

# Protein kinase CK2 governs the molecular decision between encephalitogenic T<sub>H</sub>17 cell and T<sub>reg</sub> cell development

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T helper 17 (T<sub>H</sub>17) cells represent a discrete T<sub>H</sub> cell subset instrumental in the immune response to extracellular bacteria and fungi. However, T<sub>H</sub>17 cells are considered to be detrimentally involved in autoimmune diseases like multiple sclerosis (MS). In contrast to T<sub>H</sub>17 cells, regulatory T (T<sub>reg</sub>) cells were shown to be pivotal in the maintenance of peripheral tolerance. Thus, the balance between T<sub>reg</sub> cells and T<sub>H</sub>17 cells determines the severity of a T<sub>H</sub>17 cell-driven disease and therefore is a promising target for treating autoimmune diseases. However, the molecular mechanisms controlling this balance are still unclear. Here, we report that pharmacological inhibition as well as genetic ablation of the protein kinase CK2 (CK2) ameliorates experimental autoimmune encephalomyelitis (EAE) severity and relapse incidence. Furthermore, CK2 inhibition or genetic ablation prevents T<sub>H</sub>17 cell development and promotes the generation of T<sub>reg</sub> cells. Molecularly, inhibition of CK2 leads to reduced STAT3 phosphorylation and strongly attenuated expression of the IL-23 receptor, IL-17, and GM-CSF. Thus, these results identify CK2 as a nodal point in T<sub>H</sub>17 cell development and suggest this kinase as a potential therapeutic target to treat T<sub>H</sub>17 cell-driven autoimmune responses.

multiple sclerosis | T<sub>H</sub>17 cells | regulatory T cells | FOXP3 | MS therapy

It is widely accepted that IL-17-producing T<sub>H</sub>17 cells infiltrating the central nervous system (CNS) play a critical role in experimental autoimmune encephalomyelitis (EAE), and multiple sclerosis (MS) (1–4), whereas CD4<sup>+</sup> forkhead box protein (FOXP3)<sup>+</sup> regulatory T (T<sub>reg</sub>) cells are pivotal for the prevention of autoimmune responses (5). Although direct involvement of IL-17 in EAE remains elusive, inhibition of IL-17 using monoclonal antibodies led to reduced EAE outcome measures (1, 6, 7). IL-17 production by T<sub>H</sub>17 cells is triggered by IL-23, and CNS-infiltrating T cells in *Il23*-deficient mice do not produce IL-17. Hence, it was concluded that IL-23 mediates T<sub>H</sub>17 cell pathogenicity. Initial responsiveness to IL-23 needs to be acquired during T<sub>H</sub>17 cell differentiation, as naive T cells do not express the IL-23 receptor (IL-23R). Up-regulation of IL-23R is induced by IL-6 and IL-1 and/or TGF- $\beta$  (8–10) and guided by the transcription factor RAR-related orphan receptor (ROR) $\gamma$ t (11–13). It is of note that IL-6 signaling is conferred by STAT3 phosphorylation, and in *Stat3*-deficient animals T<sub>H</sub>17 cell differentiation is impaired and EAE development is absent (14, 15). Thus, IL-6 and STAT3 phosphorylation initiate T<sub>H</sub>17 cell differentiation, and IL-23 is needed for manifestation of the T<sub>H</sub>17 cell pathogenicity. Another cytokine induced by IL-23 in T<sub>H</sub>17 cells is granulocyte-macrophage colony-stimulating factor (GM-CSF), which essentially contributes to CNS inflammation (16). In adoptive T-cell transfer EAE, antibodies to GM-CSF led to amelioration of the clinical score (17) and *Il23*-deficient animals were resistant to EAE (18). Thus, analyzing

the molecular pathway leading to GM-CSF production by T<sub>H</sub>17 cells is important to better understand the disease.

Recently, different biologics interfering with the IL-17 pathway have been introduced in the clinic. For example, ustekinumab, an antibody targeting the IL-12p40 subunit of IL-23, is used as a therapy for psoriasis (19), and secukinumab, a human anti-IL-17A antibody, is in phase II clinical trial for the treatment of psoriasis (20–22) and relapsing-remitting MS (3, 23).

Given the limitations of protein-based biologics, it is interesting that, in rheumatoid arthritis, importance is placed on protein kinase inhibitors interfering at different tiers at subcellular levels modifying signal transduction during differentiation. Cytokine function, in particular, was suggested to be manipulated by small molecules (24). Originally investigated as a target for combatting cancer (25–27), the role of protein kinase CK2 (CK2) signaling in neurodegenerative diseases has recently been discussed (28). CK2 was believed to influence the survival of lymphocytes (29); however, recent work has suggested an involvement of CK2 in lymphocyte differentiation.

The aim of the current study was to determine whether pharmacological inhibition of protein kinase CK2 could interfere with T<sub>H</sub>17 cell development and T-cell-driven EAE outcome.

## Significance

**Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system in the western world and leads to devastating disability in young adults, with only limited treatment options currently available. Our recent work demonstrates that pharmacological inhibition of the protein kinase CK2 (CK2) results in inhibition of encephalitogenic human and mouse T helper 17 (T<sub>H</sub>17) cell development and effector function while at the same time promoting development of induced regulatory T (iT<sub>reg</sub>) cells. Hence, modulation of CK2 activity might represent a promising approach for the treatment of MS and other T<sub>H</sub>17 cell-driven inflammatory diseases.**

