Identification of *Orch3*, a Locus Controlling Dominant Resistance to Autoimmune Orchitis, as Kinesin Family Member 1C

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Abstract

Experimental autoimmune orchitis (EAO), the principal model of non-infectious testicular inflammatory disease, can be induced in susceptible mouse strains by immunization with autologous testicular homogenate and appropriate adjuvants. As previously established, the genome of DBA/2J mice encodes genes that are capable of conferring dominant resistance to EAO, while the genome of BALB/cByJ mice does not and are therefore susceptible to EAO. In a genome scan, we previously identified *Orch3* as the major quantitative trait locus controlling dominant resistance to EAO and mapped it to chromosome 11. Here, by utilizing a forward genetic approach, we identified kinesin family member 1C (*Kif1c*) as a positional candidate for *Orch3* and, using a transgenic approach, demonstrated that *Kif1c* is *Orch3*. Mechanistically, we showed that the resistant *Kif1c*D2 allele leads to a reduced antigen-specific T cell proliferative response as a consequence of decreased MHC class II expression by antigen presenting cells, and that the L578→P578 and S1027→P1027 polymorphisms distinguishing the BALB/cByJ and DBA/2J alleles, respectively, can play a role in transcriptional regulation. These findings may provide mechanistic insight into how polymorphism in other kinesins such as *KIF21B* and *KIF5A* influence susceptibility and resistance to human autoimmune diseases.

Introduction

Experimental autoimmune orchitis (EAO) is a model of idiopathic male infertility mediated by autoreactive T cells [1,2]. It can be induced in mice by active immunization with mouse testicular homogenate (TH) emulsified in complete Freund's adjuvant (CFA) and *Bordetella pertussis* toxin (PTX) [3]. In genetically susceptible mice, the inflammatory lesions comprised of monocytes, macrophages, lymphocytes, neutrophils, and eosinophils are mainly found in the seminiferous tubules of the testes in association with aspermatogenesis [3]. We previously have shown that MHC class II restricted CD4+ T cells are the primary effectors in autoimmune orchitis [4,5]. However, recent evidence suggests the involvement of CD8+ T cells during the onset and maintenance of chronic inflammation [6,7].

Various strains of inbred mice respond differently to EAO induction, indicating that susceptibility is genetically controlled. Previously, it was shown that BALB/cByJ (CByJ) mice are highly susceptible to EAO [8] whereas DBA/2J (D2) and (CByJ×D2F1) hybrid (CD2F1) are resistant [3,9]. This demonstrates that resistance to EAO is inherited as a dominant phenotype in this strain combination. Additionally, resistance can be adoptively transferred to CByJ mice with CD2F1 primed splenocytes [10]. Therefore, the factors that regulate EAO resistance appear to be governed by an immune-mediated dominant negative mechanism.

Genome exclusion mapping was utilized to map the immune-suppressive genes regulating dominant resistance to EAO [10] with significant linkages to multiple loci residing on chromosomes (Chr) 1 and 11 [10]. Of these, *Orch3* on Chr11 displayed the most significant linkage and accounted for the majority of disease resistance seen in D2 mice.

In this study, congenic mapping was employed to restrict *Orch3* to a ~1.3 Mb interval that identified *Kif1c* (kinesin family member 1c) as a positional candidate. By generating CByJ.CD11B-Kif1cD2 transgenic (Tg) mice, we demonstrated that *Kif1c* underlies *Orch3*. Mechanistically, we showed that the resistant *Kif1c*D2 allele leads to reduced antigen (Ag)-specific T cell responsiveness as a consequence of decreased MHC class II expression by myeloid cells, and that the L578→P578 and S1027→P1027 polymorphisms distinguishing the CByJ and D2 alleles, respectively, can play a role in regulating gene transcription.
Author Summary

Although the etiology of autoimmunity is not well known, a variety of studies have demonstrated that genetic predisposition is a major contributor to disease susceptibility and resistance. The major histocompatibility complex (MHC) is the primary genetic determinant of autoimmune disease susceptibility with multiple additional interacting loci required. However, the identification and characterization of non–MHC genes has been problematic, since most autoimmune diseases are polygenic with the individual genes exhibiting only partial or minimal penetrance. We previously identified Orch3 (mouse chromosome 11) as the most important immune-suppressive locus controlling dominant resistance to autoimmune orchitis, the principal animal model of non-infectious testicular inflammatory/autoimmune disease. Here, using congenic mapping, we identified kinesin family member 1C (Kif1c) as a positional candidate for Orch3. Furthermore, over-expression of the Kif1c resistant allele in susceptible mice rendered animals autoimmune orchitis resistant, demonstrating that Kif1c is Orch3. We propose that Kif1c plays an immunoregulatory role by controlling the levels of MHC class II in antigen presenting cells and consequently impacting autoreactive orchitogenic T cell responses. These findings are particularly relevant since polymorphism in other kinesins such as Kif21B and Kif5A have been associated with susceptibility and resistance to human autoimmune disease.

