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Proof of Principle for a T Lymphocyte Intrinsic Function of Coronin 1A*

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Coronins are evolutionarily conserved proteins that were originally identified as modulators of actin-dependent processes. Studies analyzing complete *Coronin 1a* knock-out mice have shown that this molecule is an important regulator of naive T cell homeostasis and it has been linked to immune deficiencies as well as autoimmune disorders. Nevertheless, because Coronin 1A is strongly expressed in all leukocyte subsets, it is not conclusive whether or not this phenotype is attributed to a T cell-intrinsic function of Coronin 1A. To address this research question, we have generated a T cell-specific *Coronin 1a* knock-out mouse (*Coro1a*$^{fl/fl}$ × Cd4[Cre]). Deletion of Coronin 1A specifically in T cells led to a strong reduction in T cell number and a shift toward the effector/memory phenotype in peripheral lymphoid organs when compared with Cd4[Cre] mice expressing wild-type Coronin 1A. In contrast to peripheral lymphoid tissue, thymocyte number and subsets were not affected by the deletion of *Coronin 1a*. Furthermore, T cell-specific *Coronin 1a* knock-out mice were largely resistant to the induction of autoimmunity when tested in the myelin oligoglycoprotein-induced EAE mouse model of multiple sclerosis. Thus, the phenotype of T cell-specific *Coronin 1a* deletion resembles the phenotype observed with conventional (whole body) *Coronin 1a* knock-out mice. In summary, our findings provide formal proof of the predominant T cell-intrinsic role of Coronin 1A.

Coronins are an evolutionarily conserved family of intracellular proteins comprising seven members in mammals. The actin-binding Coronin 1A (Coro1A)[3] is highly expressed in all leukocytes with the exception of Kupffer cells, a liver-resident macrophage subset (for review see Ref. 1). Initially, Coro1A was identified as a factor facilitating *Mycobacteria tuberculosis* survival in macrophages through the modulation of endosomal-lysosomal fusion (2, 3). However, other macrophage functions such as phagocytosis and chemotaxis appeared normal in the absence of Coro1A expression (3, 4). Coro1A also seems to be largely dispensable for the functionality of neutrophils, mast cells, dendritic cells, and B cells, even though some controversy exists concerning the role of Coro1A in neutrophils and mast cells (5–10).

The cell type mainly affected by the germline deletion of *Coro1a* in mice is T cells, whereas no gross defect in other leukocyte subsets has been observed, which is somewhat surprising given the high expression of Coro1A in almost all leukocyte subpopulations. Common to all four *Coro1a*-deficient mice strains, which were generated in different laboratories, are strongly reduced peripheral T cell counts (11–14). Naive T cells are especially impaired, whereas effector/memory and regulatory T cells (Treg) are far less affected by the deletion of *Coro1a*. The distinct studies concurringly report that *Coro1a*-deficient naive T cells show survival defects and an increased apoptosis rate. Different explanations are provided regarding the mechanism of how Coro1A regulates peripheral T cell homeostasis, including impaired signaling downstream of the T cell receptor (TCR) (12, 13, 15) and perturbed actin dynamics affecting mitochondrial membrane potential (11). In addition to impaired survival, migration defects, egress from the thymus as well as homing to the lymph nodes, have been reported to affect naive T cell homeostasis in the absence of functional Coro1A (11, 16). In contrast to peripheral T cells, thymic development in *Coro1a*-deficient mice has been reported to be either normal (12) or only barely impaired, affecting merely T cell terminal differentiation and leading to reduced single positive thymocytes (11, 13).

In mice, Coro1A has been associated with resistance to autoimmunity. A nonsense mutation of *Coro1a* has been identified to suppress disease development in a mouse model of lupus erythematosus (15). Furthermore, *Coro1a*-deficient mice develop no or only mild symptoms of autoimmunity when immunized with the myelin oligoglycoprotein (MOG) peptide to induce experimental autoimmune encephalomyelitis (EAE), a common murine model of multiple sclerosis (14, 17).

MOG-induced EAE is considered a predominantly T cell-mediated disease, in which MOG-specific naive T cells are activated and expanded during the early phase of the immune response in lymph nodes draining the immunization site. Subsequently, the differentiated effector T cells invade the central nervous system (CNS), where they initiate the recruitment of other inflammatory immune cells such as neutrophils and macrophages. Nevertheless, B cells are also discussed to contribute to the pathogenesis either by secreting autoantibodies, presenting antigens to T cells, or by suppressing effector responses. Furthermore, dendritic cells as professional antigen-presenting cells (APC) are crucial for the priming of T cells in lymphoid organs and in addition to reactivate T cells in the CNS. Thus, besides T

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[3] The abbreviations used are: Coro1A, Coronin 1A; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligoglycoprotein; APC, antigen-presenting cell; ANOVA, analysis of variance; Treg, regulatory T cell; TCR, T cell receptor.

