

Protein kinase CK2 governs the molecular decision between encephalitogenic T_H17 cell and T_{reg} cell development

Alexander Ulges^{a,1}, Esther J. Witsch^{b,1}, Gautam Pramanik^b, Matthias Klein^a, Katharina Birkner^b, Ulrike Bühler^b, Beatrice Wasser^b, Felix Luessi^b, Natascha Stergiou^a, Sarah Dietzen^a, Till-Julius Brühl^a, Toszka Bohn^a, Georg Bündgen^a, Horst Kunz^c, Ari Waisman^d, Hansjörg Schild^a, Edgar Schmitt^a, Frauke Zipp^{b,2}, and Tobias Bopp^{a,2,3}

^aInstitute for Immunology, University Medical Center of the Johannes Gutenberg University of Mainz, 55131 Mainz, Germany; ^bDepartment of Neurology, University Medical Center Mainz of the Johannes Gutenberg University of Mainz, 55131 Mainz, Germany; ^cInstitute of Organic Chemistry, Johannes Gutenberg University of Mainz, 55128 Mainz, Germany; and ^dInstitute for Molecular Medicine, University Medical Center of the Johannes Gutenberg University of Mainz, 55131 Mainz, Germany

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T helper 17 (T_H17) cells represent a discrete T_H cell subset instrumental in the immune response to extracellular bacteria and fungi. However, T_H17 cells are considered to be detrimentally involved in autoimmune diseases like multiple sclerosis (MS). In contrast to T_H17 cells, regulatory T (T_{reg}) cells were shown to be pivotal in the maintenance of peripheral tolerance. Thus, the balance between T_{reg} cells and T_H17 cells determines the severity of a T_H17 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_H17 cell development and promotes the generation of T_{reg} 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_H17 cell development and suggest this kinase as a potential therapeutic target to treat T_H17 cell-driven autoimmune responses.

multiple sclerosis | T_H17 cells | regulatory T cells | FOXP3 | MS therapy

It is widely accepted that IL-17-producing T_H17 cells infiltrating the central nervous system (CNS) play a critical role in experimental autoimmune encephalomyelitis (EAE), and multiple sclerosis (MS) (1–4), whereas CD4⁺ forkhead box protein (FOXP3)⁺ regulatory T (T_{reg}) 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_H17 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_H17 cell pathogenicity. Initial responsiveness to IL-23 needs to be acquired during T_H17 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- β (8–10) and guided by the transcription factor RAR-related orphan receptor (ROR) γ t (11–13). It is of note that IL-6 signaling is conferred by STAT3 phosphorylation, and in *Stat3*-deficient animals T_H17 cell differentiation is impaired and EAE development is absent (14, 15). Thus, IL-6 and STAT3 phosphorylation initiate T_H17 cell differentiation, and IL-23 is needed for manifestation of the T_H17 cell pathogenicity. Another cytokine induced by IL-23 in T_H17 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_H17 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_H17 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_H17) cell development and effector function while at the same time promoting development of induced regulatory T (iT_{reg}) cells. Hence, modulation of CK2 activity might represent a promising approach for the treatment of MS and other T_H17 cell-driven inflammatory diseases.

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¹A.U. and E.J.W. contributed equally to this work.

²F.Z. and T. Bopp contributed equally to this work.

³To whom correspondence should be addressed. Email: bopppt@uni-mainz.de.

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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-tetra-bromo-1*H*-benzimidazole (DMAT) (30) or the respective vehicle control with or without addition of TGF- β . Although treatment with DMAT had no effect on cell viability (Fig. S1A), proliferation (Fig. S1B), or IL-2 production (Fig. S1C), IL-17 production was substantially reduced in both the absence and presence of TGF- β (Fig. 1A and B) in a dose-dependent manner (Fig. S1D). ROR γ t mRNA (Fig. S1E) as well as protein expression (Fig. S1F) was significantly reduced between day 4 and 5 upon T_H17 cell differentiation. Remarkably, in the presence of TGF- β 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. 1C 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. S2A) or proliferation (Fig. S2B), it resulted in a dose-dependent inhibition of T_H17 differentiation as evidenced by a strongly reduced ability to produce IL-17 (Fig. S2C) with only marginal effects on ROR γ t mRNA (Fig. S2D) or protein expression (Fig. S2E). Again, addition of TGF- β resulted in a comparable inhibition of T_H17 differentiation in the presence of CX4945 (Fig. S2F and G) and in a concomitant induction of FOXP3 expression (Fig. S2F and H) under T_H17 cell-polarizing conditions. Importantly, CK2 inhibition by either DMAT (Fig. S3A) or CX4945 (Fig. S3B) 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 μ M DMAT and 1 μ 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*^{-/-}) host mice (Fig. S4A). Concomitantly, we detected a reduced proportion of CNS-infiltrating CD4⁺ T cells (Fig. S4B) but no effect on peripheral CD4⁺ T-cell numbers (Fig. S4C). Among the CNS-infiltrating CD4⁺ T cells, the percentages and absolute numbers (Fig. S4D) of IL-17⁺ROR γ t⁺ T_H17 cells were strongly reduced in favor of CNS-infiltrating FOXP3⁺ iT_{reg} cells (Fig. S4E). 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. S5A), whereas hardly any demyelination was found after transfer of CK2 inhibitor DMAT-treated T_H17 cells (Fig. S5A). Furthermore, the density of mononuclear cells within the white matter region was significantly reduced in mice that received DMAT-treated T_H17 cells (Fig. S5B).