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## Results

### CK2 Inhibition Suppresses $T_H17$ in Favor of Induced $T_{reg}$ Cell Development.

To analyze the suppressive capacity of CK2 inhibitors on  $T_H17$  cell development in vitro, we stimulated murine naïve ( $CD62L^{high} CD44^{low}$ )  $CD4^+$  T cells under  $T_H17$  cell-polarizing conditions in the presence of the CK2 inhibitor 2-dimethylamino-4,5,6,7-tetrabromo-1*H*-benzimidazole (DMAT) (30) or the respective vehicle control with or without addition of TGF- $\beta$ . Although treatment with DMAT had no effect on cell viability (Fig. S1*A*), proliferation (Fig. S1*B*), or IL-2 production (Fig. S1*C*), IL-17 production was substantially reduced in both the absence and presence of TGF- $\beta$  (Fig. 1*A* and *B*) in a dose-dependent manner (Fig. S1*D*). ROR $\gamma$ t mRNA (Fig. S1*E*) as well as protein expression (Fig. S1*F*) was significantly reduced between day 4 and 5 upon  $T_H17$  cell differentiation. Remarkably, in the presence of TGF- $\beta$  inhibition of CK2 resulted in enhanced induced  $T_{reg}$  ( $iT_{reg}$ ) cell development as well as in a strong induction of FOXP3 expression even under  $T_H17$ -skewing conditions (Fig. 1*C* and *D*). To demonstrate specificity and importance of CK2 for  $T_H17$  cell differentiation, we additionally cultured naïve  $CD4^+$  T cells in the presence of another CK2 inhibitor, named CX4945 (31). Although inhibition of CK2 by CX4945 showed no effect on cell viability (Fig. S2*A*) or proliferation (Fig. S2*B*), it resulted in a dose-dependent inhibition of  $T_H17$  differentiation as evidenced by a strongly reduced ability to produce IL-17 (Fig. S2*C*) with only marginal effects on ROR $\gamma$ t mRNA (Fig. S2*D*) or protein expression (Fig. S2*E*). Again, addition of TGF- $\beta$  resulted in a comparable inhibition of  $T_H17$  differentiation in the presence of CX4945 (Fig. S2*F* and *G*) and in a concomitant induction of FOXP3 expression (Fig. S2*F* and *H*) under  $T_H17$  cell-polarizing conditions. Importantly, CK2 inhibition by either DMAT (Fig. S3*A*) or CX4945 (Fig. S3*B*) only marginally affected  $T_H1$  cell differentiation and had no effect on differentiation of  $T_H2$  and  $T_H9$  cells. For subsequent in vitro experiments, we chose 1.5  $\mu$ M DMAT and 1  $\mu$ M CX4945 because, at these concentrations, both CK2 inhibitors did not show

any adverse effects on cell viability but maximally inhibited the differentiation of  $T_H17$  cells.

**Encephalitogenicity of  $T_H17$  Cells Is Dependent on CK2 Activity.** Upon CK2 inhibition,  $T_H17$  cells were inferior in inducing EAE after transfer into recombination-activating gene 1-deficient (*Rag1*<sup>-/-</sup>) host mice (Fig. S4*A*). Concomitantly, we detected a reduced proportion of CNS-infiltrating  $CD4^+$  T cells (Fig. S4*B*) but no effect on peripheral  $CD4^+$  T-cell numbers (Fig. S4*C*). Among the CNS-infiltrating  $CD4^+$  T cells, the percentages and absolute numbers (Fig. S4*D*) of IL-17<sup>+</sup>ROR $\gamma$ t<sup>+</sup>  $T_H17$  cells were strongly reduced in favor of CNS-infiltrating FOXP3<sup>+</sup>  $iT_{reg}$  cells (Fig. S4*E*). Concomitantly to the altered T-cell infiltration into the CNS, mice that received vehicle-treated  $T_H17$  cells displayed demyelination in multiple widespread areas in the white matter region (Fig. S5*A*), whereas hardly any demyelination was found after transfer of CK2 inhibitor DMAT-treated  $T_H17$  cells (Fig. S5*A*). Furthermore, the density of mononuclear cells within the white matter region was significantly reduced in mice that received DMAT-treated  $T_H17$  cells (Fig. S5*B*).

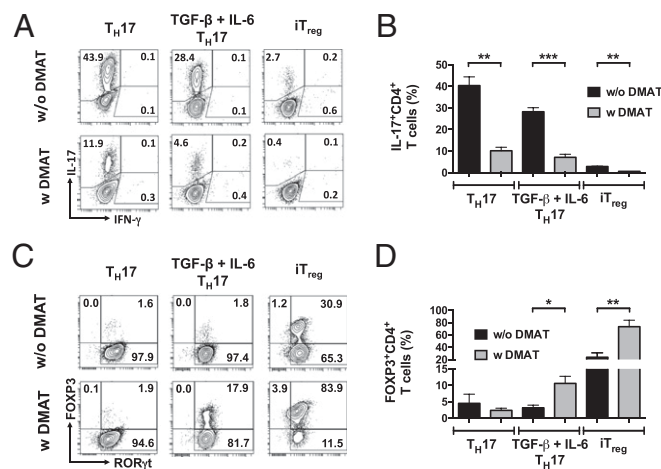
Together, these data further corroborate that CK2 participates in differentiation of  $T_H17$  cells and limits generation of  $iT_{reg}$  cells in vitro and in vivo.

