We then examined binding of endogenously expressed C/EBPβ to the MS risk-associated CBLB allele rs12487066 in CD4+ T cells of homozygote risk versus non-risk allele carriers with and without exposure to IFN-β by chromatin immunoprecipitation (ChiP). C/EBPβ binds to the rs12487066 risk allele sequence in human CD4+ T cells only upon exposure to IFN-β, whereas binding could not be detected in homozygous non-risk allele carriers under this condition and could not be quantified accordingly (Fig. 2D, 2E). When analyzing RNA of PBMCs from patients with untreated RR-MS, we found that C/EBPβ RNA expression was significantly upregulated already during remission and clearly elevated during relapse (Fig. 2G). Consistent with this observation and previously published experiments, serum levels of IFN-β are significantly elevated in patients with untreated relapsing RR-MS (Fig. 2H) (28, 29). We then hypothesized that C/EBPβ decreases CBL-B expression in risk allele carriers and thus lowers the activation threshold or activation requirements, or both, of CD4+ T cells in the presence of IFN-β. Consequently, CBLB risk allele carriers might less stringently control T cell activation, cytokine production, and proliferation in the presence of IFN-β.

Genotyping of 60 healthy donors for the MS risk alleles rs12487066 (T, non-risk allele C) and rs9657904 was performed to identify donors for functional CD4+ T cell studies. We identified four individuals per group for the rs12487066 SNP (Supplemental Table I) who were matched by gender and age. CBL-B expression in CD3/CD28-stimulated purified CD4+ T cells did not differ significantly in healthy SNP rs12487066 risk (TT) versus non-risk (CC) allele carriers (Supplemental Fig. 2C). C/EBPβ is a key transcription factor for CD4+CD25+ T regulatory cells (32, 33), the key transcription factor for CD4+CD25+ T regulatory cells. No upregulation of CBL-B is seen in risk allele carriers after exposure of activated CD4+ T cells to IFN-β (Fig. 3A), whereas non-risk allele carriers express significantly higher levels of CBL-B in the presence of IFN-β. To ensure that this effect is dependent on C/EBPβ, we used siRNA to diminish the expression level of C/EBPβ in primary human CD4+ cells (Supplemental Fig. 2A, 2B). siRNA for C/EBPβ abrogated the IFN-β–mediated upregulation of CBLB in non-risk allele carriers (Fig. 3A, right panel). Therefore, C/EBPβ expression appears to depend on C/EBPβ in non-risk allele carriers. IFN-β inhibits the proliferation of T cells in vitro through multiple pathways and mechanisms (30). After observing that CBLB risk allele carriers fail to upregulate CBL-B upon exposure to IFN-β, and since CBL-B deficiency causes increased phosphorylation of PLC-γ1, elevated production of IL-2 as well as hyperproliferation (11), we studied the influence of IFN-β on these functional aspects in CD4+ T cells in risk versus non-risk allele carriers. In the presence of IFN-β, CD4+ T cells of non-risk allele carriers showed less phosphorylation of PLC-γ1 and diminished secretion of IL-2 cytokine (Fig. 3B, 3C). The differences between the carriers of the non-risk versus risk allele in all parameters could be abrogated by the use of siRNA against C/EBPβ, whereas the nontargeting siRNA had no effect. Consistent with this result, proliferation was inhibited by IFN-β only in CBLB non-risk allele carriers (Fig. 3D), indicating that inhibition of T cell proliferation by IFN-β is compromised in CBLB risk allele carriers.

Consequently, we tested whether the rs12487066 is associated with treatment responsiveness to IFN-β in RR-MS patients and therefore might be clinically relevant. We genotyped 125 patients with RR-MS who received IFN-β for at least 2 y with regular follow-up at our outpatient unit. We found that the rs12487066 non-risk allele (C) was significantly more prominent in responders to IFN-β treatment (Supplemental Fig. 3A) and that homozygote non-risk allele carriers (CC) showed a lower degree of disability as measured by the EDSS (Supplemental Fig. 3B).