Results

Congenic mapping of Orch3

In the genome scan in which Orch3 was identified, D11Mit219, D11Mit18, and D11Mit118 exhibited the most significant linkage [10]. As the first step in the positional-candidate gene cloning of Orch3, we used marker-assisted selection to introgress the orch3 allele onto the susceptible CByJ background. Next, we generated overlapping interval specific recombinant congenic (ISRC) lines (Figure S1 and Figure S2) and studied them in a stepwise fashion for susceptibility and resistance to EAO (Figure 1). Importantly, since resistance to EAO is inherited as a dominant trait in CD2F1 hybrid mice [10], and the pathology indices (PI) between homozygous and heterozygous congenic lines were not significantly different (data not shown), the data were pooled for each line. Control parental CByJ mice were clearly susceptible to EAO, with an average PI of 4.0, whereas D2 and CD2F1 hybrid mice were resistant, with an average PI of 0.1 and 0.8, respectively (Figure 2). C.D2-Ev3/Hba, C.D2-3.1, C.D2-5, C.D2-8.4, C.D2-8.5, and C.D2-9 mice were also susceptible with average scores of 3.6, 4.9, 2.6, 3.7, 3.8, and 4.0, respectively. In contrast, C.D2-Ev2, C.D2-3, C.D2-3.2, C.D2-8 and C.D2-8.1 thru -8.3 were resistant with average scores of 0.2, 0.6, 1.3, 1.7, and ≤1.4, respectively.

These data placed Orch3 within the interval between D11Mit298 (69339966–69340164) and NLR (nucleotide-binding domain and leucine rich repeat containing) family, pyrin domain containing 1A, B, C (Nlpr1a/b/c) at 70.9–71.0 Mb (70904699–71098734 bp). Importantly, this excluded transient receptor potential cation channel, subfamily V, member 1 (Trpv1) at 73.0 Mb (73047794–73074744) underlying Idh1, a quantitative trait loci (QTL) controlling susceptibility to type 1 diabetes in the NOD mouse [11], and inducible nitric oxide synthase (Nos2/iNos), important in inflammatory diseases including autoimmunity [12,13], as candidate genes for Orch3. Nlpr1a/b/c is one of two highly polymorphic positional candidate loci of immunological relevance within the interval, the second gene being kinesin family member 1C (Kif1c). However, Nlpr1a could be excluded as a candidate since it is a pseudogene (www.informatics.jax.org) and Nlpr1a and -b are less likely to be relevant to Orch3 than Kif1c due to discordance between EAO susceptibility and Nlpr1a and -b alleles among CByJ, BALB/cJ and D2 mice [www.informatics.jax.org] [14].

CBByJ.CD11B-Kif1cD2 Tg (Tg-Kif1cD2) mice are resistant to EAO

To confirm that Kif1c was the most likely candidate gene for Orch3 and to definitively exclude Nlpr1a/b as a positional candidate, we generated overlapping sub-ISRC congenic lines across the C.D2-3.2 interval and studied them for susceptibility to EAO (Figure 3). Statistically significant differences in EAO susceptibility between C.D2-3.2, C.D2-3.2c and CByJ mice were observed (Figure 3, right panel). In contrast, the severity of EAO in C.D2-3.2a and C.D2-3.2b was not significantly different from that of CByJ mice. Moreover, dominant resistance co-segregated with Orch3 as evidenced by the fact that no significant difference in the PI between homozygous and heterozygous mice was detected across all congenic lines studied (Figure 2 and Figure 3). Taken...
Figure 2. Congenic mapping places Orch3 within the Kif1c/Nlrp1a/b/c interval. For convenience, D2 alleles have been shaded. The significance of differences in severity of EAO among CByJ, CD2F1 hybrids and CD2-ISRC lines was determined using the Kruskal-Wallis test (overall p-value < 0.0001) followed by Dunn's multiple comparison test.