### STAT3-Mediated Transcriptional Changes During $T_H17$ Cell Development Are Controlled by CK2 Activity.

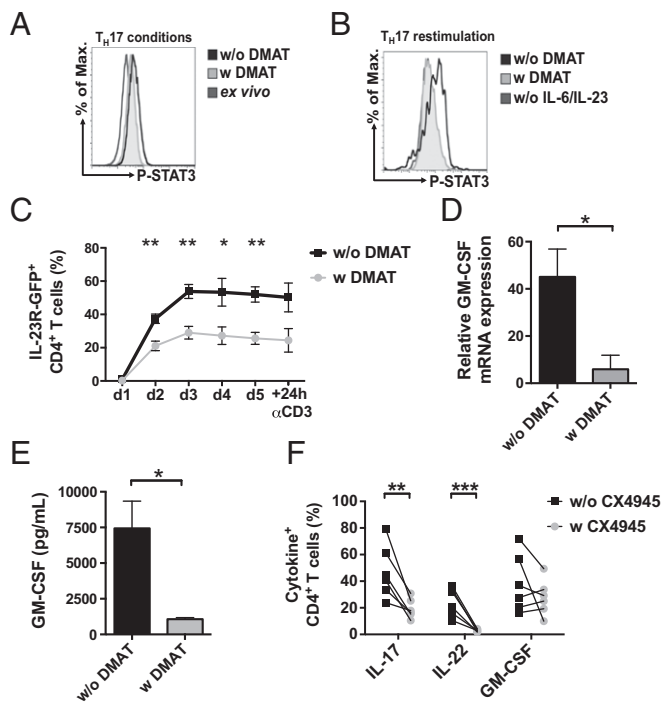
Cytokine receptor signaling results in phosphorylation of the STAT family of transcription factors, contributing to the differentiation of different  $T_H$  cell subsets. For instance, STAT3 phosphorylation in response to IL-6, IL-21, and IL-23 regulates expression of the orphan nuclear receptor ROR $\gamma$ t, a signature transcription factor for  $T_H17$  cells. Therefore, we analyzed phosphorylation of STAT3 upon inhibition of CK2 by flow cytometry. To this end, we stimulated naïve  $CD4^+$  T cells under  $T_H17$  cell-polarizing conditions in the presence of DMAT or vehicle control. Although vehicle-treated  $T_H17$  cells showed strong phosphorylation of STAT3 (Fig. 2*A*), the CK2 inhibitor DMAT (Fig. 2*A*) as well as the CK2 inhibitor CX4945 (Fig. S6*A*) strongly attenuated phosphorylation of STAT3, suggesting a crucial role of CK2 in STAT3 phosphorylation, and therefore in the differentiation of  $T_H17$  cells. The encephalitogenicity of  $T_H17$  cells strongly relies on IL-23R-mediated signaling, which also leads to phosphorylation of STAT3 in differentiated  $T_H17$  cells. Hence, we also analyzed STAT3 phosphorylation upon restimulation of  $T_H17$  cells in the presence of IL-6, IL-23, and DMAT (Fig. 2*B*) and CX4945 (Fig. S6*B*) or the respective vehicle controls. Although stimulation of  $T_H17$  cells in the presence of IL-6 and IL-23 led to strong phosphorylation of STAT3, inhibition of CK2 completely prevented cytokine-induced phosphorylation of this transcription factor. These data suggest that CK2 plays a decisive role in cytokine receptor signaling resulting in activation of the transcription factor STAT3.

In search of the resulting transcriptional changes evoked by CK2 inhibition, we stimulated naïve  $CD4^+$  T cells under  $T_H17$ -polarizing conditions in the absence and presence of DMAT or CX4945 for 24 h and comparatively analyzed the transcriptome of these cells by using next-generation sequencing-based RNA sequencing (RNA Seq). These analyses revealed a significant down-regulation of mRNAs known to contribute to the encephalitogenicity of  $T_H17$  cells (Fig. S6*C*). Next to *Il17a* and *Il17f*, expression of *Il23r* was significantly reduced upon inhibition of CK2.

To confirm the results obtained by RNA Seq and to further analyze IL-23R expression on the protein level, we made use of the IL-23R-GFP reporter mouse generated previously (32). Although culture of naïve  $CD4^+$  T cells from IL-23R-GFP reporter mice under  $T_H17$  cell-polarizing conditions resulted in strong expression of IL-23R peaking on day 3 of stimulation, inhibition of CK2 by either DMAT (Fig. 2*C* and Fig. S6*D*) or CX4945 (Fig. S6*E*) strongly reduced the percentage of IL-23R-expressing  $T_H17$  cells. To address this reduced IL-23R expression upon CK2 inhibition, we restimulated  $T_H17$  cells differentiated in the



**Fig. 1.** Inhibition of protein kinase CK2 interferes with  $T_H17$  lineage commitment and favors  $iT_{reg}$  cell differentiation. (A) Flow-cytometric analysis of IL-17 and IFN- $\gamma$  expression in ionomycin/PMA-stimulated  $T_H17$ , TGF- $\beta$  + IL-6  $T_H17$ , and  $iT_{reg}$  cells, differentiated in the presence (w DMAT) or absence (w/o DMAT) of the CK2 inhibitor DMAT. (B) Percentage of IL-17<sup>+</sup> among  $CD4^+$   $T_H$  cells differentiated toward  $T_H17$ , TGF- $\beta$  + IL-6  $T_H17$ , and  $iT_{reg}$  cells in the presence (w DMAT) or absence (w/o DMAT) of the CK2 inhibitor DMAT. (C) Flow-cytometric analysis of FOXP3 and ROR $\gamma$ t expression in ionomycin/PMA-stimulated  $T_H17$ , TGF- $\beta$  + IL-6  $T_H17$ , and  $iT_{reg}$  cells, differentiated in the presence (w DMAT) or absence (w/o DMAT) of the CK2 inhibitor DMAT. (D) Percentage of FOXP3<sup>+</sup> cells among  $CD4^+$   $T_H17$ , TGF- $\beta$  + IL-6  $T_H17$ , and  $iT_{reg}$  cells. Representative (A and C) or combined (B and D) data are shown of at least three independent experiments. Numbers in plots indicate percentage of cells in each quadrant (A and C). Error bars show SEM, and *P* values were evaluated using unpaired Student's *t* test: \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001 (B and D).



