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RESEARCH ARTICLE

Characterization of Mice with a Platelet-Specific Deletion of the Adapter Molecule ADAP

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ABSTRACT The adhesion and degranulation-promoting adapter protein (ADAP) is expressed in T cells, NK cells, myeloid cells, and platelets. The involvement of ADAP in the regulation of receptor-mediated inside-out signaling leading to integrin activation is well characterized, especially in T cells and in platelets. Due to the fact that animal studies using conventional knockout mice are limited by the overlapping effects of the different ADAP-expressing cells, we generated conditional ADAP knockout mice (ADAP$^{fl/fl}$ PF4-Cre^{tg}) (PF4, platelet factor 4). We observed that loss of ADAP restricted to the megakaryocytic lineage has no impact on other hematopoietic cells even under stimulation conditions. ADAP$^{fl/fl}$ PF4-Cre^{tg} mice showed thrombocytopenia in combination with reduced plasma levels of PF4 and transforming growth factor β1 (TGF-β1). *In vitro*, platelets from these mice revealed reduced P-selectin expression, lower levels of TGF-β1 release, diminished integrin αIIbβ3 activation, and decreased fibrinogen binding after stimulation with podoplanin, the ligand of C-type lectin-like receptor 2 (CLEC-2). Furthermore, loss of ADAP was associated with impaired CLEC-2-mediated activation of phospholipase Cγ2 (PLCγ2) and extracellular signal-regulated kinase 1/2 (ERK1/2). Induction of experimental autoimmune encephalomyelitis (EAE) in mice lacking ADAP expression in platelets caused a more severe disease. *In vivo* administration of TGF-β1 early after T cell transfer reduced EAE severity in mice with loss of ADAP restricted to platelets. Our results reveal a regulatory function of ADAP in platelets *in vitro* and during autoimmune disease EAE *in vivo*.

KEYWORDS adapter protein, signal transduction

ADAP—adhesion and degranulation-promoting adapter protein—is involved in intracellular signaling cascades leading to cell activation, adhesion, and motility in immune cells (1, 2). This adapter molecule is expressed within the hematopoietic lineage that includes T cells, NK cells, myeloid cells, and platelets but excludes mature B cells (3). In addition, ADAP expression was detected in neurons (4). ADAP was first described as SLAP-130 (SLP76 associated phosphoprotein of 130 kDa) in human and mouse T cells (5, 6). Using conventional ADAP knockout (k.o.) mice, it was shown that ADAP-deficient T cells exhibit impaired T cell proliferation, migration, and interleukin-2 (IL-2) production as well as reduced NF-κB activation (7, 8). More importantly, ADAP was the first adapter protein described linking T cell receptor (TCR)- and chemokine-mediated signaling to integrin activation, a process called inside-out signaling (7, 9, 10).

In murine platelets, the deletion of ADAP decreased the activation of αIIbβ3 integrin and attenuated fibrinogen binding by reducing the interaction with the integrin regulatory adapter proteins talin and kindlin-3 (11). In addition, ADAP was shown to participate in collagen-induced platelet activation mediated by collagen receptor GPVI

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and integrin $\alpha 2\beta 1$ (12). ADAP-deficient mice showed mild thrombocytopenia and normal bleeding time but prolonged rebleeding from tail wounds (13). Thrombus formation after carotoid artery occlusion was also impaired (14). Only recently, a nonsense mutation in the human gene encoding for ADAP was reported to cause small-platelet thrombocytopenia and bleeding tendency in patients (15, 16).

During the last 15 years, a key role of platelets in the formation of lymphatic vessels during embryogenesis was established. By forming aggregates, platelets are involved in the separation of newly formed lymphatic vessels from blood vessels (17). The interaction of platelets and lymphatic endothelial cells is mediated by the molecule podoplanin on lymphatic endothelial cells and the C-type lectin receptor CLEC-2 on the surface of platelets. Recently, a role of platelet CLEC-2 receptor interaction with podoplanin in maintaining high endothelial venule integrity was shown (18). CLEC-2 was originally described as the platelet receptor for the snake venom protein rhodocytin purified from the Malayan pit viper (19). CLEC-2 is a platelet-activating receptor as stimulation by agonists results in integrin activation, aggregation, and secondary mediator release. The CLEC-2 receptor contains a hemi-immunoreceptor tyrosine activation motif (hemi-ITAM). Upon receptor dimerization, this allows binding and activation of the Syk kinase. The adapter proteins LAT and SLP-76 were also shown to be involved in CLEC-2 signaling (20). Loss of CLEC-2, Syk, SLP-76, and phospholipase Cγ2 (PLCγ2) in platelets leads to severe malformation of lymphatics during embryogenesis (21–23). However, CLEC-2-deficient mice show only minimal bleeding disorder. As ADAP is an inducible direct binding partner of SLP-76 in T cells (5, 6) and platelets (14), a possible modulating effect of ADAP on CLEC-2 signaling is hypothesized. Indeed, detailed proteomic analysis of the CLEC-2 signalosome after rhodocytin stimulation identified (among others) the adapter protein ADAP (24).

In our previous work, we reported that ADAP-deficient mice undergo milder experimental autoimmune encephalomyelitis (EAE), a mainly T cell-mediated neuroinflammatory disease. This was shown for active EAE as well as in a passive-transfer EAE model. The induction of EAE in ADAP-deficient recipient mice reconstituted with wild-type bone marrow also revealed milder EAE, suggesting a mainly T cell-independent mechanism. These data indicated possible effects of radioresistant cells or indirect effects of other hematopoietic cells (25).

The role of platelets in EAE is controversial. It has been clearly shown that platelets are present in the inflamed spinal cord of mice after EAE induction (26). Platelet depletion during the early immunization phase did not affect EAE development. In contrast, depletion of platelets suppressed EAE when applied in the effector-inflammatory phase of the disease. This observation was explained by the fact that platelets secrete chemokines and proinflammatory cytokines, thus activating invading leukocytes or resident microglia (27). Another series of studies demonstrated that application of antithrombocyte serum during late stages of EAE exacerbated the disease. It was shown that activated platelets form aggregates with CD4 T cells, leading to decreased levels of CD4 T cell activation (28). Summarizing, it is well accepted that platelets and platelet mediators are involved in inflammation during EAE. However, the exact role of platelets during different phases of the disease remains to be elucidated.

The impact of ADAP deficiency restricted to platelets on the course of EAE has not been investigated so far. Experimental animal studies using conventional ADAP knockout mice are limited by the overlapping effects of different ADAP-expressing immune cells. To overcome this problem, we used the Cre recombinase expressed under the control of the PF4 promoter to generate conditional knockout mice with lineage-specific deletion of ADAP (29). In contrast to other reports (30, 31), we found no Cre recombination beyond the megakaryocytic lineage in myeloid and lymphoid cells even under inflammatory conditions.

In this study, we demonstrated that loss of ADAP restricted to the megakaryocytic lineage (i) has no impact on other hematopoietic cells, (ii) causes thrombocytopenia and reduced plasma levels of PF4 and transforming growth factor $\beta 1$ (TGF-$\beta 1$), (iii)

