IL-1 Receptor-Associated Kinase 1 Regulates Susceptibility to Organ-Specific Autoimmunity

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Infections often precede the development of autoimmunity. Correlation between infection with a specific pathogen and a particular autoimmune disease ranges from moderately strong to quite weak. This lack of correspondence suggests that autoimmunity may result from microbial activation of a generic, as opposed to pathogen-specific host-defense response. The Toll-like receptors, essential to host recognition of microbial invasion, signal through a common, highly conserved pathway, activate innate immunity, and control adaptive immune responses. To determine the influence of Toll/IL-1 signaling on the development of autoimmunity, the responses of wild-type (WT) mice and IL-1R-associated kinase 1 (IRAK1)-deficient mice to induction of experimental autoimmune encephalomyelitis were compared. C57BL/6 and B6.IRAK1-deficient mice were immunized with MOG 35–55/CFA or MOG 35–55/CpG DNA/IFA. WT animals developed severe disease, whereas IRAK1-deficient mice were resistant to experimental autoimmune encephalomyelitis, exhibiting little or no CNS inflammation. IRAK1-deficient T cells also displayed impaired Th1 development, particularly during disease induction, despite normal TCR signaling. These results suggest that IRAK1 and the Toll/IL-1 pathway play an essential role in T cell priming, and demonstrate one means through which innate immunity can control subsequent development of autoimmunity. These findings may also help explain the association between antecedent infection and the development or exacerbations of some autoimmune diseases.

Multiple sclerosis (MS) is a demyelinating CNS disease that affects 250,000–350,000 persons in the United States. Although pathologic specimens from MS patients document the presence of inflammation and cellular infiltration, the exact cause of the disease remains unknown. Infections can trigger or exacerbate the course of MS, but many different agents, both bacterial and viral, have been implicated (1, 2). How acute infections precipitate or worsen suspected autoimmune diseases is also uncertain, although many mechanisms have been proposed. Agents from pathogens may resemble autoantigens and activate T cells, leading to clinical autoimmune disease (3, 4). Molecular mimicry, however, has found limited empiric support as a general mechanism to explain the development of autoimmune diseases. Alternatively, infections with Thieher’s murine encephalomyelitis virus can stimulate APCs that activate autoreactive T cells that do not cross-react with Thieher’s murine encephalomyelitis virus Ags (5). The diversity of pathogens associated with clinical MS and other human autoimmune diseases, the paucity of pathogen-associated Ags that activate autoreactive T cells, and the demonstration that microbiobly mediated APC activation can trigger autoimmunity suggest that the host response to infection may influence the development of autoimmunity more than the specific pathogen.

Experimental autoimmune encephalomyelitis (EAE) models several aspects of MS pathogenesis (6). EAE can be induced by immunization with myelin Ags and certain adjuvants (7). Adjuvants enhance specific immune responses (8, 9). Some adjuvants, particularly those containing microbial components tend to promote a Th1 effector response (9). Recent evidence suggests that activation of the Toll-like receptors (TLRs) promote Th1 responses (10). One of the most commonly used adjuvants for induction of EAE is CFA, a lipid-based emulsion containing killed mycobacteria. Individual microbial components such as DNA from insect and microbial and synthetic sources, which signal through TLR9 and the Toll/IL-1 pathway, can also function as adjuvants, and promote a Th1 response and EAE (11–15). Following immunization with a myelin Ag and adjuvant, T cells differentiate into Th1 effectors. IL-12 and IL-18 drive expansion of myelin reactive Th1 lymphocytes as well as the production of IFN-γ, which together with IL-2 and TNF-α, participate in the cellular effector immune responses (16–19). Activated T cells then migrate to the CNS to orchestrate myelin destruction, either directly or by the recruitment of additional inflammatory cells, resulting in clinical disease.

Toll/IL-1 signaling may play a critical role in the development of autoimmunity. The pathway, which consists of the molecules of the myeloid differentiation marker (MyD) 88, the IL-1R-associated

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kinase (IRAK) 1 and IRAK4, and the TNFR-associated factor 6, transduces signals from multipleTLRs that sense infectious and noninfectious injury (12, 20–24) and from IL-1R family members, including the IL-1R1 and IL-1R2 (22, 25–28). Pathway function downstream of theTLRs is required for development of an adaptive immune response to foreign Ag (29). Furthermore, mice lacking the type 1 IL-1R or IL-18 fail to develop EAE (17, 30). The central role played by this pathway during both injury-sensing and effector functions led to the hypothesis that Toll/IL-1 signaling influences the development of autoimmune disease.