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 γ 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. 2A), the CK2 inhibitor DMAT (Fig. 2A) as well as the CK2 inhibitor CX4945 (Fig. S6A) 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. 2B) and CX4945 (Fig. S6B) 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. S6C). 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. 2C and Fig. S6D) or CX4945 (Fig. S6E) 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

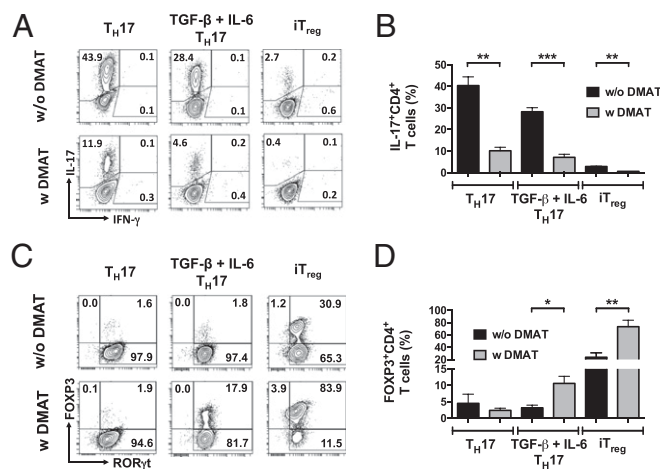


Fig. 1. Inhibition of protein kinase CK2 interferes with T_H17 lineage commitment and favors iT_{reg} cell differentiation. (A) Flow-cytometric analysis of IL-17 and IFN- γ expression in ionomycin/PMA-stimulated T_H17, TGF- β + IL-6 T_H17, and iT_{reg} cells, differentiated in the presence (w DMAT) or absence (w/o DMAT) of the CK2 inhibitor DMAT. (B) Percentage of IL-17⁺ among CD4⁺ T_H cells differentiated toward T_H17, TGF- β + IL-6 T_H17, and iT_{reg} cells in the presence (w DMAT) or absence (w/o DMAT) of DMAT. (C) Flow-cytometric analysis of FOXP3 and ROR γ t expression in ionomycin/PMA-stimulated T_H17, TGF- β + 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⁺ cells among CD4⁺ T_H17, TGF- β + 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).