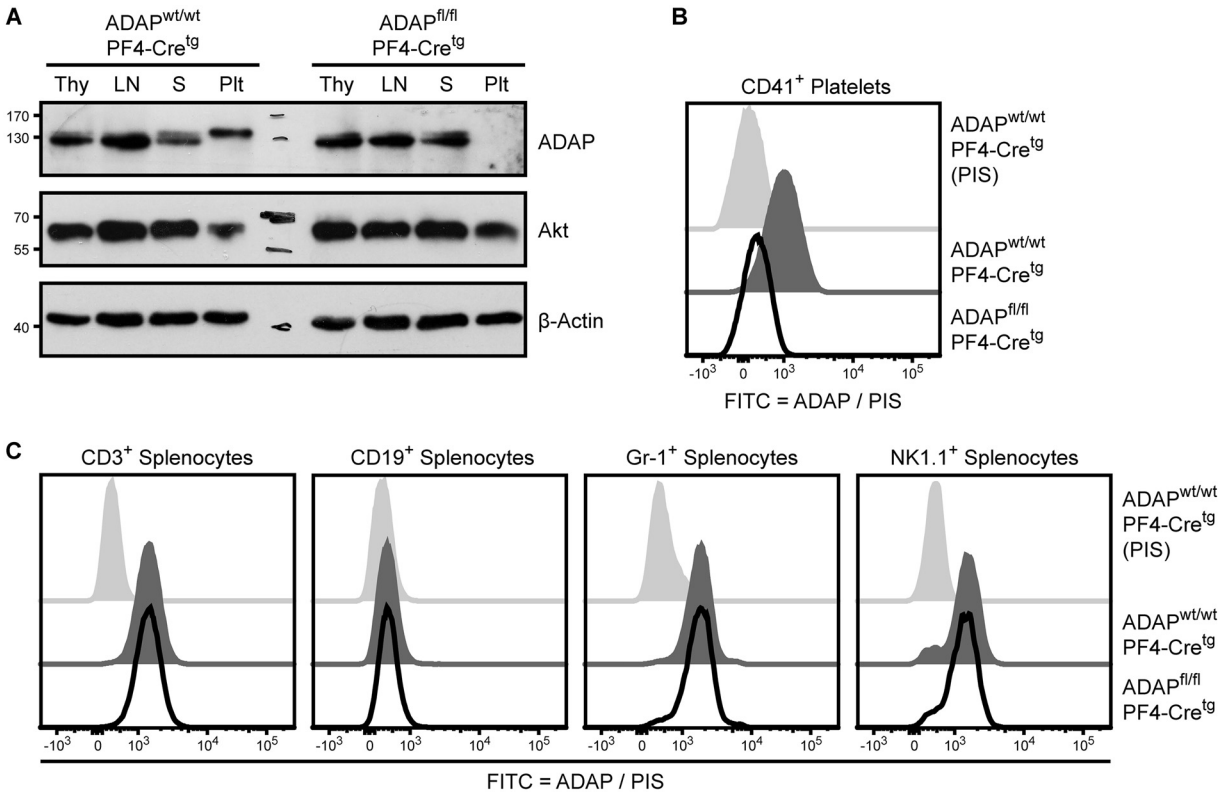

FIG 1 PF4-Cre-mediated recombination specifically inactivates *Fyb* gene in platelets. (A) Lysates of thymus (Thy), lymph nodes (LN), spleen (S), and platelets (Plt) isolated from platelet-rich plasma from conditional knockout mice ($ADAP^{fl/fl}$ $PF4\text{-}Cre^{tg}$) and Cre control mice ($ADAP^{wt/wt}$ $PF4\text{-}Cre^{tg}$) were separated by SDS-PAGE, and Western blots were probed with the indicated antibodies. Data represent one experiment representative of three independent experiments. (B) Flow cytometric analysis of washed platelets from conditional knockout mice and Cre control mice stained with anti-ADAP or preimmune sera (PIS). FITC, fluorescein isothiocyanate. (C) Anti-ADAP or preimmune sera (PIS) were used to detect ADAP levels in T cells ($CD3^+$), B cells ($CD19^+$), granulocytes ($Gr1^+$), and NK cells ($NK1.1^+$) of the spleen from conditional knockout mice and Cre control mice by flow cytometry. Fluorescence-activated cell sorter (FACS) plots are representative of results from three independent experiments.

impairs CLEC-2-mediated platelet activation *in vitro*, and (iv) results in enhanced EAE severity.

RESULTS

Platelets of conditional knockout mice are devoid of ADAP. To generate a platelet-specific ADAP k.o. mouse, we made use of the commercially available C57BL/6N-$Fyb^{tm1a(EUCOMM)Hmgu}$/Cnrm (EUCOMM) mouse (32). In this so-called "knockout-first strategy" mouse, the ADAP allele is modified upstream and downstream of critical exon 2, the largest exon of ADAP. Crossing this mouse to a mouse ubiquitously expressing the Flp recombinase removed the "LacZ-Neo cassette" inserted into the intron region between exons 1 and 2, leaving the latter flanked by *loxP* sites ("floxed" ADAP allele/$ADAP^{fl/fl}$). This $ADAP^{fl/fl}$ strain was then crossed to the C57BL/6-Tg(Pf4-icre)Q3Rsko/J mouse expressing the Cre recombinase under the control of megakaryocyte-specific promoter PF4. Subsequent intercrossing of $ADAP^{wt/fl}$ $PF4\text{-}Cre^{tg}$ mice generated littermates of $ADAP^{wt/wt}$ $PF4\text{-}Cre^{tg}$ (Cre control) and $ADAP^{fl/fl}$ $PF4\text{-}Cre^{tg}$ (conditional k.o.) mice at normal Mendelian frequencies (data not shown).

The platelet-specific ADAP k.o. was first confirmed by Western blotting. Comparison of lysates of thymus, lymph nodes, spleen, and platelets showed a specific and complete loss of ADAP expression in platelets of $ADAP^{fl/fl}$ $PF4\text{-}Cre^{tg}$ (conditional k.o.) mice (Fig. 1A). Interestingly, as observed in $ADAP^{wt/wt}$ $PF4\text{-}Cre^{tg}$ (Cre control) mice, the platelets expressed only the 130-kDa isoform of ADAP, whereas cells from all other organs/tissues displayed expression of both 120-kDa (predominantly) and 130-kDa isoforms. A more detailed analysis by flow cytometry in which ADAP was intracellularly

stained by a sheep antiserum confirmed the specific ADAP k.o. in CD41$^+$ (integrin αIIb) platelets in ADAP$^{fl/fl}$ PF4-Cre^{tg} (conditional k.o.) mice (Fig. 1B). Splenocytes of ADAP$^{fl/fl}$ PF4-Cre^{tg} (conditional k.o.) mice stained for CD3, Gr-1, or NK1.1 showed ADAP expression as strong as that seen with those derived from ADAP$^{wt/wt}$ PF4-Cre^{tg} (Cre control) mice. Since mature B cells are known to lack ADAP expression, ADAP was not detectable in CD19$^+$ splenocytes (Fig. 1C).

PF4-Cre-mediated recombination does not occur beyond the megakaryocyte lineage even after stimulation. Two recent publications raised concerns about the specificity of the PF4 promoter for megakaryocyte/platelet-restricted Cre expression (30, 31). It was demonstrated that PF4-Cre was activated in up to 30% of splenic myeloid and lymphoid cells. Therefore, we generated a reporter strain by crossing C57BL/6-Tg(Pf4-icre)Q3Rsko/J mice to Rosa26-tdRFP (tdRFP, tandem-dimer red fluorescent protein) reporter mice. In Rosa26-tdRFP mice, red fluorescent protein (RFP) expression is prevented by a "stop cassette" and an inverted coding sequence of tdRFP. Due to the presence of *loxP* and inverted *loxP* sites flanking the stop cassette and the tdRFP sequence, Cre expression results in excision of the stop cassette and inversion of the tdRFP sequence allowing its expression. CD41$^+$ platelets served as positive control in flow cytometric analyses of Rosa26-tdRFP^{het} PF4-Cre^{tg} and Rosa26-tdRFP^{het} PF4-Cre^{ntg} littermates, and only mice carrying the PF4-Cre transgene showed RFP expression (Fig. 2A). In contrast, splenocytes of mice of both genotypes stained for CD3, CD19, Gr-1, or NK1.1 displayed no RFP signal at steady state (data not shown). Splenocytes of Rosa26-tdRFP^{het} PF4-Cre^{tg} and Rosa26-tdRFP^{het} PF4-Cre^{ntg} littermates were additionally stimulated for 72 h with either phytohemagglutinin (PHA) (Fig. 2B to D) or PMA plus ionomycin (data not shown) to control for PF4 promoter activity under stimulatory/inflammatory conditions. Afterward, cells were stained for CD69, CD3, CD19, Gr-1, or NK1.1 and analyzed for RFP expression by flow cytometry; however, no effect of Cre recombinase could be detected (Fig. 2C and D). Equal levels of stimulation of splenocytes derived from mice of the two genotypes by the presence of PHA (Fig. 2B) or PMA plus ionomycin (data not shown) were shown by virtually identical frequencies of CD69-positive cells (Fig. 2B, left panel) and CD69 expression levels (Fig. 2B, right panel). In immune cell subsets, only a slight and statistically not significant increase of the mean fluorescence intensity of RFP in the case of PHA stimulated splenocytes of PF4-Cre^{tg} versus PF4-Cre^{ntg} reporter mice was observed (Fig. 2D, left panel). In total, less than 3% of each cell population showed recombination, with only CD19$^+$ B cells showing a statistically significant increase in the frequency of recombined cells (Fig. 2D, right panel). However, since B cells lack ADAP expression (see also Fig. 1C), PF4-Cre-mediated recombination has no consequences in the case of our conditional ADAP k.o. mouse.

On the basis of the results described above, we conclude that expression of Cre controlled by the PF4 promoter in our mice and under our experimental conditions was megakaryocyte/platelet specific and resulted in loss of ADAP expression exclusively in platelets.