To test this hypothesis, we compared the responses of WT and IRAK1-deficient mice and of immune cells to immunization with the immunodominant myelin-derived epitope MOG 35–55. Results from our studies suggest that IRAK1 function, and by extension Toll/IL-1 signaling, is critical for the induction of EAE as well as the activation and expansion of autoreactive T cells.

Materials and Methods

Mice
C57BL/6 (B6) WT mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IRAK1-deficient mice (B6:IRAK1), produced by backcrossing the Irak1 null allele onto a B6 background for 14 generations, were used for these experiments. These mice were bred and maintained in the animal colony at University of Texas Southwestern Medical Center (Dallas, TX). The use of mice in these experiments was approved by the Institutional Animal Care and Research Advisory Committee. All mice were 7–10 wk of age when experiments were initiated.

Immunization and evaluation of EAE
For induction of EAE, mice were immunized s.c. with 200 μg MOG 35–55 (BioSource International, Camarillo, CA) in an emulsion with either CFA or IFA and 60 μg CpG (15, 31). Synthetic phosphorothioate-modified oligonucleotides were purchased from Operon Technologies (Alameda, CA). The sequence of the immunostimulatory oligonucleotide 1826 used in both in vitro and in vivo studies was: 5′-TCC ATG ACG TTC CTG ACG TT-3′. The sequence of the immunostimulatory oligonucleotide 1826 used in both in vitro and in vivo studies was: 5′-TCC ATG ACG TTC CTG ACG TT-3′. In vitro and in vivo studies was: 5′-TCC ATG ACG TTC CTG ACG TT-3′. The sequence of the immunostimulatory oligonucleotide used in both in vitro and in vivo studies was: 5′-TCC ATG ACG TTC CTG ACG TT-3′. The immunodominant myelin-derived epitope MOG 35–55. Results from our studies suggest that IRAK1 function, and by extension Toll/IL-1 signaling, is critical for the induction of EAE as well as the activation and expansion of autoreactive T cells.

Cytokine detection
IFN-γ, IL-4, and IL-10 were measured by ELISA. ELISA plates (Immunol 2; Dynatech, Franklin, MA) were coated with 2 μg/ml (50 μl well) of IFN-γ, IL-4, or IL-10 mAb (BD Pharmingen, San Diego, CA) in 0.1 M carbonate buffer (pH 9.2) overnight at 4°C. The plates were blocked with 200 μl of 10% FBS in PBS for 2 h. Supernatant (100 μl) was added to various dilutions titered to the linear portion of the absorbance/concentration curve in duplicate and incubated overnight at 4°C. After the plates were washed four times with PBS and Tween 20 (0.05%), 100-μl biotinylated anti-cytokine detecting mAbs (directed to a different determinant than the first Ab used to coat ELISA plates) at 1 μg/ml in PBS and 10% FBS were added for 45 min at room temperature. Then 100 μl of avidin-peroxidase (2.5 μg/ml) was added and incubated for 30 min. Subsequently, the peroxidase substrate ABTS (2,2′-azino-di-[3-ethylbenzthiazoline-sulfonate] (6)) in 0.1 M citric buffer (pH 4.35) in the presence of H2O2 was added and the absorbance was measured at 405 nm.

Immunohistochemistry
Mice were deeply anesthetized with sodium pentobarbinate and transcardially perfused with sterile PBS, followed by 4% paraformaldehyde in phosphate buffer. Brain and spinal cords were removed, fixed overnight in 4% paraformaldehyde and cryoprotected in 30% sucrose at 4°C before being embedded in Tissue-Tek (Ted Pella, Redding, CA) and quickly frozen in isopentane. Immunohistochemistry was performed on adjacent sections. After pretreatment with 0.3% hydrogen peroxide in absolute methanol, sections were blocked with 1% BSA for 2 h at room temperature and then incubated with the primary Ab (1/20 dilution of a 200 μg/ml stock) overnight at 4°C. MOM2 mAb (BioSource International) was used to stain macrophages. The binding of the primary Abs was detected using a biotinylated secondary Ab and an avidin-biotin-peroxidase method under humidified conditions (ABC Elite kit; Vector Laboratories, Burlingame, CA). Peroxidase was visualized with 3,3′-diaminobenzidine (DAB kit; Vector Laboratories) as a substrate or with methyl green counterstain. Omission of primary Ab served as a negative control. The specificity of staining was also controlled on sections of peripheral lymphoid organs. The tissue area was measured by a Scion image analysis system (Scion, Frederick, MD). Positive cells were counted by automatic video scanning using a Leica Q500 MC (Zeiss, Oberkochen, Germany), and the number of stained cells per 104 square pixels tissue area was calculated.