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Figure 3. Identification of Orch3 as Kif1c. (CD2-3.2 × CByJ) × CByJ backcross mice were screened from recombinants using microsatellite markers spanning the Orch3 interval. Three sub-ISRC lines were identified, fixed and homozygous progeny studied for susceptibility to EAO (D = D2 allele; C = CByJ allele). The significance of differences in EAO among CByJ, CD2-3.2a, CD2-3.2b, CD2-3.2c and Tg-Kif1cD2 transgenic mice was determined using the Kruskal-Wallis test (overall p-value < 0.0001) followed by Dunn's multiple comparison test (**p < 0.01). Region outlined in red reflects location of Orch3 based on high resolution congenic mapping relative to the lower resolution mapping outlined in blue.

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together, these data restrict *Onk3* to a ~1.3 Mb interval distal of *D11Mit298* (69339966–69340164) and proximal of *D11Mit30* (70552627–70552762) which includes *Kif1c* but not *Nlpl* and *Nhph1b* (Figure 3, left panel), thereby excluding them as positional candidates for *Onk3*.

Given the role of *Kif1c* in macrophage function [15], and that kinesins have been implicated in antigen processing and presentation [16], we decided to directly test the hypothesis that *Onk3* is *Kif1c*. We generated a transgenic mouse line that selectively expressed the resistant *Kif1c* allele on the susceptible CByJ background using the human *CD11B/ITGAM* regulatory elements for macrophage/myeloid-specific expression of *Kif1c* (Figure 4A). The expression of the transgene did not affect macrophage/myeloid cell generation or homeostasis as similar percentages of splenic F4/80+ (Figure 4B) and CD11b+ (Figure 4C) cells were detected on Tg- *Kif1c* mice compared to negative littermate control (NLC) mice. In addition, no differences in the expression of CD40 or CD86 were observed between strains at baseline (data not shown). Compared to NLC, greater Kif1c protein expression was seen in thioglycollate-induced Tg- *Kif1c* cells (Figure 4D). Despite the existence of polymorphisms upstream of *Kif1c* in potential regulatory regions (http://phenome.jax.org/), we did not observe differences in *Kif1c* expression at the mRNA level between the *Kif1c* and *Kif1c* alleles (Figure 4E). NLC and Tg- *Kif1c* mice were studied for susceptibility to EAO. The expression of *Kif1c* in CD11b+ cells protected susceptible CByJ mice from EAO (Figure 3, right panel). This finding establishes *Kif1c* as being *Onk3*.

Tg expression of *Kif1c* downregulates MHC class II expression and antigen presenting function of CD11b+ cells

To better understand the mechanism of resistance to EAO conferred by *Kif1c*, microarray analyses were performed on CD11b+ cells from NLC and Tg- *Kif1c* mice. Using a false discovery rate (FDR) cutoff of 0.05, we determined that 164 genes were differentially expressed between NLC and Tg- *Kif1c* CD11b+ cells (Table S1). An analysis for functional inference using Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com) revealed that T helper cell differentiation was the most significant pathway influenced by *Kif1c* (p<2.80 E-10; Figure S3 and Table S2). In addition, 18 of the top 20 pathways implicated a role for MHC class II, including antigen presentation. Indeed, compared to NLC CD11b+ cells, we observed a marked down regulation in MHC class II gene expression by Tg- *Kif1c* CD11b+ cells (Table S1 and Figure 5A, dark blue dots). This is consistent with the role of kinesin as the motor that drives MHC class II to the plus end of microtubules toward the cell surface [16].

To corroborate diminished class II expression, flow cytometric analysis was performed using naive TCRβ+CD19+CD11b+ splenocytes. The results presented in Figure 5B show lower MHC class II expression on Tg- *Kif1c* cells compared to NLC, D2, and C.D2-3.2 mice. Despite the differences in MHC II expression, no significant difference in the proportion of total splenic CD11b+ cells was observed (Figure 4C). Therefore, expression of the transgene in CD11b+ cells negatively regulates MHC II protein levels.