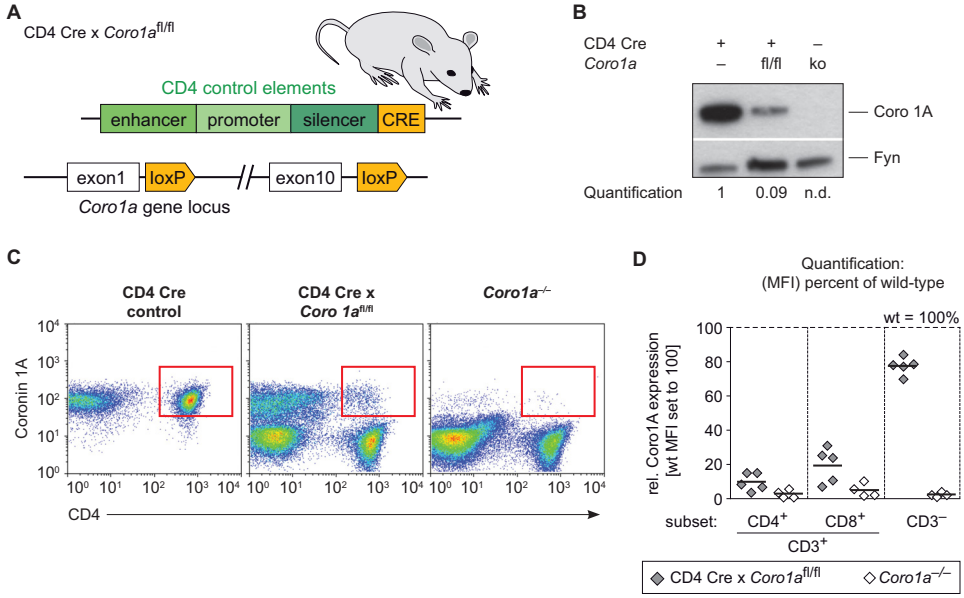

FIGURE 1. **Generation of a T cell-specific conditional *Coro1a* knock-out mouse.** *A*, schematic presentation of genetic modification leading to T cell-specific deletion of *Coro1a*. *B*, efficiency of T cell-specific deletion of *Coro1a* was determined on protein level by SDS Page and immunoblotting. Protein lysate from 1×10^5 MACS-sorted $CD4^+$ and $CD8^+$ cells of the three genotypes (Cd4-Cre transgenic mice harboring wild-type or floxed *Coro1a* gene and germline $Coro1a^{-/-}$ mice) was applied per lane. One representative Western blot of two independent experiments is shown. C, intracellular Coro1A expression was analyzed in splenocytes by flow cytometry; representative dot blots depict Coro1A and CD4 staining (gated on $CD3^+$ cells). *D*, for quantification and comparison between the three different genotypes the mean fluorescent intensity of Coro1A staining in wild-type (CD4-Cre) cells was set to 100%. Results for $CD4^+$ and $CD8^+$ T cells and non-T cells ($CD3^-$) are presented as means and single values of 4 independent experiments with a total of 4 or 5 mice. *n.d.*, not detectable.

cells, several different populations of immune cells may contribute to the inflammatory response leading to neuronal damage and resulting in symptoms of ascending flaccid paralysis (see Ref. 18 for a comprehensive review).

Given the abundant expression of Coro1A in many different immune cell types, the question remains of whether the phenotype of *Coro1a*-deficient mice can be attributed predominately to its role in T cells. Moreover, it has not been proven that the paucity of peripheral T cells observed in conventional *Coro1a*-deficient mice is caused by a T cell intrinsic role of Coro1A or a lack of Coro1A expression in other leukocyte subsets. To address these points, we have generated mice lacking Coro1A expression specifically in T cells and analyzed their immune status as well as their susceptibility to the induction of EAE by immunization with the MOG peptide. Our results suggest a predominant T cell-intrinsic role of Coro1A responsible for peripheral T cell homeostasis and resistance to the development of severe EAE.