Loss of ADAP in the megakaryocytic lineage has no impact on immune cell distribution. We next analyzed the constitution of primary (thymus and bone marrow) and secondary (spleen) lymphoid organs of ADAP$^{wt/wt}$ PF4-Cre^{tg} (Cre control) and ADAP$^{fl/fl}$ PF4-Cre^{tg} (conditional k.o.) mice. As expected, the total numbers of cells in bone marrow, thymus, and spleen were identical in the mice of the two genotypes (Fig. 3A, C, and E). In addition, neither the distribution of thymic doubly negative cells (CD4$^-$ CD8$^-$), doubly positive cells (CD4$^+$ CD8$^+$), and CD4$^+$ and CD8$^+$ singly positive cells (Fig. 3B) nor the distribution of the precursor pro-B cell/pre-B cell, immature, and mature B cells in bone marrow (Fig. 3D) was affected in ADAP$^{fl/fl}$ PF4-Cre^{tg} (conditional k.o.) mice. The same holds true for the proportions of T cells (CD3$^+$ CD4$^+$, CD3$^+$ CD8$^+$, CD3$^+$, and CD3$^+$ CD69$^+$), NK cells (NK1.1$^+$ NKp46$^+$), and B cells (B220$^+$ IgM$^+$) in the spleen (Fig. 3F). Included in these data sets are total cell numbers and percentages of the conventional ADAP k.o. mouse in which the known reduction of total thymocytes and the changed distributions of doubly negative cells (representing increases) and singly

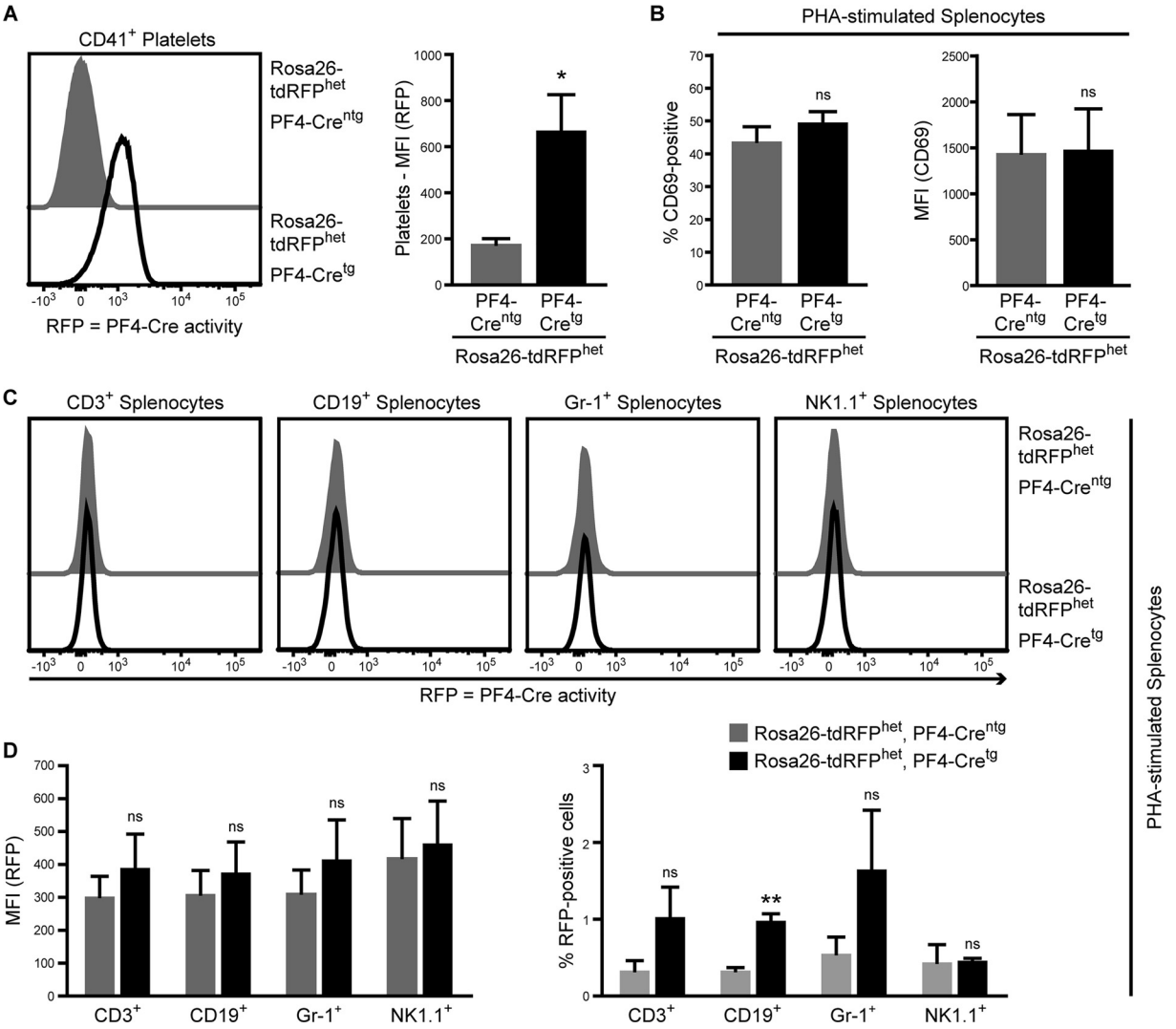

FIG 2 PF4-Cre-mediated recombination does not occur beyond the megakaryocyte lineage. (A) Flow cytometric analysis of washed platelets from Rosa26-tdRFP^{het} PF4-Cre^{ntg} and Rosa26-tdRFP^{het} PF4-Cre^{tg} littermates for RFP expression indicative of PF4-Cre-mediated recombination (left panel) and quantification (mean fluorescence intensity [MFI], right panel). Results are expressed as means plus SEM of data from five independent experiments (*, $P < 0.05$). (B) Flow cytometry-based quantification of frequency (left panel) and magnitude (MFI, right panel) of CD69 expression of splenocytes from Rosa26-tdRFP^{het} PF4-Cre^{ntg} and Rosa26-tdRFP^{het} PF4-Cre^{tg} littermates after stimulation with PHA (4 μg/ml) for 72 h (means plus SEM, $n = 3$; ns, not significant). (C) Flow cytometric analysis of RPF expression in CD3$^+$, CD19$^+$, Gr-1$^+$, and NK1.1$^+$ splenocytes after stimulation with PHA (4 μg/ml) for 72 h. (D) Quantification of magnitude (MFI, left panel) and frequency (right panel) of RFP expression of the cells indicated in panel C (means plus SEM, $n = 3$).

positive cells (representing decreases) can be seen (33). In the spleen, conventional ADAP k.o. mice display reduced percentages of T cells (CD3$^+$), which is mainly due to a reduction in the levels of CD3$^+$CD8$^+$ T cells. In contrast, the percentage of B cells (B220$^+$ IgM$^+$) increased, thereby leaving the total number of splenocytes unaffected.

Thus, these data demonstrate that the conditional ADAP k.o. in platelets (ADAP$^{fl/fl}$ PF4-Cre^{tg}) has no effect on the cellular constitution of primary and secondary lymphoid organs.

Loss of ADAP in the megakaryocytic lineage causes thrombocytopenia. We continued with the analysis of the blood of ADAP$^{wt/wt}$ PF4-Cre^{tg} (Cre control) and ADAP$^{fl/fl}$ PF4-Cre^{tg} (conditional k.o.) littermates. Using a hematology analyzer, we found identical total numbers of white blood cells, lymphocytes, monocytes, and granulocytes in the mice of the two genotypes (Fig. 4A). A reduction of cell numbers could be detected only in the conventional ADAP k.o. mouse. However, the specific loss of ADAP expression in platelets (ADAP$^{fl/fl}$ PF4-Cre^{tg}) caused thrombocytopenia virtually identical

