Coronin 1-mediated Naïve T cell Survival is Essential for the Development of Autoimmune Encephalomyelitis

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Abstract

Autoimmune encephalomyelitis is a disease of the central nervous system that can develop when an initial peripheral inflammatory stimulus is followed by infiltration and reactivation of T lymphocytes in the central nervous system. We here report a crucial role for coronin 1, which is essential for maintenance of the naïve T cell pool, for the development of murine experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis. In the absence of coronin 1, immunization with MOG_{35-55} peptide largely failed to induce EAE symptoms, despite normal mobilization of leukocyte subsets in the blood as well as effector cytokine expression comparable to wild-type T cells upon polyclonal stimulation. Susceptibility of coronin 1-deficient mice to EAE induction was restored by transfer of wild-type $CD4^+$ T cells, consistent with a failure of coronin 1-deficient mice to generate a MOG-specific T effector cell response. Importantly, while coronin 1-deficient regulatory T cells (Tregs) showed a suppressor activity comparable to wild-type Tregs, Treg depletion failed to restore EAE development in coronin 1-deficient animals. These results suggest a hitherto unrecognized role of naïve T cells in the development of autoimmune encephalomyelitis and reveal coronin 1 as a crucial modulator of EAE induction.

Introduction

The immune system has evolved mechanisms to respond to a diverse set of foreign antigens while minimizing the risk for auto-reactivity. T cells play an important role in ensuring a successful immune response; however, under still ill defined circumstances, T cells can cause harm to the body's own constituents and mediate autoimmune pathology. Multiple sclerosis (MS) is an inflammatory demyelinating brain disease, in which autoreactive T cells are thought to play a major role in disease pathogenesis (for review see (1)). This immune response leads to an inflammation in the central nervous system (CNS) and subsequent demyelination and axonal injury. Experimental autoimmune encephalomyelitis (EAE) is the commonly used animal model to study immunopathogenesis of MS. To induce active EAE, C57BL/6 mice are immunized with peptides of a CNS-derived autoantigen e.g. myelin oligoglycoprotein, MOG (2, 3). In this form of EAE, MOG-specific naïve T cells are activated and expand during the initiation phase of the immune response in the draining lymph node. At later time points the expanded T cells invade the CNS where they are reactivated by local and infiltrating antigen-presenting cells (4, 5) and initiate the recruitment of inflammatory cells such as neutrophils and macrophages to induce the effector phase. In this rodent model of EAE, inflammation predominantly targets the spinal cord resulting in the classic symptoms of ascending flaccid paralysis.

While Th1 cells were believed to be the main effector cell subset in this particular EAE model, recent work has highlighted the importance of Th17 cells for the pathology of EAE. To date, several studies indicate that Th1 as well as Th17 cells can induce autoimmunity in the CNS and play complementary roles in pathogenesis of EAE (6, 7), while Th2 response are protective (8, 9). In contrast to these effector T cell subsets, regulatory T cells have been implicated in the prevention and resolution of EAE. Increasing the number of Tregs has shown beneficial effects on EAE pathology, while decreasing Treg numbers worsened disease outcome (10-13). It thus appears that the balance between different T cell subsets, in particular Th1, Th17, Th2 and Tregs may be dictating disease outcome following immunization with myelin-derived peptides.

In healthy mice, the different T cell populations such as naïve, memory and regulatory T cells occupy their own niche in the periphery, and T cell numbers are fairly well maintained. The mechanisms underlying the maintenance of the peripheral T cell pool are incompletely understood. Naïve T cell survival in the periphery seems to depend on continuous low-level stimulation of their T cell receptor (TCR) by self-MHC molecules in secondary lymphoid organs as well as IL-7 signaling (reviewed in (14)). In the absence of appropriate stimuli such as in mice lacking major histocompatibility complex (MHC) expression, the naïve T cell pool is greatly diminished and lymphopenia develops, that favors the enrichment of memory-like T cells (15-18). Similarly, ablation of the T cell receptor expression leads to a reduction in peripheral T cells, underscoring that peripheral T cell survival relies on TCR-dependent stimulation.

