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A Single Nucleotide Polymorphism in Tyk2 Controls Susceptibility to Experimental Allergic Encephalomyelitis

Karen M. Spach,* Rajkumar Noubade,* Ben McElvany,* William F. Hickey,‡ Elizabeth P. Blankenhorn,§ and Cory Teuscher2*†

Genes controlling immunopathologic diseases of differing etiology may also influence susceptibility to autoimmune disease. B10.D1-H2q/SgJ mice with a 2538 G→A missense mutation in the tyrosine kinase-2 gene (Tyk2) are susceptible to Toxoplasma gondii yet resistant to autoimmune arthritis, unlike the wild-type B10.Q/Ai substrain. To understand whether Tyk2 is also important in a second autoimmune model, experimental allergic encephalomyelitis (EAE) was induced in B10.D1-H2q/SgJ (Tyk2A) and B10.Q/Ai (Tyk2C) mice with the myelin oligodendrocyte glycoprotein peptide 79–96. B10.D1-H2q/SgJ mice were resistant to EAE whereas B10.Q/Ai mice were susceptible, and a single copy of the Tyk2 allele conferred EAE susceptibility in F1 hybrids. Furthermore, EAE resistance in B10.D1-H2q/SgJ mice was overridden when pertussis toxin (PTX) was used to mimic the effects of environmental factors derived from infectious agents. Numerous cytokines and chemokines were increased when PTX was included in the immunization protocol. However, only RANTES, IL-6, and IFN-γ increased significantly with both genetic compensation and PTX treatment. These data indicate that Tyk2 is a shared autoimmunity disease susceptibility gene whose genetic contribution to disease susceptibility can be modified by environmental factors. Single nucleotide polymorphisms like the one that distinguishes Tyk2 alleles are of considerable significance given the potential role of gene-by-environment interactions in autoimmune disease susceptibility. The Journal of Immunology, 2009, 182: 7776–7783.

Autoimmune diseases have common features and understanding the commonalities underlying them may aid in the design of rational treatments. One approach to understanding these commonalities is to identify genes, termed shared autoimmune disease genes, whose alternately expressed alleles influence susceptibility to multiple autoimmune diseases. Bphs/Hrh1, which controls susceptibility to both experimental allergic encephalomyelitis (EAE) (1) and orchitis (2) was the first non-Ag-dependent, shared autoimmune disease susceptibility gene to be identified and positionally cloned in the mouse (3). Another example of a potential shared autoimmune disease susceptibility gene is Eae3/Idd3/Aod2/Cia5 (4–7), which controls susceptibility to EAE, autoimmune insulin-dependent type I diabetes mellitus, day 3 thymectomy-induced autoimmune ovarian dysgenesis, and collagen-induced arthritis. In both mice and humans the genetic clustering of autoimmune disease quantitative trait loci supports the hypothesis that susceptibility to autoimmune disease may be controlled by shared genes (8). Moreover, relatives of multiple sclerosis (MS) and celiac disease patients are at increased risk for other autoimmune diseases, suggesting a shared genetic susceptibility (9–12). Similarly, multiple autoimmune diseases are often observed within a single patient (13).

Tyrosine kinase-2 gene/protein (Tyk2/Tyk2) participates in the signaling pathways of multiple cytokines in innate and acquired immunity (14, 15). Tyk2 is a member of the JAK/STAT signaling pathway (16) and contributes to the signaling of IFN-γ (17), IL-6 (18), IL-10 (19), IL-12 (20), IL-13 (21), and IL-23 (22). Depending on the ligand, cytokine receptor aggregation activates Tyk2, leading to phosphorylation of STAT-1, STAT-3, STAT-4, or STAT-5 (16). The phosphorylated STAT dimerizes and translocates to the nucleus to promote gene transcription.