![Figure 4. Generation of BALB/cByJ-CD11B-Kif1c transgenic (Tg-Kif1c) mice.](image-url)

**Figure 4. Generation of BALB/cByJ-CD11B-Kif1c transgenic (Tg-Kif1c) mice.** (A) Schematic representation of the *Kif1c* gene used to generate the transgenic mice showing the promoter (CD11B/ITGAM), and the *Kif1c* gene, followed by the hGH/polyA signal sequence. Arrows indicate PCR-primers for screening. (B and C) Percentage of splenic F4/80+ (B) and CD11b+ (C) cells of Tg- *Kif1c* and NLC. The analysis was performed on gated live cells according to their FSC vs. SSC profile. Statistical significance was determined using the Mann-Whitney *U* test. Data represent the mean ± SEM of at least 5 individual mice. (D) Kif1c expression in thioglycollate-induced adherent cells by Western blotting using whole-cell extracts and the anti-Kif1c mAb. Actin was used as a loading marker. (E) mRNA expression of *Kif1c* was measured from sorted TCRβ+ CD19+ CD11b+ myeloid cells of CByJ mice and compared with TCRβ+ CD19+ CD11b+ myeloid cells of D2 mice. [2-microglobulin and GAPDH were used as an endogenous control. Data represent the mean ± SEM of two experiments (pool of 5 animals/each).

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To further establish a functional role for the differential expression of MHC class II, we assessed antigen presentation by examining Ag-specific T cell proliferation. NLC and Tg-Kif1cD2 mice were immunized with ovalbumin (OVA)+CFA or proteolipid protein (PLP) 190–199 peptide (PLP190-199)+CFA on d0 and d7. Spleen and lymph nodes (LN) were harvested at d10 and the proliferative responses evaluated. Compared to NLC antigen presenting cells (APCs), T cell proliferation in response to OVA was significantly reduced when T cells were stimulated in the presence of Tg-Kif1cD2 APCs (Figure 6A). Similar results were observed for PLP190-199-dependent T cell responses (Figure 6B). These data show that expressing the Kif1cD2 allele in CD11b+ cells confers resistance to EAO by modulating APC function. Taken together our data suggest that kif1c coding region polymorphism controls susceptibility to autoimmune orchitis.

Figure 5. Analysis of MHC II expression on splenic CD11b+ myeloid cells. (A) Scatterplot of genes differentially expressed in splenic TCRβ+ CD19+ CD11b+ myeloid cells of Tg-Kif1cD2 and NLC mice as determined by microarray. There were 164 genes differentially expressed (FDR<0.05) and for each gene, the log2 fold change was plotted on the ordinate against the -log10 p-value, plotted on the abscissa. Each data point represents the log2 fold change (Tg-Kif1cD2 minus NLC) for each gene. Dark blue data points indicate H2 genes that were downregulated in splenic CD11b+ cells of Tg-Kif1cD2 mice. (B) Flow cytometric analysis of the frequency of TCRβ+ CD19+ CD11b+ myeloid cells expressing MHC II in the spleen of Tg-Kif1cD2, D2, D2-C3.2, and NTC. Statistical significance was determined using the Kruskal-Wallis test (overall ***p-value<0.0001) followed by Dunn’s multiple comparison test (**p<0.01, *p<0.05). Data represent the mean ± SEM of at least 5 individual mice.

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Amino acid polymorphisms at residues 578 and 1027 on the C-terminal end of kif1c regulate its function

Kif1c alleles possess amino acid substitutions at residues 578, 1027, and 1066 [17]. Four haplotypes have been identified: LSS (Kif1cL578S), PSS, PPS, and PPY (Kif1cP1027Y). In addition, it has been shown that the C-terminal region of KIF1c is involved in protein-protein interactions and cargo function [17–20]. Therefore, substitutions at 578, 1027, and/or 1066 may have a significant impact on KIF1c function. Given that KIF17b has been shown to control CREM-dependent transcription by regulating the intracellular location of the transcriptional coactivator ACT (activator of CREM in testis) [21,22], and CREM binding to the Il2 promoter suppresses its activity [23], we evaluated the effect of LSS Kif1cL578S and PPY Kif1cP1027Y alleles on Il2 transcriptional activity as an in vitro assay of KIF1c allelic function. Jurkat cells were co-transfected with a plasmid containing the PPY Kif1cP1027Y allele, the LSS Kif1cL578S allele, or an empty plasmid, and an Il2-promoter luciferase reporter. Cells were then activated with phorbol myristate acetate (PMA) and calcimycin, a calcium ionophore, and the luciferase activity quantified. Jurkat cells that were transfected with the plasmid containing the PPY Kif1cP1027Y allele displayed significantly decreased luciferase activity (mean decrease 31.46±5.59%, P = 0.03) as compared to the plasmid containing the LSS Kif1cL578S allele or the control plasmid (Figure 7A). These data demonstrate the functionality of the KIF1c structural polymorphism.