Recently, coronin 1 (also known as P57 or TACO, for tryptophan aspartate containing coat protein) has been identified as a molecule essential for naïve T cell maintenance in the periphery (19-23). Coronin 1, which is a leukocyte-specific molecule of the coronin protein family of WD repeat containing proteins, is essential for intracellular calcium mobilization following T cell receptor stimulation and survival of naïve T cells. Mice lacking coronin 1 have strongly reduced naïve T cell numbers in the periphery, while thymic cellularity and selection is relatively normal (19-23). Interestingly, despite strongly reduced naïve T cell numbers and the observed *in vitro* activation defect, coronin 1-deficient mice show relatively normal antibody responses to thymus-dependent and independent antigens (24).

We here show that upon immunization with MOG_{35-55} peptide, coronin 1-deficient mice are largely resistant to EAE. Failure to induce EAE in the absence of coronin 1 occurred despite the fact that upon polyclonal stimulation, coronin 1-deficient $CD4^+$ T cells expressed effector cytokines comparable to wild-type T cells. The transfer of wild-type $CD4^+$ T cells prior to EAE induction could restore susceptibility to EAE. Furthermore, despite normal regulatory T cell function in the absence of coronin 1, Tregs were not involved in EAE resistance.

These results suggest an important role for coronin 1 in the development of EAE and furthermore reveal the importance of an intact naïve T cell pool as a prerequisite for autoimmune induction.

Material and methods

Mice

Coronin 1-deficient mice were generated in our laboratory as described (25) and backcrossed to C57BL/6 for eight generations. The corresponding littermates (knockout and wild-type) were bred in-house as homozygous lines. For the EAE experiments females of age 8 to 16 weeks were used. All experiments were approved by the Kantonales Veterinäramt Basel-Stadt.

Induction of active EAE

EAE was induced by active immunization with MOG_{35-55} peptide based on established protocols (26, 27). To that end, 8-12 weeks old the mice were immunized subcutaneously on the back at day 0 with 200 µg MOG_{35-55} peptide (Genscript) emulsified in 100 µl complete Freund´s adjuvant (Sigma) at a final volume of 200 µl. On day 0 and 2 the animals were injected with 250 ng pertussis toxin (Sigma) in 100 µl PBS intra peritoneally. In some experiments, wild-type $CD4^+$ T cells were transferred i.v. to coronin 1-deficient mice one day prior to EAE induction. For the $CD4^+$ T cell sort, erythrocyte-depleted cell suspensions of lymph node and spleen from naïve wild-type mice were stained with anti–CD4-FITC antibody (clone RM4-5, Biolegend) and subsequently labeled with anti-FITC microbeads (Miltenyi Biotec). Thereafter, the $CD4^+$ T cells were isolated using LS columns and magnets (Miltenyi Biotec). The sort purity was above 95% on reanalysis. For Treg depletion experiments, the mice were treated with 0.5 mg anti-CD25 antibody (PC61, purified according to (28)) i.p. 5 and 3 days before induction of EAE, which corresponds to day 0. Wild-type and coronin 1-deficient mice not treated with antibody were used as controls. The efficiency of CD25 depletion was monitored in the blood by flow cytometry at day -8, -1 and 7 by staining with an anti-CD25 antibody that recognizes an epitope different from that recognized by PC61 to avoid interference of the depleting antibody with the staining.

To monitor immune response in peripheral blood and to determine the success of T cell transfer or Treg depletion, mice were bled prior to EAE induction and during disease progression. Not more than 60 µl blood was taken from the tail vein by puncture with a 22G needle at each time point and collected in heparinized tubes.