Although Tyk2 activation has been implicated in the signaling of multiple cytokines (23), Tyk2H2q/SgJ (Tyk2−/−) mice primarily exhibit defects in response to IL-12 and type I IFN (15, 24). B10.D1-H2q/SgJ (B10.D1) mice are a strain of B10.Q mice that have a naturally occurring mutation in Tyk2, designated Tyk2A (25). The 2538 G→A base substitution is predicted to result in a nonconservative amino acid substitution (E775K) within a critical APE motif of the JH2 (pseudokinase) domain of Tyk2. The JH2 domain is required for Tyk2 activation via ligand-activated cytokine receptors (17). Although Tyk2A-specific transcripts are present at normal levels in B10.D1 mice, an immunoreactive protein cannot be detected (25). B10.D1 splenocytes exhibit impaired STAT phosphorylation in response to IL-12, IL-23, and IFN-α stimulation (25). In addition, neither T cells nor NK cells from B10.D1 mice produce IFN-γ when stimulated with IL-12; however, this defect can be overcome by increasing the concentration of IL-12 and the incubation time or by stimulation through an IL-12-independent pathway (26, 27).

Importantly, although B10.Q/Ai mice, which express a wild-type Tyk2C allele, are susceptible to collagen-induced arthritis (CIA) and resistant to Toxoplasma gondii infections, the Tyk2C mutation renders B10.D1 mice resistant to CIA and highly...
susceptible to T. gondii (25–28), demonstrating that Tyk2 is a shared immunopathology gene. We tested the hypothesis that Tyk2 is a shared autoimmune disease gene by assessing the susceptibility of B10.D1 and B10.Q/Ai mice to EAE, the principal animal model of MS. We found that Tyk2 is a critical genetic regulator of EAE susceptibility and that the resistant Tyk2^d allele can be compensated by one copy of the wild-type Tyk2^G allele and by environmental factors such as pertussis toxin (PTX). These results are of particular significance given that TYK2 polymorphisms are associated with an increased risk of systemic lupus erythematosus (29, 30) and that Tyk2 has been confirmed through independent replication (76).

Materials and Methods

Mice

B10.D1-H2^d/Sgp1 (B10.D1) (strain no. 002024) mice bearing the Tyk2^2538-A (Tyk^a) allele were purchased from The Jackson Laboratory and B10.Q/Ai (line no.4059) mice with the Tyk2^2538-C (Tyk^c) allele were purchased from Taconic Farms through the National Institute of Allergy and Infectious Diseases Animal Supply Contract. Reciprocal F_1 hybrids (B10.Q/Ai × B10.D1) (open triangle; n = 31) and (B10.D1 × B10.Q/Ai) F_1 hybrids (open diamond; n = 18) were also fully susceptible to EAE, indicating that Tyk2^c is recessive. Regression lines and mean clinical scores are shown.

Induction and evaluation of EAE

Mice were immunized for the induction of EAE using either the double injection or the single injection protocol. For the double injection protocol mice were immunized for the induction of EAE using either the double injection protocol. For the double injection protocol mice were immunized for the induction of EAE using either the double injection protocol. For the single injection protocol mice were injected s.c. in the posterior right and left flanks with a sonicated H37Ra (Difco Laboratories); 1 wk later the mice received the same injection on the right and left flanks anterior of the original injection with an emulsion of myelin oligodendrocyte glycoprotein peptide 79–96 (GKVALRIQNVRFSDEGGY) (MOG79–96) (32) and PBS/oil emulsion containing 50 μg of mycobacterial H37Ra on the posterior right and left flank and the scruff of the neck. Immediately afterward, each mouse received 200 μg PTX (List Biological Laboratories) in 0.2 ml by i.v. injection (33). EAE was evaluated daily beginning at day 5 as follows: 0, no clinical expression of disease; 1, flaccid tail without hind limb weakness; 2, hind limb weakness; 3, complete hind limb paralysis and floppy tail; 4, hind leg paralysis accompanied by a floppy tail and urinary or fecal incontinence; 5, moribund. Clinical quantitative trait variables were generated as previously described (34). Mice were considered positive for incidence if they showed any clinical signs ≥ 1 for two or more consecutive days. The severity index is the cumulative disease score per day affected. Histological assessment of EAE neuropathology was done as previously described (34–37). Briefly, brains and spinal cords were dissected from calvaria and vertebral columns, respectively, and fixed by immersion in phosphate-buffered (pH 7.2) 10% formalin. Representative areas of the brain and the spinal cord, including brainstem, cerebrum, cerebellum, and the cervical, thoracic, and lumbar segments of the spinal cord, were selected for histopathological evaluation. The type and severity of the EAE lesions were evaluated in each animal and scored according to a semiquantitative scale as previously described (32, 33). Two types of lesions occur in these mice. There were the typical inflammatory lesions of EAE containing lymphocytes, macrophages, activatedmicroglial cells, and reactive astrocytes (Fig. 2A). In addition areas of intense, acute inflammation were noted (Fig. 2, B and/or C). In these latter foci of suppuration, >50% of the infiltrating leukocytes were neutrophils; they were admixed with the typical cells of EAE lesions. Axonal damage and focal destruction of the CNS parenchyma itself were frequently seen.