To further characterize the amino acid(s) responsible for the observed differences on Il2-promoter activity associated with the alleles, we replaced the D2-P1027→L578 (LPY-KIF1c) or D2-P1027→S1027 (PSY-KIF1c). Jurkat cells were co-transfected with the plasmids containing the wild type D2 PPY-KIF1c allele, LPY-KIF1c (P1027→L578) or PSY-KIF1c (P1027→S1027) mutant alleles, or a control plasmid, and Il2-promoter luciferase reporter. Cells were activated with PMA and calcimycin and luciferase activity was assessed. As shown in Figure 7B, LPY-KIF1c and PSY-KIF1c mutants resulted in increased Il2-promoter luciferase activity compared to the D2 PPY-KIF1c allele. Taken together, our data demonstrate that structural polymorphisms at position 578 and 1027 are critical for KIF1c allelic functions.

Discussion

EAO is an organ-specific autoimmune disease that is a model of immunological male infertility [1,2]. We previously demonstrated
that genetic control of EAO is complex and involves both H2-linked (Orch1) and non-H2-linked (Orch3, Orch4, and Orch5) genes [24,25]. The H2-linked immune response genes primarily control susceptibility to EAO, whereas the non-H2-linked genes suppress the phenotypic expression of disease associated with a susceptible Orch1/H2 allele [9]. Here we report the identification of Orch3 as Kif1c that suppresses EAO by decreasing MHC class II expression and resistant to EAE, respectively [28].

With the exception of tyrosine kinase-2 (Tyk2), in which a rare single nucleotide polymorphism in a well conserved APE motif within the pseudokinase domain is fully penetrant in controlling susceptibility to autoimmune diseases [29,30], the vast majority of non-MHC autoimmune loci identified to date are QTL that exhibit only partial to minimal penetrance. This has proven to be problematic as researchers have attempted to positionally clone and characterize such genes [31]. The fact that Orch3/Kif1c controls a dominant negative immunoregulatory mechanism that suppresses autoimmune orchitis with a high degree of penetrance is unique. Because EAO resistance is conferred in a dominant fashion in this model, an animal must be Orch3<sup>2B/J</sup>/Kif1c<sup>2B/J</sup> homozygous to permit disease progression. By using a forward genetic approach, we have now established that Orch3 is Kif1c which, in isolation, controls resistance to EAO with a remarkable degree of penetrance.

Using a transgenic approach we demonstrated that Kif1c is Orch3, and that expression of the resistant Kif1c<sup>2D</sup> allele by CD11b<sup>+</sup> cells of CByJ mice confers complete protection from the development of EAO. Our data are consistent with the growing number of CD11b<sup>+</sup> myeloid cell types with immunosuppressive activity [32,33]. Indeed, resistance to autoimmune type 1 diabetes in NOD mice and EAE correlated with the presence of immunomodulatory CD11b<sup>+</sup> myeloid cells [34–36] and the capacity of these cells to maintain a proper T regulatory cell function [37].

Kinesin family members are involved in the activation of immune cells and inflammatory responses [38,39], and autoimmune disease GWAS identified KIF21B and KIF5A as candidates for autoimmune disease genes [40,41], suggesting an immunoregulatory role for kinesin family members. In addition, kinesin proteins have been identified as the major molecular motor of microtubule-based intracellular transport [42]. Kif1c is expressed in a variety of tissues [43] and overexpression of a dominant negative form disrupts molecular motor-dependent Golgi-to-Endoplasmic Reticulum (ER) retrograde vesicular transport [18]. It is known that Kif1c alleles possess amino acid substitutions at residues 578, 1027, and 1066 [17]. Here, we demonstrated that residues 578 and 1027 are functionally significant. Although the amino acid polymorphism at residue 1027 is not in an evolutionarily conserved domain [17], it is in the C-terminal region believed to participate in cargo binding. In fact, alterations of this domain have been shown to modify in vivo kinesin protein function [19]. Moreover, it has been shown that the C-terminal tail domain of KIF1c (amino acids 811–1090) is involved in the interaction with bicaudal-D-related protein 1 (BDRP1) and this interaction regulates secretory transport required for neurite development [20]. Therefore, the ability of KIF1c to bind and transport cargo may be altered by polymorphism in this region. However, motor-dependent Golgi-to-ER transport functions normally in Kif1c knockout mice [44]. Immunohistochemical staining partially co-localized KIF1c with the Golgi marker CTR433, suggesting that KIF1c may also be involved in transport around the Golgi apparatus rather than only Golgi-to-ER transport. Accordingly, Wubbolts, et. al. [16] showed that kinesin plays a role in the vesicular transport of MHC II-containing lysosomes from the microtubule organizing center region towards the cell surface. Here, we provide evidence that the resistant Kif1c<sup>2D</sup> allele negatively regulates the expression of MHC II proteins on APCs, since Tg-Kif1c<sup>2D</sup> CD11b<sup>+</sup> cells express lower mRNA and protein levels. The reduction in MHC II expression by CD11b<sup>+</sup> Tg-Kif1c<sup>2D</sup> cells was directly correlated with impaired antigen presentation as reflected by diminished Ag-specific T cell proliferative response. Whether amino acids at position 578 and 1027 on Kif1c are involved in MHC II expression is currently under investigation. Taken together, our results nevertheless provide mechanistic insight into how polymorphism in other kinesins including KIF21B and KIF5A influence human autoimmune disease susceptibility.

