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Aspirin ameliorates experimental autoimmune encephalomyelitis through interleukin-11–mediated protection of regulatory T cells

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Abstract

Multiple sclerosis (MS) is a human disease that results from autoimmune T cells targeting myelin protein that is expressed within the central nervous system. In MS, the number of FoxP3-expressing regulatory T cells (T_{regs}) is reduced, which facilitates the activation of autoreactive T cells. Because aspirin (acetylsalicylic acid) is the most widely used nonsteroidal anti-inflammatory drug, we examined its immunomodulatory effect in mice with experimental autoimmune encephalomyelitis (EAE), an animal model of MS. We found that low-dose aspirin suppressed the clinical symptoms of EAE in mouse models of both relapsing-remitting and chronic disease. Aspirin reduced the development of EAE driven by myelin basic protein (MBP)–specific T cells and the associated perivascular cuffing, inflammation, and demyelination. The effects of aspirin required the presence of CD25⁺FoxP3⁺ T_{regs}. Aspirin increased the amounts of Foxp3 and interleukin-4 (IL-4) in T cells and suppressed the differentiation of naïve T cells into T helper 17 (T_H17) and T_H1 cells. Aspirin also increased the transcription of IL11 mediated by the transcription factor CREB, which was necessary for the generation of T_{regs}. Neutralization of IL-11 negated the effects of aspirin on T_{reg} development and exacerbated EAE. Furthermore, we found that IL-11 alone was sufficient to maintain the percentage of FoxP3⁺ T_{regs} and protect mice from EAE. These results identify a previously uncharacterized mode of action of aspirin.

INTRODUCTION

Multiple sclerosis (MS) is a debilitating disease in adults. Although the etiology of MS is not completely understood, in patients with MS, a T cell–mediated autoimmune response that targets the central nervous system (CNS) results in demyelination and associated disability (1). A misguided and overactive immune response against myelin and nonmyelin

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SUPPLEMENTARY MATERIALS

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antigens may underlie the development of MS (2–4). Whereas regulatory T cells (T_{regs}) suppress the activation and proliferation of self-reactive T cells under normal conditions (5–7), during MS disease, there is a substantial decrease in the activity and the number of T_{regs} . This may lead to the proliferation of self-reactive T cells and subsequent autoimmune attack (3, 4, 6, 8). Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS and is associated with a deficiency in T_{reg} numbers and function (9–11). Accordingly, T_{regs} play a critical role in protection and recovery from EAE (12). Therefore, maintenance and/or protection of T_{regs} under autoimmune conditions is an important area of research.

Aspirin, also known as acetylsalicylic acid, is one of the most widely used medications in the world. A nonsteroidal anti-inflammatory drug, it is often used as an analgesic to relieve minor aches and pains. It also effectively reduces both fever and inflammation. Here, we found that oral low-dose aspirin was capable of ameliorating both relapsing-remitting EAE (RR-EAE) and chronic EAE. Aspirin treatment promoted the development of T_{regs} but suppressed T helper 1 (T_{H1}) and T_{H17} responses in vitro and inhibited the encephalitogenicity of T cells in vivo. Because interleukin-11 (IL-11) is an immunoregulatory cytokine with anti-autoimmune properties (13, 14) and inhibits the activation of nuclear factor κB (NF- κB) and inflammation (15), we examined whether aspirin affected IL-11. We found that IL-11 alone was also sufficient to increase the frequency of T_{regs} in EAE and that aspirin rapidly increased CREB [cAMP (adenosine 3', 5'-monophosphate) response element-binding protein]-mediated IL-11 to promote T_{reg} development. Accordingly, IL-11-neutralizing antibodies inhibited the effects of aspirin on T_{regs} and abrogated aspirin-mediated protection from EAE in mice. These findings raise a possibility that low-dose aspirin may find further application in MS and other autoimmune disorders.

RESULTS

Low-dose aspirin inhibits the development of EAE in multiple mouse models

We examined the effect of aspirin on RR-EAE induced in female SJL/J mice by adoptive transfer of myelin basic protein (MBP)-primed T cells (16–18). When we treated these EAE mice with aspirin by oral gavage starting at 8 days after T cell transfer (dpt), we found that even at the lowest dose, aspirin significantly inhibited clinical symptoms of EAE (Fig. 1A). Stronger inhibition of clinical EAE symptoms was observed in acute as well as relapse phases of EAE at a dose of 2 mg/kg body weight per day (Fig. 1A). Whereas female SJL/J mice were used to induce RR-EAE, the chronic form of EAE was modeled in male C57/BL6 mice after immunization with MOG35–55 peptide. When we examined the efficacy of aspirin in this model of chronic EAE, we found that similar to its effect on RR-EAE in female SJL/J mice, aspirin significantly inhibited the clinical symptoms of EAE (Fig. 1B).