Statistical analysis
Differences between pairs of groups in clinical score were assessed by the Mann-Whitney U test sum or ranks. Differences among the three groups were tested by Kruskal-Wallis one-way ANOVA. All significance tests were two-sided.

Results
IRAK1-deficient mice are resistant to EAE and have impaired T cell responses
Because Toll/IL-1 signaling is critical to both afferent (injury-sensing) and efferent (proinflammatory) limbs of the innate immune response, we hypothesized that IRAK1-deficient mice would be resistant to EAE. To test this hypothesis we immunized both C57BL/6 (WT) and B6:IRAK1 (IRAK1-deficient) mice (13 mice/group) with MOG 35–55 (200 μg) emulsified in CFA. Pertussis toxin (200 ng, i.p.) was injected on days 0 and 2. Clinical signs of EAE were monitored and scored as previously described (32). WT mice began to exhibit signs of EAE 10 days after immunization...
and displayed a monophasic disease course described previously in C57BL/6 mice (33). All 13 of the WT mice developed EAE with a maximum mean EAE score of 2 (Fig. 1). B6.IRAK1 mice, in contrast, were almost completely resistant to EAE, although three animals developed mild disease, characterized by less severe symptoms (only mild tail weakness) and a shorter disease course than their WT counterparts (Fig. 1). The difference in EAE severity between WT and IRAK1-deficient mice was highly significant (p < 0.0001). Furthermore, EAE onset was also delayed in affected IRAK1-deficient animals. These mice first displayed signs of disease 18, 23, and 28 days after immunization, respectively. These results indicate that IRAK1 is required for normal EAE development following immunization with Ag and a complex adjuvant (CFA).

EAE is a Th1-mediated autoimmune disease (34). Development of a Th1 effector response may involve TLR/IL-1 signaling at several different stages. The TLR/IL-1 pathway controls development of a Th1 response through its action downstream of the TLRs (10). IL-1 and IL-18, which signal through IRAK1 (26–28), also contribute to T cell activation and regulate pathogenesis of autoimmune diseases (17, 18, 27, 35, 36). To examine the role of IRAK1 in T cell activation, both WT and IRAK1-deficient mice were immunized with MOG 35–55/CFA as previously described. Ten days later, draining LNC were isolated and cultured in the presence of MOG 35–55. The T cell proliferative and cytokine responses were then examined. WT T cells exhibited a dose-dependent proliferative response to increasing Ag concentrations (Fig. 2A). IRAK1-deficient cells, in contrast, failed to proliferate at all Ag doses tested (Fig. 2A). This failure to proliferate was also accompanied by defective IFN-γ production. Although WT LNC secreted increasing amounts of IFN-γ following exposure to MOG 35–55, IRAK1-deficient cells exhibited marked impairment in production of this cytokine (Fig. 2B). Cytokines associated with a Th2 response (IL-4 and IL-10) were undetectable in supernatants from either WT or IRAK1-deficient LNC (data not shown). Thus, the absence of Ag-specific proliferation and IFN-γ production in T cells from IRAK1-deficient mice 10 days following immunization indicates that IRAK1 may be required for early T cell priming and development of a Th1 effector response.

**FIGURE 1.** IRAK1-deficient mice are resistant to EAE. C57BL/6 (WT) and B6.IRAK1 (IRAK1 KO) mice (13 mice per group) were immunized with MOG 35–55 emulsified in CFA. Pertussis toxin was injected on days 0 and 2. Clinical signs of EAE were monitored and scored as described in Materials and Methods. Thirteen of 13 B6 mice developed EAE beginning 10 days after immunization. In contrast, only 3 of 13 B6.IRAK1 mice showed signs of EAE, which was both milder and delayed with respect to WT animals. The difference of EAE severity between WT and IRAK1-deficient mice was significant (p < 0.0001). Results are displayed as mean values and error bars represent ±SEM.