Materials and Methods

Ethics statement

Mice were housed at 25°C with 12/12-h light-dark cycles and 40–60% humidity. The experimental procedures performed in this study were under the guidelines of the Animal Care and Use Committees of the University of Vermont (Burlington, VT) and University of Illinois at Urbana-Champaign (Urbana, IL).

Animals

BALB/cByJ (CByJ), DBA/2J (D2), and (BALB/cByJ×DBA/2J)F1 hybrid (CD2F1) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The congenic lines in this study were generated using (BALB/cAnPt×DBA/2Ncr)F<sub>6</sub> BALB/cAnPt backcross mice [45]. Third generation backcross mice heterozygous at Es<sub>2</sub> or at Hba and Es<sub>3</sub> were selected and backcrossed for six generations to BALB/cAnPt mice and fixed by brother-sister mating to generate the C.D2-Es<sub>2</sub> and C.D2-Hba/Es<sub>3</sub> lines. Overlapping interval specific recombinant congenic (ISRC) lines were generated by crossing C.D2-Es<sub>2</sub> mice to CByJ mice. F<sub>2</sub> hybrids were genotyped using tail snip DNA and PCR with Chr11 microsatellite markers discriminating CByJ and D2 mice across the Orch3 candidate interval. Founders were analyzed for background contamination at a density of 2–5 cM and mice
carrying CByJ alleles at all background marker loci were backcrossed an additional two generations to CByJ mice. The lines were fixed by brother-sister mating to generate the C.D2-3, C.D2-5, C.D2-8, and C.D2-9 ISRC lines. Similarly, higher order resolution mapping panels of ISRC lines were generated by screening (C.D2-3×CByJ)×CByJ, (C.D2-8×CByJ)×CByJ, (C.D2-9×CByJ)×CByJ and (C.D2-2×CByJ)×CByJ backcross mice for recombinants. The genome and complete genotypes of the C.D2 congenic mice used in this study are given in Figure S1 and Figure S2, respectively.

The CByJ.CD11B-Kif1cD2 transgenic (Tg-Kif1cD2) mice were generated by microinjection with a construct containing the human CD11B/ITGAM promoter [46], Kif1c exon2 cDNA, and the human growth hormone (hGH) polyA signal [47] into C57BL/6 fertilized eggs at the University of Vermont Transgenic/Knockout Facility. Mice were screened for hGH gene by PCR using hGH Fwd 5′-TAG GAA GAA GCC TAT ATC CCA AAG G 3′, hGH Rev 5′ ACA GTC TCT CAA AGT CAG TGG GG 3′ primers. Proinsulin Fwd 5′-CTA GTT GCA GTA GTT CTC CAG 3′ and proinsulin Rev 5′-CCT GCC TAT CTT TCA GGT C 3′ primers were used as internal control.

**PCR-based restriction fragment length polymorphism (RFLP)**

Genomic DNA was PCR-amplified using standard conditions and the following primers designed around a polymorphism in Nip11a: 5′-GGGGCACATGTTCAGGAGAT-3′, 5′-AGAGACCCCCACCAAATTCG3′. 10 μl of PCR reaction was digested using 5 units of ApaLI in 50 μl of 1× NEBuffer 4 (New England BioLabs, Inc., Ipswich, MA) for 1 hour at 37°C. Resulting fragments were electrophoresed in 2% agarose gels and visualized by ethidium bromide.

**EAO induction**

Six-12 week old mice were immunized as previously described [9] with 10 mg of TH plus CFA (Sigma-Aldrich, St. Louis, MO) supplemented with 200 μg of M. tuberculosis H37Ra (Difco Laboratories, Detroit, MI) in conjunction with PTX (List Biological Laboratories Inc., Campbell, CA). EAO was evaluated (Difco Laboratories, Detroit, MI) in conjunction with PTX (List Biological Laboratories Inc., Campbell, CA), anti-TCRβ-FITC, and anti-IA/IE-PE (eBioscience, San Diego, CA), and sorted on a FACSaria (BD Biosciences, San Jose, CA) by gating in the TCRβ/IA/IE+CD11c+CD11b+ myeloid cell population. Antibodies against B220 and CD19 (eBioscience) were also used for flow cytometry.