Cell culture conditions and lymphokine assays

For ex vivo cytokine and chemokine analysis, spleens and draining lymph nodes were obtained from mice immunized by both methods 10 days earlier for EAE as described above. Single cell suspensions at 1 × 10^6 cells/ml in RPMI 1640 medium (Cellgro Mediatech) plus 5% FBS (HyClone) were stimulated with 50 μg of MOG_{35–55}. Cell culture supernatants were recovered at 72 h and 23 different cytokine and chemokine levels were quantified in duplicate by BioPlex multiplex cytokine assay (BD Biosciences), including IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, cTAK, G-CSF, GM-CSF, IFN-γ, keratinocyte, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α. To confirm the IL-17 and IFN-γ results, ELISAs were performed as described (33) using primary anti-IL-17 and anti-IFN-γ Abs and their corresponding biotinylated secondary Abs (BD Biosciences Pharmingen). Other ELISA reagents were generated and bred at the University of Vermont (Burlington, VT). Mice were housed at 25°C with 12/12-h light-dark cycles and 40–60% humidity. Naive, age-matched male and female mice were used throughout. The experimental procedures performed in this study were approved by the Institutional Animal Care and Use Committee of the University of Vermont.

### Table I. Summary of clinical disease traits in B10.D1, B10.Q/Ai, and reciprocal F_1 hybrids following MOG_{35–55}-CFA immunization

<table>
<thead>
<tr>
<th>Strain</th>
<th>Overall Incidence</th>
<th>Day of Onset</th>
<th>Cumulative Disease Score</th>
<th>Peak Score</th>
<th>Severity Index</th>
<th>Days Affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.D1</td>
<td>0/46</td>
<td>N/A</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>B10.Q/Ai</td>
<td>31/56</td>
<td>14.8</td>
<td>20.5</td>
<td>1.7</td>
<td>1.3</td>
<td>13.0</td>
</tr>
<tr>
<td>(Ai × D1)</td>
<td>26/31</td>
<td>14.9</td>
<td>21.5</td>
<td>1.5</td>
<td>1.5</td>
<td>13.5</td>
</tr>
<tr>
<td>(D1 × Ai)</td>
<td>16/18</td>
<td>15.4</td>
<td>18.7</td>
<td>1.3</td>
<td>1.3</td>
<td>13.7</td>
</tr>
<tr>
<td>89% (χ^2)</td>
<td>415±3.6 (F)</td>
<td>32.0±3.6 (F)</td>
<td>43.5±3.6 (F)</td>
<td>74.2±3.6 (F)</td>
<td>67.6±3.6 (F)</td>
<td></td>
</tr>
</tbody>
</table>

*Trait values among B10.Q/Ai and the reciprocal F_1 hybrids were not significantly different, whereas all were significantly different from B10.D1 mice p < 0.0001. Means are shown. The significance of differences for the trait values among the strains was assessed by χ^2 or Kruskal-Wallis followed by Dunn’s post hoc multiple comparisons test (F), p < 0.0001.

*Animals were considered affected that showed clinical signs of ≥ 1 for two or more consecutive days.