**FIGURE 2.** Impaired T cell proliferation and IFN-γ production in IRAK1-deficient mice 10 days after immunization. WT and IRAK1-deficient mice were immunized with MOG 35–55/CFA as previously described. Ten days later, draining LNC were cultured with MOG 35–55. A, Proliferative response to increasing peptide concentrations was determined by [3H]thymidine incorporation. IRAK1-deficient cells exhibit a significantly lower proliferative response to Ag stimulation than WT cells at all doses. B, IFN-γ responses by LNC cultured for 24, 48, and 72 h in the presence of MOG 35–55 (10 μg/ml) were measured by ELISA of supernatants. Cells lacking IRAK1 demonstrate marked impairment in Ag-induced IFN-γ production compared with WT mice. Th2 cytokines (IL-4 and IL-10) were undetectable in supernatants of lymphocytes from both IRAK1-deficient and WT mice. Results shown are mean values and error bars represent ±SEM. *p < 0.01, **p < 0.005.

Similar responses in WT and IRAK1-deficient T cells after prolonged Ag stimulation

Although IRAK1-deficient mice display absent early T cell priming to MOG 35–55/CFA, a few still developed a mild variant of EAE. The delayed onset of mild disease in a subset of IRAK1-deficient animals could be due to prolonged Ag stimulation, which in some systems can overcome initial hyporesponsiveness and result in a near-normal response to Ag (37). We therefore hypothesized that T cells in IRAK1-deficient mice might become activated with longer exposure to the MOG 35–55 Ag. To test this hypothesis, proliferative and IFN-γ responses were examined in LNCs and splenocytes from mice immunized with MOG 35–55/CFA ~1 mo (26–35 days) earlier. As seen in Fig. 3, both T cell proliferation and IFN-γ production were comparable in cells from both the draining lymph nodes and spleens from WT and IRAK1-deficient mice, except for IRAK1-deficient splenocytes, which secreted significantly less IFN-γ at 72 h than WT cells. Moreover, reimmunization of three disease-free IRAK1-deficient mice with MOG 35–55 in IFA 40 days after initial challenge caused grade 2 EAE in one animal. Together, these data suggest that longer or repeated antigenic stimulation may partially overcome defective early T cell activation in IRAK1-deficient mice.
FIGURE 3. Comparable proliferative and IFN-γ responses between WT and IRAK1-deficient mice 4 wk after MOG 35–55/CFA immunization. Twenty-six to 35 days after immunization, draining LNC and splenocytes were cultured in the presence of MOG 35–55. LNC (A) and splenocyte (B) proliferation were determined by measuring [3H]thymidine incorporation as described. There was no difference between the T cell proliferative responses of WT and IRAK1-deficient mice. LNC (C) and splenocyte (D) IFN-γ production were assayed by ELISA. Responses of WT and B6.IRAK LNC were similar at all times, except after 72 h of Ag stimulation where WT splenocytes secreted significantly more IFN-γ than splenocytes from IRAK1-deficient mice. Results are displayed as mean values and error bars represent ±SEM. *, p < 0.0005.

Impaired CpG DNA responsiveness of naive IRAK1-deficient splenocytes

Disease resistance and early T cell hyporesponsiveness in IRAK1-deficient mice could also be due to impaired response to adjuvant. CFA is a complex, ill-defined mixture of mycobacterial components. CpG-containing ODN are synthetic nucleic acids, with well-defined immunostimulatory properties that depend on the presence of specific sequence motifs (38). CpG DNA can also function as an adjuvant to promote development of an adaptive immune response (38). Previous reports have indicated that CpG-containing DNA signals through the Toll/IL-1 pathway (11, 12). The role of IRAK1 in CpG responsiveness, however, has not been examined. Spleens were harvested from unimmunized WT and IRAK1-deficient mice and splenocytes were cultured in the presence of increasing concentrations of oligonucleotide 1826, containing 2 CpG motifs. IL-12 p40 and TNF-α concentrations were measured in 24-hour supernatants using ELISA. As seen in Fig. 4, WT splenocytes respond to CpG-containing DNA beginning at 0.25 μg/ml. Peak responses of both IL-12 p40 (Fig. 4A) and TNF-α (Fig. 4B) production occurred with 1.25 μg/ml CpG ODN. IRAK1-deficient splenocytes, in contrast, display a markedly attenuated response at all doses tested. The responses of naive WT and knockout (KO) splenocytes to nonimmunostimulatory oligonucleotides (1982, negative control) and LPS (positive control) were also compared. As seen in Fig. 4, A and B, neither WT nor IRAK-deficient cells secrete appreciable amounts of TNF-α or IL-12 p40 following treatment with oligonucleotide 1826. Following LPS treatment, in contrast, WT splenocytes produce more TNF-α and IL-12 p40 than IRAK1-deficient cells (Fig. 4, A and B), consistent with previous findings in IRAK1-deficient macrophages (23). Thus, IRAK1, like MyD88, an adapter protein that interacts with this kinase at the activated receptor complex, is required for the response of naive splenocytes to CpG DNA (12).