**Microarray analysis**

Total RNA was extracted and purified from Tg-Kif1cD2/IE+CD11c+CD11b+ myeloid cells from naive NLC and Tg-Kif1cD2 mice (n=6 to 10 mice/strain) using RNAeasy isolation reagent (Qiagen Inc.). Purified RNA was quantified using a Nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and quality was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, California). The RNA integrity number of all samples was greater than 8. For microarray analysis, two RNA pools were created so that each pool contained RNA from 3 to 5 mice, and two arrays per strain were analyzed.

**FACS sorting and flow cytometric analysis**

NLC and Tg-Kif1cD2 myeloid cells from erythrocyte-free spleens were first enriched by negative selection (using magnetic beads, Qiagen, Hilden, Germany) to deplete cells expressing CD8, CD4, and IgM. For FACS isolation, negatively selected enriched myeloid cells were stained with anti-CD11b–APC/PEγ7 (BD Pharmingen, Franklin Lakes, NJ), anti-CD11c–PE-5.5 (Invitrogen, Carlsbad, California), anti-TCRβ–FITC, and anti-IA/IE–PE (eBioscience, San Diego, CA), and sorted on a FACSaria (BD Biosciences, San Jose, CA) by gating in the TCRβ/IA/IE+CD11c+CD11b+ myeloid cell population. Antibodies against B220 and CD19 (eBioscience) were also used for flow cytometry.

**Cell preparation and Western blotting**

Spleens were collected from CByJ and Tg-Kif1cD2 mice, and single cell suspensions were prepared by passing the cells through a 50 μm nylon mesh (Small parts Inc, Miami Lakes, FL). Erythrocytes were lysed by incubation with 0.025% Trypsin-EDTA (Invitrogen, Carlsbad, California), washed three times and pelleted. Whole-cell lysates were prepared in Triton lysis buffer and equal amounts of protein were then separated via SDS-PAGE and transferred to nitrocellulose membranes as described previously [48]. Primary antibodies used for Western blot include anti-Kif1c and anti-Actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Bound antibody was visualized by peroxidase-conjugated secondary antibody and detected by chemiluminescence (Kirkegaard and Perry Laboratories, Gaithersburg, MD).
depletion and treated with mitomycin C (25 μg/ml; Sigma-Aldrich). Responder CD4 T cells from LN and spleens were isolated by negative selection as previously described [40]. Single cell suspensions of OVA- or PLP180–199-APCs (2 × 10⁵ cells/well) and Ag-specific responder CD4 T cell (1 × 10⁶ cells/well) suspensions were transfecting a plasmid encoding IFN-γ plasmid for transfection in RPMI 1640 (5% FBS) and plated on standard 96-well U-bottom tissue culture plates. Cells were stimulated with 1, 10, and 25 μg/ml of OVA or 2.5, 10, and 50 μg/ml of PLP180–199 for 72 h at 37°C. During the last 18 h of culture, 1 μCi of [³H] thymidine (PerkinElmer, Santa Clara, CA) was added. Cells were harvested on glass fiber filters and thymidine uptake was determined with a liquid scintillation counter.

Preparation of Jurkat cells, transfection, stimulation, and luciferase assays

Jurkat cells were cultured in RPMI containing 10% FBS without stimulation for 24 hours at a concentration of 1 × 10⁶ cells/ml. Plasmids encoding kif1cD2, kif1cCByJ alleles, LPV-KIF1c and PSY-KIF1c mutants, corresponding empty vector (pCDNA, Invitrogen, Carlsbad, CA), IL2 promoter (−575 to +57 base pairs) luciferase reporter, and control pGL2 luciferase reporter (Promega, Madison, WI) were used for transfection. Five micrograms of each plasmid were used for the transfection of approximately 5 × 10⁶ Jurkat cells by electroporation at 250 mV and 900 μF in 250 μl of RPMI with a BioRad electroporator (BioRad, Hercules, CA). Cells were subsequently cultured in RPMI and 10% FBS for 24 hours and then stimulated with PMA (10 ng/ml) and calcium ionophore calcimycin (0.5 μg/ml) for 3 hours. Cell lysates were prepared and supernatants collected to quantify luciferase activity. The luciferase activity was normalized using the β-galactosidase. The luciferase activity was normalized using the β-galactosidase.