Leukocyte counts in the erythrocyte-depleted blood were determined with a Neubauer chamber and further analyses were performed by flow cytometry.
Clinical signs of EAE were assessed daily according to the standard scoring protocol using the following scoring system: (29, 30) 0, no clinical signs; 0.5, tip tail weakness; 1, tail weakness; 1.5, mild monoparesis; 2, monoparesis or monoplegia; 2.5, mild paraparesis; 3, paraparesis, paraplegia or mild hemiplegia; 3.5, paraplegia with spasticity; 4, quadriparesis; 5, morbid stage. The mice were euthanized at score 4 or when a clinical score of 3.5 lasted for more than 2 days. In some experiments, the body weight was determined as well.

Proliferation analyses, intracellular cytokine expression and ELISA

At different time points after EAE induction the mice were scarified using high concentrations of CO_2 and the spleen was removed for further *in vitro* analyses. Erythrocyte-depleted splenocytes were cultured with different concentrations of MOG_{35-55} peptide or anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) antibodies in RPMI-1640 (Sigma) supplemented with MEM non-essential amino acids (Invitrogen), 1 mM sodium pyrovate (Sigma), Penicillin (100 units)-Streptomycin (100 μg/ml) (Sigma), 2-mercaptoethanol (Invitrogen), 10% foetal bovine serum (PAA laboratories). For proliferation analysis, splenocytes (4x 10^5 per well) were cultured in 150 μl in 96-well round bottom plates. At 72 hours of culture, 1 μCi [^{3}H]thymidine (Hartmann) was added per well and the cells were harvested 16 hours later and assessed for incorporation of the radioisotope. For determination of cytokine secretion, splenocytes (2x 10^6/ml, 500μl in 24 well plate) were stimulated with 50 μg/ml MOG_{35-55} peptide for 48 hours and then the IL-4, IFN-γ and IL-17A concentration in the supernatant was measured by ELISA (Biolegend). Intracellular cytokines were analyzed in vitro after 4 hours of stimulation with anti-CD3 and anti-CD28 antibodies in the presence of 10μg/ml brefeldin A (Sigma) for the last 2 hours.

Flow cytometry

The following antibodies were used for staining cells for flow cytometric analyses: anti-CD4 Pacific blue (Invitrogen), CD25-PE-Cy7 (clone PC61, BD Biosciences), CD25-PE (clone 7D4, Miltenyi Biotech), IL-4-PE (Biolegend), IL-17A-APC (Biolegend), IFN-γ (Biolegend). For Foxp3 detection the Foxp3-Alexa Fluor 647 flow kit from Biolegend

was used und the staining performed according to the manufacturers instructions. For surface staining the cells were incubated with the labeled antibodies for 15 minutes on ice in the dark and then washed once with PBS/0.5%BSA. For intracellular staining, cells were first stained for surface antigens as described and then fixed with 2% paraformaldehyde for 20 minutes at room temperature. After a washing step with PBS/0.5%BSA the cells were permeabilized using 0.5% saponin (Sigma) in PBS/0.5% BSA and stained in this buffer for 15 minutes with anti-cytokine antibodies at room temperature. This was followed by one washing step with 0.5% saponin/PBS/0.5%BSA and one washing step with PBS/0.5%BSA. Incubation with FcR block (anti-CD16/32 antibody, Biolegend) was included. The samples were measured on a FACS Canto II (BD Biosciences) and the data were analyzed with the FlowJo software (Tree Star).