Results

Characterization of a T Cell-specific Coronin 1A Knock-out Mouse—Coro1A, which has been implicated as an important regulator of T cell functions by several studies analyzing conventional (whole body) *Coro1a* knock-out mice or natural mutants, is highly expressed in all leukocyte subsets (with the exception of Kupffer cells). Nevertheless, it is currently not clear whether the expression of Coro1A in T cells or other leukocyte subsets such as APCs accounts for the strong reduction of T cell counts observed with conventional knock-out mice. To address this research question, T cell-specific *Coro1a* knock-out mice were generated in our laboratory by crossing $Coro1a^{flox/flox}$ mice harboring loxP sites after exon 1 and 10 of the *coro1a* gene to Cd4-Cre transgenic mice (Tg(Cd4-cre)1Cwi/BfluJ) containing *CD4* enhancer, promoter, and silencer sequences driving the expression of a Cre recombinase gene (Fig. 1*A*).

To determine the efficiency as well as specificity of *Coro1a* deletion, offspring expressing the Cre recombinase together with either a wild-type ("Cd4-Cre") or floxed version of the *Coro1a* gene ("Cd4-Cre × $Coro1a^{flox/flox}$") along with conventional *Coro1a* knock-out mice ("$Coro1a^{-/-}$") were analyzed for Coro1A protein expression. The Coro1A protein expression level in sorted $CD4^+/CD8^+$ T cells isolated from Cd4-Cre × $Coro1a^{flox/flox}$ mice was strongly reduced (to less than 10%) when compared with Cd4-Cre mice by SDS-PAGE gel and Western blotting analysis using a monoclonal antibody against Coro1A (Fig. 1*B*). Remarkably, as few as 1×10^5 T cells isolated from Cd4-Cre mice gave a very strong band for Coro1A on immunoblots, emphasizing that Coro1A is abundantly expressed in T cells. As expected, no Coro1A protein was detected with T cells of $Coro1a^{-/-}$ mice, also confirming the specificity of the monoclonal antibody, which is raised against human CORO1A but also detects its highly conserved mouse homologue.

To determine Coro1A expression on the single cell level, and thus the specificity of *Coro1a* deletion by the Cre recombinase in lymphocyte subsets, splenocytes of all three genotypes were analyzed by flow cytometry. Because no fluorochrome-labeled anti-Coro1A antibody was commercially available, we used the same monoclonal antibody that has been applied for immunoblotting in combination with Zenon technology (see "Experimental Procedures"). We chose this approach because it should result in less unspecific background staining than using fluorochrome-labeled secondary antibodies. The results with splenocytes from $Coro1a^{-/-}$ mice, showing negligible "false Coro1A-positive" $CD4^+$ T cells, and a clear positive population with

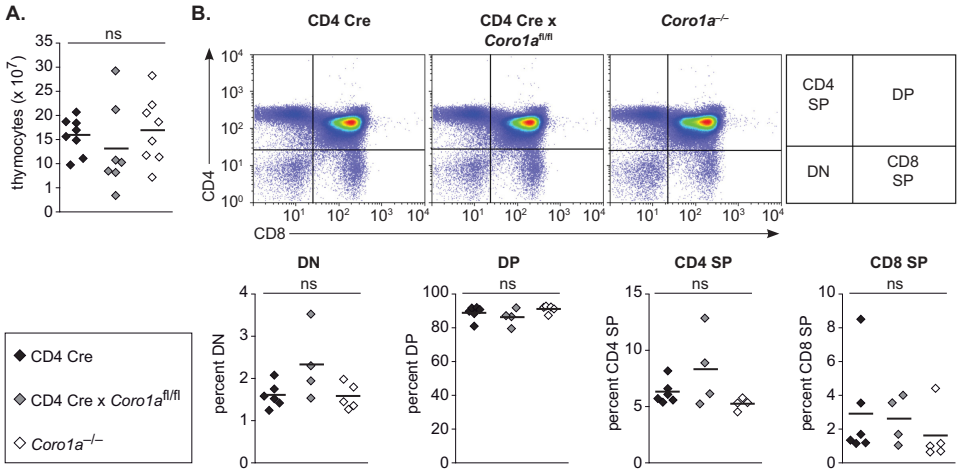

FIGURE 2. **Normal thymic development of T cells in the absence of *Coro1a*.** *A*, total thymocyte counts were determined using a Luna cell counter. Each *symbol* represents data of an individual mouse and the mean values are indicated in the graph. Results of 6 independent experiments with a total of 7–9 mice per genotype are shown. *B*, the frequency of thymocyte subsets (DN, DP, $CD4^+$ and $CD8^+$ SP) was analyzed by flow cytometry. Representative FACS dot plots of CD4 and CD8 staining together with graphs summarizing three experiments with a total of 4–6 mice are shown. *DN*, double negative; *DP*, double positive; *SP*, single positive. Statistical analyses were performed using one-way ANOVA and Bonferroni's multiple comparison test.