Discussion

Our study indicates an important role of the CBLB SNP rs12487066 risk allele in lowering the activation threshold of CD4+ T cells and provides an interesting link to a genetic variant of a gene that is involved in immune regulation, which might be modulated by environmental factors such as viral infections. During the course of RR-MS, CBLB expression is diminished only during relapses in CD4+ T cells and, while more pronounced in naïve than in effector CD4+ T cells, it is also observed in CD4+CD25+ regulatory T cells. Previously, diminished CBL-B mRNA and protein expression in whole PBMCs of patients with RR-MS have both been described (16). In experimental models, the absence of CBLB or a functional deficiency of its E3-ligase activity triggers diabetes in rats (13, 14) or mice carrying a TCR transgene specific for a foreign Ag expressed in the pancreas (14). Because autoreactive CD4+ T cells are critical for the development of MS, it is notable that we found a reduction of CBL-B during MS relapses in CD4+ T cells. A comparably reduced CBL-B expression in CD3+ T cells has been observed in patients with systemic lupus erythematosus (31). CBL-B–deficient animals show increased susceptibility to peptide-induced autoimmune thyroiditis (15) that is caused by removing the requirement for CD28-mediated costimulation and results in disproportionate T cell activation even to weak stimuli. Our data indicate reduced dependence of CD4+ T cells on CD28-mediated costimulation and survival in disproportionate T cell activation even to weak stimuli. Our data indicate reduced dependence of CD4+ T cells on CD28-mediated costimulation and survival in disproportionate T cell activation even to weak stimuli. Our data indicate reduced dependence of CD4+ T cells on CD28-mediated costimulation and survival in disproportionate T cell activation even to weak stimuli. Our data indicate reduced dependence of CD4+ T cells on CD28-mediated costimulation and survival in disproportionate T cell activation even to weak stimuli.
Figure 3. CBLB rs12487066 risk allele carriers differ in their phenotype of CBLB and CBLB-dependent T cell functions in the presence of IFN-β. (A) CBLB protein expression in FACS analysis is significantly enhanced in the presence of IFN-β in CD4+ T cells of CBLBrs12487066 non-risk allele carriers (CC), whereas risk allele carriers (TT) fail to upregulate CBLB. The upregulation of CBLB can be abrogated by the use of siRNA for C/EBPβ (right). One-way ANOVA, p = 0.0004. (B) Phosphorylation of PLC-γ1 in flow cytometric analysis is significantly decreased in the presence of IFN-β in CD4+ T cells of CBLB rs12487066 non-risk allele carriers (CC) in contrast to the risk allele carriers (TT). The effect of IFN-β can be abrogated by the use of siRNA for C/EBPβ (right). One-way ANOVA, p = 0.0029. (C) Production of IL-2 is significantly decreased in the presence of IFN-β in CD4+ T cells of CBLB rs12487066 non-risk allele carriers (CC), whereas IFN-β fails to decrease IL-2 production in risk allele carriers (TT). The effect of IFN-β can be abrogated by the use of siRNA for C/EBPβ (right). One-way ANOVA, p = 0.0078. (D) Proliferation of CD4+ T cells (3H-thymidine incorporation) of CBLB rs12487066 non-risk allele carriers (CC) is significantly diminished in the presence of IFN-β compared with risk allele carriers (TT). One-way ANOVA, p < 0.0001. Risk allele carriers (TT, n = 4) for the rs12487066 are indicated in black. Non-risk allele carriers (CC, n = 4) for the rs12487066 are indicated in white. Data are presented as δ between CD4+ T cells stimulated with anti-CD3, anti-CD2, and anti-CD28-coated beads without IFN-β versus CD4+ T cells stimulated with anti-CD3, anti-CD2, and anti-CD28-coated beads with IFN-β from four independent experiments for the untreated and for three independent experiments for the siRNA with a nonsense/scrambled sequence (ssRNAi) and siRNA C/EBPβ conditions. Response to stimulation with anti-CD3, anti-CD2, and anti-CD28-coated beads between the different allele carriers showed equal results in mean, SEM, and variances (Supplemental Fig. 2C–F). Statistical methods used one-way ANOVA followed by Bonferroni multiple comparison test. *p < 0.05, **p < 0.01, n.s., not significant.
T cells of CBLB risk allele carriers might express lower levels of CBL-B during viral infections because of a type I IFN-induced repression by C/EBPβ. As a result, T cells of CBLB risk allele carriers probably have a lower T cell activation threshold and can be activated more easily, even by low affinity interactions with self-antigens (e.g., myelin peptides in MS) (40). Interestingly, it is well known from epidemiologic studies that viral infections frequently precede MS relapses (41) and that at least half of the patients with RR-MS show an increase in the expression of type I IFN-related genes (42), corresponding to our data showing elevated IFN-γ serum levels during relapse and our observed association with a more frequent allele presence of the risk allele in non-responders to IFN-β therapy. Risk allele carriers might be more prone to T cell activation by low-affinity interactions because of their lower CBL-B expression levels in CD4+ T cells during viral infections that induce a type I IFN host response and consequently RR-MS patients carrying the risk allele seem to respond less to IFN-β therapy, which should be validated in a larger cohort of patients with IFN-β–treated RR-MS. Accordingly, we propose a model in which C/EBPβ binds selectively to the rs12487066 MS risk allele upstream of CBLB and thereby alters the transcription and induction of CBL-B by C/EBPβ in the promoter region (Fig. 4). We were not able to show a preferential induction of the repressive LIP isoform of C/EBPβ that has been discussed previously as a possible mechanism for C/EBPβ induced repression (43). The C/EBPβ long isoforms LAPα and LAP, which contain transactivation domains, can also act as context-dependent repressors (e.g., by recruitment of chromatin remodeling complexes) (44) of repressor complexes, such as N-CoR (45) or SMRT (46), of histone-modifying enzymes like lysine or arginine methyltransferases (21, 47) or by alteration of homodimerization or heterodimerization states of C/EBP complexes (48). It remains to be determined whether one of the above or yet another mechanism is involved in the altered transcription of CBLB in the context of the rs12487066 MS risk allele. In the context of the treatment of RR-MS with IFN-β, it is interesting that only approximately half respond to IFN-β, while ~50% are incomplete responders or nonresponders (18). A study that investigated SNPs that might contribute to IFN-β treatment insufficiency or failure did not include the CBLB SNP rs12487066 (49), for which we have seen differences in the response to IFN-β. This study revealed SNPs in genes of the IL-6 signaling pathway, which is consistent with its deregulation in the monocytes of RR-MS patients who respond weakly to IFN-β (28). A major transcription factor in the IL-6 signaling pathway is C/EBPβ (50), for which we found differential binding to the CBLB MS-associated rs12487066 risk allele. Supporting our hypothesis, a recent report shows that CBLB RNA expression is significantly lowered in regulatory T cells of RR-MS patients and that IFN-β treatment was not able to reconstitute CBLB RNA expression in this patient cohort, for whom no genotyping information is available (51). Therefore, our experiments indicate that genetic variants of CBLB are relevant in controlling T cell–mediated immune mechanisms.