Complete resistance to EAE and impaired primary immune response in IRAK1-deficient mice immunized with a CpG DNA-containing adjuvant

When immunized with MOG 35–55 in CFA, three B6.IRAK1 mice developed EAE. This heterogeneous could be due to the variability of mutant animals’ responsiveness, although these mice are virtually identical genetically. Alternatively, it could be due to something in the immunization itself, in particular the presence of microbial components in CFA that could trigger autoimmunity via an IRAK1-independent means. CpG oligonucleotides are potent adjuvants for the activation of autoreactive encephalitogenic T cells and EAE induction in vivo (15). Because results from the preceding experiment indicated that CpG DNA signals through IRAK1, the responses of WT and IRAK1-deficient animals to a MOG 35–55 in IFA with CpG ODN was tested.

Both B6 (WT; 15 mice/group) and B6.IRAK1 (KO; 17 mice/group) mice were immunized with MOG 35–55 (200 μg) in IFA with oligonucleotide 1826 (60 μg/mouse) (15). Pertussis toxin (200 ng i.p.) was injected on days 0 and 2. Clinical signs of EAE were monitored and scored as previously described (32). WT mice began to exhibit signs of EAE 10 days after immunization and displayed typical monophasic disease (33). Twelve of 15 WT mice developed EAE and 3 died due to severe disease (Fig. 5). In contrast, no IRAK1-deficient mice developed signs of EAE (Fig. 5). This difference in clinical EAE score between B6 and IRAK1-deficient mice was highly significant (p < 0.0001). Furthermore, four IRAK1-deficient mice were reimmunized with MOG 35–55 and CpG DNA (60 μg) in IFA 40 days after the initial immunization and none developed EAE during the 4 wk after the boost. This finding provides additional support that CpG DNA signals through IRAK1 and demonstrates that IRAK1 is absolutely required for EAE development when CpG DNA is used as an adjuvant.

Early Ag-specific T cell responses were then compared. Ten days after immunization with MOG 35–55/CpG/IFA, LNC from WT and IRAK1-deficient mice were harvested and cultured in the presence of peptide. T cells from WT mice exhibited a dose-dependent proliferative response to increasing Ag concentrations (Fig. 6A). IRAK1-deficient cells, on the other hand, displayed virtually no expansion at all peptide doses (Fig. 6A). Similarly, WT cells produce markedly higher IFN-γ upon MOG 35–55 stimulation compared with LNCs from B6.IRAK1 mice at both 48 and 96 h (Fig. 6B). IFN-γ concentrations were lower in 72-h cultures for WT cells (than at either 48 or 96 h) and thus not different from
IRAK1-deficient LNCs. But at all three time points, Ag-driven IFN-γ production by IRAK1-deficient cells was severely attenuated. IL-4 and IL-10 were undetectable in supernatants from either WT or IRAK1-deficient LNC. These findings parallel those seen in LNC from WT and IRAK1-deficient mice immunized with Ag and CFA as adjuvant and indicate that IRAK1 is required for Ag-specific T cell expansion and Th1 development.

**IRAK1-deficient mice exhibit impaired macrophage CNS infiltration**

The differential sensitivity of WT and IRAK1-deficient mice to EAE induction prompted examination of CNS inflammation in sensitized animals. Spinal cords from WT or IRAK1-deficient mice immunized with MOG 35–55/CFA or MOG 35–55/CpG/IFA and sacrificed 35–38 days postimmunization were stained for infiltrating macrophages, and the number of stained cells was quantified using a Scion image analysis system (Fig. 7, A–G). As seen in Fig. 7A, WT mice immunized with MOG 35–55/CFA exhibit
FIGURE 7. Reduced CNS inflammation in IRAK1-deficient mice. Sections from the lumbar spinal cord were examined for immunohistochemical evidence of infiltrating macrophages on frozen sections (10 μm) of WT mice immunized with MOG 35–55/CFA (A and B), IRAK1-deficient mice immunized with MOG 35–55/CFA (C and D), or IRAK1-deficient mice immunized with MOG 35–55/CpG/IFA (E and F). Magnification ×50 (A, C, and E) and ×400 (B, D, and F). Samples were obtained 35 days after immunization with Ag plus CpG DNA and 38 days after immunization with Ag plus CFA. D, Numbers of infiltrating macrophages (expressed as cells/10^6 square pixels) in spinal cord sections of mice induced to develop EAE. Details are described in Results. Infiltrating cells were quantified as described. The mean number of infiltrating cells are depicted, and error bars represent ±SEM. **, p < 0.001 compared with WT.
marked macrophage infiltration into the CNS. WT animals challenged with MOG 35–55/CpG/IFA also exhibited pronounced CNS inflammation, although there was no significant difference in MOM2-positive cells between WT animals immunized with Ag plus CFA and those immunized with Ag plus CpG/IFA (data not shown). IRAK1-deficient mice, in contrast, exhibit marked impairment in recruitment of macrophages to CNS lesions when compared with WT mice. The number of lesion-associated macrophages was reduced by 85% in IRAK1-deficient mice immunized with MOG 35–55/CFA compared with WT mice (Fig. 7, A and C; p < 0.001). Furthermore, macrophages were virtually absent from the CNS of IRAK1-deficient mice immunized with MOG 35–55/CpG/IFA (Fig. 7, E and F), with a 99% reduction in macrophages in IRAK1-deficient compared with WT spinal cords (Fig. 7, A and F; p < 0.001)