splenocytes from Cd4-Cre mice, indicated that the intracellular Coro1A staining was working well (Fig. 1*C*). Although all $CD3^+$ ($CD4^+$ and $CD4^-$) from Cd4-Cre control mice express Coro1A, Coro1A-positve $CD4^+$ T cells were almost absent in $CD4^+$ T cells from Cd4-Cre × *Coro1a*$^{flox/flox}$ mice. To allow for a better comparison between the genotypes, the mean fluorescent intensity of Coro1A staining of $CD4^+$ and $CD8^+$ T cells as well as $CD3^-$ splenocytes from control Cd4-Cre control mice was set to 100%. The Coro1A expression of $CD4^+$ T cells from conditional knock-out mice reached almost full knock-out level. Furthermore, $CD8^+$ T cells showed a strong reduction in Coro1A expression with on average 20% of the value obtained for control mice. In contrast, $CD3^-$ splenocytes, which are not expected to express the Cre recombinase, had Coro1A expression levels close to the control Cd4-Cre mice. With the generation of this T cell-specific *Coro1a* knock-out line a valuable tool to address the role of Coro1A in T cells is now available.

T Cell-specific Deletion of Coro1a Does Not Affect Thymic T Cell Development, But Leads to a Strong Reduction of Peripheral T Cell Counts—The major phenotype of conventional *Coro1a* knock-out mice is a strong reduction of naive $CD4^+$ T cells in peripheral organs, whereas thymic counts and subset frequency are not affected (12). Although comparing conventional and T cell-specific *Coro1a* knock-out mice along with Cd4-Cre control mice, no significant difference in thymic counts was observed (Fig. 2*A*). Also, subset frequencies (DN, DP, and CD4/8 SP), which were analyzed by flow cytometry, were comparable between the three genotypes (Fig. 2*B*). Thus, similar to the conditional *Coro1a* knock-out mice, deletion of *Coro1a* in thymocytes at the CD4/8 double-positive stage does not affect thymocyte development.

Nevertheless, the deletion of *Coro1a*, either the germline or specifically in T cells, strongly reduced the frequency of $CD3^+$ T cells in the spleen even though total splenocyte numbers were not affected (Fig. 3*A*). On the other hand, the percentage of $CD19^+$ B cells in both *Coro1a* knock-out lines was enhanced; most likely attributed to the lymphopenic environment in *Coro1a*-deficient animals.

Additional flow cytometric analyses of the peripheral T cell compartment revealed that especially the $CD4^+$ T cell compartment was affected by deletion of *Coro1a*, leading to a reduced percentage of $CD4^+$ T cells in both knock-out lines (Fig. 3*B*). Interestingly, whereas the percentage of $CD4^+$ T cells was not significantly different between germline and T cell-specific *Coro1a* deletion, the frequency of $CD8^+$ T cells was increased in the spleen of conventional but not of T cell-specific *Coro1a* knock-out mice. This difference between both *Coro1a* knock-out lines in regard to $CD4^+$ and $CD8^+$ cell frequency is further illustrated by the significantly lower CD4:CD8 ratio in the spleen of conventional *Coro1a* knock-out mice.

As mentioned above, the main subset affected by *Coro1a* deletion seems to be naive $CD4^+$ T cells. Therefore, this subset was analyzed by flow cytometry using CD62L and CD44 to distinguish naive and effector/memory T cells. T cell-specific deletion of *Coro1a* resulted in a strong reduction of naive $CD4^+$ T cells and induced a shift toward an effector/memory phenotype. However, these changes were less pronounced than observed for $CD4^+$ T cells from conventional *Coro1a* knock-out mice (Fig. 4).

Deletion of Coro1a in T Cells Protects from the Development of EAE—The most striking phenotype of conventional *Coro1a* knock-out mice was their resistance to EAE induction using the MOG(35–55) peptide-based protocol (14, 17). Given the similar phenotype of mice harboring either T cell-specific or germline deletion of *Coro1a*, we expected that deletion of *Coro1a* in T cells would also confer protection in this model of autoimmunity. This was indeed observed when the three genotypes were immunized with the MOG(35–55) peptide and disease progression was monitored: mice lacking *Coro1a* only in T cells were as well protected as mice lacking *Coro1a* in all their cells (Fig. 5). In summary, our results show a predominant T cell intrinsic role of Coro1A with regard to T cell function.