Because MBP-primed T cells are encephalitogenic and adoptive transfer of these T cells induces EAE, we examined whether aspirin exposure was sufficient to inhibit EAE development in this transfer model. T cells from the lymph nodes of MBP-primed mice were stimulated with MBP in the presence of aspirin, and these aspirin-treated MBP-primed T cells were adoptively transferred to recipient mice. The data showed that mice that received aspirin-treated MBP-primed T cells exhibited significantly reduced clinical symptoms and

disease severity when compared to mice that received only MBP-primed T cells (Fig. 1C). Even at the lowest dose, aspirin strongly inhibited the encephalitogenic property of MBP-primed T cells (Fig. 1C). Sometimes, the effects caused by in vitro treatment may not be maintained for a long time after transfer. However, in this case, aspirin-treated MBP-primed T cells remained unable to induce clinical symptoms of EAE in acute as well as chronic phases of EAE (Fig. 1C).

Low-dose aspirin suppresses spinal cord demyelination and immune cell infiltration in mice with EAE

EAE and MS are associated with infiltration of autoreactive T cells and mononuclear phagocyte cells into the CNS. Therefore, we investigated whether low-dose aspirin treatment attenuated immune cell infiltration and demyelination in mice with RR-EAE. As expected, we found widespread infiltration of inflammatory cells into the spinal cords of mice that had RR-EAE by hematoxylin and eosin (H&E) staining (Fig. 2A). However, aspirin treatment markedly inhibited the infiltration of inflammatory cells into the spinal cord of RR-EAE mice (Fig. 2A). Blinded quantitation of spinal cord histology indicated that aspirin reduced the appearance of cuffed vessels and immune cell infiltration in spinal cord of mice with RR-EAE (Fig. 2, B and C). We also examined whether aspirin treatment reduced the expression of proinflammatory mediators in the spinal cord of RR-EAE mice by reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR. We observed marked expression of proinflammatory molecules like *iNOS* and *IL-1 β* in spinal cord tissue from untreated RR-EAE mice when compared to control mice (fig. S1A and Fig. 2, D and E). However, aspirin treatment markedly suppressed the mRNA expression of *iNOS* and *IL-1 β* in spinal cord tissue from mice with RR-EAE (fig. S1A and Fig. 2, D and E).

Although the mechanisms that lead to demyelination in MS are poorly understood, the infiltration of blood mononuclear cells and associated neuroinflammation may promote CNS demyelination. To examine demyelination, we stained spinal cord sections with luxol fast blue, which reacts with myelin. We observed widespread demyelination zones in the white matter in mice with EAE (Fig. 2, F and G). However, aspirin treatment prevented the loss of myelin in the spinal cords of mice with RR-EAE (Fig. 2, F and G). We found similar results when we monitored spinal cord tissue from mice with EAE for mRNA expression of *MBP*, *MOG*, and *PLP* myelin-specific molecules by qRT-PCR (fig. S1B and Fig. 2, H and I). Together, these results indicated that aspirin reduced mononuclear cell infiltration, inflammation, and demyelination in mice with EAE.

Aspirin prevents loss of T_{regs} in MBP-primed splenocytes and in EAE mice

To understand how aspirin protects from EAE disease, we examined the effect of aspirin on T_{regs}, an inhibitory T lymphocyte subset characterized by the transcription factor FoxP3 (8) that protects mice from EAE (19, 20). We found that MBP priming reduced the expression of FoxP3 when compared to control splenocytes (fig. S2). Because FoxP3⁺ T_{regs} usually express CD25, CTLA4, and CD62L, we also analyzed the mRNA expression of these molecules. Similar to *Foxp3*, the mRNA expression of *CD25*, *CD62L*, and *CTLA4* also decreased in MBP-primed splenocytes as compared to unstimulated splenocytes (fig. S2).

However, aspirin treatment prevented the loss of *Foxp3*, *CD25*, *CD62L*, and *CTLA4* (fig. S2 and Fig. 3, A and B). However, neither the MBP priming nor aspirin treatment had an effect on the mRNA expression of *CD4* (fig. S2), which suggests that these results are not due to global changes in the frequency of CD4⁺ T cells. When we performed fluorescence-activated cell sorting (FACS) analysis, we found a significant reduction in the frequency of Foxp3⁺CD4⁺ and CD25⁺CD4⁺ T cells in MBP-primed splenocytes when compared to unstimulated splenocytes (Fig. 3, C and D). However, aspirin treatment prevented this loss in the Foxp3⁺CD4⁺ and CD25⁺CD4⁺ populations (Fig. 3, C and D). Analysis of the mean fluorescence intensity (MFI) of Foxp3⁺ and CD25⁺ populations also indicated that aspirin prevented the activation-induced loss of T_{regs} (Fig. 3, E and F).

We examined the effects of aspirin treatment in mice with EAE that received aspirin from 8 to 16 dpt. We found that there was a significant reduction in frequency and MFI of the Foxp3⁺CD4⁺ population of T cells in splenocytes from mice EAE when compared to control splenocytes. However, aspirin treatment of mice with EAE increased the frequency of the Foxp3⁺CD4⁺ population in splenocytes (Fig. 3, G and H). Therefore, we investigated whether aspirin protected mice from clinical symptoms of EAE through its effects on T_{regs} by depleting T_{regs} in mice with an antibody against CD25. Whereas aspirin ameliorated the clinical symptoms of RR-EAE, T_{reg} depletion with anti-CD25 markedly abrogated the protective effects of aspirin on EAE in mice (Fig. 3I). In contrast, control immunoglobulin G (IgG) had no such effect. These results implied that T_{regs} play an important role in how aspirin protects from EAE disease.