Immunofluorescence and microscopy

Spinal cord tissue was removed, embedded in O.C.T. Tissue-Tek (Sakura) and frozen on dry ice. Cryostat sections (10 µm) were air-dried for 30 minutes and fixed for 30 minutes in 4% paraformaldehyde for staining with anti-F4/80 antibodies (macrophages and microglia), anti-CD4 (T cells) or anti-coronin 1 antibodies. Sections were first incubated for 1h in blocking buffer (5% normal donkey serum (NDS), 0.1% Triton X-100, 0.05% Tween-20) followed by incubation with primary antibodies in blocking buffer overnight at 4°C. After washing with PBS the sections were incubated with the secondary antibodies for one hour at room temperature. Slides were mounted with Fluorosave (Calbiochem) and kept at 4°C. Confocal microscopy was performed using a Zeiss LSM 710 microscope with ZEN 2009 software (Carl Zeiss, Inc.). Lesion quantification was done using the Cell^P software (Olympus). Lesion area was determined by F4/80 staining and analyzed using three sections (approx. cervical segment 6, thoracal segment 6 and lumbar segment 1) per animal. Lesion load was displayed as percentage of whole spinal cord analysed. The following antibodies were used for staining of spinal cord sections: anti-F4/80 (kindly provided by V.H. Perry, University of Southhampton, (31, 32), anti-CD4 (clone RM4-5, Biolegend) and polyclonal anti-coronin 1 rabbit antiserum (Gatfield et al., 2005). The following secondary antibodies were used (all from Jackson ImmunoResearch

Laboratories, Inc.): donkey-anti-rabbit DyLight 488 (1:500), donkey-anti-rabbit DyLight 549 (1:500), donkey-anti-rat-Cy3 (1:500), donkey anti-rat-Cy5 (1:500).

Treg suppression assay

T cell subsets were isolated from spleen and peripheral and mesenteric lymph nodes of wild-type and coronin 1-deficient mice. Erythrocyte-depleted cell suspensions were stained with anti-CD25 APC and anti-CD4 PE antibody (Biolegend). Prior to sorting of $CD25^+$ and $CD25^+$ $CD4^+$ T cells with FACS ARIA (BD Biosciences), $CD4^+$ T cells were isolated using anti-PE microbeads and a magnetic separation system (Miltenyi Biotec). Single cell suspensions from C57BL/6 spleens, erythrocyte-lysed and depleted of $CD4^+$ and $CD8^+$ T cells, were irraditated and used as antigen presenting cells (APCs). Suppression assays were performed in 96-well round bottom plates (3x 10^4 $CD25^-CD4^+$ responder cells plus 6x 104 irradiated APCs plus titrated numbers of $CD25^+CD4^+$ Treg cells) in triplicates. As controls $CD25^+CD4^+$ and $CD25^-$ $CD4^+$ T cells were cultured without Tregs. The cells were stimulated with 1 µg/ml anti-CD3 antibody (clone 2C-11, Biolegend) and pulsed after 60 hours for 16 hours with 1 µCi [^{3}H]thymidine per well (Hartmann).

Statistical analyses

The data represent the mean (± SD or SEM, indicated in the figure legends); FACS dot plots and microscopic pictures show representative results. The number of mice (n) used per experiment and the number of experiments performed are listed in each figure legend. Statistically significance of the ELISA and flow cytometry results as well as the EAE day of onset, maximal disease score and lesion size was analyzed by Mann-Whitney statistical test and the EAE score and weight versus time was analyzed by Wilcoxon signed rank test using GraphPad Prismsoftware (GraphPad).

Results

Role for coronin 1 in the development of EAE

Coronin 1 is a leukocyte-specific regulator of Ca^{2+}-dependent signaling processes and essential for the maintenance of the naïve T cell pool in the periphery (19-22). Despite diminished numbers of naïve T cells (20), when coronin 1-deficient mice are immunized with a variety of antigens, normal antibody responses are generated (24). To investigate the role of coronin 1 in the pathogenesis of T cell mediated autoimmunity, coronin 1-deficient and wild-type C57Bl/6 mice were immunized with MOG_{35-55} peptide emulsified in complete Freund´s adjuvant (CFA) subcutaneously together with pertussis toxin intra peritonealy leading to induction of active EAE (see *materials and methods*). The development of clinical signs of disease was monitored over several weeks. Initial signs of EAE, starting with tail weakness, were observed ten days (on average 11.6 ± 2.7 days) after immunization of wild-type mice while at this time point none of the coronin 1-deficient mice showed any signs of disease (Figure 1A). Onset and progression of disease were also reflected by weight loss (Figure 1B). While by day 17, all wild-type mice had developed full-blown disease with a mean maximal clinical score of 3.1 ± 0.4, only two out of 32 coronin 1-deficient mice showed clinical signs of EAE. During the entire period of observation, only 13 out of 32 coronin 1-deficient mice developed EAE symptoms, which were characterized by a significantly delayed onset of disease (23.6 ± 6 days) and a reduction in disease severity (maximal clinical score of 1.8 ± 0.9) in comparison to wild-type mice (Figure 1C, D and E). Prolonged monitoring of the EAE development up to 60 days revealed a continuous slow progressing remission in wild-type mice, whereas clinical scores of the coronin 1-deficient mice stayed low also at these late time points after immunization (Suppl. Figure 1).