*Not applicable.
Tyk2 alleles determine EAE susceptibility

Alleles determine EAE susceptibility

Proliferation assays

Statistical analysis

Results

B10.D1 mice are resistant to MOG<sub>79-96</sub>-CFA-induced EAE

To determine whether Tyk2 is a shared autoimmune disease gene, EAE susceptibility was assessed in B10.D1 and B10.Q/Ai mice using the MOG<sub>79-96</sub>-CFA double-injection protocol. B10.D1 mice were resistant (0/46) to EAE whereas B10.Q/Ai mice were susceptible (31/36; p < 0.001) (Fig. 1A and Table I). On day 30, EAE pathology in the brain and spinal cord was assessed using previously defined neuropathologic trait variables (34, 36). The susceptible B10.Q/Ai mice had marked EAE pathology in the spinal cord while B10.D1 had no EAE pathology (Fig. 2). Neither strain showed significant EAE pathology in the brain.

FIGURE 2. EAE pathology in B10.Q/Ai, B10.D1, and reciprocal F<sub>1</sub> hybrid mice. EAE was elicited by immunization with MOG<sub>79-96</sub>-CFA on days 0 and 7. A, A typical submeningeal inflammatory EAE lesion in a murine spinal cord is shown that corresponds to a lesion score of 3. The infiltrate is composed of lymphocytes, macrophages, and activated microglial cells (H&E staining; original magnification: ×225). B, An area of suppuration beneath the spinal cord meninges is shown corresponding to a suppuration score of 3. In contrast to typical EAE lesions (as in A), these lesions contain large numbers of neutrophils in addition to other leukocytes, and axonal and parenchymal damage has occurred (H&E staining; original magnification: ×225). C, Foci of suppuration such as this contain large numbers of neutrophils in addition to the other infiltrating leukocytes. Parenchymal damage, nuclear debris, and axonal spheroids were frequently found at these sites and were given the highest suppuration score (H&E staining; original magnification: ×400). D, Quantification of EAE pathology in B10.Q/Ai, B10.D1, and reciprocal F<sub>1</sub> hybrid mice. EAE pathology in the spinal cords of B10.Q/Ai mice was more severe on day 30 compared with that in B10.D1 mice for lesion severity, monocyte/lymphocyte infiltration, and total EAE pathology (p < 0.001) with B10.D1 < (B10.D1 × B10.Q/Ai) F<sub>1</sub> = (B10.Q/Ai × B10.D1) F<sub>1</sub> = B10.Q/Ai (n = 4–11/group). Significance of differences was assessed using the Kruskal-Wallis test followed by Dunn’s multiple comparisons.

FIGURE 3. Ex vivo MOG<sub>79-96</sub>-specific cytokine profiles and proliferation of MOG<sub>79-96</sub>-CFA-immunized B10.D1, B10.Q/Ai, and F<sub>1</sub> hybrid mice. A–F, Cytokine production was assessed by stimulating splenocytes and lymphocytes from draining lymph nodes on day 10 after MOG<sub>79-96</sub>-CFA immunization with 50 μg of MOG<sub>79-96</sub> for 72 h and by measuring levels in supernatants using a BioPlex assay (n = 8–10/group). The significance of differences was determined using the Kruskal-Wallis test followed by Dunn’s post hoc multiple comparisons; *, p < 0.05; **, p < 0.01; and ***, p < 0.001. IL-4 (A), TNF-α (B), and IL-17 (C) did not differ significantly with EAE susceptibility whereas IFN-γ (D) (F = 4.1; p < 0.05), RANTES (E) (F = 9.9; p < 0.01), and IL-6 (F) (F = 7.3; p < 0.01) increased significantly with increasing EAE susceptibility. Data for B10.D1 (open bars), B10.Q/Ai (filled bars), and F<sub>1</sub> hybrids (gray bars) are from one representative experiment of two is shown. G, Proliferation was assessed by stimulating splenocytes and lymphocytes from draining lymph nodes on day 10 after MOG<sub>79-96</sub>-CFA immunization with and without MOG<sub>79-96</sub> for a total of 72 h, and [<sup>3</sup>H]thymidine incorporation was measured in the final 18 h. Stimulation indices were calculated as average counts/background counts. B10.D1 (open circle), B10.Q/Ai (filled circle), and reciprocal F<sub>1</sub> hybrid (gray circle) samples (n = 8–10/group) were analyzed by two-way ANOVA (comparison: concentration of MOG<sub>79-96</sub> p < 0.0001; strain differences, p = 0.11; interaction, p = 0.49). Data from one representative experiment of two are shown.
Tyk2<sup>A</sup>-mediated resistance is abrogated by PTX