Aspirin suppressed the development of T_H17 and T_H1 responses

T_H17 cells may play a more active role than T_H1 cells in the disease process of MS and EAE (21, 22). Our data indicated that aspirin treatment promoted the development of T_{regs} (Fig. 3 and fig. S2), which inhibit T_H17 cell development (23). Therefore, we examined how aspirin affected T_H17 cell development. In addition to the trademark cytokine IL-17, T_H17 cells are also characterized by the transcription factor ROR γ T. Whereas MBP priming increased the mRNA expression of *ROR γ t* and *IL-17* (fig. S3 and Fig. 4, A and B) and enriched CD4⁺ROR γ t⁺ (Fig. 4, C and E) and CD4⁺IL-17⁺ (Fig. 4, D and F) T cell populations in splenocytes, aspirin markedly suppressed antigen-induced expression of *ROR γ t* and *IL-17* mRNAs (fig. S3 and Fig. 4, A and B) as well as CD4⁺ROR γ t⁺ (Fig. 4, C and E) and CD4⁺IL-17⁺ (Fig. 4, D and F) cells. These results indicated that aspirin suppressed the development of T_H17 cells.

Similar to T_H17 cells, T_H1 cells are also inflammatory and are characterized by expression of interferon γ (IFN γ) and transcription factor Tbet. In contrast, T_H2 cells are anti-inflammatory and display GATA3-dependent IL-10 and IL-4 release. When we stimulated splenocytes from MBP-primed mice in vitro with MBP, we found that antigen activation increased the mRNA expression of *Tbet* (figs. S3 and S4A) but decreased the mRNA expression of *Gata3* (figs. S3 and S4B) when compared to MBP stimulation of splenocytes from unprimed mice. Similarly, antigen activation increased the frequency of CD4⁺Tbet⁺ (fig. S4C and Fig. 4G) and CD4⁺IFN γ ⁺ cells (fig. S4D and Fig. 4H) but decreased the frequency of CD4⁺IL-4⁺ (fig. S4E and Fig. 4I) cells. However, aspirin treatment prevented

increased expression of *Tbet* (figs. S3 and S4A) and reduced the frequency of CD4⁺Tbet⁺ (fig. S4C and Fig. 4G) and CD4⁺IFN γ ⁺ (fig. S4D and Fig. 4H) T cell populations after antigen stimulation of MBP-primed splenocytes. In contrast, aspirin treatment prevented the loss of *Gata3* mRNA expression (figs. S3 and S4B) and increased the frequency of CD4⁺IL-4⁺ T cells (fig. S4E and Fig. 4I) in antigen-activated splenocytes. Together, these results suggested that aspirin prevents the increased T_H1 response and decreased T_H2 response stimulated by antigen activation.

Aspirin increased the CREB-mediated transcription of IL-11 in splenocytes

Because aspirin increased IL-11 abundance in astrocytes (24), we tested whether aspirin stimulated IL-11 expression in splenocytes. We found that aspirin dose-dependently increased the mRNA expression of *IL-11* in normal splenocytes (fig. S5A and Fig. 5A). Aspirin promoted *IL-11* mRNA expression in normal splenocytes from 4 hours after treatment and reached the maximum induction at 6 hours (fig. S5B and Fig. 5B). Western blot analysis indicated that IL-11 protein abundance was also increased by aspirin treatment of splenocytes (Fig. 5, C and D). In addition, immunofluorescence microscopy showed that aspirin increased the amount of IL-11 in CD3⁺ T cells (Fig. 5E). When we examined the effect of aspirin on the induction of IL-11 in MBP-primed splenocytes, we found that aspirin prevented the marked reduction in *IL-11* mRNA expression that we observed after stimulation of MBP-primed splenocytes (fig. S5C and Fig. 5F). Using MatInspector analysis of the *IL-11* gene promoter, we found the presence of a consensus cAMP response element (CRE) (fig. S6A). Therefore, we examined whether aspirin stimulated CREB-mediated transcription of IL-11. By Western blot, we found that aspirin stimulated the phosphorylation of CREB (Fig. 5H and fig. S6B). In contrast, aspirin did not increase the amount of total CREB (Fig. 5G). When we knocked down CREB expression in splenocytes using small interfering RNA (siRNA), we found that reduced expression of CREB prevented aspirin-stimulated IL-11 production (Fig. 5H and fig. S6C). These data suggested that aspirin stimulated IL-11 production by splenocytes through CREB. To investigate whether CREB was directly involved in the transcription of the *IL-11* gene in aspirin-treated splenocytes, we examined the recruitment of CREB to the *IL-11* gene promoter in aspirin-treated splenocytes by chromatin immunoprecipitation (ChIP) assay (fig. S6A). After immunoprecipitation of aspirin-treated splenocyte chromatin fragments using antibodies against CREB, we were also able to amplify 199-base pair fragments from the *IL-11* gene promoter (fig. S6D and Fig. 5I). Because CREB-binding protein (CBP), a histone acetyltransferase, plays an important role in multiple CREB-mediated transcriptional activities, we investigated whether CBP was also involved in aspirin-induced transcription of the *IL-11* gene. We found that aspirin treatment induced the recruitment of CBP to the *IL-11* gene promoter (Fig. 5I and fig. S6D). In contrast, aspirin did not recruit another histone acetyltransferase, p300, to the CRE of *IL-11* promoter (fig. S6D and Fig. 5I), which indicated that the recruitment of CBP may be specific. Consistent with the recruitment of CREB and CBP to the *IL-11* gene promoter, aspirin also re-recruited RNA polymerase to the promoter of *IL-11* gene (fig. S6D and Fig. 5I).