**Fig. 2.** CK2 inhibition results in diminished expression of molecules associated with pathogenicity of TH17 cells. (A) Flow-cytometric analysis of Y705 STAT3 phosphorylation in naive T cells, differentiated toward TH17 cells for 24 h in the presence (w DMAT) or absence (w/o DMAT) of CK2 inhibitor DMAT. Representative overlays from three independent experiments are shown. (B) Analysis of Y705 STAT3 phosphorylation in TH17 cells on day 5, differentiated in the presence (w DMAT) or absence (w/o DMAT) of DMAT and restimulated with anti-CD3 for 24 h in the absence (w/o IL-6/IL-23) or presence of IL-6 and IL-23 (10 ng/mL each). Representative overlays from two independent experiments are shown. (C) Percentage of IL-23R-GFP<sup>+</sup> among CD4<sup>+</sup> T cells differentiated toward TH17 cells in the presence (w DMAT) or absence (w/o DMAT) of DMAT at indicated time points. Error bars show SEM. *P* values were calculated for each time point using unpaired Student's *t* test: \**P* < 0.05; \*\**P* < 0.01. Relative expression of GM-CSF mRNA (D) and GM-CSF concentration in culture supernatants (E) of TH17 cells differentiated in the presence (w DMAT) or absence (w/o DMAT) of DMAT and restimulated for 24 h using plate-bound anti-CD3 (4 µg/mL). Data are combined from *n* = 4 independent experiments. Error bars show SEM. *P* values were calculated using unpaired Student's *t* test: \**P* < 0.05. (F) Flow-cytometric analysis of IL-17, IL-22, and GM-CSF expression in human TH17 cells, differentiated in the presence (w CX4945) or absence (w/o CX4945) of CK2 inhibitor CX4945. Data are combined from *n* = 5 independent experiments. *P* values were calculated using Student's *t* test; \*\**P* < 0.01; \*\*\**P* < 0.001.

presence or absence of DMAT in combination with IL-23. Although stimulation of TH17 cells in the presence of IL-23 resulted in enhanced IL-17 expression, IL-23-mediated stimulation of TH17 cells generated in the presence of DMAT had no effect on IL-17 production (Fig. S6F), mechanistically demonstrating the inhibition of IL-23R expression upon CK2 inhibition.

In an attempt to identify an IL-23-dependent soluble factor that mediates the encephalitogenicity of TH17 cells, it was demonstrated that IL-23 signaling in cooperation with the transcription factor RORγt drives expression of the proinflammatory cytokine GM-CSF, which serves a nonredundant function in the initiation and manifestation of autoimmune neuroinflammation (16, 17). Hence, we analyzed the production of GM-CSF upon inhibition of CK2 in TH17 cells. CD4<sup>+</sup> T cells stimulated under TH17 cell-polarizing conditions showed vigorous GM-CSF mRNA expression (Fig. 2D and Fig. S6G) and cytokine secretion (Fig. 2E) with kinetics correlating to expression of the IL-23R. In contrast, TH17 cells treated with DMAT (Fig. 2D and E) or CX4945 (Fig. S6G) showed strongly reduced expression of GM-CSF.

To determine the potential of CX4945 to prevent effector function of human TH17 cells, we isolated IL-17-secreting CD4<sup>+</sup> T cells from human peripheral blood mononuclear cells (PBMCs) and stimulated these cells in the presence of IL-1β and IL-23 and in presence and absence of CX4945. After 6 d of culture, phorbol 12-myristate 13-acetate (PMA)- and ionomycin-stimulated T cells were analyzed for their ability to produce signature cytokines of TH17 cells. Flow-cytometric analyses of these cultures demonstrated that CX4945 efficiently inhibited the production of IL-17 and IL-22 as well as GM-CSF in some of the donors (Fig. 2F).

Collectively, these data demonstrate the vital function of CK2 in the development of encephalitogenic TH17 cells and suggest CK2 inhibitors as potential drugs to treat TH17-driven autoimmune diseases.

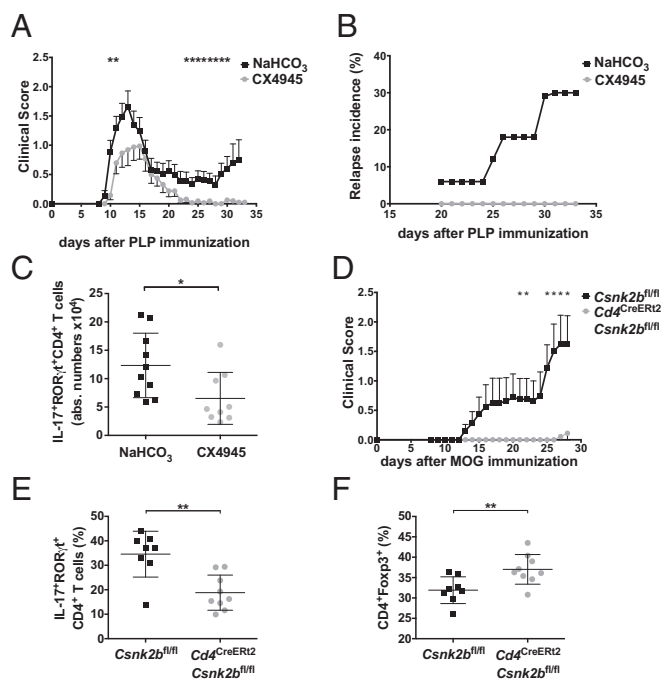
**Inhibition of CK2 Suppresses TH17 Cell Differentiation in Vivo and Ameliorates EAE.** In proteolipid protein (PLP)-induced EAE in the SJL mouse, disease is characterized by a relapsing–remitting course of paralysis, and is probably the preferred in vivo model to assess efficacy of immunoregulatory strategies. Using the CK2 inhibitor DMAT, treating every 3 d with i.p. administration (10 mg/kg body weight; Fig. S7A), we did not observe major differences in EAE outcome measures in the acute phase; however, DMAT treatment significantly ameliorated disease severity in the relapse (Fig. S7B) as well as the relapse incidence (Fig. S7C). This amelioration of the incidence and severity of the relapse was also found when DMAT was administered in a late therapeutic treatment regimen starting at the peak of the first wave of paralysis (Fig. S7D and E).