PTX is an example of an environmental factor derived from an infectious agent that influences susceptibility to EAE and is capable of overriding genetic checkpoints in this autoimmune disease (38). Therefore, we included PTX in the immunization protocol and tested the susceptibility of B10.D1 and B10.Q/Ai mice to EAE. In the MOG<sub>79–96</sub>-CFA plus PTX single-injection protocol (33), mice receive the same total amount of MOG<sub>79–96</sub> in CFA as in the double-injection protocol but also receive an i.v. injection of PTX on day 0. In B10.Q/Ai mice injected with MOG<sub>79–96</sub>-CFA plus PTX (Fig. 4B) there was increased incidence and more severe disease compared with B10.Q/Ai mice injected with MOG<sub>79–96</sub>-CFA alone (Table II). A more dramatic difference was seen in B10.D1 mice, which are much more susceptible (33/37) to EAE induced using PTX (Fig. 4A and Table II) than with MOG<sub>79–96</sub>-CFA alone. Clearly, environmental factors derived from infectious agents such as PTX can override the genetic checkpoint, resulting in EAE susceptibility in B10.D1 mice.

EAE susceptibility, modified by PTX, correlates with increased cytokine production

When PTX was included in the immunization protocol it bypassed the Tyk2<sup>A</sup> mutation, leading to EAE susceptibility in B10.D1 mice; but this was not reflected in their Ag-specific ex vivo proliferative responses, which were not significantly different between immunization protocols (Fig. 5B). Inclusion of PTX in the immunization protocol, however, resulted in increased cytokine secretion by cultured cells from both strains, consistent with the increased severity of EAE induced by the PTX protocol. PTX is known to induce many cytokines that are proinflammatory and pathogenic in EAE, of which IL-1β, IL-6, TNF-α, IL-17, and RANTES were produced in greater amounts in PTX-treated animals as compared with MOG<sub>79–96</sub>-CFA alone (Fig. 5).

Table II. Summary of EAE clinical traits in B10.D1 and B10.Q/Ai mice with and without PTX included in the immunization protocol<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Immunization Protocol</th>
<th>Overall Incidence</th>
<th>Day of Onset</th>
<th>CDS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Peak Score</th>
<th>Severity Index</th>
<th>Days Affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.D1</td>
<td>MOG&lt;sub&gt;79–96&lt;/sub&gt;-CFA</td>
<td>0/46</td>
<td>N/A&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>B10.D1</td>
<td>MOG&lt;sub&gt;79–96&lt;/sub&gt;-CFA + PTX</td>
<td>33/37*</td>
<td>13.8*</td>
<td>27.1*</td>
<td>2.3*</td>
<td>1.9*</td>
<td>12.9*</td>
</tr>
<tr>
<td>B10.Q/Ai</td>
<td>MOG&lt;sub&gt;79–96&lt;/sub&gt;-CFA</td>
<td>31/36</td>
<td>14.8</td>
<td>20.5</td>
<td>1.7</td>
<td>1.3</td>
<td>13.0</td>
</tr>
<tr>
<td>B10.Q/Ai</td>
<td>MOG&lt;sub&gt;79–96&lt;/sub&gt;-CFA + PTX</td>
<td>44/44*</td>
<td>10.1*</td>
<td>38.8*</td>
<td>3.1*</td>
<td>2.0*</td>
<td>19.3*</td>
</tr>
</tbody>
</table>

<sup>a</sup> Clinical traits were calculated as detailed in the Table I legend. Within each strain, trait values for both B10.D1 and B10.Q/Ai were significantly different between MOG<sub>79–96</sub>-CFA plus PTX and MOG<sub>79–96</sub>-CFA protocols as assessed by χ<sup>2</sup> or Student’s t test; *, p < 0.001. Means are shown.