Discussion

Several studies have associated mutations in the *Coro1a* gene with resistance to autoimmunity. A nonsense mutation that had arisen spontaneously has been identified as a disease-sup-

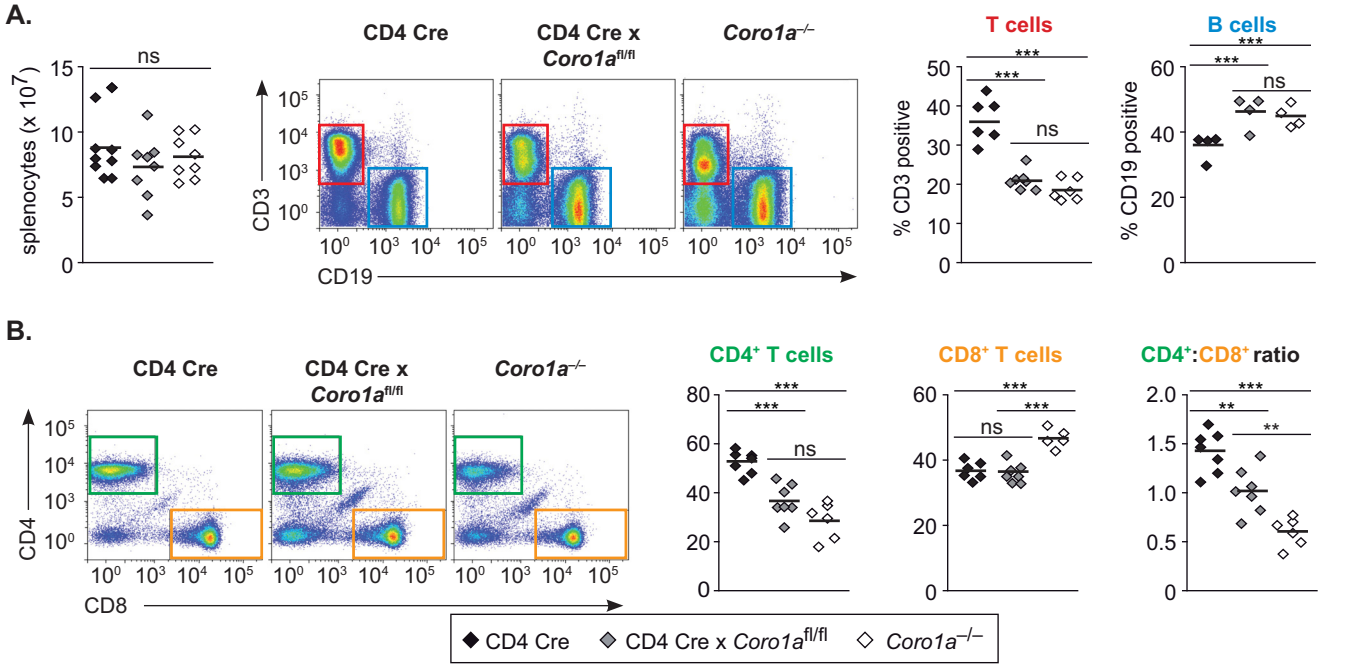

FIGURE 3. **Reduced peripheral T cell counts in *Coro1a* knock-out mice.** *A*, total splenocyte counts and frequency of T and B lymphocytes in spleens of Cd4-Cre transgenic and *Coro1a*-deficient mouse strains (conventional and CD4-specific). Representative dot blots of CD3 and CD19 staining are depicted together with graphs summarizing the frequency of T and B cells. *B*, the graphs show flow cytometric analyses of $CD4^+$ and $CD8^+$ T cell frequency (representative dot blot and quantification) along with the CD4 to CD8 ratio. Results of 6 independent experiments with a total of 7–9 mice per genotype are shown. Statistical analyses were performed using one-way ANOVA and Bonferroni's multiple comparison test.

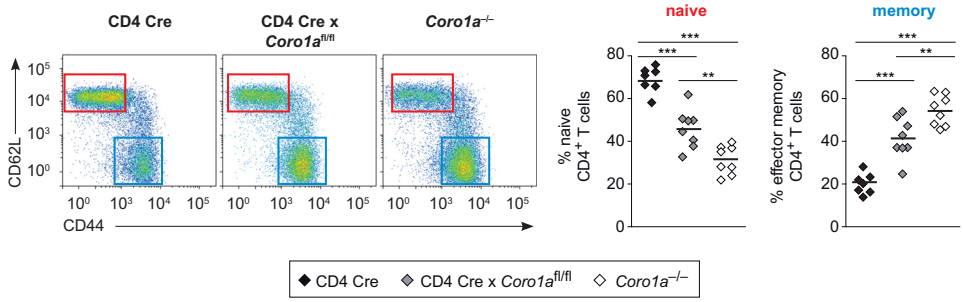

FIGURE 4. **Deletion of *Coro1a* mainly affects naive T cells.** Frequencies of naive and effector/memory $CD4^+$ T cells in the spleens of Cd4-Cre transgenic and both *Coro1a*-deficient mouse strains were analyzed by flow cytometry (using CD62L and CD44 as markers). Results of 6 independent experiments with a total of 7–9 mice per genotype are shown. Statistical analyses were performed using one-way ANOVA and Bonferroni's multiple comparison test.