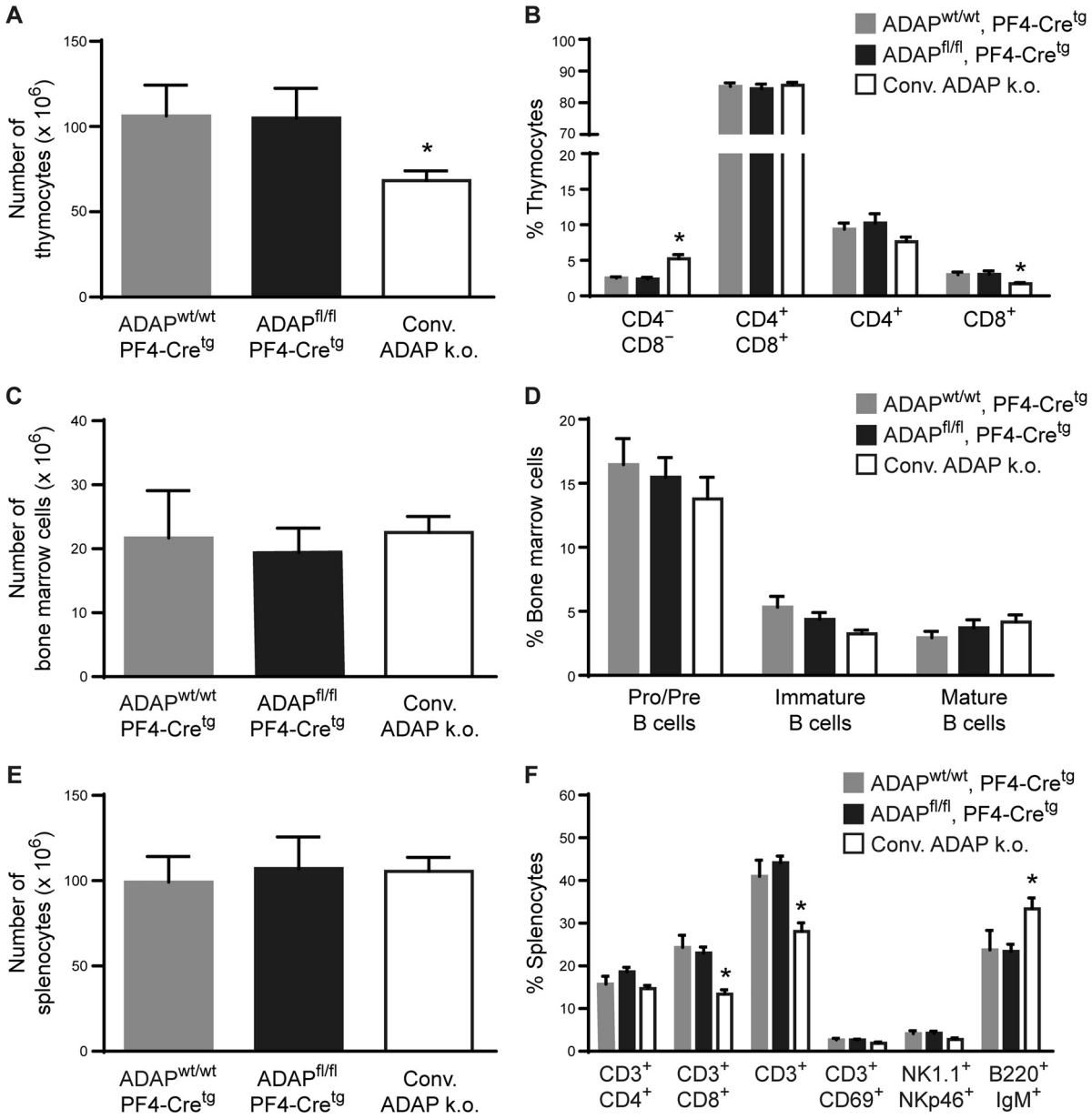

FIG 3 Conditional deletion of ADAP in platelets has no impact on the immune cell distribution. Cells were isolated cells from thymus (A and B), bone marrow (C and D), and spleen (E and F) of ADAP$^{fl/fl}$ PF4-Cre^{tg}, ADAP$^{wt/wt}$ PF4-Cre^{tg}, and conventional ADAP knockout mice (Conv. ADAP k.o.). The absolute cell numbers (left panel) and the percentages of cells (right panel) are given. The lymphocyte subpopulations were stained with the indicated antibodies. Pro/Pre B cells, B220^{low} IgM^{-}; Immature B cells, B220^{low} IgM^{+}; Mature B cells, B220^{high} IgM^{+}. Results are expressed as means plus SEM of data from six independent experiments (*, $P < 0.05$).

to what was observed in the conventional ADAP k.o. mouse, reducing the number of platelets to 38% of the level seen with the ADAP$^{wt/wt}$ PF4-Cre^{tg} (Cre control) mouse (Fig. 4B). It was previously reported that conventional ADAP k.o. mice show normal bleeding time but rebleed from mouse tail wounds (13). Therefore, we measured bleeding time in the conditional ADAP k.o. mice. As shown in Fig. 4C, the ADAP$^{fl/fl}$ PF4-Cre^{tg} conditional k.o. mice and ADAP$^{wt/wt}$ PF4-Cre^{tg} control littermates exhibited comparable bleeding times. Rebleeding occurred more frequently in the platelet-specific ADAP k.o. mice than in the control animals (Fig. 4C, lower panel). Thus, the thrombocytopenia and the higher frequency of secondary bleeding observed in conditional ADAP k.o. mice parallel the phenotype of conventional ADAP k.o. mice.

Next, we measured the plasma levels of PF4 and TGF-β1, two components of α-granules of platelets. In accordance with the reduced platelet numbers, we found

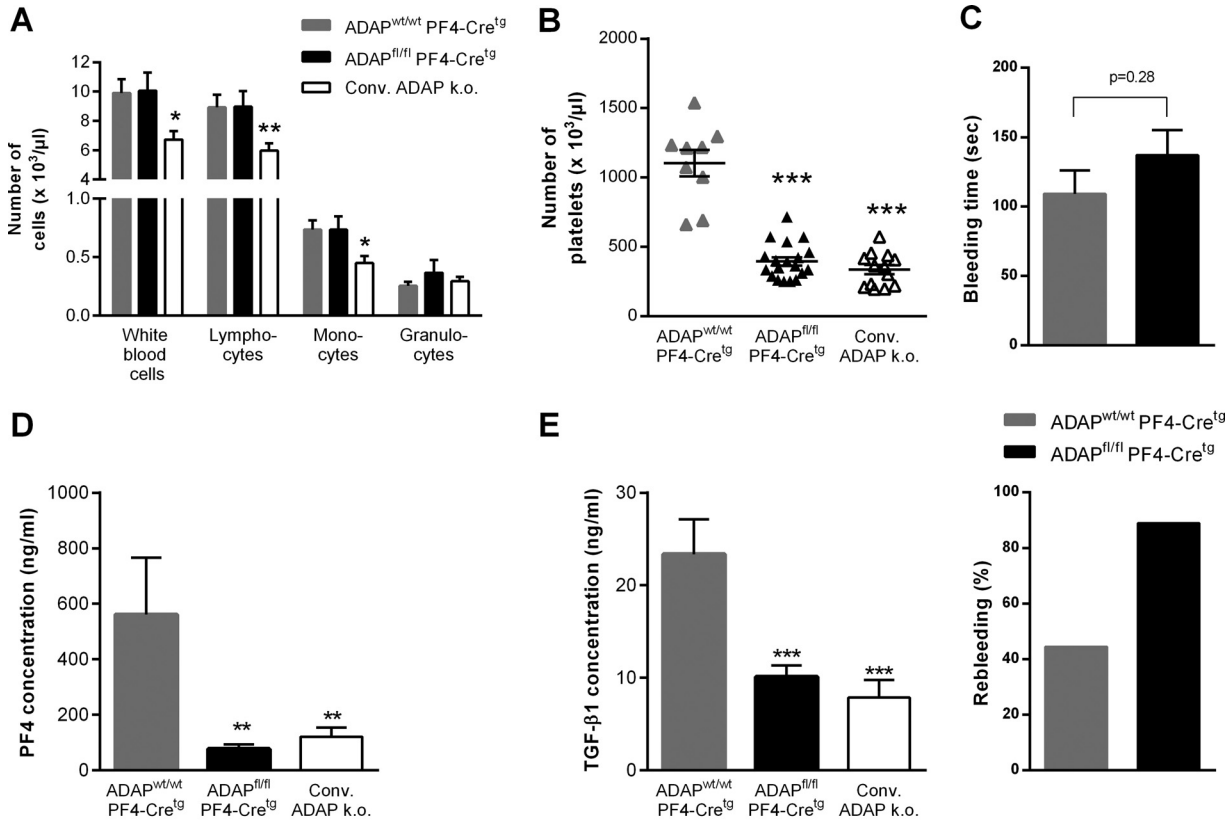

FIG 4 Conditional ADAP knockout mice (ADAP$^{fl/fl}$ PF4-Cre^{tg}) show thrombocytopenia. (A) White blood cell count and leukocyte differential from ADAP$^{fl/fl}$ PF4-Cre^{tg} mice and ADAP$^{wt/wt}$ PF4-Cre^{tg} mice as well as from conventional ADAP knockout mice (Conv. ADAP k.o.) were measured. Data are shown as means plus SEM ($n = 6$). (B) Platelet levels are given as means ± SEM ($n = 9$). (C) (Upper panel) Tail bleeding times of ADAP$^{fl/fl}$ PF4-Cre^{tg} conditional k.o. mice and ADAP$^{wt/wt}$ PF4-Cre^{tg} control animals were compared (means plus SEM, $n = 9$ per group). The percentages of mice that showed rebleeding within 5 min are presented in the lower panel. (D and E) Cytokine concentrations of PF4 (D) and latent TGF-β1 (E) were determined in platelet-free plasma from these mice using ELISA. Shown are means plus SEM (minimum $n = 5$). *P* values are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

strong reductions of PF4 and TGF-β1 levels (14% and 43%, respectively, compared to ADAP$^{wt/wt}$ PF4-Cre^{tg} [Cre control]) in plasma of ADAP$^{fl/fl}$ PF4-Cre^{tg} (conditional k.o.) mice (Fig. 4D and E). The concentration of PF4 and TGF-β1 in platelet lysates did not show any differences between the conditional k.o. mice and corresponding control animals (data not shown), suggesting that the reduced PF4 and TGF-β1 plasma levels were likely due to thrombocytopenia.