Low-dose aspirin stimulates IL-11 to promote T_{regs} and protect mice from EAE

Because aspirin rapidly increased expression of IL-11 (Fig. 5A), we examined whether IL-11 was also involved in aspirin-mediated protection of mice with EAE. We found that IL-11, which was present in splenic sections of control mice, was markedly decreased in mice with EAE (fig. S7, A and B). However, aspirin treatment prevented the loss of IL-11 in spleens of mice with EAE (fig. S7, A and B). These results were further confirmed by Western blot analysis of splenic homogenates (fig. S7, C and D). Additionally, when we used an IL-11–neutralizing antibody in mice that received MBP-primed T cells, we found that blocking IL-11 abrogated the protective effect of aspirin on EAE disease development (Fig. 5J). This result was specific because control IgG had no such effect (Fig. 5J). These results suggested that aspirin protected mice from EAE through IL-11.

Strong induction of T_{regs} occurs independently of gp130-mediated signaling in other models of EAE (25). We examined whether IL-11 played any role in aspirin-mediated protection of T_{regs}. Whereas antigen stimulation decreased the mRNA expression of *Foxp3*, *CD62L*, and *CD25* in MBP-primed splenocytes, aspirin treatment prevented the loss of these T_{reg} markers (fig. S8A and Fig. 6, A and B). Anti-IL-11–neutralizing antibodies, but not control IgG, abrogated the protective effect of aspirin on the expression of *Foxp3*, *CD62L*, and *CD25* in antigen-stimulated MBP-primed splenocytes (fig. S8A and Fig. 6, A and B). These results were specific because neither MBP stimulation, aspirin treatment, nor neutralization of IL-11 had an effect on the expression of CD4 (fig. S8A and Fig. 6, A and B). FACS dot plot for Foxp3 and CD4 (Fig. 6C) and MFI analysis of Foxp3 (Fig. 6D) also confirmed that aspirin-stimulated IL-11 promoted the expression of Foxp3 and increased the frequency of T_{regs} after antigen stimulation.

Because IL-11 was necessary for the effects of aspirin on T_{regs}, we examined whether IL-11 alone was sufficient to promote the differentiation of T_{regs}. In *in vitro* studies, we stimulated splenocytes isolated from MBP-immunized mice with MBP in the presence of recombinant mouse IL-11. By RT-PCR (fig. S8B), real-time PCR (Fig. 7, A and B), flow cytometry (Fig. 7C), and MFI analysis (Fig. 7D), we found that IL-11 alone increased *Foxp3*, *CD25*, and *CD62L* mRNA expression and the frequency of CD4⁺Foxp3⁺ T cells in antigen-activated MBP-primed splenocytes. We also treated mice with EAE with recombinant mouse IL-11 (50 ng per mouse) for 8 days after disease induction (Fig. 7, E and F). As in our earlier studies, we found that aspirin restored T_{reg} frequency in mice with EAE and IL-11–neutralizing antibodies negated this effect of aspirin (Fig. 7, E and F). Although aspirin may induce many other molecules, IL-11 alone prevented the loss of the Foxp3⁺CD4⁺ T cell populations in EAE mice (Fig. 7, E and F). These data indicated that IL-11 alone was sufficient to increase T_{regs} in EAE mice.

DISCUSSION

Although few drugs are available, it is important to identify a safer and an effective drug for MS. Aspirin is one of the most widely used drugs throughout the world for various purposes. On the one hand, it is a household analgesic for controlling pain, fever, and inflammation; on the other hand, it is also used to lower heart attack and stroke in patients with cardiovascular disease. It has been reported that aspirin can also reduce the incidence of cancer and cancer

mortality, especially in gastrointestinal cancers (26, 27). Aspirin increases the production of ciliary neurotrophic factor from astrocytes and that applying the supernatant of aspirin-treated wild-type, but not *Cntf*^{-/-}, astrocytes to oligodendrocytes increases the abundance of myelin-associated proteins and protected oligodendrocytes from tumor necrosis factor- α (TNF- α) insult (28). Here, we describe a new function of aspirin in which this drug inhibits the autoimmune disease EAE. Oral use of low-dose aspirin reduced the progression of both adoptively transferred and chronic EAE in mice. Aspirin treatment of mice with EAE also inhibited the invasion of mononuclear cells into the spinal cord as well as the expression of inflammatory molecules [inducible nitric oxide synthase (iNOS) and IL-1 β] and restored myelination and the expression of myelin-specific genes within the CNS. A recent double-blind randomized controlled pilot trial suggests that aspirin may represent an effective pretreatment for exercise in MS (29). Fatigue is very common in MS, and low-dose aspirin is also being considered for treating MS-related fatigues (30). Here, our preclinical data suggest that low-dose aspirin may be repurposed for disease modification in MS patients.