pressing allele in lupus-prone Fas^{lpr} mice (15). This point mutation introduces a stop codon in the middle of the protein, leading to a truncated version of Coro1A, which fails to localize to the plasma membrane. Furthermore, conventional *Coro1a*-deficient mice, generated independently in distinct laboratories, were protected against the development of severe signs of EAE induced by immunization with the MOG peptide (14, 17). This phenotype has been attributed to alterations of intrinsic T cell function, namely impaired migration, cell activation, and survival. Of note, however, those mouse strains lack functional Coro1A in all their cells. Our results obtained with mice lacking *Coro1a* specifically in T cells support the notion of a predominant T cell intrinsic role of Coro1A underlying the resistance to autoimmunity. Thus, similarly to conventional *Coro1a*-deficient mice, Cd4-cre × $Coro1a^{flox/flox}$ mice show strongly reduced peripheral $CD4^+$ and $CD8^+$ T cell counts and are largely protected from the induction of EAE.

Before conditional *Coro1a*-deficient mice were generated, the conclusion that T cell intrinsic Coro1A accounts for reduced susceptibility of autoimmunity was drawn mainly from transfer experiments. On one hand, the transfer of T cells baring the point mutation described above was sufficient to reduce lupus-like diseases (15). On the other hand, the transfer of wild-type $CD4^+$ T cells before immunization did restore the susceptibility of *Coro1a*-deficient mice to EAE induction (17). The later experimental approach suggests that *Coro1a*-deficient APCs are fully capable of priming T cells and promoting effector T cell differentiation as long as those T cells express Coro1A. The assumption that Coro1A is dispensable for antigen presentation and T cell activation by APCs is further supported by the observation that bone marrow-derived dendritic cells induce antigen-specific T cell proliferation regardless of Coro1A expression (8). Thus, it seems that Coro1A expression in T cells rather than in APCs is essential for the activation of autoantigen-specific T cells and the development of symptoms of EAE. This is in accordance with our results of T cell-specific *Coro1a*-deficient mice being largely resistant to EAE induction.

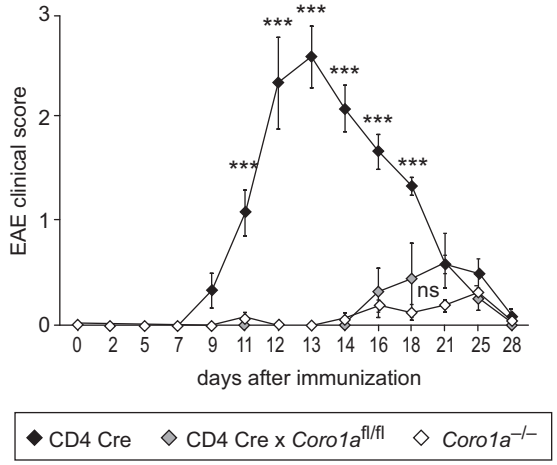

FIGURE 5. ***Coro1a* deficiency protects from EAE pathology.** Cd4-Cre, Cd4-Cre × *Coro1a*$^{flox/flox}$, and *Coro1a*$^{-/-}$ mice were immunized with the MOG(35–55) peptide in CFA to induce active EAE. Disease development and progression were monitored over time using the scoring system as published (17). One representative result of two independent experiments (total $n = 7/8$) is shown. *Error bars* represent the S.E. Statistical analyses were performed using two-way ANOVA.

It is believed that myelin-specific T cells, which have escaped central tolerance, are present in the peripheral T cell pool in a state of ignorance (19) and, in addition, their activation is prevented by Tregs (17, 20–22). Therefore, the balance between distinct T cell subsets may dictate disease outcome after immunization with myelin-derived peptides. In this regard, it is worth noting that an increased Treg/naive T cell ratio was observed in the absence of *Coro1a*, in conditional as well as T cell-specific knock-out mice (see Ref. 17; data not shown). However, whereas the transfer of wild-type CD4$^+$ T cells did impact on EAE development, the depletion of CD25$^+$ Tregs did not restore disease susceptibility (17), indicating that resistance to EAE in the absence of *Coro1a* is most likely caused by disturbed naive T cell priming.