The number of infiltrating macrophages also differed significantly between IRAK1-deficient mice depending on the adjuvant used for immunization. IRAK1-deficient mice immunized using the CpG/IFA adjuvant exhibited a 94% reduction in infiltrating macrophages compared with IRAK1-deficient mice immunized using CFA (Fig. 7, C and E; p < 0.05). Thus, IRAK1 is critical to the development of CNS inflammation in EAE induced with CFA, but it is absolutely required when CpG DNA is used as an adjuvant.

**Impaired memory T cell IFN-γ production but normal proliferation in IRAK1-deficient mice immunized with a CpG DNA-containing adjuvant**

Memory responses were similar in WT and IRAK1-deficient T cells from mice immunized with CFA as an adjuvant. This lack of difference could be explained by the presence of mycobacterial determinants that exert an adjuvant effect independent of IRAK1. Alternatively, it could be due to the ability of prolonged Ag stimulation to override the effect of IRAK1 deficiency. To examine this latter possibility, the memory T cell responses in WT and IRAK1-deficient mice were compared. B6 and B6.IRAK1 animals were immunized with MOG 35–55/CpG/IFA as before. Thirty days later, draining LNC and splenocytes were harvested and cultured in the presence of MOG 35–55. Proliferative responses of LNC from WT and IRAK1-deficient mice were similar (Fig. 8A), as were those of cultured spleen cells (Fig. 8C). IFN-γ production, in contrast, was impaired in IRAK1-deficient LNC (Fig. 8B). This difference was statistically significant (p < 0.005). Cytokine production by mutant splenocytes was also attenuated in comparison to WT cells, although the differences were more noticeable at 24 and 48 h (Fig. 8D). These data indicate that while IRAK1 may have little direct effect on Ag-specific memory T cell proliferation, it contributes to Ag-stimulated IFN-γ production in these same cells.

**IRA1K1-deficient T cells respond normally to TCR activation**

Impaired T cell responses in IRAK1-deficient mice could be due to one or more defects, including impaired Ag presentation to T cells, defective T cell responsiveness to presented Ag, or attenuated sensitivity of T cells to cytokines critical to the development of effector response. Recent reports indicate a role for receptor-interacting protein (Rip) 2, a death domain-containing serine-threonine kinase involved in TNFR family signaling, in TCR signaling; Rip2-deficient T cells exhibit impaired responses to TCR activation (39). To determine whether IRAK1-deficient T cells exhibit analogous defects in response to TCR activation, purified T cells from immunized WT and IRAK1-deficient mice were stimulated with anti-CD3 and anti-CD28 Abs or anti-CD3 Ab in the presence of irradiated WT splenocytes. There were no differences in T cell proliferation and IFN-γ secretion to anti-CD3 and anti-CD28 Ab stimulation between WT and IRAK1-deficient mice (Fig. 9, A and C). Similarly, proliferation of WT and IRAK1-deficient T cells treated with anti-CD3 and WT APC was comparable (Fig. 9B). Furthermore, WT and IRAK1-deficient T cells stimulated with anti-CD3 Ab in the presence of either WT or KO irradiated APCs produce equivalent amounts of IFN-γ (Fig. 9D). These data demonstrate that IRAK1 deficiency does not affect T cell responses to