ADAP is involved in CLEC-2-mediated integrin activation. Next, we investigated the impact of specific loss of ADAP in the megakaryocytic/platelet lineage on platelet function *in vitro*. It was previously shown that ADAP-deficient platelets have defects in integrin αIIbβ3 activation after stimulation of different receptors, including collagen receptor GPVI, glycoprotein GPIb-V-XI, and thrombin receptor PAR1/4 (13, 14). As ADAP was found to participate in CLEC-2 signaling (24), we focused on the issue of whether ADAP is involved in integrin αIIbβ3 activation after stimulation of platelet CLEC-2 receptor. To test this, we preincubated washed platelets from conditional k.o. mice and control mice with Fc-podoplanin chimeric protein, the endogenous ligand of CLEC-2 receptor. Activation was performed by cross-linking with anti-Fc IgG antibody. The activated high-affinity conformation of αIIbβ3 and the degranulation-dependent P-selectin surface expression were simultaneously detected by flow cytometry. ADAP-deficient platelets from conditional k.o. mice as well as from the conventional ADAP k.o. mice exhibited reduced integrin activation as well as reduced P-selectin surface exposure after stimulation with podoplanin (Fig. 5A). Quantification revealed significant differences in mean fluorescence intensity (Fig. 5B and C). In contrast,

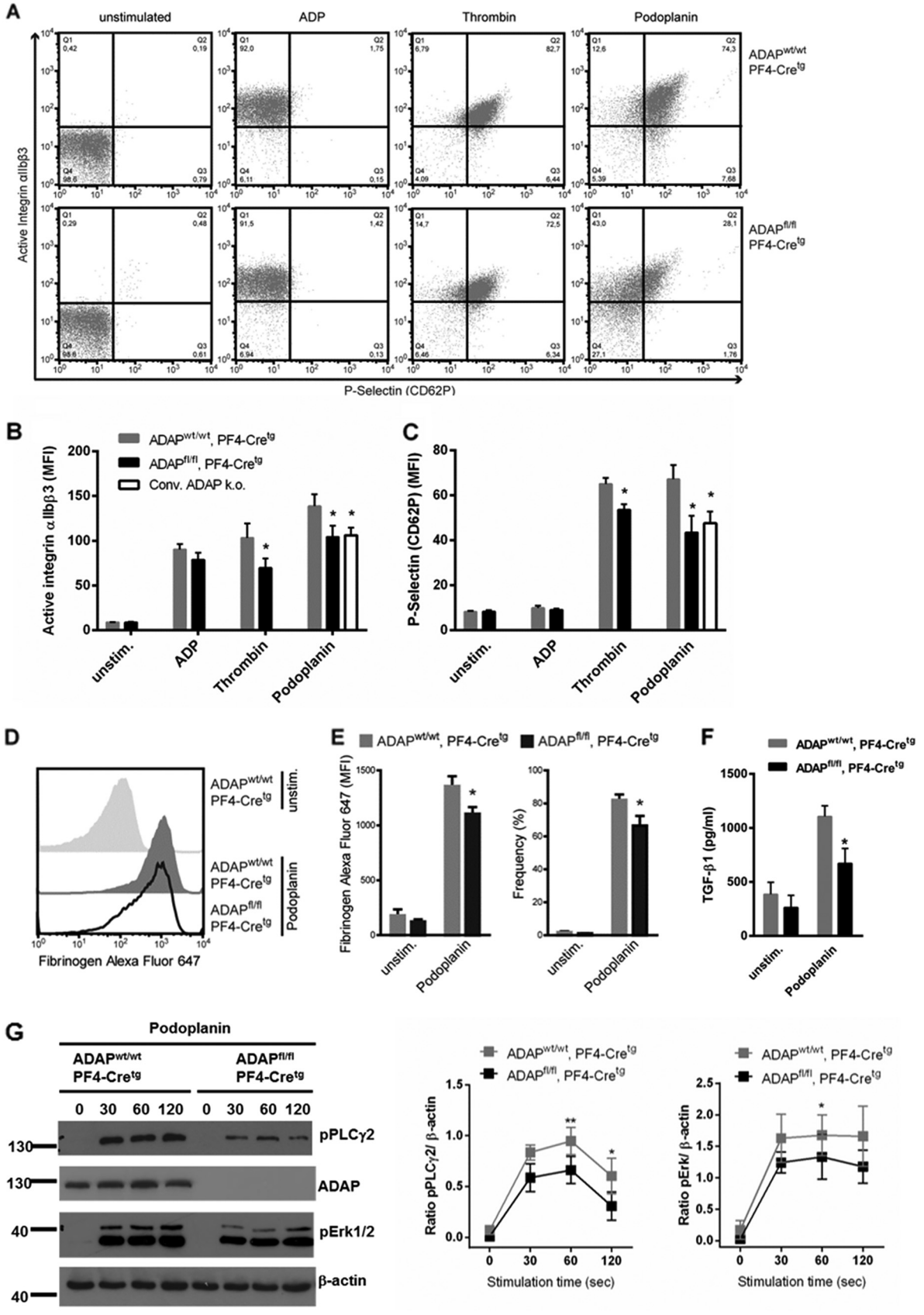

FIG 5 ADAP is involved in activation of αIIbβ3 after stimulation of CLEC-2 receptor. (A) Flow cytometric analysis of αIIbβ3 integrin activation and P-selectin surface expression was performed. Washed platelets were preloaded with Fc-podoplanin chimeric protein and activated by

(Continued on next page)

platelets from conditional k.o. mice showed overall normal activation after stimulation with ADP (Fig. 5A to C). Activated integrin αIIbβ3 binds fibrinogen, which can cross-link adjacent platelets into aggregates. Therefore, we used the same stimulation conditions in the presence of soluble Alexa Fluor 647-fibrinogen. After stimulation of CLEC-2 receptor with podoplanin, a statistically significant decrease in fibrinogen binding was found in ADAP-deficient platelets compared to ADAP-sufficient platelets (Fig. 5D and E). Accordingly, ADAP-deficient platelets displayed diminished αIIbβ3 integrin activation and reduced fibrinogen binding upon stimulation with podoplanin. As shown in Fig. 4, conditional ADAP k.o. mice had reduced TGF-β1 plasma concentrations compared to control mice. In addition, P-selectin surface expression was diminished in platelets of conditional ADAP k.o. mice after specific stimulation of the CLEC-2 receptor *in vitro*. Consequently, we hypothesized that TGF-β1 secretion after stimulation of CLEC-2 might also be affected by the absence of ADAP. To prove this hypothesis, we stimulated the CLEC-2 receptor as described above and quantified TGF-β1 in the supernatant. The concentration of TGF-β1 was significantly decreased in platelet supernatants from conditional k.o. mice (Fig. 5F). This result indicates that ADAP is involved in CLEC-2-mediated TGF-β1 release from platelets.

To gain insight into CLEC-2 signaling in the absence of ADAP, we investigated downstream effector enzymes PLCγ2 and extracellular signal-regulated kinase 1/2 (ERK1/2). Tyrosine phosphorylation of PLCγ2 and ERK1/2 was markedly reduced in platelets from conditional k.o. mice (Fig. 5G). Taken together, these results clearly indicate that ADAP is involved in signaling leading to platelet activation upon stimulation of CLEC-2 receptor with podoplanin.

Passive EAE is more severe in conditional ADAP knockout mice. Experimental autoimmune encephalomyelitis (EAE) is a strongly T cell-dependent demyelinating disease of the central nervous system (CNS) and represents the accepted animal model of the human disease multiple sclerosis. We have previously shown that conventional ADAP k.o. mice develop much milder EAE than wild-type mice, however, most likely mainly due to a T cell-independent mechanism (25). Therefore, on the basis of our observed effects of specific ADAP k.o. in platelets (namely, thrombocytopenia, reduced PF4 and TGF-β1 plasma levels, and reduced CLEC-2-mediated integrin αIIbβ3 activation and CD62P cell surface expression) and the controversial role of platelets in EAE (26–28), we explored whether the exclusive loss of ADAP expression in platelets had any impact on the course and/or severity of the disease.

EAE was induced passively by adoptive transfer of *in vitro* activated and polarized myelin oligodendrocyte glycoprotein (MOG)-specific TCR transgenic T cells (2D2 T cells), leading to gradual paralysis of mice (Fig. 6A). Our findings show a surprising result. The loss of ADAP expression exclusively in platelets (ADAP$^{fl/fl}$ PF4-Cre^{tg}) caused a more severe course of the disease. The latter was characterized by a faster rise of clinical signs, higher severity at the peak of the disease, and a higher incidence within the first week of clinical symptoms. Overall, this led to a statistically significant more severe course of EAE in mice exclusively devoid of ADAP expression in platelets.

Platelet-derived TGF-β1 is known to have profound immunosuppressive effects on T cell activation and proliferation (34). We hypothesized that the reduced TGF-β1 plasma levels (as shown in Fig. 4E) might contribute to the enhanced EAE severity in

FIG 5 Legend (Continued)
cross-linking with anti-Fc IgG. Alternatively, platelets were stimulated with ADP or with thrombin or were left unstimulated. Representative dot plots are shown. (B and C) Quantification of active αIIbβ3 integrin (left panel) and P-selectin (right panel) expression is depicted as mean fluorescence intensity (MFI). Results represent means plus SEM of data from at least 5 experiments (*, $P < 0.05$). (D and E) Activation-induced Alexa Fluor 647-fibrinogen binding to αIIbβ3 was measured in response to CLEC-2 ligation with podoplanin. (D) Representative histograms are shown. (E) Results for ADAP$^{fl/fl}$ PF4-Cre^{tg} and ADAP$^{wt/wt}$ PF4-Cre^{tg} platelets are shown as MFI of Alexa Fluor 647-fibrinogen and percentages of positive cells. Data represent a summary of data from 6 independent experiments (means plus SEM; *, $P < 0.05$). (F) Washed platelets from conditional k.o. mice or control mice were stimulated with podoplanin, and the CLEC-2-mediated release of TGF-β1 was quantified in the supernatant (means plus SEM; $n = 5$; *, $P < 0.05$). (G) Washed platelets derived from Cre control and conditional k.o. mice were either left unstimulated or stimulated with podoplanin for 30, 60, 90, or 120 s and were subsequently lysed and separated by SDS-PAGE, and Western blots were probed with the indicated antibodies. Expression of the phosphorylated PLCγ2 ($n = 4$) and phosphorylated ERK1/2 ($n = 3$) was normalized to β-actin (means plus SEM; *, $P < 0.05$; **, $P < 0.01$).