Our findings also indicate that genetic risk traits can only unmask their functional role in a specific context, such as an infection and subsequent IFN production, and then maintain their effects during the disease. This finding could explain why we were not able to detect the phenotype of the rs12487066 in CD4+ T cells of healthy

![FIGURE 4. Proposed model for the influence of the CBLB variant rs12487066 on CBLB gene expression and T cell function. Upper and lower left panel, During T cell stimulation, C/EBPβ induces CBL-B independent of the CBLB allele variant for the rs12487066. T cells show enhanced PLC-γ1 phosphorylation, IL-2 production, and enhanced proliferation. Upper right panel, During T cell stimulation in the presence of a type I IFN (i.e., IFN-β), C/EBPβ is increasingly induced in the non-risk allele (C) carrier, CBL-B expression is enhanced, and consecutively T cell activation (as determined by PLC-γ1 phosphorylation, IL-2 production, and proliferation) is inhibited. Lower right panel, During T cell stimulation in the presence of a type I IFN (i.e., IFN-β), C/EBPβ is still increasingly induced in the risk allele (T) carrier, but the binding to the allelic variant inhibits CBL-B expression by a still unknown mechanism. As a result, T cell activation (PLC-γ1 phosphorylation, IL-2 production, and CD4+ T cell proliferation) is not inhibited.](http://www.jimmunol.org/)

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individuals, they lack the proinflammatory mediators that induce C/EBPB8 and thereby mediate the upregulation of CBL-B in CD4\(^+\) T cells of CC allele carriers, but only in an inflammatory setting like in CD4\(^+\) T cells of patients with RR-MS. To our knowledge, this is the first report of a differential transcriptional regulation by type I IFNs in association with an MS risk-related genetic variant. Future studies should examine whether others of the recently reported MS risk alleles act in concert with the CBLB risk SNPs and whether such gene-environment interactions also exist for other MS risk genes.

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Disclosures

The authors have no financial conflicts of interest.