<sup>b</sup> Cumulative disease score.

<sup>c</sup> Not applicable.
and IL-13 responses were elevated compared with those elicited by immunization without PTX (Fig. 5A). In addition, two members of the CC chemokine family, MIP-1α and RANTES, were elevated >4-fold compared with those induced by MOG79–96-CFA alone, suggesting that there is likely to be a distinct mobilization of Th1 cells in the PTX-exposed mice (41, 42). Notably, among the changes in cytokine and chemokine production elicited in B10.D1 mice by the inclusion of PTX in the immunization protocol, IFN-γ, IL-6, and RANTES were also significantly elevated by genetic compensation (Fig. 3).

### Discussion

A naturally occurring single nucleotide polymorphism within the pseudokinase domain of Tyk2 influences the immunopathologic outcomes of CIA (26) and T. gondii infection in B10.Q/Ai and B10.D1 mice (27), making Tyk2 a shared immunopathology gene. The data presented in this study showing that Tyk2 alleles control EAE susceptibility demonstrate that Tyk2 is also a shared autoimmune disease susceptibility gene. Specifically, B10.D1 mice that possess the Tyk2G allele are completely resistant to MOG79–96-CFA-induced EAE, whereas B10.Q/Ai strain mice expressing the Tyk2A allele are susceptible. Moreover, a single copy of the Tyk2G allele fully confers EAE susceptibility in B10.D1 F1 hybrids, clearly establishing that Tyk2 is important in controlling autoimmune disease susceptibility. When PTX was included in the immunization regime to mimic the effects of environmental agents derived from infectious agents, it overcame the genetic checkpoints resulting in EAE susceptibility, thus emphasizing the contextual role that gene-by-environment interactions play in determining susceptibility to autoimmune diseases.

It is not surprising that Tyk2 is a player in mouse autoimmunity and inflammation due to its importance in supporting IL-12-induced IFN-γ responses and its ability to down-regulate Th2-mediated Ab production, especially IgE (43). There are differences between the requirements for Tyk2 in mouse as compared with human immune responses. For example, human TYK2-deficient cell lines are completely unresponsive to type I IFN, and IL-6 and IL-10 signaling is severely impaired (14), whereas these phenotypes are leaky in Tyk2−/− mice and high concentrations of IFN-α can overcome the deficiency in MHC class I expression (24). However, evidence exists that Tyk2 shares some functions between these two species. There is a report of one patient with a mutation in Tyk2 who had a remarkably similar immune response to that seen in B10.D1 mice, with hyper-IgE syndrome, increased susceptibility to multiple microbial pathogens, an impaired STAT-4 phosphorylation pattern, and undetectable IFN-γ production in response to IL-12 (14). This supports the hypothesis that human TYK2 is also a shared immunopathology gene. Supporting the hypothesis that TYK2 is a shared human autoimmune disease susceptibility gene, it was recently identified as a strong MS susceptibility gene in a genome-wide association study (31), and this was confirmed through independent replication (76); TYK2 polymorphisms have also been associated with an increased risk of systemic lupus erythematosus (29, 30).