To analyze the relative lesion size in spinal cords from immunized wild-type and coronin 1-deficient animals, histological sections were stained with F4/80, a marker for activated microglia/macrophages. Consistent with the aforementioned clinical EAE symptoms, all wild-type mice showed massive inflammatory lesions in the spinal cord; in contrast, only some coronin 1-deficient mice developed inflammatory lesions (Figure 1F). Furthermore, also confirming the clinical observations, the lesions of

coronin 1-deficient spinal cord were smaller in size and observed mainly in the lumbar part, which reflects less progressed disease (Figure 1F). We conclude from these results that coronin 1-deficient mice are largely resistant to EAE induction.

Immune responses during EAE development in the presence and absence of coronin 1

To monitor immune responses during EAE initiation and progression in wild-type and coronin 1-deficient mice, blood samples were taken before and at several time points after immunization. Determination of leukocyte cell counts in the blood after lysis of erythrocytes revealed a strong increase in cellularity in both wild-type and coronin 1-deficient mice upon immunization, while non-immunized mice analyzed in parallel showed no changes in their immune cell composition in blood (Figure 2). Specifically, immunization led to a comparable increase in leukocyte, granulocyte and lymphocyte counts (Figure 2A and B) as well as $CD4^+$ T cells (Figure 2C) in both mice strains, although overall coronin 1-deficient mice had lower numbers of leukocyte subsets compared to wild-type mice. These results suggest an equal mobilization and activation of immune cells in the presence and absence of coronin 1 as a consequence of immunization, independent of the development of signs of autoimmune encephalomyelitis.

Since upon immunization both in the presence as well as absence of coronin 1 changes in immune cell composition in blood were observed, we addressed whether the resistance to EAE induction in the absence of coronin 1 can be explained by an altered cytokine response. To that end, the ability of coronin 1-deficient $CD4^+$ T cells to produce the Th1-cytokine IFN-γ, the Th17-cytokine IL-17A and the Th2-cytokine IL-4 was analyzed upon polyclonal stimulation with anti-CD3/CD28 antibodies *in vitro*. Interestingly, coronin 1-deficient mice had equal or even increased numbers of IFN-γ, IL-17A and IL-4-positive $CD4^+$ T cells in comparison to wild-type mice (Figure 2D). Of note is the IL-4-expression that was significantly increased in coronin 1-deficient as compared to wild-type $CD4^+$ T cells, both in naïve as well as immunized wild-type and coronin 1-deficient mice. However, immunization resulted in elevated expression of all analyzed cytokines in both mice strains. Thus, the capability to express cytokines upon polyclonal stimulation *ex vivo*, is similar between coronin 1-deficient and wild-type $CD4^+$ T cells, and therefore cannot explain the resistance of the coronin 1-deficient mice to EAE induction.