Mutagenesis

Point mutations were introduced in the plasmid encoding the Kif1c allele from the D2 mouse using the QuickChange Site-Directed mutagenesis kit (Stratagene, USA). Briefly the plasmid was denatured and then annealed with the appropriate mutagenic primer that contained the desired mutation. Using Pfu DNA polymerase, new mutagenized strands were created. The parental DNA template was digested with DpnI and the new mutated plasmid was used to transform E. coli. The plasmid DNA was extracted using the Qiagen Maxi-Prep kit (Qiagen, Valencia, CA). The primers used for mutagenesis of the nucleotide at position 1033 (amino acid 578) of the D2 allele were: forward: 5'-GCTGGTACGGAGCTGCTGTTGCTGATGTCG-3'; reverse: 5'-GACTTTAACAGCAGTCGCTGAGACGC-3' and for the nucleotide at position 3079 (amino acid 1027): Forward: 5'GGAAGACCCACCGTTTCGAGAATTCGCG-3', and Reverse: 5'GGGAAATTCTGAGGAGACGTTGCTGTC-3'.

Supporting Information

Figure S1 Genealogy of the congenic and interval specific congenic lines used in this study. Third backcross generation (BALB/cAnPt × DBA/2NCr) × BALB/cAnPt mice heterozygous at Eo2 or at Hba and Eo3 were selected and backcrossed for six generations to BALB/cAnPt mice. Homozygous lines C.D2-Eo2 and C.D2-Hba/Eo3 were fixed by brother-sister mating. Overlapping interval specific recombinant congenic (ISRC) lines were generated by crossing C.D2-Eo2 mice to CByJ mice. E2 hybrids were genotyped using tail snip DNA and PCR with Chr11 microsatellite markers discriminating CByJ and D2 mice across the Orch3 candidate interval [10]. Founders were analyzed for background contamination and mice carrying CByJ alleles at all background marker loci were backcrossed an additional two generations to CByJ mice. Homozygous C.D2-3, C.D2-5, C.D2-8, and C.D2-9 ISRC lines were fixed by brother-sister mating. Similarly, higher order resolution mapping panels of ISRC lines were generated by screening (C.D2-3×CByJ)×CByJ, (C.D2-8×CByJ)×CByJ and (C.D2-3.2×CByJ)×CByJ backcross mice for recombinants.

PDF

Figure S2 Genotypes of congenic and interval specific congenic lines used in this study. Microsatellite and SNP based genotyping was done using tail snip DNA and PCR [10]. Locations are as given on either Ensembl or MGI. Hba alleles were determined by isoelectric focusing as described in [53]. Tbe marker and deletion mutation locations are as described in [54]. Eo2 and Eo3 alleles were determined as described in [55].

PDF

Figure S3 Illustration of the potential effect of altered MHC Class II expression on T helper (TH) cell differentiation. (Figure generated using Ingenuity Pathway Analysis, Ingenuity Systems. Green = expression decreased in Tg-kif1cD2 relative to NLC). (PDF)

Table S1 Genes differentially expressed between NLC and Tg-kif1cD2 CD11b⁺ cells. TCR β/IE/IA CD11c⁻ CD11b⁺ myeloid cells were harvested from naïve NLC and Tg-kif1cD2 mice and differential gene expression was detected using Affymetrix GeneChip Mouse Genome 430A 2.0 Arrays. ¹Chr = chromosome. ²logFC = log2 signed fold change.

(PDF)

Table S2 Transgenic expression of Kif1cD2 on CD11b⁺ cells influences pathways involving MHC Class II (genes in bold). TCRβ IA/IE/CD11c⁻ CD11b⁺ myeloid cells were harvested from naïve NLC and Tg-kif1cD2 mice and differential gene expression was detected using Affymetrix GeneChip Mouse Genome 430A 2.0 Arrays. Pathway analysis was conducted using Ingenuity Pathway Analysis software (Ingenuity Systems, www.ingenuity.com). To ensure biological relevance, cell type was restricted to B-cells, dendritic cells, and macrophages.

(PDF)

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Author Contributions

Conceived and designed the experiments: RdR RDM NDM VCK. Analyzed the data: RdR RDM NDM CT CT. Performed the experiments: RdR RDM NDM VCK. Contributed reagents/materials/analysis tools: GMT CT CT. Wrote the paper: RdR EHW CT.
References