Although the CK2 inhibitor DMAT potently inhibits TH17 cell development and function in vitro and the relapse incidence and severity in EAE-diseased mice, the potential clinical use of this compound is far from being definitive. In contrast, CX4945 (silmatisertib), an orally bioavailable small-molecule inhibitor of CK2, is currently being tested in clinical trials for the treatment of patients with multiple myeloma and cholangiocarcinoma patients. Hence, we addressed the therapeutic potential of CX4945 to ameliorate the relapsing–remitting EAE. Daily administration of CX4945 by i.p. injection efficiently reduced the severity of the initial clinical wave as well as disease severity in the relapse (Fig. 3A). Strikingly, this treatment regimen almost completely protected mice from EAE relapse (Fig. 3B). Consistent with the in vitro results, administration of CX4945 not only decreased the number of CD4<sup>+</sup> T cells infiltrating the CNS (Fig. S7F) but also strongly reduced absolute numbers of IL-17<sup>+</sup>RORγt<sup>+</sup>CD4<sup>+</sup> TH17 in the CNS (Fig. 3C and Fig. S7G), without any effect on cell viability in the CNS (Fig. S7H) or in the periphery (Fig. S7I). These data highlight CX4945 as a potent inhibitor of TH17 cell-driven immunopathology in the CNS and suggest CK2 as a valuable target in the treatment of TH17 cell-driven autoimmune diseases.

To exclude any unmanageable adverse effects of the pharmacological CK2 inhibitors and to unequivocally demonstrate the crucial role of CK2 in encephalitogenicity of TH17 cells in vivo, we made use of a mutant mouse strain, carrying a transgene with tamoxifen-inducible Cre recombinase under the control of the mouse *Cd4* locus (*Cd4*<sup>CreERT2</sup>) (33). Upon crossing of these mice with mice carrying loxP-flanked *Csnk2b* (CK2 beta subunit gene) alleles to specifically ablate CK2β expression after tamoxifen-induced Cre recombinase expression in CD4<sup>+</sup> T cells (*Cd4*<sup>CreERT2</sup> *Csnk2b*<sup>fl/fl</sup>), we induced EAE by MOG<sub>35–55</sub> (myelin oligodendrocyte glycoprotein peptide fragment 35–55)/complete Freund's adjuvant (CFA) injection as previously described (34, 35). Remarkably, tamoxifen-induced ablation of CK2β in CD4<sup>+</sup> T cells resulted in an almost complete prevention of EAE induction (Fig. 3D). Concomitantly, we detected a reduced proportion of CNS-infiltrating IL-17<sup>+</sup>RORγt<sup>+</sup>CD4<sup>+</sup> TH17 cells (Fig. 3E and Fig. S7J) and enhanced percentage of FOXP3-expressing T<sub>reg</sub> cells in CNS-draining lymph nodes (Fig. 3F and Fig. S7K).

Taken together, our data demonstrate that CK2 activity represents a molecular switch between TH17 cell and iT<sub>reg</sub> cell development and that the CK2 inhibitor CX4945 might have the





**Fig. 3.** Pharmacological inhibition of CK2 prevents T<sub>H</sub>17 cell development and ameliorates EAE. (A) Clinical score of EAE in SJL mice. Animals were treated daily with 75 mg/kg body weight of CX4945 (w CX4945), or 0.1 mM NaHCO<sub>3</sub> buffer (pH 8.2) as solvent control (NaHCO<sub>3</sub>) starting from day 0. Error bars show SEM. P values were calculated using Mann-Whitney U test; \*P < 0.05. Data are combined from three independent experiments, with n = 34 until day 11 and n = 17 mice from day 12 on (NaHCO<sub>3</sub>), and with n = 33 until day 11 and n = 16 from day 12 on (CX4945). (B) Relapse incidence as percentage of SJL mice treated with solvent (NaHCO<sub>3</sub>) or CK2 inhibitor CX4945. (C) Flow-cytometric analysis of IL-17 and RORγt expression among PMA/ionomycin-stimulated CD4<sup>+</sup> T cells isolated from CNS of mice that received CX4945 (CX4945) or respective solvent (NaHCO<sub>3</sub>). Shown is the absolute number of IL-17<sup>+</sup>RORγt<sup>+</sup> among CD4<sup>+</sup> T cells. Error bars show SD. P values were calculated using unpaired Student's t test; \*P < 0.05. Data represent one experiment with at least n = 9 mice per group. (D) Clinical score of EAE in *Csnk2b<sup>fl/fl</sup>* (n = 8) and *Cd4<sup>CreER12</sup> Csnk2b<sup>fl/fl</sup>* (n = 9) mice. Animals were treated with tamoxifen at day -2 and day 7 after immunization by i.p. injection. Error bars show SEM. P values were calculated using Mann-Whitney U test; \*P < 0.05. Data are representative for one single experiment. (E) Percentage of IL-17<sup>+</sup>RORγt<sup>+</sup> cells among CD4<sup>+</sup> T cells from the CNS of *Csnk2b<sup>fl/fl</sup>* (n = 8) and *Cd4<sup>CreER12</sup> Csnk2b<sup>fl/fl</sup>* (n = 9) mice treated as described in D. Error bars show SD. P values were calculated using unpaired Student's t test; \*\*P < 0.01. (F) Percentage of FOXP3<sup>+</sup> among CD4<sup>+</sup> T cells in the CNS-draining lymph nodes of *Csnk2b<sup>fl/fl</sup>* (n = 8) and *Cd4<sup>CreER12</sup> Csnk2b<sup>fl/fl</sup>* (n = 9) mice treated as described in D. Error bars show SD. P values were calculated using unpaired Student's t test; \*\*P < 0.01.

potential to be a promising lead compound for the treatment of T<sub>H</sub>17 cell-driven autoimmune diseases in humans.