Because there is a significant decrease in the number of CD4⁺Foxp3⁺ T cells as well as the expression of Foxp3 in RR-MS and other autoimmune disorders (12, 31, 32), preventing the loss of T_{regs} may be beneficial. Although how the T_{regs} are lost during an autoimmune insult is poorly understood, our data suggest that aspirin may protect Foxp3⁺ T_{reg} stability. Whereas MBP antigen stimulation reduced the expression of Foxp3 in splenocytes (33), aspirin markedly inhibited the loss of Foxp3, which preserved CD4⁺Foxp3⁺ T cells after antigen stimulation of MBP-primed splenocytes. Foxp3⁺ T_{regs} were also characterized by CD25, CD62L, and CTLA4. Accordingly, we found that antigen stimulation promotes loss of CD25, CD62L, and CTLA4 and a reduction in the CD4⁺CD25⁺ T cell population in MBP-primed splenocytes when compared to unstimulated splenocytes. Again, aspirin treatment prevented the loss of CD25, CD62L, and CTLA4 and protected CD4⁺CD25⁺ T cell population in MBP-primed splenocytes. The unperturbed expression of CD4 suggests that either loss of T_{regs} after MBP recall may not be due to any reduction of CD4⁺ cells. T_{regs} are important for the suppression of autoreactive T cells (3, 6). Accordingly, aspirin inhibited the differentiation of T_H17 and T_H1 cells and shifted the balance toward a T_H2 response. Although IL-11 may induce T_H17 responses in patients with early RR-MS (34), this study stimulated peripheral blood mononuclear cells (PBMCs) with nonspecific immune stimuli such as phorbol 12-myristate 13-acetate and ionomycin. Thus, it remains to be determined whether IL-11 has a similar function in mice and humans.

How does aspirin prevent the loss of T_{regs}? IL-11, a member of the gp130 family of cytokines, is an immunomodulatory and neuro protective molecule (15, 35). It is expressed in PBMCs as well as brain cells such as astrocytes. IL-11 has been reported to inhibit T_H1 polarization by exerting a direct effect on human T lymphocytes and by reducing IL-12 production by macrophages (25). By inhibiting the activation of NF- κ B, it is also known to exert anti-inflammatory effect in macrophages (15). In the CNS, IL-11 may have a promyelinating effect, where myelin-producing cells (oligodendrocytes) express the receptor (IL-11R α) of IL-11 (14, 36). Our data suggested that aspirin alone was capable of up-regulating IL-11 in splenocytes. Because aspirin is a well-known cyclooxygenase 2 (COX2) inhibitor, this increase in IL-11 by aspirin suggests that other COX2 inhibitors may also increase IL-11 in splenocytes. Moreover, here, we have described an unexpected function of

IL-11, where IL-11 protects T_{regs} from autoimmune insult. Accordingly, aspirin treatment preserves T_{regs} and attenuates the disease process of EAE by increasing expression of IL-11. In contrast, loss of gp130 signaling increases the frequency of Foxp3⁺ T_{regs} and reduces EAE disease after immunization with myelin oligodendrocyte glycoprotein (MOG) peptide with complete Freund's adjuvant (25). Thus, it will be important to further understand the role of IL-11 in MS patients. However, autoimmune insults also reduce Foxp3 expression and inhibit T_{reg} activity through induction of nitric oxide (NO) production (33, 37). Additionally, recombinant human IL-11 markedly suppresses bacterial lipopolysaccharide-induced NO production from macrophages (35). Therefore, it is possible that IL-11 prevented conversion of T_{regs} in EAE mice by suppressing the production of NO.

There are several advantages of aspirin over other available therapies for MS. Aspirin has been widely used as an analgesic throughout the world for decades with well-known safety profile. Because it is an oral drug, it can be taken through the least painful route. Although at high doses aspirin is reported to exhibit some toxic effects (gastric ulcers, stomach bleeding, tinnitus, etc.) (38), in our study aspirin suppressed the disease process of EAE at low doses (1 and 2 mg/kg body weight per day). A single pill of baby aspirin containing 81 mg of aspirin is considered to be very much safe for adults for daily use, and the doses we used in mice are almost equivalent to baby aspirin. Moreover, it is possible to avoid the degradation of aspirin in the stomach by using enteric-coated tablets available for oral use. In an open randomized trial, low-dose aspirin in slow-releasing formulation showed efficacy as an antiplatelet agent (39) without much noticeable side effects. According to Laudanno *et al.* (40), *S*-adenosylmethionine, an amino acid naturally formed in the body, is capable of reducing large dose of aspirin (1300 mg)-mediated stomach damage by 90%. Although mouse results are not always translated to human, our results highlight an undiscovered property of aspirin and suggest the possibility that low-dose aspirin may be repurposed for therapeutic intervention in MS and other demyelinating conditions as an adjunct therapy.

MATERIALS AND METHODS

Reagents

Bovine MBP, L-glutamine, and β -mercaptoethanol were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) and RPMI 1640 were from Mediatech (Washington, DC). Acetylsalicylic acid (aspirin), Solvent Blue 38, cresyl violet acetate, lithium carbonate, and all molecular biology-grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Heat-killed *Mycobacterium tuberculosis* (H37RA) was purchased from Difco Laboratories. Incomplete Freund's adjuvant (IFA) was obtained from Calbiochem. Alexa Fluor antibodies used in immunostaining were obtained from Jackson ImmunoResearch, and IRDye-labeled reagents used for immunoblotting were from LI-COR Biosciences.