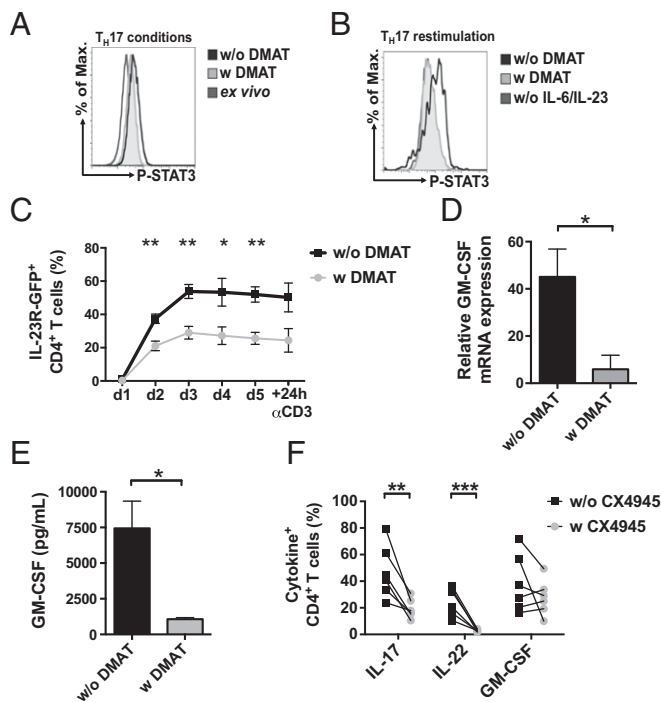


Fig. 2. CK2 inhibition results in diminished expression of molecules associated with pathogenicity of T_H17 cells. (A) Flow-cytometric analysis of Y705 STAT3 phosphorylation in naive T cells, differentiated toward T_H17 cells for 24 h in the presence (w DMAT) or absence (w/o DMAT) of CK2 inhibitor DMAT. Representative overlays from three independent experiments are shown. (B) Analysis of Y705 STAT3 phosphorylation in T_H17 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⁺ among CD4⁺ T cells differentiated toward T_H17 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 T_H17 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 T_H17 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 T_H17 cells in the presence of IL-23 resulted in enhanced IL-17 expression, IL-23-mediated stimulation of T_H17 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 T_H17 cells, it was demonstrated that IL-23R 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 T_H17 cells. CD4⁺ T cells stimulated under T_H17 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, T_H17 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 T_H17 cells, we isolated IL-17-secreting CD4⁺ 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 T_H17 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 T_H17 cells and suggest CK2 inhibitors as potential drugs to treat T_H17 -driven autoimmune diseases.

Inhibition of CK2 Suppresses T_H17 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 T_H17 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 (silmisertib), 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⁺ T cells infiltrating the CNS (Fig. S7F) but also strongly reduced absolute numbers of IL-17⁺RORγt⁺CD4⁺ T_H17 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 T_H17 cell-driven immunopathology in the CNS and suggest CK2 as a valuable target in the treatment of T_H17 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 T_H17 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*^{CreERT2}) (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⁺ T cells (*Cd4*^{CreERT2} *Csnk2b*^{fl/fl}), we induced EAE by MOG_{35–55} (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⁺ T cells resulted in an almost complete prevention of EAE induction (Fig. 3D). Concomitantly, we detected a reduced proportion of CNS-infiltrating IL-17⁺RORγt⁺CD4⁺ T_H17 cells (Fig. 3E and Fig. S7J) and enhanced percentage of FOXP3-expressing T_{reg} cells in CNS-draining lymph nodes (Fig. 3F and Fig. S7K).

Taken together, our data demonstrate that CK2 activity represents a molecular switch between T_H17 cell and iT_{reg} cell development and that the CK2 inhibitor CX4945 might have the

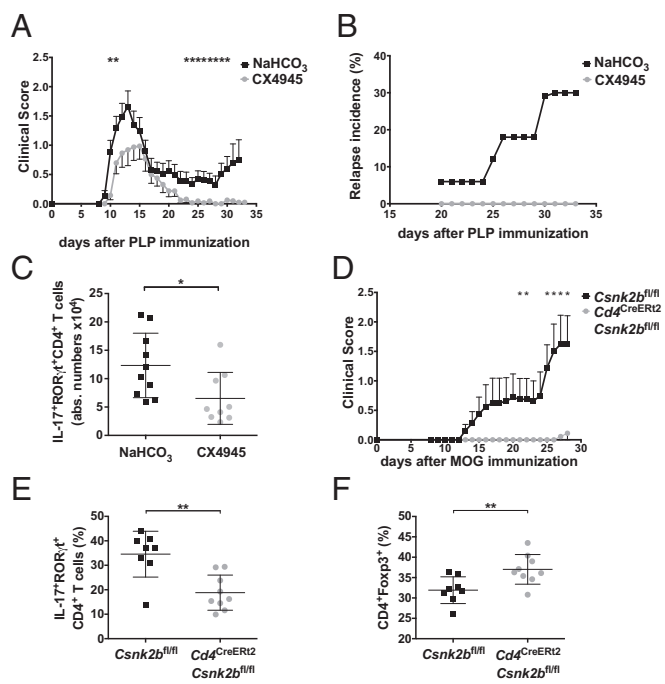


Fig. 3. Pharmacological inhibition of CK2 prevents T_H17 cell development and ameliorates EAE. (A) Clinical score of EAE in SJL mice. Animals were treated daily with 75 mg/kg body weight of CX4945 (w CX4945), or 0.1 mM NaHCO_3 buffer (pH 8.2) as solvent control (NaHCO_3) starting from day 0. Error bars 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_3), 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_3) or CK2 inhibitor CX4945. (C) Flow-cytometric analysis of IL-17 and ROR γ t expression among PMA/ionomycin-stimulated CD4^+ T cells isolated from CNS of mice that received CX4945 (CX4945) or respective solvent (NaHCO_3). Shown is the absolute number of IL-17 $^+$ ROR γ t $^+$ among CD4^+ 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 $\text{Csnk2b}^{\text{fl/fl}}$ ($n = 8$) and $\text{Cd4}^{\text{CreER12}} \text{Csnk2b}^{\text{fl/fl}}$ ($n = 9$) 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 $^+$ ROR γ t $^+$ cells among CD4^+ T cells from the CNS of $\text{Csnk2b}^{\text{fl/fl}}$ ($n = 8$) and $\text{Cd4}^{\text{CreER12}} \text{Csnk2b}^{\text{fl/fl}}$ ($n = 9$) 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 $^+$ among CD4^+ T cells in the CNS-draining lymph nodes of $\text{Csnk2b}^{\text{fl/fl}}$ ($n = 8$) and $\text{Cd4}^{\text{CreER12}} \text{Csnk2b}^{\text{fl/fl}}$ ($n = 9$) 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_H17 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_H17 cells as playing a crucial role (37). The differentiation of T_H17 cells has been shown to be dependent on the cytokines IL-6, IL-1 β 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_H17 cells, and in the absence of STAT3, differentiation of T_H17 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_{reg} 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_H17 cells. Most importantly, our findings demonstrate that CK2 is involved in controlling the lineage decision between iT_{reg} cells and T_H17 cells.