## Discussion

It has been made unambiguously evident that autoimmune diseases such as MS are instigated and perpetuated by proinflammatory T cells (36), and particular focus has been placed upon T<sub>H</sub>17 cells as playing a crucial role (37). The differentiation of T<sub>H</sub>17 cells has been shown to be dependent on the cytokines IL-6, IL-1β and IL-23 in mice and humans (38, 39) and autocrine activity of IL-21 (40). Similarly, STAT3 was found to govern the differentiation of T<sub>H</sub>17 cells, and in the absence of STAT3, differentiation of T<sub>H</sub>17 cells was abrogated (14). Moreover, animals deficient in STAT3 did not exhibit an autoimmune response in EAE (15). STAT3 phosphorylation was shown to be crucial in counteracting the development of T<sub>reg</sub> cells and was demonstrated in response to IL-6, IL-23, IL-21, and IL-27 (41).

Phosphorylation of JAK/STAT was shown to play a role in transmitting signals in response to many cytokines and growth factors, and in tumor cells, CK2 is associated with JAKs. The cross talk between CK2 and the JAK/STAT pathway is necessary for JAK phosphorylation and JAK/STAT signaling in response to survival signals (42). In developing T cells, however, the involvement of CK2 in the sequence of events leading to STAT3 phosphorylation has not yet been studied.

We show here that CK2 represents an enzyme responsible for such phosphorylation in the differentiation of T<sub>H</sub>17 cells. Most importantly, our findings demonstrate that CK2 is involved in controlling the lineage decision between iT<sub>reg</sub> cells and T<sub>H</sub>17 cells.

Recent studies have investigated the developmental checkpoints in the lineage decision and antipodal signaling pathways leading to the lineage commitment of T<sub>H</sub> cells. It has been suggested that T<sub>H</sub>17 cells depend on de novo fatty acid synthesis by acetyl-CoA carboxylase 1 (ACC1) activity, and pharmacological inhibition of ACC1 leads to inhibition of T<sub>H</sub>17 cells, but in turn favors the development of T<sub>reg</sub> cells (43).

We introduce an additional nodal checkpoint involved in this lineage decision and found that CK2 represents a central player in the developmental decision of T<sub>H</sub>17 vs. iT<sub>reg</sub> cell differentiation. However, groundbreaking studies of T-cell plasticity have led to the widely accepted model that already-differentiated T cells are also able to alter their effector function in vivo. Along this line, it was demonstrated that T cells producing IFN-γ in the spinal cord during EAE originated from IL-17-producing T<sub>H</sub>17 cells which underwent their conversion to ex-T<sub>H</sub>17 cells by the proinflammatory cytokine IL-23 (44, 45). Another study demonstrated T-cell plasticity by showing that FOXP3<sup>+</sup>RORγt<sup>+</sup> intermediate T cells are able to terminally differentiate into either FOXP3<sup>+</sup>RORγt<sup>+</sup> (iT<sub>reg</sub>) or FOXP3<sup>+</sup>RORγt<sup>+</sup> (T<sub>H</sub>17) T cells upon polarization, extending the repertoire of effector functions of T cells in regard to differentiation and function in autoimmunity (46). We correlate the finding of FOXP3<sup>+</sup> T cells emerging from IL-17<sup>+</sup>FOXP3<sup>+</sup> T<sub>H</sub>17 cells after in vivo transfer to such plasticity and suggest that CK2 inhibition before T<sub>H</sub>17 cell transfer led to a susceptible intermediate cell type responding to in vivo stimuli favoring the development of FOXP3<sup>+</sup> iT<sub>reg</sub> cells.

Deciphering the nodal checkpoints that define the lineage decision for development of T<sub>H</sub>17 and iT<sub>reg</sub> cells is crucial for understanding where and how the balance between these cell types is defined. Specifically, we show in our work that shifting the balance toward the iT<sub>reg</sub> cell compartment in the CNS leads to the absence of autoimmune pathogenicity in EAE and that this shift depends on the activity of CK2.

The effector cytokines secreted by T<sub>H</sub>17 cells are GM-CSF, IL-17, IL-21, and IL-22 (47), and even though it is not yet clear how the effector cytokines contribute to the disease, it has been shown that antibodies to IL-17 are effective in human inflammatory diseases (4) and in EAE; neutralizing IL-17 led to amelioration of disease outcome measures (1). Hints for a pathogenic role of T<sub>H</sub>17 cells comes from increased copy numbers of IL-17 transcripts found in chronic MS lesions compared with acute lesions in MS patients and from mouse models of EAE (48). Thus, the signature cytokines produced by T<sub>H</sub>17 cells are regarded as a pattern by which T<sub>H</sub>17 cells are defined; however, proof of direct involvement of IL-17 in encephalitogenicity is missing, and mice deficient in IL-17A and IL-17F still develop EAE (49).