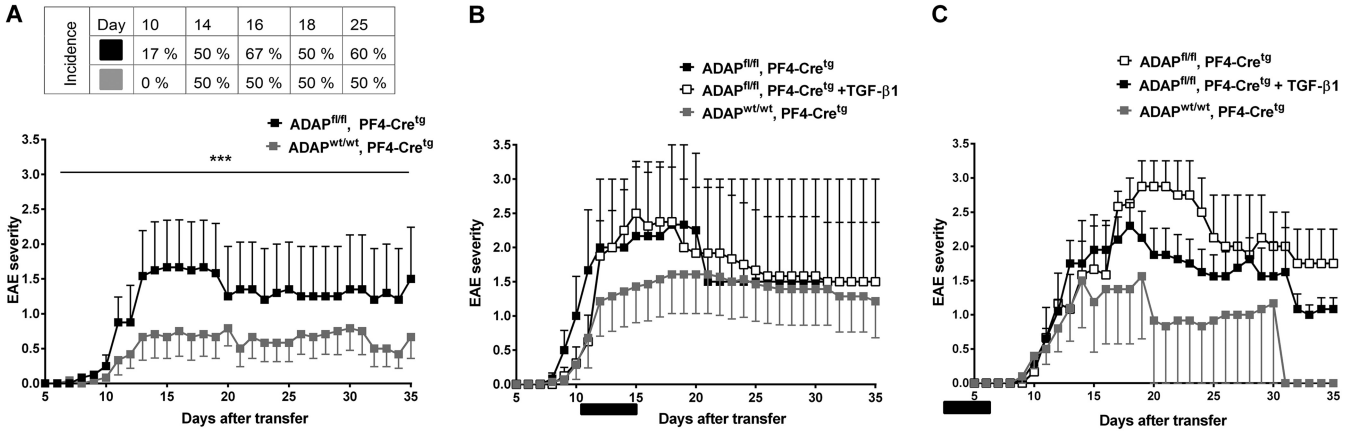

FIG 6 EAE is more severe in conditional ADAP knockout mice. (A) Splenic T cells of 2D2 mice were stimulated *in vitro* with $MOG_{35–55}$ in the presence of IL-2 and IL-7 and were reactivated with plate-bound anti-CD3 and anti-CD28 in the presence of IL-12 and IL-18. Fully activated transgenic 2D2 T cells were adoptively transferred into Cre control and conditional ADAP knockout recipients. The clinical score of passive EAE was assessed for 35 days after immunization. Data are shown as means ± SEM ($n = 6$ for Cre control and conditional ADAP knockout mice, respectively; ***, $P < 0.001$ [conditional ADAP knockout versus Cre control]). The incidence of EAE is indicated in the top panel as the percentage of diseased animals. (B) EAE was induced by adoptive transfer of fully activated 2D2 T cells. TGF-β1 (1 μg/mouse/day) or PBS alone as a control was given i.p. on days 11 to 15 after transfer to conditional ADAP knockout mice. All animals were monitored daily, and the mean EAE score was calculated for each group of six mice. (C) Activated 2D2 T cells were transferred at day 0. On day 1 after transfer, TGF-β1 (1 μg/mouse/day) or PBS was given i.p. to conditional ADAP knockout mice for 7 days. The clinical score was assessed daily. Data represent means ± SEM ($n = 5$ animals per group). The difference in mean EAE scores between TGF-β1-treated and untreated conditional ADAP knockout mice was significant ($P = 0.048$).

mice lacking ADAP in platelets. In the first experiment, conditional k.o. mice received TGF-β1 or phosphate-buffered saline (PBS) alone as a control for 5 days beginning at day 11 after transfer, when the animals exhibit first clinical signs of EAE. The mean levels of EAE severity of the two treatment groups showed no differences (Fig. 6B). In the next experiment, we tested the ability to ameliorate EAE severity by injection of TGF-β1 or of PBS alone as a control starting at day 1 after transfer. Both groups of treated mice developed EAE. However, there was a significant suppression of EAE severity in the TGF-β1-treated group compared to the PBS-treated group (Fig. 6C). These results show that systemic injection of TGF-β1 can attenuate EAE severity in platelet-specific ADAP k.o. mice in a preventive but not in a therapeutic manner.

DISCUSSION

In this study, we investigated the consequences of the loss of the adapter protein ADAP specifically in platelets *in vitro* and *in vivo*. To achieve this aim, we generated a conditional knockout mouse. We found that the absence of ADAP causes thrombocytopenia, modulates CLEC-2-mediated platelet activation, and enhances the severity of the T cell-mediated autoimmune disease EAE.

We used a codon-optimized Cre recombinase expressed as a transgene under the control of the PF4 promoter. This mouse strain was established in 2007 and has been widely used to delete genes of interest in the megakaryocytic lineage (29) (see overview in reference 31). Different reports revealed expression of the PF4-Cre transgene beyond the megakaryocytic lineage. A lineage tracing study demonstrated that the PF4-Cre was activated in around 30% of mature splenic T cells, B cells, and granulocytes (30). This is in clear contrast to the findings obtained with our mouse line under our experimental conditions where expression of Cre under the control of the PF4 promoter was megakaryocyte/platelet specific and resulted in loss of ADAP expression exclusively in platelets as detected by Western blotting and intracellular flow cytometry analyses. In addition, recombination outside the megakaryocyte/platelet lineage was shown *in vitro* under inflammatory conditions. Pertuy et al. reported recombination events in splenocytes after stimulation with PHA or PMA plus ionophore A23187 in up to 40% of cells (31). We could not confirm these data under similar stimulatory conditions. In addition, the reported recombination in approximately 20%

of unstimulated (PBS-treated) splenocytes could not be detected with our reporter system. One explanation might be the use of different reporter mouse strains (EGFP-tdTomato doubly fluorescent versus tdRFP Cre reporter mice). Abram et al. observed recombination in small immune cell subpopulations like peritoneal macrophages and blood monocytes (35). We cannot completely exclude the possibility that recombination events occurred in rare immune cell subsets. A possible impact of these Cre-mediated deletion events in our disease model appears to be rather unlikely.

Altogether, our data confirm that the PF4-Cre mouse line is an effective and specific tool for Cre-mediated deletion of *loxP*-flanked genes in the megakaryocyte/platelet lineage. However, care must be taken in choosing the right control for studies using the PF4 Cre-mediated conditional knockout mice. This is important because of the presence of additional copies of the CXCL5, CXCL7, and CXCL15 chemokine genes in the bacterial artificial chromosome (BAC)-derived PF4-Cre transgene (29). Therefore, the use of PF4 Cre littermates that are transgenic but with an unfloxed gene of interest as controls is necessary.

Using this approach, we compared the levels of maturation and distribution of immune cells in the primary and secondary lymphoid organs. The absolute numbers and the percentages of cells were found to be equal in $ADAP^{fl/fl}$ PF4-Cre^{tg} (conditional k.o.) and $ADAP^{wt/wt}$ PF4-Cre^{tg} (Cre control) mice. This indicates that the loss of ADAP in platelets has no impact on the immune cell distribution. In contrast, the conventional ADAP knockout impairs thymic development and leads to a reduction in the levels of splenic T cells (7, 9).

White blood cell counts and differentials were normal in the platelet-specific ADAP knockout mice. However, we found clearly evident thrombocytopenia with a reduction in the platelet count to 38% of the level seen with control mice. This result was expected, as, for conventional ADAP knockout mice, a reduction in platelet number to 60% of the level seen with wild-type mice was reported (7). However, the reason for the presence of thrombocytopenia in mice with ADAP-deficient platelets is not completely understood. Only recently, Spindler et al. provided evidence that the reduced platelet count is caused by ectopic release of (pro)platelets into the bone marrow compartment and by shortened platelet life span in the periphery. Those authors describe defective F-actin organization and impaired podosome formation of megakaryocytes leading to reduced integrin activation and impaired spreading on collagen I and fibronectin. This intrinsic defect of megakaryocytes was shown in conventional ADAP knockout mice and confirmed in conditional ADAP-deficient mice (36). A possible role of podoplanin-stimulated CLEC-2 signaling during thrombopoiesis is unsolved and requires further investigation.

To address the issue of whether the reduction in platelet numbers is accompanied by a reduced concentration of platelet mediators, we measured levels of latent TGF-β1 and PF4. Platelets represent the major source of TGF-β1 and PF4 in circulation, and both cytokines are stored in α-granules. We found a strongly reduced concentration of TGF-β1 and PF4 in the plasma of both conditional and conventional ADAP knockout mice. These findings suggest that thrombocytopenia is the main cause of the diminished plasma concentration of TGF-β1 and PF4.