However, the manner by which the Tyk2G allele restores autoimmunity is not known. T cell proliferation induced by IL-12 is not Tyk2 dependent (24) and, likewise, no differences in Ag-specific proliferation were observed between B10.D1 and wild-type B10.Q/Ai mice following immunization with MOG79–96-CFA in this study. B10.D1 mice make IL-12 in normal abundance (27), and both IL-12 and TNF-α levels were similar between B10.D1 and B10.Q/Ai and F1 hybrids after the induction of EAE without PTX. Therefore, differences in proliferation and/or levels of these cytokines are not likely important mechanisms by which B10.D1 mice are resistant to EAE. The Tyk2A mutation impairs signaling through both the IL-12R and the IL-23R pathways (25). It is more likely therefore that their EAE resistance is due to this signaling defect, leading to an inability to up-regulate encephalitogenic levels of IFN-γ (via IL-12R; reviewed in Ref. 44) and IL-17 (via IL-23R; reviewed in Ref. 44) or to activate the T cells that make these cytokines. We observed differences between B10.D1 and B10.Q/Ai mice for both of these effector molecules, although the difference in IL-17 was only a trend. In addition, B10.D1 mice had a significantly impaired ability to produce proinflammatory molecules such as IL-6 and RANTES. IL-17 and IFN-γ have well-documented roles in EAE (45–48), and the development of EAE is blocked by Abs or antagonists of IL-6 and RANTES (49, 50). It is important to note that the effects of the Tyk2G allele may not be exclusively in T cells, as

![FIGURE 5. MOG79–96-CFA plus PTX immunization increased ex vivo MOG79–96-specific cytokine and chemokine production compared with MOG79–96-CFA in B10.D1 mice, but proliferative responses did not differ between strains. Cytokine production and proliferation were assessed by stimulating splenocytes and lymphocytes from draining lymph nodes on day 10 after immunization with MOG79–96-CFA or MOG79–96-CFA plus PTX. A, Single-cell suspensions were stimulated with 50 μg MOG79–96 for 72 h and the levels in supernatants were measured by cytometric bead assay. Samples (n = 10/group) were analyzed by one-way ANOVA. Cytokines that changed significantly have their average fold change represented in the graph; p < 0.05. B, Proliferation was assessed by stimulating splenocytes and lymphocytes with and without MOG79–96 for a total of 72 h and [3H]thymidine incorporation was measured in the final 18 h. Stimulation indices were calculated as average counts/background counts. Samples (n = 10/group) from B10.D1 mice immunized with MOG79–96-CFA with PTX (open circle) or without PTX (filled circle) were analyzed by two-way ANOVA (comparison: concentration of MOG79–96, p = 0.002; strain, p = 0.3; interaction, p = 0.44). Data from one representative experiment of two are shown.](http://www.jimmunol.org/Downloadedfrom)
Tyk2 is also required in dendritic cells for IL-12, IL-23, and IFN-γ production (51).

In the present study, PTX was included in the EAE induction protocol to reveal gene-by-environment interactions and especially the effects of an environmental factor derived from an infectious agent. B10.D1 mice were susceptible to EAE only when it was induced with MOG\textsubscript{35–55}-CFA plus PTX, and they produced significantly higher levels of multiple Th1-, Th2-, and Th17-type cytokines and chemokines compared with the MOG\textsubscript{35–55}-CFA-inoculated mice (Fig. 5A). Of interest, both GM-CSF and IL-5 showed large increases with PTX treatment. MS patients in the active phase of the disease have elevated levels of GM-CSF compared with patients in remission (52), and mice lacking GM-CSF are resistant to MOG\textsubscript{35–55}-induced EAE (53). Taken together, these reports and the present study suggest that GM-CSF may be critically important in EAE and MS pathogenesis. A small study of MS patients and controls found that patients with highly proliferating myelin basic protein-specific T cells produced higher levels of IL-5 and IL-17, and this correlated with number of magnetic resonance imaging-identified active plaques (54). However IL-5 has also been shown to increase in MS patients treated with IFN-β (55) or glatiramer acetate (56).

Although it is not known how PTX restores EAE susceptibility in B10.D1 mice, several possibilities exist. PTX may act intrinsically in T cells because they express PTX-sensitive G\textsubscript{i/o} proteins. Inhibitory G\textsubscript{i/o} proteins are inactivated by PTX-mediated ADP ribosylation (57). Following direct TCR stimulation, T cells from B10.D1 mice produce more IL-2, IL-4, and IFN-γ than wild-type mice (58). Thus, the PTX-mediated abrogation of G\textsubscript{i/o} coupled inhibitory signals in T cells would increase the magnitude of the cytokine responses. This may be one mechanism by which PTX overcomes the Tyk2\textsuperscript{A} allele to restore EAE susceptibility in B10.D1 mice. Accordingly, PTX also increases the expression of the costimulatory molecule CD80 on T cells (40), which would therefore potentiate the immune response.