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A Multiple Sclerosis-Associated Variant of CBLB Links Genetic Risk with Type I IFN Function

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Supplementary Figure 1: CBL-B Expression in different lymphocyte subsets of relapsing-remitting multiple sclerosis patients in remission (RR-MS, n = 10) or during relapse (RR-MS/R*, n = 10) in comparison to healthy donors (HD, n = 15). n.s. - not significant.

All values are presented as Median Fluorescence Intensity (MedFI) normalized to the Isotype control (MedFI/Isotype Control x 100).

(A) CBL-B expression is not altered in CD8+ T cells of RR-MS patients.
(B) CBL-B expression is not altered in CD19+ B cells of RR-MS patients.
(C) CBL-B expression is not altered in Natural Killer Cells of RR-MS patients.
(D) CBL-B expression is not altered in CD14+ monocytes of RR-MS patients.
(E) CBL-B expression is significantly diminished in naive but only by trend in effector CD4+ T cells of RR-MS patients.
(F) CBL-B expression is significantly diminished during relapse in regulatory CD4+ CD25high CD127low T cells of RR-MS patients.

Control staining with Foxp3 showed that the CD4+ T cell population identified by this surface staining was >93% Foxp3 positive.

(G) CBL-B expression decreases stepwise in the development of CD4+ naive towards CD4+ effector cells; exemplary data are shown from n = 5 healthy donors (Repeated measures one-way Anova, p < 0.0001, n = 5).

(F) CBL-B expression in CD4+ T cells is not associated with age (n = 50).
**Supplementary Figure 2**

(A, B) C/EBPβ induction by IFN-β is inhibited equally and effectively in non-risk and risk allele carriers of the CBLB rs 12487066 variant by the C/EBPβ siRNA (real-time quantitative PCR). All values are presented as Fold Induction.

(C) CBL-B expression in CD4+ T cells is equal in CC and TT allele carriers of the rs12487066 after stimulation with anti-CD2/anti-CD3 and anti-CD28. One-way Anova, p = 0.3863.

(D) Phosphorylation of PLC-γ1 in CD4+ T cells is equal in CC and TT allele carriers of the rs12487066 after stimulation with anti-CD2/anti-CD3 and anti-CD28. One-way Anova, p = 0.087.

(E) IL-2 production of CD4+ T cells is equal in CC and TT allele carriers of the rs12487066 after stimulation with anti-CD2/anti-CD3 and anti-CD28. One-way Anova, p = 0.9989.

(F) Proliferation of CD4+ T cells is equal in CC and TT allele carriers of the rs12487066 after stimulation with anti-CD2/anti-CD3 and anti-CD28. One-way Anova, p = 0.7376.

Non-risk allele carriers (CC, n = 4) for the rs12487066 are indicated in white, risk allele carriers (TT, n = 4) for the rs12487066 are indicated in black. Data are presented from four independent experiments for the untreated and for three independent experiments for the ssRNAi and siRNA C/EBPβ conditions. Statistics were carried out using one-way Anova followed by Bonferroni’s multiple comparison test. n.s. - not significant.
Supplementary Figure 3

Supplementary Figure 3: CBLB Genotype in a cohort of IFN-β-treated RR-MS patients (n = 125).

(A) Percentages of non-responders, intermediate responders and responders for each genotype of the rs12487066. Data are presented as percent of non-responders (NR), intermediate responders (IR) or responders (R) to IFN-β-treatment.

(B) Allele frequency of the CBLB risk allele rs12487066 is higher in non-responders to IFN-β-treatment in RR-MS patients.
Allele frequency of the T allele in non-responders is 0.7339 and 0.5778 in responders, respectively.
Allele frequency of the C allele in non-responders is 0.2661 and 0.4222 in responders, respectively. (Chi-Square p < 0.05).
Intermediate Responders were excluded from analysis due to the low patient number.

(C) The non-risk CBLB genotype CC is associated with lower degree of disability in a cohort of IFN-β-treated RR-MS patients (p < 0.05).
Supplementary Table 1

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age (years)</th>
<th>Gender</th>
<th>CBLB rs12487066</th>
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<td>CT</td>
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<tr>
<td>D8</td>
<td>25</td>
<td>male</td>
<td>TT (risk)</td>
<td>CT</td>
</tr>
</tbody>
</table>

**Supplementary Table 1**: Data of healthy donors studied in the functional experiments; donors were paired for all experiments as following:
- D1 and D5
- D2 and D6
- D3 and D7
- D4 and D8