Induction of EAE

Animal maintenance and experiments were in accordance with National Institutes of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee of the Rush University of Medical Center, Chicago, IL. Adoptively transferred EAE was induced in 4- to 5-week-old female SJL/J mice (Harlan Sprague Dawley, Indianapolis, IN),

as described (16–18, 40, 41). Donor mice were immunized subcutaneously with 400 µg of bovine MBP and 60 µg of *M. tuberculosis* in IFA (16–18, 41). Animals were euthanized 10 to 12 days after immunization, and the draining lymph nodes were harvested and single-cell suspensions were cultured in RPMI 1640 supplemented with 10% FBS, MBP (50 µg/ml), 50 µM 2-mercaptoethanol, 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). On day 4, cells were harvested and resuspended in Hanks' balanced salt solution. A total of 2×10^7 viable cells in a volume of 200 µl were injected into the tail vein of naïve mice. Pertussis toxin (150 ng per mouse; Sigma-Aldrich) was injected intraperitoneally on 0 dpt of cells. Animals were observed daily for clinical symptoms. Experimental animals were scored by a masked investigator, as follows: 0, no clinical disease; 0.5, piloerection; 1, tail weakness; 1.5, tail paralysis; 2, hindlimb weakness; 3, hindlimb paralysis; 3.5, forelimb weakness; 4, forelimb paralysis; 5, moribund or death. To induce chronic EAE, C57BL/6 mice were immunized with 100 µg of MOG35–55, as described (40). Mice also received two doses of pertussis toxin (150 ng per mouse) on 0 and 2 days post-immunization.

Aspirin treatment

Aspirin was mixed in 0.5% methyl cellulose, and EAE mice were gavaged with 100 µl of aspirin-mixed methyl cellulose once daily using a gavage needle. Therefore, control EAE mice were also gavaged with 100 µl of 0.5% methyl cellulose as vehicle.

Treatment with neutralizing antibodies against IL-11—EAE mice were treated with neutralizing antibodies (20 µg per mouse) against IL-11 (rat monoclonal IgG_{2A}; R&D Systems; catalog no. MAB418; clone: 188520) by intraperitoneal injection once at the onset of acute phase (6 to 8 dpt).

Histological microscopy

Mice were perfused with phosphate-buffered saline (PBS) (pH 7.4) and then with 4% (w/v) paraformaldehyde solution in PBS followed by dissection of cerebellum and whole spinal cord. The tissues were further fixed and then divided into halves: One-half was used for histological staining, whereas the other half was used for myelin staining as described earlier (16–18, 41). For histological analysis, routine histology was performed to obtain perivascular cuffing and morphological details of CNS tissues. Paraformaldehyde-fixed tissues were embedded in paraffin, and serial sections (4µm) were cut. Sections were stained with conventional H&E staining method. Digital images were collected under bright-field setting using a 40× objective. Slides were assessed in a blinded fashion by three examiners for inflammation in different anatomical compartments (meninges and parenchyma). Inflammation was scored using the following scale as described: for meninges and parenchyma: 0, no infiltrating cells; 1, few infiltrating cells; 2, numerous infiltrating cells; and 3, widespread infiltration. For vessels: 0, no cuffed vessel; 1, one or two cuffed vessels per section; 2, three to five cuffed vessels per section; and 3, more than five cuffed vessels per section. For scoring, we used at least six serial sections of each spinal cord from each of five mice per group.

Staining for myelin

Sections were stained with luxol fast blue for myelin as described earlier (16, 18). Slides were assessed in a blinded fashion for demyelination by three examiners using the following scale: 0, normal white matter; 1, rare foci; 2, a few areas of demyelination; and 3, large areas of demyelination. At least six serial sections of each spinal cord from each of five mice per group were scored and statistically analyzed by analysis of variance (ANOVA).

Semiquantitative RT-PCR analysis

Total RNA was isolated and semiquantitative RT-PCR was carried out as described earlier (16, 18, 42) using an RT-PCR kit (Clontech, Mountain View, CA) and primers (table S1).

Real-time PCR analysis

Real-time PCR analysis was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) as described earlier (16, 18, 42).

ChIP assay

Recruitment of CREB to IL-11 gene promoter was determined by ChIP assay as described previously (24, 43, 44). Briefly, normal splenocytes were treated with aspirin under serum-free conditions, and after 3 hours of stimulation, cells were fixed by adding formaldehyde (1% final concentration), and cross-linked adducts were resuspended and sonicated. ChIP was performed on the cell lysate by overnight incubation at 4°C with 2µg of anti-CREB, anti-CBP, anti-RNA polymerase II, or anti-p300 antibodies followed by incubation with protein G agarose (Santa Cruz Biotechnology) for 2 hours. The beads were washed and incubated with elution buffer. To reverse the cross-linking and purify the DNA, precipitates were incubated in a 65°C incubator overnight and digested with proteinase K. DNA samples were then purified and precipitated, and precipitates were washed with 75% ethanol, air-dried, and resuspended in tris-EDTA (ethylenediaminetetraacetic acid) buffer. The following primers were used for amplification of chromatin fragments of mouse IL-11 gene: 5'-CCGGGCGGGCTTCCCTCTCCCTCC-3' (sense) and 5'-GGCTAGGGCTCCCGGGGCAGGGGA-3' (antisense).