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**FIGURE 8.** Impaired memory IFN-γ production in IRAK1-deficient T cells. Four to 5 wk after immunization with MOG 35–55/CpG/IFA, draining LNC and splenocytes were cultured in the presence of MOG 35–55. LNC (A) and splenocyte (B) proliferation were determined by measuring [3H]thymidine incorporation as described. Proliferative responses were similar in either LN or splenocytes derived from WT or IRAK1-deficient mice. IFN-γ production by LNC (C) and splenocytes (D) was determined using ELISA. IRAK1-deficient LNC exhibit markedly impaired IFN-γ production in response to Ag stimulation compared with LNC from B6 mice. Splenocytes lacking IRAK1 also display impaired IFN-γ production after 24 and 48 h of stimulation, but produce as much cytokine as WT splenocytes after 72 h of Ag exposure. Results are displayed as mean values and error bars represent ± SEM. *p < 0.005.
FIGURE 9. Normal TCR-triggered responses in IRAK1-deficient cells. A. Proliferative responses of WT and IRAK1-deficient T cells stimulated with anti-CD3 and anti-CD28 Abs. WT and IRAK1-deficient T cells were purified as described and cultured in the presence of increasing concentrations of anti-CD3 and anti-CD28 Abs. B. WT and IRAK1-deficient T cell proliferative responses to TCR stimulation in the presence of WT APC. APC were isolated from WT and irradiated as described. These were cocultured with either WT or IRAK1-deficient T cells stimulated with anti-CD3 and CD28 Abs as previously described (both 10 μg/ml), and IFN-γ production was measured by ELISA at the indicated times. C. TCR-triggered IFN-γ production by WT and IRAK1-deficient T cells. T cells were collected and stimulated with anti-CD3 and CD28 Abs as previously described (both 10 μg/ml), and IFN-γ production was measured by ELISA at the indicated times. D. IFN-γ response to TCR stimulation in the presence of APC. Naive WT or IRAK1-deficient splenocytes were isolated, irradiated and cocultured with either WT or IRAK1-deficient T cells treated with anti-CD3 Ab (10 μg/ml). IFN-γ concentration was determined at the indicated times by ELISA. Results are shown as mean values and error bars represent ±SEM.

Discussion

These results demonstrate an absolute requirement for IRAK1 in the development of EAE, a prototypic, organ-specific autoimmune disease. Moreover, they also suggest that IRAK1, like MyD88, may be required for a Th1 response (10). Immunization of mice with Ag plus IFA alone fails to induce EAE (40, 41), whereas addition of CpG ODN causes severe disease in C57BL/6 mice and restores a Th1 response. IRAK1-deficient mice with the same genetic background, in contrast, were completely resistant to EAE. Thus, EAE induced by Ag plus CpG ODN is completely IRAK1-dependent, reinforcing the in vitro splenocyte data that IRAK1 is required for host responsiveness to CpG DNA. Parallel experiments using CFA as an adjuvant yielded analogous results, but three IRAK-deficient animals developed a variant of disease with later onset. Development of EAE in these animals does not exclude a role for IRAK in autoimmunity, but rather suggests that CFA may contain components that induce autoimmune disease via an IRAK-independent mechanism.

Although the findings presented in this study establish IRAK1 as critical to EAE development, how it does so is still unknown. IRAK1 may operate at a discrete step or in a single-cell type in disease development, such as in APCs during Ag presentation. Conversely, autoimmunity may result from IRAK1 involvement at multiple stages. IRAK1-deficient T cells exhibit impaired priming, failing to proliferate or secrete IFN-γ in response to Ag, regardless of the adjuvant used. This particular defect may be due to IRAK1 function downstream of the TLRs in APCs, as IL-18-deficient T cells still exhibit Ag-driven proliferation under similar conditions (17), and IL-1 is dispensable for development of a Th1 response in C57BL/6 mice (42). Inadequate costimulatory molecule induction in IRAK1-deficient APCs could, for example, prevent selection and expansion of MOG 35–55-specific T cells. The disease resistance of IRAK1-deficient mice to rechallenge with Ag further supports the existence of a priming defect.

Whether impaired Th1 development in IRAK1-deficient mice is sufficient to block disease remains to be determined. Lower or absent IL-12p40 expression due to diminished CpG-induced IL-12 production could further exacerbate IL-18 hyporesponsiveness already present in IRAK1-deficient cells (27, 28) thereby preventing development of an effector response. Impaired IL-18 responsiveness (17, 27) could lead to insufficient IFN-γ production by NK cells to cause clinically apparent disease (17). It is also possible that the cumulative impact of multiple defects at several steps in the developing immune response causes disease resistance in IRAK1-deficient mice. Moreover, chronic IRAK1 deficiency may produce an unexpected phenotype, such as a reduced starting pool of autoreactive T cells.