However, no obvious bleeding was observed in mice lacking ADAP specifically in platelets. Presumably, the threshold in platelet number for a clinically apparent bleeding phenotype is even lower, and mild thrombocytopenia is not sufficient to induce bleeding (37). In addition, coagulation defects or platelet dysfunctions prolong bleeding. For ADAP-deficient mice, an increased time to rebleeding from tail wounds was shown previously to be caused by impaired activation of αIIbβ3 integrin (13). We were able to confirm these data as we observed the same rebleeding phenotype in the conditional ADAP knockout mice. Therefore, we used our mouse model to extend the functional studies of ADAP-deficient platelets.

The interaction of platelet CLEC-2 with podoplanin on the surface of lymphatic endothelial cells facilitates blood-lymphatic vessel separation during development. Conditional knockout mice with deletion of ADAP in platelets showed normal devel-

opment without obvious blood-lymphatic mixing phenotype. The exact mechanism underlying the blood-lymphatic vessel separation is still controversially debated. Different hypotheses are discussed, including the "platelet plug theory," the "platelet granule content theory," and the "prevention of backflow at the lymphovenous junction theory" (38). Soluble mediators released from activated platelets, including TGF-β1 in particular, inhibit the proliferation and migration of lymphatic endothelial cells (39). The reduced TGF-β1 concentrations and the observed CLEC-2-mediated functional defects in platelets described in this study might be not sufficient to induce a manifest blood-lymphatic vessel mixing phenotype, especially in the light of the different suggested mechanisms.

It is well known that stimulation of hemi-ITAM receptor CLEC-2 induces strong platelet activation (19). Involvement of ADAP in CLEC-2 signaling was first hypothesized after proteomic analysis of rhodocytin-stimulated platelets (24). However, no functional studies have been described so far. For stimulation of the CLEC-2 receptor, we used the natural ligand podoplanin. Indeed, we found significantly reduced levels of high-affinity αIIbβ3 integrin, decreased P-selectin surface exposure and TGF-β1 release, and diminished fibrinogen binding. However, αIIbβ3 integrin activation was not completely abolished, suggesting that additional molecules/pathways are involved in CLEC-2 signaling. The discovered mechanism of the most remotely downstream signaling events was a decreased association of talin with the αIIbβ3 integrin complex in the absence of ADAP (11). It seems obvious that this common final signaling event is independent of the receptor engaged and is therefore also involved in CLEC-2-mediated αIIbβ3 activation. One of the first signaling events after CLEC-2 receptor stimulation is activation of Syk, which phosphorylates the ITAMs (40). The more remote downstream signaling cascade involves several adapter proteins, including LAT, SLP-76, and Grb2, leading to the activation of PLCγ2, phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein (MAP) kinases (41). Indeed, we could demonstrate clearly reduced activation of the downstream effector molecules phospholipase Cγ2 and MAP kinase ERK1/2. These findings reveal for the first time that ADAP is involved in CLEC-2-mediated inside-out signaling leading to αIIbβ3 integrin activation.

The rationale for the generation of conditional ADAP knockout mice was our previously published finding that the milder course of EAE in conventional ADAP knockout mice is most likely mainly due to T cell-independent effects (25). Therefore, it was surprising at first to see platelet-specific ADAP knockout mice developing more-severe EAE than control littermates. However, the role of platelets in EAE has been intensively studied and shown to be differential in the initiation and progression phases of this neurodegenerative disease. Most importantly, platelets have been found in the CNS of mice after the induction of EAE and in human chronic active multiple sclerosis lesions (27, 28). Langer and colleagues additionally showed that platelet depletion during the immunization phase of EAE had no effect on the course of the disease. In contrast, depletion of platelets as well as blocking the platelet-specific integrin αIIbβ3 during the induction phase ameliorated EAE (27). Importantly, depletion of platelets at the peak of the disease and afterward prevented the decline of EAE symptoms otherwise observed in diseased animals (28). Exactly the same phenomenon was observed when blocking platelet P-selectin CD62P. Starossom and colleagues showed that blocking CD62P prevented platelet-CD4 T cell aggregate formation and downmodulation of T cell activation (28). This finding might be one reason for the exacerbated EAE in conditional ADAP knockout mice since the CD62P expression levels on activated platelets and the frequency of CD62P-positive platelets are significantly reduced upon loss of ADAP expression. The slightly reduced active integrin αIIbβ3 levels might even counteract the effect of reduced CD62P levels with regard to EAE severity. Moreover, the observed thrombocytopenia along with the diminished TGF-β1 concentration in blood of platelet-specific ADAP knockout mice might contribute to the more severe EAE by preventing T cell inactivation by direct platelet-T cell interactions (28) as well as by preventing regulatory T cell (Treg) functions.

As platelet α-granules contain many immunomodulatory constituents, we focused

on the effect of systemic TGF-β1 injection on the EAE severity. Indeed, our experiments provide evidence that systemic application of the immunosuppressive cytokine TGF-β1 can partially ameliorate EAE symptoms in mice lacking ADAP exclusively in platelets. These results are in agreement with earlier published data demonstrating that *in vivo* administration of TGF-β1 can reduce the disease severity of EAE (42, 43).

Recently, it was demonstrated that the interaction between platelet CLEC-2 receptor and lymphatic endothelial cell podoplanin regulates murine lung development. This direct interaction leads through platelet activation to TGF-β1 release, which is necessary for normal lung cell differentiation during late embryonic development (44). Our *in vitro* data provide additional evidence of CLEC-2-mediated TGF-β1 release from platelets.

It is known that podoplanin becomes upregulated on inflammatory macrophages and Th17 cells during inflammation (45, 46), enabling direct interaction with platelet CLEC-2 receptor. The CLEC-2-podoplanin pathway seems to play an anti-inflammatory role that limits the severity of sepsis in mice. The specific deletion of CLEC-2 resulted in enhanced systemic inflammation (47). In our EAE experiments the specific deletion of ADAP as part of the CLEC-2 signaling pathway in platelets exerts a similar effect, leading to enhanced EAE severity. On the basis of these reports and of our experimental data, a possible immunosuppressive, anti-inflammatory role of CLEC-2-mediated platelet-derived TGF-β1 might be assumed.

Taking the data together, we have shown that the PF4-Cre-mediated deletion of exon 2 of the *fyb* gene results in a complete loss of ADAP expression exclusively in platelets, which leads to thrombocytopenia and diminished PF4 and TGF-β1 plasma levels in mice. We were able to demonstrate *in vitro* for the first time a role of ADAP in CLEC-2-mediated signaling leading to affinity modulation of the platelet-specific integrin αIIbβ3 as well as P-selectin and TGF-β1 release from α-granules. This, in combination with the more severe course of EAE of platelet-specific ADAP knockout mice, provides novel insights into ADAP-dependent signal transduction and its role in platelet-mediated modulation of this complex autoimmune demyelinating disease.

MATERIALS AND METHODS

Mice. Mice containing the C57BL/6N-Fyb$^{tm1a(EUCOMM)Hmgu}$/Cnrm knockout first allele were sourced from the European Conditional Mouse Mutagenesis Program (EUCOMM) project and were purchased from the European Mouse Mutant Archive (EMMA) (32). The *lacZ* and neomycin resistance cassettes were both removed by breeding those mice with transgenic mice expressing the Flp recombinase resulting in floxed alleles (containing *loxP* sites) and restoring the wild type. To generate mice with the deletion of ADAP exclusively in the megakaryocytic lineage, the mice with floxed alleles were crossed with mice carrying the Cre recombinase under the control of the platelet factor 4 (PF4) promoter kindly provided by Bernhard Nieswandt, Würzburg, Germany (29). The presence or absence of the *FRT* sites, the *loxP* sites, the gene of interest, and the Cre transgene was analyzed by PCR using genomic DNA isolated from tail tissue.

For all experiments, 8- to 14-week-old animals were used. To investigate specific effects of ADAP deletion in platelets, ADAP$^{wt/wt}$ PF4-Cre^{tg} (Cre control) and ADAP$^{fl/fl}$ PF4-Cre^{tg} (conditional k.o.) mice were always used as littermates.

Reporter mice were generated by crossing PF4-Cre mice to Rosa26tdRFP mice kindly provided by Thomas Schüler (Magdeburg). The tandem-dimer red fluorescent protein (tdRFP) is expressed after Cre-mediated excision of a neomycin/stop codon cassette inserted in the *rosa26* locus.

Conventional ADAP-deficient mice (29) were backcrossed to C57BL/6JBom for at least 10 generations. MOG_{35-55}-specific TCR transgenic mice (2D2 mice) were kindly provided by Vijay Kuchroo (48).

All mice were bred and maintained under specific-pathogen-free conditions in the central animal facility of the medical faculty of the University of Magdeburg. All procedures were conducted according to protocols approved by the local authorities (Landesverwaltungsamt Sachsen-Anhalt; reference number 42502-2-1273 UniMD).