By contrast, the enormous importance of IL-23 in EAE is obvious from mice deficient in IL-23, which are completely resistant to EAE (18). Hence, IL-17 seems to be involved in EAE severity, but IL-23 is dominant in determining EAE outcome. IL-23 produced by activated microglia and macrophages enables T<sub>H</sub>17 cells to differentiate and to produce GM-CSF through engagement of IL-23R on T cells, and thus IL-23 and GM-CSF play a dominant role in CNS inflammation (16, 17, 50). Our work shows that both IL-17 and GM-CSF production as well as responsiveness to IL-23 is affected by pharmacological inhibition of CK2 in developing T<sub>H</sub>17 cells. Previously reported resistance

toward experimental neuroinflammation in CD5-deficient mice was attributed to an abrogation of binding to CK2 and considered to be relevant for activation and persistence of effector T cells (51). Here, we sought to understand the molecular basis for the inability of T<sub>H</sub>17 cells under the influence of CK2 inhibition to induce disease, and measured the activity of possible candidates important for EAE induction. Indeed, inhibiting CK2 completely prevented cytokine-induced phosphorylation of STAT3 indicating its decisive role in cytokine-mediated activation. Notably, the gene locus encoding STAT3 belongs to the susceptibility areas identified by our genome-wide association study analysis in MS (52). Recently, it was reported that STAT3 expression by T<sub>reg</sub> cells is crucial for the ability to suppress T<sub>H</sub>17 cell responses (53), further demonstrating the crucial importance of this transcription factor in the regulation of T<sub>H</sub>17 cell responses *in vivo*.

Overall, our data indicate that CK2 has an important influence on T<sub>H</sub>17 cell fate, and thus beneficial effects on T<sub>H</sub>17 cell-driven pathology, such as that in MS, could result from blocking this kinase. With its role in the lineage decision of T<sub>H</sub>17/iT<sub>reg</sub> cell development, for which we provide compelling evidence *in vitro* and *in vivo* in EAE, CK2 could also be a significant drug target in autoimmune neuroinflammation where T<sub>H</sub>17 cell-mediated pathology is crucial.

## Methods

**Mice.** C57BL/6J, myelin oligodendrocyte glycoprotein (MOG)-specific T-cell receptor transgenic mice (2D2), C57BL/6 *Rag1*<sup>−/−</sup> and SJL/J mice were purchased from The Jackson Laboratory. *Cd4*<sup>CreERT2</sup> mice (33) were provided by A. Diefenbach, Department of Microbiology, University Medical Center Mainz, Mainz, Germany. Mice carrying a loxP-flanked *Csnk2b* allele (*Csnk2b*<sup>fl</sup>) on C57BL/6 background (54) were provided by B. Boudryeff, KinaseDetect, Aarslev, Denmark. To obtain *Cd4*<sup>CreERT2</sup>*Csnk2b*<sup>fl/fl</sup> mice, *Cd4*<sup>CreERT2</sup> and *Csnk2b*<sup>fl</sup> mice were crossed. IL-23R-GFP reporter mice (32) were kindly provided by M. Oukka, Department of Immunology, University of Washington, Seattle, WA. Animal procedures were performed in accordance with the European Union normative for care and use of experimental animals and were approved by the Landesuntersuchungsamt Rheinland-Palatinate, Germany. Mice were housed in a specific pathogen-free colony at the animal facility of Johannes Gutenberg University.

**Antibodies.** The following antibodies were used in cell isolation procedures: anti-CD4 (H129.19 and RM4-5), anti-CD62L (MEL-14), anti-CD44 (IM7), and anti-CD25 (PC61). For *in vitro* stimulation of T cells, anti-CD3 (145-2C11) and anti-CD28 (37.51) were used at 4 μg/mL each. The following fluorescent dye-labeled antibodies were used for flow cytometry: anti-CD4 (RM4-5 and GK1.5), anti-CD8 (SK1), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-RORγt (B2D), anti-FOXP3 (FJK-16s), anti-T-bet (ebio4B10), anti-GATA-3 (TWAJ), anti-IL-17 (ebio17B7), anti-IL-4 (11B11), anti-IFN-γ (XMG1.2), anti-IL-9 (D930-2C12), anti-STAT3-Y705P (4/P-STAT3; from BD Biosciences), anti-human-CD4 (L200), anti-human-GM-CSF (GM2F3), anti-human-IL-17a (N49.653; both BD Biosciences), and anti-human-IL-22 (22URT1). All antibodies were purchased from eBioscience or Biolegend, unless otherwise specified. For ELISA experiments, anti-IL-2 (JE56-1A12) was used as the primary capture antibody and biotinylated anti-IL-2 (JE56-5H4) as the secondary detection antibody.

**EAE.** EAE was induced using PLP<sub>139–151</sub>/CFA or MOG<sub>35–55</sub>/CFA emulsion in prefilled syringes (Hooke Laboratories) as described (34, 35, 55). For preventive therapy with CK2 inhibitors, 10 mg/kg body weight DMAT (solubilized in DMSO) was injected i.p. in a volume of 50 μL every 72 h, or alternatively 75 mg/kg body weight CX4945 (solubilized in 0.1 mM NaHCO<sub>3</sub>) was injected i.p. in 100 μL daily throughout the course of the EAE beginning on the day of immunization. For therapeutic treatment of established EAE with CK2 inhibitor DMAT, 20 mg/kg body weight was administered daily by i.p. injection in 100 μL. To induce genetic ablation of *Csnk2b* during EAE *in vivo*, 100 mg/kg tamoxifen dissolved in peanut oil was administered into *Cd4*<sup>CreERT2</sup>*Csnk2b*<sup>fl/fl</sup> and *Csnk2b*<sup>fl/fl</sup> control mice by i.p. injection in 40 μL at day −2 and 7 of immunization. Clinical symptoms were converted into disease scores as described elsewhere (56). Relapse incidence was defined for each mouse when the score reached at least one full number higher than the lowest score during remission.

**Cell isolation during EAE.** Brains, spinal cords, and spleens were removed, and immune cells were isolated as described previously (57).

**Naïve T-cell isolation.** CD4<sup>+</sup> T cells (H129.19) were isolated from spleens using MACS separation (Miltenyi Biotec) as described (58). CD4<sup>+</sup>CD25<sup>−</sup>CD62L<sup>+</sup>CD44<sup>−</sup> naïve T cells were stained and FACS purified from the preenriched fraction using BD FACSAria II Cell sorter to a purity of >98%.