IRAK1 deficiency may protect animals against disease through an impaired IL-12 response, although the essential role of this cytokine in EAE induction has been questioned (43). Naive splenocytes lacking IRAK1 display defective CpG responsiveness, as determined by impaired IL-12 p40 (and TNF-α) production. This lower IL-12 response could have both direct and indirect effects on subsequent EAE development. IL-12 is a direct inducer of Th1 responses and has a direct effect on IFN-γ production by both NK and Th1 cells (19, 44–50). Lower IL-12 production by IRAK1-deficient APC and effector cells could impair autoreactive T cell development and expansion. Second, IL-12 induces expression of the receptor for IL-18 (51), which synergizes with IL-12 for IFN-γ production in Th1 and NK cells (52, 53). Diminished or absent IL-18R expression due to attenuated IL-12 production could further exacerbate IL-18 hyporesponsiveness already present

TCR stimulation. Moreover, irradiated IRAK1-deficient APC can also provide adequate costimulatory signals to T cells.
in IRAK1-deficient cells (27, 28), thereby preventing development of an effector response.

Recent reports indicate that Rip2 is required for signaling through both TLRs and TCRs (39). This finding prompted us to determine whether IRAK1, another death domain-containing serine-threonine kinase, might also be involved in TCR-triggered responses. Our data show that WT and IRAK1-deficient T cells have comparable responses to TCR stimulation. This occurs independently of how a costimulatory signal is delivered, whether by anti-CD28 stimulation or by coculture with irradiated splenocytes. In fact, both irradiated WT and IRAK1-deficient splenocytes can provide costimulatory signals to T cells. Appropriate costimulation by KO APC occurs despite the fact that these cells exhibit impaired CpG DNA-induced IL-12 production, a crucial stimulus for T cell differentiation. It is possible that irradiation induces an appropriate costimulatory signal in APC via an IRAK1-independent route. Thus, the muted responses of IRAK1-deficient T cells to MOG 35–55 and resistance of IRAK1-deficient mice to EAE are not due to intrinsic TCR-mediated defects in T cells lacking IRAK1.

These studies establish this model system as a useful tool to examine the contributions of established and putative Toll/IL-1 signaling pathway members to autoimmunity. Use of a simplified adjuvant that signals through TLR9 obviates the need for CFA, a complex ill-defined mixture of microbial-derived immunostimulatory molecules. This has at least two important implications for future research. First, it provides a platform from which to assess the roles of different pathway molecules and their mutant forms in a model disease process. For example, based on existing data, one would expect that MyD88 KO mice would also be resistant to EAE (12). On the other hand, disease resistance in mice expressing a catalytically inactive form of IRAK would indicate a requirement for IRAK catalytic activity in the development of autoimmunity. Second, use of a TLR-specific adjuvant system also permits investigation of the participation of Toll/IL-1 signaling in mouse models of other autoimmune diseases such as rheumatoid arthritis, myasthenia gravis, or postinfectious carditis.

Finally, these findings may help shed light on the relationship between infection and autoimmunity. Although the association between antecedent infection—with organisms that activate IRAK1 and the Toll/IL-1 pathway—and autoimmunity is well established, a mechanistic link between Toll/IL-1 signaling and human autoimmune disease has yet to be elucidated. In some instances, such as rheumatic fever or Lyme arthritis, the connections between specific pathogen and subsequent autoimmunity are particularly strong (54, 55). In other diseases, for example MS or ankylosing spondylitis, the precise connection between infection and autoimmune disease has proven more difficult to establish (56, 57). Activation and expansion of autoreactive lymphocytes, however, are essential shared features of many autoimmune diseases (58). In this regard, IRAK1 and Toll/IL-1 signaling may play a central role. Triggering of this pathway by determinants from a broad class of pathogens (CpG DNA, for example) could lead to activation of autoreactive lymphocytes while bypassing a requirement that microbial Ags structurally resemble the endogenous Ag recognized by the adaptive immune response. Furthermore, persistent or recurrent activation of IRAK1 and Toll/IL-1 signaling by different infectious and inflammatory stimuli could help explain the genesis of epitope spreading and the relapsing and remitting nature of some autoimmune diseases. Until more is known about functional polymorphisms in pathway components and barring serendipitous findings, however, the links between Toll/IL-1 signaling and human autoimmunity remain speculative. Confirmation of a role for this pathway in autoimmunity may ultimately provide the basis for selective IRAK1 inhibition that could decrease the frequency of relapses and slow the progression of some autoimmune diseases, such as MS.

References


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