Flow cytometry

Two-color flow cytometry was performed as described previously (33, 37). Briefly, 1×10^6 splenocytes suspended in flow staining buffer were incubated at 4°C with appropriately diluted fluorescein isothiocyanate (FITC)-labeled antibody to CD4 for 30 min, washed, and resuspended in fixation and permeabilization solution. After incubation in the dark for 30 min, cells were washed, blocked with test Fc block (anti-mouse CD16/32) in permeabilization buffer, and subsequently incubated with appropriately diluted phycoerythrin-labeled antibodies to Foxp3 at 4°C in the dark. After incubation, the cell suspension was centrifuged, washed thrice, and resuspended in flow staining buffer. The cells were then analyzed through FACS (BD Biosciences, San Jose, CA). Cells were gated on the basis of morphological characteristics. Apoptotic and necrotic cells were not accepted for FACS analysis.

Immunoblotting

Western blotting was conducted as described earlier (45, 46).

Densitometric analysis

Protein blots were analyzed using ImageJ (NIH, Bethesda, MD), and bands were normalized to their respective β -actin loading controls.

Immunofluorescence analysis

Immunofluorescence analysis was performed as described earlier (47–49). Briefly, coverslips containing 100 to 200 cells/mm² were fixed with 4% paraformaldehyde followed by treatment with cold ethanol and two rinses in PBS. Samples were blocked with 3% bovine serum albumin (BSA) in PBS–Tween 20 (PBST) for 30 min and incubated in PBST containing 1% BSA and anti-IL-11 or anti-CD3. After three washes in PBST (15 min each), slides were further incubated with Cy2 (Jackson ImmunoResearch Laboratories Inc.). For negative controls, a set of culture slides was incubated under similar conditions without the primary antibodies. The samples were mounted and observed under an Olympus IX81 fluorescence microscope. Counting analysis was performed using Olympus MicroSuite V software with the help of a touch counting module.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Low-dose aspirin suppresses clinical symptoms of EAE.

(A) Clinical EAE disease scores of mice after adoptive transfer of MBP-primed T cells and daily treatment with aspirin, as indicated. Data are means $\pm$ SEM of seven mice per group from two independent experiments. (B) Clinical EAE disease scores of mice after immunization with MOG35–55 and daily treatment with aspirin, as indicated. Data are means $\pm$ SEM of seven mice per group from two independent experiments. (C) Clinical EAE disease scores of mice after adoptive transfer of MBP-primed T cells stimulated in vitro with the indicated concentrations of aspirin. Data are means $\pm$ SEM of seven mice per

group from two independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ by Sidak's multiple comparisons test.

Fig. 2. Low-dose aspirin inhibits demyelination and suppresses immune cell infiltration of the spinal cord in EAE.

(A) H&E staining of spinal cord sections from mice after the indicated treatments. Data are representative of five mice per group from two independent experiments. Scale bars, 100 μm (top) or 20 μm (bottom). (B and C) Histology scores of immune cell infiltration and cuffed blood vessels in spinal cord sections from mice after the indicated treatments. Data are means ± SEM of five mice per group from two independent experiments. (D and E) qRT-PCR analysis of *Nos2* and *Il1β* mRNA expression in spinal cord tissue of mice after the

indicated treatments. Data are means $\pm$ SEM of five mice per group from two independent experiments. (**F** and **G**) Luxol fast blue staining for myelin in spinal cord sections from mice after the indicated treatments. Images (F) are representative of two independent experiments. Quantified scores from tissue sections (G) are means $\pm$ SEM of five mice per group. (**H** and **I**) qRT-PCR analysis of *Mbp* and *Cnp* mRNA expression in spinal cord tissue of mice after the indicated treatments. Data are means $\pm$ SEM of five mice per group from two independent experiments. ** $P < 0.01$ and *** $P < 0.005$ by two-sample t tests.

Fig. 3. Aspirin treatment stabilizes Tregs and protects mice from EAE.

(A and B) qRT-PCR analysis of *Foxp3* and *IL2ra* mRNA expression in splenocytes from MBP-immunized mice stimulated with MBP with or without aspirin. Data are means $\pm$ SEM from three independent experiments performed in duplicate. (C to F) Flow cytometry analysis of FoxP3 and CD25 abundance in CD4 T cells from MBP-immunized mice stimulated as indicated. Dot plots are representative of three independent experiments performed in duplicate. Quantified mean fluorescence intensity (MFI) values are means $\pm$ SEM from all experiments. (G and H) Flow cytometry analysis of FoxP3 abundance in

CD4⁺ T cells from mice that received MBP-primed T cells and were treated with aspirin as indicated. Dot plots (G) are representative of five mice per group from two independent experiments. Quantified MFI data (H) are means $\pm$ SEM from all experiments. (I) Clinical EAE disease scores of mice after adoptive transfer of MBP-primed T cells and daily treatment with aspirin and anti-CD25 or control IgG, as indicated. Data are means $\pm$ SEM of seven mice per group from two independent experiments. ** P < 0.01 and *** P < 0.005 by two-sample t tests (A, B, D, and H) or Sidak's multiple comparisons test (I). Ab, antibody.