Preparation of washed platelets. Whole blood was collected by cardiac puncture in tubes containing heparin and analyzed on an automatic hematology counter (AcT Diff2; Beckman Coulter). Platelet-rich plasma was obtained by centrifugation at 300 $\times$ *g* for 5 min. The pellet was resuspended in modified Tyrode´s buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 0.35% bovine serum albumin [BSA]) supplemented with prostacyclin (Sigma Aldrich) (0.5 μM). After centrifugation at 1,300 $\times$ *g* for 5 min, the platelet pellet was resuspended in Tyrode´s buffer containing apyrase (Sigma Aldrich) (0.02 U/ml) and prostacyclin (0.5 μM). After incubation for 5 min at 37°C, platelets were collected by centrifugation. Prior to analysis, platelets were resuspended in Tyrode´s buffer containing apyrase and allowed to rest 30 min at 37°C. Determination of the absolute

platelet count was performed on a FACSCalibur flow cytometer using BD Trucount tubes (BD Biosciences).

***In vitro* platelet activation.** To simultaneously determine the surface expression levels of activated integrin αIIbβ3 and P-selectin (CD62P), we used the respective antibody sets from Emfret Analytics. Briefly, 1×10^6 washed platelets in a total volume of 26 μl of Tyrode´s buffer containing 1 mM $CaCl_2$ were left unstimulated or were stimulated with ADP (Sigma) (50 μM) or thrombin (Roche Applied Science) (0.2 U/ml) for 15 min at room temperature. For stimulation of CLEC-2 receptor, washed platelets were preincubated with recombinant mouse podoplanin Fc chimera (R&D Systems) (2 μg/ml) for 60 min at room temperature and then stimulated by cross-linking with $F(ab´)_2$ fragment of goat anti-mouse IgG Fcγ (Dianova) (20 μg/ml) for 15 min.

For fibrinogen binding, 1×10^6 platelets in a total volume of 25 μl washed platelets were left unstimulated or were stimulated as described above in the presence of Alexa Fluor 647-fibrinogen (Molecular Probes) (100 μg/ml). Measurement was performed on a FACSCalibur flow cytometer set up with CaliBrite beads and using Cellquest software (BD Biosciences). Platelets and platelet aggregates were acquired using forward and side scatter gating.

For TGF-β1 release assays, 10×10^6 washed platelets in a total volume of 125 μl were left unstimulated or were stimulated with podoplanin as described above and TGF-β1 was quantified in the supernatant by enzyme-linked immunosorbent assay (ELISA).

***In vitro* leukocyte activation.** Splenocytes from ROSA26-tdRFP^{het} PF4-Cre^{ntg} or ROSA26-tdRFP^{het} PF4-Cre^{tg} mice were isolated as previously described (49). Cells were stimulated by incubation with phytohemagglutinin (PHA; 4 μg/ml) or phorbol myristate acetate (PMA; 250 ng/ml) plus ionomycin (500 ng/ml) mixed with RPMI medium containing 10% fetal calf serum (FCS), 1% penicillin-streptomycin, and 50 μM 2-mercaptoethanol for 72 h and stained for flow cytometry.

Flow cytometry. Cells were stained with the following antibodies: anti-CD3 (clone 145-2C11), anti-CD4 (clone RM4-5), anti-CD69 (clone H1.2F3), anti-CD11c (clone N418), anti-NKp46 (clone 29A1.4), anti-NK1.1 (clone PK136), anti-B220 (clone RA3-6B2), anti-IgM (clone RMM-1), anti-IgD (clone 11-26c.2a), and the respective isotype controls (all BioLegend). Flow cytometric measurements were performed as 4-color analyses using FACSCalibur and CellQuest software (BD Biosciences).

In the case of *in vitro* stimulated splenocytes from ROSA26-tdRFP reporter mice (Fig. 2) as well as intracellular ADAP staining (Fig. 1; see below) in splenocytes of ADAP$^{wt/wt}$ PF4-Cre^{tg} and ADAP$^{fl/fl}$ PF4-Cre^{tg} mice, cells were first stained with the fixable viability dye eFluor 506 (eBioscience) and treated with a Fc block; subsequently stained with anti-CD3–allophycocyanin (anti-CD3–APC; clone 145-2C11), anti-CD19–APC (clone 6D5), anti-Gr-1–APC (clone RB6-8C5), and anti-NK1.1–phycoerythrin (PE)-Cy7 (clone PK136; all BioLegend); and fixed with 4% paraformaldehyde (PFA)–PBS. All steps were performed on ice with incubations at 4°C in the dark. Platelets were stained with anti-CD41–APC (clone MWReg30; BioLegend) without prior stainings and with all steps performed at room temperature.

For intracellular staining, we used the polyclonal sheep anti-ADAP antiserum and the corresponding preimmune serum kindly provided by Gary Koretzky (University of Pennsylvania). The staining procedure was previously described (3). Measurements were performed using BD LSRFortessa and BD FACSDiva software (BD Biosciences) and analyzed with FlowJo v10. Analyses were performed on viable and single cells.

Western blotting. Lysates (25 μg total protein) of thymocytes, lymph node cells (pooled inguinal, axial, brachial, and mesenteric lymph nodes), splenocytes, and platelets were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. ADAP was detected with the polyclonal sheep antiserum. Membranes were reprobed with the following antibodies: rabbit polyclonal anti-phospho-ERK1/2 (Cell Signaling Technology), mouse monoclonal anti-β-actin (Proteintech), and rabbit polyclonal anti-total PLCγ2 and anti-phospho-PLCγ2 (tyrosine 1217; both Cell Signaling Technology). Rabbit monoclonal horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Dianova. Immunoreactivity was visualized by chemiluminescence (Signal Fire ECL; Cell Signaling Technology). For quantifications of the Western blots, densitometric analysis was performed. The intensity of bands of phosphorylated PLCγ2 and ERK1/2 was normalized to the intensity of bands of β-actin.

Cytokine concentration. Platelet-poor plasma was obtained by double centrifugation ($400 \times g$ for 5 min and subsequently $6.000 \times g$ for 15 min). Plasma concentrations of PF-4 and latent TGF-β1 were measured using specific ELISA kits (R&D Systems).

Bleeding time. Bleeding time assays were performed using a disposable surgical blade to transect the mouse tail 1 mm from the tip. Immediately after transection, the tail was immersed in 0.9% NaCl at 37°C. The time for initial cessation of blood flow was noted as the bleeding time. The tail was further monitored for 5 min to determine rebleeding.

EAE induction. Passive induction of EAE by adoptive transfer of polarized myelin oligodendrocyte glycoprotein (MOG)-specific T cells (TCR Vα3.2) was performed as described previously (25). Briefly, splenocytes from 2D2 mice were cultured and stimulated with MOG_{35-55} (20 ng/ml) in the presence of 5 ng/ml IL-2/IL-7 (Miltenyi Biotech) for 2 days. At the end of this incubation period, cells were expanded with IL-2 and IL-7 for another 4 days. Subsequently, cells were reactivated for 24 h with plate-bound anti-CD3 and anti-CD28 (1 μg/ml) in the presence of 20 ng/ml IL-12 (R&D Systems) and IL-18 (Biozol). Activated T cells were collected and washed, and 4×10^6 to 6×10^6 cells were transferred in PBS intraperitoneally (i.p.) into recipient mice. Successful activation and polarization were monitored via flow cytometric analysis of CD62L and CD69 cell surface levels as well as of CD4 and TCR Vα3.2 expression. Mice were examined daily for signs of disease and graded on a scale of increasing severity from 0 to 5 as follows: 0, no signs; 0.5, partial tail weakness; 1, limp tail or slight slowing of righting from supine position; 1.5, limp tail and slight slowing of righting; 2, partial hind limb weakness or marked slowing of

righting; 2.5, dragging of hind limb(s) without complete paralysis; 3, complete paralysis of at least one hind limb; 3.5, hind limb paralysis and slight weakness of forelimbs; 4, severe forelimb weakness; 5, moribund or dead (50). For reasons of animal welfare, mice were killed when they reached a score of 3 or above. Mean clinical scores at each day were calculated by adding disease scores of individual mice divided by the number of mice in each group.

For prevention and treatment of EAE with TGF-β1, human recombinant TGF-β1 protein was purchased from R&D Systems and was reconstituted according to the instructions of the manufacturer. Mice received 1 μg TGF-β1–PBS or PBS alone as a control by daily intraperitoneal injection. In the experiments assessing the prevention of EAE, TGF-β1 or PBS was applied on days 1 to 7 after transfer. In the experiments assessing the treatment of EAE, TGF-β1 or PBS was given on days 11 to 15 after transfer.

Statistical analysis. Results are expressed as means $\pm$ standard errors of the means (SEM). Unpaired Student´s *t* tests were used to assess the statistical significance of the differences. Statistical comparisons of levels of EAE disease severity between different two groups of animals were accomplished by performing nonparametric Wilcoxon matched-pair tests using GraphPad Prism software.

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We state that we have no conflicts of interest.

J.M.R. and A.R. designed and performed the research, analyzed data, and wrote the manuscript. K.G., G.W., and C.A.M. performed research and analyzed data. S.K., D.R., and B.S. contributed to interpretation of data, critical writing, and revising the paper.

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