Recent studies have investigated the developmental checkpoints in the lineage decision and antipodal signaling pathways leading to the lineage commitment of T_H cells. It has been suggested that T_H17 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_H17 cells, but in turn favors the development of T_{reg} 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_H17 vs. iT_{reg} 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_H17 cells which underwent their conversion to ex- T_H17 cells by the proinflammatory cytokine IL-23 (44, 45). Another study demonstrated T-cell plasticity by showing that FOXP3 $^+$ ROR γ t $^+$ intermediate T cells are able to terminally differentiate into either FOXP3 $^+$ ROR γ t $^-$ (iT_{reg}) or FOXP3 $^+$ ROR γ t $^+$ (T_H17) 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 $^+$ T cells emerging from IL-17 $^+$ FOXP3 $^-$ T_H17 cells after in vivo transfer to such plasticity and suggest that CK2 inhibition before T_H17 cell transfer led to a susceptible intermediate cell type responding to in vivo stimuli favoring the development of FOXP3 $^+$ iT_{reg} cells.

Deciphering the nodal checkpoints that define the lineage decision for development of T_H17 and iT_{reg} 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_{reg} 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_H17 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_H17 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_H17 cells are regarded as a pattern by which T_H17 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_H17 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_H17 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_H17 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_{reg} cells is crucial for the ability to suppress T_H17 cell responses (53), further demonstrating the crucial importance of this transcription factor in the regulation of T_H17 cell responses in vivo.

Overall, our data indicate that CK2 has an important influence on T_H17 cell fate, and thus beneficial effects on T_H17 cell-driven pathology, such as that in MS, could result from blocking this kinase. With its role in the lineage decision of T_H17/iT_{reg} 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_H17 cell-mediated pathology is crucial.

Methods

Mice. C57BL/6J, myelin oligodendrocyte glycoprotein (MOG)-specific T-cell receptor transgenic mice (D2D2), C57BL/6 *Rag1*^{-/-} and SJL/J mice were purchased from The Jackson Laboratory. *Cd4*^{CreERT2} mice (33) were provided by A. Diefenbach, Department of Microbiology, University Medical Center Mainz, Mainz, Germany. Mice carrying a loxP-flanked *Csnk2b* allele (*Csnk2b*^{fl/fl}) on C57BL/6 background (54) were provided by B. Boudryeff, KinaseDetect, Aarslev, Denmark. To obtain *Cd4*^{CreERT2}*Csnk2b*^{fl/fl} mice, *Cd4*^{CreERT2} and *Csnk2b*^{fl/fl} 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 Rhineland-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 (JES6-1A12) was used as the primary capture antibody and biotinylated anti-IL-2 (JES6-5H4) as the secondary detection antibody.

EAE. EAE was induced using PLP_{139–151}/CFA or MOG_{35–55}/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 NaHCO₃) 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*^{CreERT2}*Csnk2b*^{fl/fl} and *Csnk2b*^{fl/fl} 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).

Naive T-cell isolation. CD4⁺ T cells (H129.19) were isolated from spleens using MACS separation (Miltenyi Biotec) as described (58). CD4⁺CD25⁻CD62L⁺CD44⁻ naive T cells were stained and FACS purified from the pre-enriched 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_H1, T_H2, and T_H9 cell differentiation was initiated as described (59). T_H17 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_H17 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_{reg} cells were differentiated by porcine TGF-β (5 ng/mL) and mrlL-2 (250 ng/mL).

Measurement of cytokine production. T_H 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_H 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_H17 and iT_{reg} 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 NaHCO₃ buffer (pH 8.2). Before use, toxicity of CK2 inhibitors on cultured CD4⁺ 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_H 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 TGC TGT CCT 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_H17 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_H17 and iT_{reg} 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⁺CD4⁺ cells were cultured using IL-1β and IL-23, and expanded by anti-CD3/CD28/CD137 Dynabeads (Dyna). 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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