## In Vitro Stimulation.

**T-cell differentiation.** For *in vitro* experiments, naïve T cells were cultured and stimulated as described (59). T<sub>H</sub>1, T<sub>H</sub>2, and T<sub>H</sub>9 cell differentiation was initiated as described (59). T<sub>H</sub>17 cells were differentiated by IL-1β (10 ng/mL), mrlL-6 (10 ng/mL), mrlL-23 (20 ng/mL), anti-IL-4 (10 μg/mL; clone 11B11), and anti-IFN-γ (XMG1.2; 10 μg/mL). Alternatively, “TGF-β + IL-6” T<sub>H</sub>17 cells were differentiated by TGF-β (1 ng/mL), mrlL-6 (5 ng/mL), anti-IL-4 (10 μg/mL; clone: 11B11), and anti-IFN-γ (XMG1.2; 10 μg/mL). On day 3, cells were harvested and cultured in medium supplemented without cytokines for 48 h. iT<sub>reg</sub> cells were differentiated by porcine TGF-β (5 ng/mL) and mrlL-2 (250 ng/mL).

**Measurement of cytokine production.** T<sub>H</sub> cells were harvested on day 5 from cultures or isolated from the CNS, washed with PBS, counted, and resuspended in medium. For flow cytometry, cells were stimulated with 1 μM ionomycin (Sigma) and 20 ng/mL PMA (Sigma) for 5 h in the presence of Monensin (eBioscience/Biolegend). For analysis of cytokine production using ELISA or mRNA expression using quantitative real-time PCR (qRT-PCR), T<sub>H</sub> cells were stimulated for 24 h with anti-CD3 (4 μg/mL).

**Flow-Cytometric Analysis.** Flow-cytometric experiments were performed on a BD LSR II and analyzed using BD FACSDiva software 6.0 and FlowJo software (Tree Star). For surface staining, cells were incubated with antibodies against the indicated markers and fixable viability dye 780 (eBioscience) for live/dead cell determination for 30 min at 4 °C. Intracellular staining was carried out using the FOXP3 staining kit (eBioscience). To determine cytokine production, cells were stimulated as indicated above.

**ELISA.** Cytokine production of stimulated T<sub>H</sub>17 and iT<sub>reg</sub> was measured by ELISA. Measurement of IL-17 was performed using mouse IL-17 DuoSet Kits (R&D). Detection of IL-2 was carried out as described (60).

**CK2 Inhibitors.** The CK2 inhibitor DMAT was purchased from Merck Millipore and dissolved in DMSO. CK2 inhibitor CX4945 (silmilasertib) was purchased from Selleckchem and dissolved in DMSO, or as sodium salt dissolved in 0.1 mM NaHCO<sub>3</sub> buffer (pH 8.2). Before use, toxicity of CK2 inhibitors on cultured CD4<sup>+</sup> T cells was titrated, as determined by flow-cytometric analysis using fixable viability dye 780 (eBioscience). DMAT was used at a final concentration of 1.5 μM and CX4945 at 1 μM *in vitro*.

**mRNA Detection by qRT-PCR.** Isolation of total RNA from T<sub>H</sub> cells, reverse transcription (RT), as well as qRT-PCRs to quantify expression of mRNAs were described (59). The following oligonucleotides were used: IL-23R\_forw, CAG TTT CCC AGG TTA CAG C; IL-23R\_rev, GCA GGA TGT CCT CTG AGG A; GM-CSF\_forw, TGT GGT CTA CAG CCT CTC AGC AC; GM-CSF\_rev, ACC TTT CTG ACT GAT ATC CCC TTT G; RORγt\_forw, GTG TGT GCT GCT GGG CTA CC; RORγt\_rev, AGC CCT TGC ACC CCT CAC AG; HGPRT\_forw, GTT GGA TAC AGG CCA GAC TTT GTT G; and HGPRT\_rev, GGG TAG GCT GGC CTA TAG GCT.

**Measurement of GM-CSF.** Analysis of GM-CSF production by T<sub>H</sub>17 cells was carried out in cell culture supernatants using CBA Mouse GM-CSF Flex Set and the Cytometric Bead Array Mouse/Rat Soluble Protein Master Buffer Kit (BD Biosciences).

**Carboxyfluorescein Succinimidyl Ester Proliferation Assay.** To determine proliferation of T<sub>H</sub>17 and iT<sub>reg</sub> cells in the absence and presence of CK2 inhibitors, naïve T cells were labeled with 1 μM carboxyfluorescein succinimidyl ester as described (60). Proliferation was measured on day 3 using flow cytometry.

**Human Th17 Culture.** Human PBMCs were isolated as described (61) and stimulated with Cytostim (Miltenyi Biotec). IL-17-secreting T cells were isolated using IL-17 Secretion Assay (Miltenyi Biotec). The IL-17<sup>+</sup>CD4<sup>+</sup> cells were cultured using IL-1β and IL-23, and expanded by anti-CD3/CD28/CD137 Dynabeads (Dyna) cells. Cells were treated with 10 μM CX4945 or with DMSO only. After 6 d, cells were restimulated with PMA (50 ng/mL) and ionomycin (0.5 μg/mL) and incubated for 5 h. Brefeldin A was added to all samples. Cells were stained with Fixable Viability Dye eFluor 450 (eBioscience) and the following antibodies: anti-CD4-PerCP, anti-IL-17-AF640, anti-IL-22-PECy7, and anti-GM-CSF-PE. Surface antigen expression and intracellular cytokine production were measured by flow cytometry.

**Statistical Analysis.** Data are shown as the mean values ± SEM, unless otherwise specified. Student's *t* test, one-way ANOVA, or Mann-Whitney *U* test was employed, using the software GraphPad Prism to calculate statistical significance of the mean values. In all figures, asterisks indicate the following: \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

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