Unexpectedly, considering the decreased EAE symptoms upon primary immunization with the MOG peptide, *Coro1a*-deficient mice not only overcome the defect observed during initial induction, but also demonstrate hypersusceptibility upon the reinduction of EAE (14). The authors explain this phenotype by defective SMAD3-meditated TGFβ receptor signaling, resulting in enhanced interferon-γ and interleukin-17 production when T cells lack *Coro1a*. These findings are consistent with the observation that *Coro1a*-deficient T cells are fully competent to express Th1, Th2, and Th17 effector cytokines upon polyclonal stimulation with antibodies recognizing CD3 and CD28 (17). Therefore, a decreased disease severity in the absence of Coro1A expression cannot be attributed to a general defect of effector T cell differentiation, but might rather be caused by the reduced overall CD4$^+$ T cell number. One could further speculate that there are too few MOG-specific T cells to act as pioneer cells and to induce disease (23); however, the threshold number of effector T cells needed for substantial inflammatory response might be reached after expansion following immunization. The conclusion that the major defect lies in the naive T cell compartment while effector/memory T cells are functional is supported by a study analyzing *Coro1a*-deficient mice in a model of concanavalin A-induced liver injury, which showed a similar inflammation score for wild-type and knock-out mice (24). The response to intravenous concanavalin A occurs within a few hours after intravenous administration and it is therefore very unlikely that naive T cells are responsible for acute liver injury in this model. One could speculate that the different dependence of naive and effector/memory T cells on Coro1A is due to different requirements of TCR signal strength for their activation, which would be consistent with the role of Coro1A in signaling downstream of the TCR (12, 13, 15, 16, 25). Of note, the paucity of naive T cells in T cell-specific *Coro1a*-deficient mice was not as strong as in conventional knock-out mice. This finding suggests that naive T cell homeostasis, at least to some extent, also depends on Coro1A expression in non-T cells. However, another explanation could be some degree of residual expression of Coro1A in naive T cells of Cd4-Cre × Coro1a$^{flox/flox}$ mice.

An important role of Coro1A for an intact immune system has also been described for humans. Intriguingly, similar to the murine system, all 7 *CORO1A*-deficient patients reported to date have severe T cell lymphocytopenia, whereas B cells and natural killer cells were present in normal to low numbers (26–31). Those reports emphasize the importance of Coro1A for T cell homeostasis not only in mice but also in humans. Common to all patients were recurrent respiratory infections and the inability to control Epstein-Barr virus, associated with lymphoma at a young age. In this regard, *Coro1a*-deficient mice were highly susceptible to vesicular stomatitis virus, most likely due to the delayed production of neutralizing antibodies, which requires CD4$^+$ T cell help (32). Interestingly, lymphocytic choriomeningitis virus clearance, which depends on CD8$^+$ T cells, was comparable with wild-type mice, suggesting a different requirement for Coro1A in CD8$^+$ *versus* CD4$^+$ function. Moreover, in the current study we observed a shift toward a lower CD4:CD8 ratio in the absence of Coro1A, which was more pronounced in conventional than conditional *Coro1a*-deficient mice. This difference might be attributed to the slightly less efficient deletion of Coro1A expression in CD8$^+$ than CD4$^+$ T cells.

Besides its primary expression in the hematopoietic lineage, Coro1A was also detected in neuronal tissue (33, 34). However, based on our data obtained with T cell-specific Coro1A-deficient mice, we exclude that neuronal Coro1A plays any role in neuronal pathology and axonal injury, which was not very likely. Furthermore, in both mice and humans, a lack of functional Coro1A causes severe neurobehavioral defects, including social deficits, increased aggression, and learning disabilities (26, 33). We observed that, in contrast to conventional *Coro1a*-deficient mice, Cd4-cre × *coro1a*$^{flox/flox}$ did not display enhanced aggression, which is consistent with the specific absence of Coro1A from T cells (data not shown). Taken together, our results demonstrate a crucial T cell-intrinsic role of Coro1A in the development of EAE.