Fig. 4. Aspirin suppresses T_H17 and T_H1 , but not T_H2 , responses.

(A and B) qRT-PCR analysis of *Rorγt* and *Il17* mRNA expression in splenocytes isolated from MBP-immunized donor mice stimulated with MBP with the indicated concentrations of aspirin for 24 hours. Data are means $\pm$ SEM of three independent experiments performed in duplicate. (C to F) Flow cytometry analysis of ROR γ t and IL-17 in CD4⁺ T cells from MBP-immunized donor mice treated as indicated. Dot plots (C and D) are representative of three independent experiments performed in duplicate. Quantified MFI data are means $\pm$ SEM from all experiments. (G to I) Flow cytometry analysis of Tbet, IFN γ , and IL-4 in

CD4⁺ T cells from MBP-immunized donor mice treated as indicated. Quantified MFI data are means $\pm$ SEM from three independent experiments performed in duplicate. * P < 0.05, ** P < 0.01, and *** P < 0.005 by two-sample t tests.

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Fig. 5. Aspirin promotes CREB-mediated IL-11 expression, which protects mice from EAE. (A) qRT-PCR analysis of *Il11* mRNA expression in splenocytes from normal mice treated with increasing doses of aspirin for 4 hours. Data are means $\pm$ SEM from three independent experiments performed in duplicate. (B) qRT-PCR analysis of *Il11* mRNA expression in splenocytes from normal mice treated with aspirin for up to 8 hours. Data are means $\pm$ SEM from three independent experiments performed in duplicate. (C and D) Western blot analysis of IL-11 abundance in lysate of splenocytes from normal mice treated with the indicated doses of aspirin for 8 hours. Blots (C) are representative of three independent experiments

performed in duplicate. Quantified data (D) are means $\pm$ SEM from all experiments. (E) Immunofluorescence microscopy analysis of IL-11 and CD3 expression in splenocytes from normal mice treated with and without aspirin. Images are representative of three independent experiments performed in duplicate. DAPI, 4',6-diamidino-2-phenylindole. (F) qRT-PCR analysis of *Il11* mRNA expression in splenocytes from MBP-immunized mice that were stimulated with MBP and increasing doses of aspirin for 4 hours. Data are means $\pm$ SEM from three independent experiments performed in duplicate. (G) Western blot analysis of pCREB abundance in lysates of splenocytes treated with aspirin for the indicated times. Blots are representative of three independent experiments performed in duplicate. (H) Western blot analysis of CREB and IL-11 abundance in lysates of splenocytes transfected with either control or CREB siRNA and treated with aspirin for 6 hours. Blots are representative of three independent experiments performed in duplicate. (I) qRT-PCR analysis of the *Il-11* promoter association with CREB, CBP, p300, and RNA polymerase after ChIP from lysate of splenocytes stimulated with aspirin for 2 hours. Data are means $\pm$ SEM from three independent experiments performed in duplicate. (J) Clinical EAE disease scores of mice after adoptive transfer of MBP-primed T cells and daily treatment with aspirin and control IgG, IL-11-neutralizing anti-body, or recombinant mouse IL-11, as indicated. Data are means $\pm$ SEM of seven mice per group from two independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ by two-sample *t* tests (A, B, D, F, and I) or Sidak's multiple comparisons test (J).

Fig. 6. Aspirin-induced IL-11 stimulates T_{reg} differentiation.

(A and B) qRT-PCR analysis of *Foxp3* and *Il2ra* mRNA expression in splenocytes from MBP-immunized mice stimulated with MBP and aspirin, with or without IL-11-neutralizing antibody. Data are means ± SEM from three independent experiments performed in duplicate. (C and D) Flow cytometry analysis of FoxP3 abundance in CD4⁺ T cells from MBP-immunized mice that were stimulated with MBP, aspirin, and IL-11-neutralizing antibody, as indicated. Dot plots (C) are representative of three independent experiments performed in duplicate. Quantified MFI data (D) are means ± SEM from all experiments.

** $P < 0.01$ and *** $P < 0.005$ by two-sample t tests

Fig. 7. IL-11 alone is capable of stabilizing T_{regs} in MBP-primed splenocytes and in vivo in EAE mice.

(A and B) qRT-PCR analysis of *Foxp3* (A) and *IL2ra* (B) mRNA expression in splenocytes from MBP-immunized mice stimulated with MBP and IL-11. Data are means ± SEM from three independent experiments performed in duplicate. (C and D) Flow cytometry analysis of FoxP3 abundance in CD4⁺ T cells from MBP-immunized mice that were stimulated with MBP and recombinant IL-11, as indicated. Dot plots (C) are representative of three independent experiments performed in duplicate. Quantified MFI data (D) are means ± SEM

from all experiments. (**E** and **F**) Flow cytometry analysis of FoxP3 abundance in CD4⁺ T cells from mice after adoptive transfer of MBP-specific T cells and treatment with aspirin and IL-11–neutralizing antibody or recombinant mouse IL-11. Dot plots (E) are representative of three independent experiments performed in duplicate. Quantified MFI data (F) are means $\pm$ SEM from all experiments. ** $P < 0.01$ and *** $P < 0.005$ by two-sample t tests.