PTX could also increase T cell cytokine responses and lead to EAE susceptibility through its effects on APCs. PTX increases the secretion of proinflammatory cytokines such as IL-1β (40), IL-6 (59), IL-12 (60), and TNF-α (61). Increased IL-6 production by PTX-treated APCs promotes the generation of IL-17-producing T cells (59). Indeed, IL-6 and IL-17 were up-regulated by PTX in both B10.D1 and B10.Q/Ai mice. PTX also enhances the ability of the APC to activate T cells by inducing dendritic cell maturation (61) and up-regulating expression of the costimulatory molecules B7-1 and B7-2 (40).

In B10.D1 mice IFN-γ and IL-17 levels were both elevated, indicating that PTX may enhance their production via Tyk2-independent pathways, perhaps via IL-18R-mediated IFN-γ production (27). Within the type I IFN pathway, alternate routes of STAT-4 phosphorylation have also been noted (62). Additionally, PTX intoxication may produce sufficient IL-12 stimulation as to cause receptor aggregation, leading to phosphorylation of Jak2 that can stand in for Tyk2. We observed high levels of active IL-12 after PTX inoculation (Fig. 5A), and it has been shown that greater exposure to IL-12 in vitro can overcome the effects of the Tyk2\textsuperscript{A} allele as measured by STAT-4 phosphorylation (26). The PTX-induced changes in EAE susceptibility of B10.D1 mice cannot be attributed solely to effects on either T cells or APCs based on these experiments. Although it is tantalizing to speculate that PTX increases costimulatory molecules on APCs, which then increases both APC maturation and T cell differentiation and clonal expansion and thus leads to the cytokine differences we observed, additional experimentation including the use of bone marrow chimeras will be required for incisive analysis.

The ability of environmental factors derived from infectious agents to alter autoimmune disease susceptibility controlled by a single nucleotide polymorphism highlights the contextual importance of gene-by-environment interactions in determining autoimmune disease susceptibility. There is increasing evidence that human autoimmune disease results from a complex interaction of environmental effects in genetically susceptible individuals. The 75% discordance rate for MS in monozygotic twins (63) suggests that environmental factors are important in MS. In particular, low UV light exposure (64–67) and the resulting low serum 25-(OH)\textsubscript{2}D\textsubscript{3} levels correlate with increased MS risk (68–71). It is therefore interesting in this regard that in vivo treatment of mice with 1,25-dihydroxyvitamin D\textsubscript{3} ameliorates EAE (72), and treatment of activated T cells in vitro with 1,25-dihydroxyvitamin D\textsubscript{3} inhibits IL-12-induced tyrosine phosphorylation of Tyk2, thereby reducing T cell responses to Ag (73). Another important finding is that the sexual dimorphism observed in MS is increasing in the last 50 years, suggesting that emergent factors such as environmental estrogen could selectively promote MS in women (74). The phytosterogen quercetin, known to reduce signs of EAE in mice, is also capable of blocking IL-12-induced phosphorylation of Tyk2 (75). In preliminary work we note that EAE susceptibility in B10.D1 and B10.Q/Ai mice is in fact sexually dimorphic (E. P. Blankenhorn, K. M. Spach, and C. Teuscher, submitted for publication). In the context of the present report, therefore, the mutant Tyk2\textsuperscript{A} allele is a good candidate for an environmentally sensitive genetic modifier of demyelinating diseases, responding to a wide variety of environmental factors including 1,25-dihydroxyvitamin D\textsubscript{3}, estrogenic compounds, and toxins produced by microorganisms, such as PTX.

In summary, we demonstrate that Tyk2 is both a shared immunopathology gene and also a shared autoimmune disease susceptibility gene in mice. We further demonstrate that the genetic contribution of Tyk2 to disease susceptibility can be modified by environmental factors. As such, this model provides a unique opportunity to identify additional environmental factors impacting a core genetic network underlying susceptibility to autoimmune disease.

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