We next determined whether the observed resistance of coronin 1-deficient mice to EAE induction was due to defective peripheral T cell responses to the initial immunization with MOG_{35-55} peptide. MOG-specific immune responses were analyzed *ex vivo* at different time points after immunization. Cytokine expression as well as proliferation was determined at day 6, 12, 16 and at day 40 after immunization, reflecting time points before disease induction, at time of disease induction, at the peak of clinical signs and during chronic disease state, respectively. While coronin 1-deficient mice did not show any signs of EAE up to day 16, some of the wild-type mice had already developed disease symptoms at day 12, and all of them by day 16 (Figure 1 and asterisks in Figure 3A). Polyclonal re-stimulation *in vitro* at the indicated time points revealed that the frequency and cell counts of IFN-γ and IL-17A-expressing $CD4^+$ T cells increased in wild-type as well as in coronin 1-deficient mice upon immunization (Suppl. Figure 2). However, while the cytokine expression peaked in wild-type mice around day 12, the number of cytokine-positive $CD4^+$ T cells of coronin 1-deficient mice increased progressively, indicating different kinetics of cytokine induction between wild-type and coronin 1-deficient mice. The analyses of MOG-specific cytokine expression by ELISA revealed that similar to the results of polyclonal cytokine expression, coronin 1-deficient $CD4^+$ T cells showed delayed MOG-specific cytokine responses. But in contrast to the cytokine expression upon polyclonal stimulation, IFN-γ and IL-17A secretion was overall lower in MOG-stimulated splenocyte cultures from coronin 1-deficient mice (Figure 3A). However, some of the immunized coronin 1-deficient mice secreted IFN-γ and IL-17A upon stimulation with MOG at levels as high as wild-type mice; but not in all of those coronin 1-deficient animals a correlation with the clinical score was observed. This is exemplified by an increased IL-17A secretion in two mice without any signs of disease (Figure 3A). In contrast to the high IL-4-expression observed upon polyclonal stimulation (Figure 2D and Suppl. Figure 2), in none of the samples any MOG-specific IL-4 was detected (data not shown).

To analyze the MOG-specific proliferative response, splenocytes from immunized wild-type and coronin 1-deficient mice were isolated at different time points after immunization and stimulated *ex vivo* with either MOG_{35-55} peptide or anti-CD3 and anti-CD28 antibodies. None of the mice, neither wild-type nor coronin 1-deficient,

showed MOG-induced proliferation when analyzed at day six after immunization or if immunized with CFA solely (Figure 3B and data not shown). Overall, MOG-specific proliferation of coronin 1-deficient splenocytes was greatly reduced in contrast to splenocytes isolated from immunized wild-type mice, which showed a dose-dependent proliferation, which was strongest at onset of EAE (day12, Figure 3B). In contrast to the diminished MOG-induced proliferation of coronin 1-deficient splenocytes, proliferation upon polyclonal stimulation was equivalent to that of wild-type splenocytes, especially at later time points after immunization. Interestingly, coronin 1-deficient splenocytes showed less proliferation upon polyclonal stimulation than wild-type cells at day 6 and day 12 after immunization, but this difference was not observed anymore at day 16, suggesting that immunization increased the proliferation capacity of coronin 1-deficient T cells to wild-type levels.

Immune cell infiltration in spinal cords of wild-type and coronin 1-deficient mice upon induction of active EAE

Experimental autoimmune encephalomyelitis is initiated by infiltration of MOG-specific $CD4^+$ T cells into the central nervous system, where they subsequently induce the recruitment of other immune cells. To analyze time-dependent infiltration of immune cells into the spinal cord of wild-type and coronin 1-deficient mice, active EAE was induced as described and spinal cord sections were analyzed at different time-points thereafter by histology. No infiltration of immune cells was observed before disease onset (day 6), but an increasing number of infiltrating immune cells ($F4/80^+$ macrophages and $CD4^+$ T cells) were detected at day 12 and 16 in the spinal cords of wild-type but not coronin 1-deficient mice (Figure 4, lesions marked by arrowheads). In contrast, no infiltration was visible in coronin 1-deficient mice at any of those time points.

Coronin 1-positive leukocytes were abundantly present in spinal cord lesions of wild-type mice, while the coronin 1 antibody did not stain the remaining area of the spinal cord sections (Figure 4). Overall, the degree of immune cell infiltration correlated with the degree of disease severity in both mice strains.