Experimental Procedures

Mice—Coronin 1a-deficient mice (*Coro1a*$^{-/-}$) were generated using Cre/LoxP-mediated deletion as described previously by Kaminski *et al.* (14). To obtain mice harboring a T cell-

specific knock-out of *Coro1a*, the same floxed *Coro1a* mouse line was crossed in our laboratory to Cd4-Cre transgenic mice (Tg(Cd4-cre)1Cwi/BfluJ) purchased from Charles River Laboratories. The corresponding littermates (Cd4-cre × Coro1a$^{flox/flox}$ and Cd4-cre) were bred in-house as separate lines homozygous for the floxed *Coro1a* allele and heterozygous for the Cd4-cre transgene. All mouse lines were maintained under specific pathogen-free conditions. Animal experiments were conducted in accordance with the Austrian Animal Welfare Law and Animal Experimental Act (BGBI number 501/1988 and BGBI number 114/2012), and were approved by the Committee of the Animal Care of the Austrian Federal Ministry of Science and Research (BM:WFW-66.011/0064-WF/V/3b/2016). Genotyping was performed by PCR following the protocol published on the JAX homepage (for Cd4-Cre) or using the following primers and thermocycler protocol (for *Coro1a*): gtcctcagtagctgactg (forward), tagcagaaaaccccaagc (reverse); 35 cycles of 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min. The expected PCR product size is 140 bp for wild-type *Coro1a* and 250 bp for the floxed *Coro1a* allele.

Thymocyte and Splenocyte Isolation and T Cell Sort—Single cell suspensions of spleens and thymi were prepared by mechanical disintegration using metal sieves and cell strainers (Falcon), followed by the removal of erythrocytes by lyses (Mouse Erythrocyte Lysing Kit, R&D Systems). After a washing step with PBS, 0.5% BSA, 2 mM EDTA (viable), cell counts were determined with a LUNA Automated Cell Counter (Logos Biosystems). $CD4^+$ and $CD8^+$ cells were positively selected using a mix of CD4 (L3T4) and CD8a (Ly-2) MicroBeads together with LS columns and a QuadroMACS Separator (all Miltenyi Biotec) according to the manufacturer's instructions. Sort purity was checked by flow cytometry.

SDS-PAGE and Immunoblotting—Cell lysates from splenocytes were prepared and subjected together with precision plus protein standard (Bio-Rad) to SDS-gel electrophoresis and immunoblotting as described previously (35). Unspecific binding was blocked with 5% nonfat milk powder. Monoclonal antibodies detecting Coronin 1A (sc-100925) or Fyn (sc-16) were purchased from Santa Cruz Biotechnology. HRP-conjugated secondary antibodies against mouse and rabbit IgG were purchased from Thermo Scientific. For detection, SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used and the bands were visualized using hyperfilm (Amersham Biosciences) and Superfix Enviro Safe 25 together with Roentrol Enviro Safe AC (Tental). Quantification was performed using the ImageJ software.

Flow Cytometry—Surface staining, including preincubation with FcR block; anti-CD16/32 (BD Biosciences), was performed as described previously (17). The following antibodies were used: anti-CD4 PE, anti-CD3 PECy7, anti-CD8 PerCP Cy5.5, anti-CD62L APC, and anti-CD4 bv605 (all from Biolegend).

Intracellular Coro1A was stained using a mouse monoclonal anti-Coro1A antibody (Santa Cruz Biotechnology) together with the Zenon Alexa 488 mouse IgG2a Labeling Kit (Thermo Fisher Scientific) after the cells had been stained for surface antigens and subsequently fixed and permeabilized using fixation buffer and intracellular staining permeabilization wash buffer obtained from Biolegend. The samples were subjected to an additional poststaining fixation step with 2% paraformaldehyde for 20 min at room temperature. Data were acquired on a FACSCalibur (CellQuest) or Fortessa (Diva) instrument (both BD Biosciences) and analyzed with FlowLogic software (eBioscience).

Induction of Active EAE—EAE was induced by active immunization with the MOG(35–55) peptide based on established protocols (35). To that end, the 9–13-week-old mice were immunized subcutaneously in the back on day 0 with 400 μg of MOG(35–55) peptide (NeoSystems, Strasbourg, France) emulsified in 100 μl of complete Freund's adjuvant (Thermo Scientific) at a final volume of 200 μl in PBS. On days 0 and 2, the animals were injected with 400 ng of pertussis toxin (Sigma) in 100 μl of intraperitoneal PBS. Clinical signs of EAE were assessed daily according to the standard scoring protocol as published (17).

Statistical Analysis—The data are presented as single values plus the mean or represent the mean ± S.E.; FACS dot plots and EAE disease scores are shown as representative results (indicated in the figure legends). The number of mice (n) used per experiment and the number of experiments performed are listed in each figure legend. The data were analyzed for statistical significance by one- or two-way ANOVA with the Bonferroni post hoc test using GraphPad Prism software (GraphPad Software Inc.) as indicated in the figure legends.

Author Contributions—K. S. designed, performed, and analyzed the experiments and wrote the manuscript. G. B. conceived and coordinated the study. V. K. and N. H. K. performed the experiments shown in Fig. 5. All authors reviewed the results and approved the final version of the manuscript.

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