EAE development in coronin 1-deficient mice after transfer of wild-type $CD4^+$ T cells and subsequent immunization with MOG_{35-55} peptide

To analyze whether the resistance of coronin 1-deficient mice towards EAE is T cell intrinsic, $CD4^+$ T cells isolated from naïve wild-type mice were adoptively transferred to coronin 1-deficient mice prior to immunization with MOG_{35-55} peptide. The presence of the transferred wild-type $CD4^+$ T cells in the blood of coronin 1-deficient mice was monitored by flow cytometry (Suppl. Figure 3). While the adoptive transfer of one and five million wild-type cells did not and the transfer of ten million did only slightly change the EAE outcome, the transfer of twenty million wild-type $CD4^+$ T cells restored EAE-susceptibility in all coronin 1-deficient recipients (with the exception of one mouse that lost the transferred cells; Suppl. Figure 3 and Figure 5). The presence of twenty million wild-type $CD4^+$ T cells in coronin 1-deficient mice increased the average disease incidence from 43% to 100%, the average maximal clinical score from 1.3 to 2.8 and the average day of onset from 22.7 to 17.8 (Table I). Following transfer of wild type $CD4^+$ T cells, the disease outcome showed a mixed phenotype: four out of six mice developed signs of EAE that were equal in severity and day of onset to that of wild-type mice, while two recipients developed EAE, which resembled the lower grade disease observed for coronin 1-deficient mice. We conclude from these results that the resistance of coronin 1-deficient mice to EAE induction is caused by a defective T-cell response.

Frequency and functionality of regulatory T cells in the presence and absence of coronin 1

Development of autoimmune encephalomyelitis depends on the activation, expansion and migration of myelin-specific $CD4^+$ T cells into the CNS and is controlled by regulatory T cells that suppress autoreactive T cell responses (reviewed by (33)). Given the severely depressed EAE responses in coronin 1-deficient mice, the impact of coronin 1-deficiency on the number and functionality of Tregs was investigated. Flow cytometric analyses revealed an increased frequency of FOXP3-positive ($CD25^+$ and $CD25^-$) $CD4^+$ T cells in peripheral lymphoid organs of coronin 1-deficient mice, while $CD25^+$ $CD4^+$ effector T cells numbers were comparable between wild-type and coronin 1-deficient mice (Figure 6A). However, the total Treg counts were approximately half of that of wild-type mice, likely as a result of the overall reduced $CD4^+$ T cell counts in coronin 1-deficient mice.

To determine the suppressive function of coronin 1-deficient Tregs, an *in vitro* suppression assay was performed with FACS-sorted $CD25^+$ and $CD25^-$ $CD4^+$ T cells. The results revealed that coronin 1-deficient $CD25^+CD4^+$ T cells were anergic similar to wild-type $CD25^+CD4^+$ T cells and did suppress the proliferation of $CD25^-CD4^+$ responder T cells in the co-cultures to a similar degree (Figure 6B). To address the contribution of Tregs to the increased EAE resistance observed for coronin 1-deficient mice, Tregs were depleted in both wild-type and coronin 1-deficient mice by injection of the anti-CD25 antibody PC61 accordingly to the treatment schedule shown in *Suppl. Figure 4*. The efficiency of the treatment was monitored by flow cytometry of blood samples before and at two time points after injection of the depleting antibody. PC61 treatment led to a strong decrease of $CD25^+CD4^+$ T cells as well as $Foxp3^+CD4^+$ T cells ($CD25^+$ and $CD25^-$) in coronin 1-deficient as well as wild-type mice that was observed already one week after treatment and became more evident later (Suppl. Figure 4). While Treg depletion resulted in a more severe EAE in wild-type mice compared to mice that were not treated with antibodies, it had no effect on disease severity of coronin 1-deficient mice that remained resistant to EAE induction (Figure 6C). Thus, these results indicate that resistance to EAE induction in the absence of coronin 1 is not due to deregulated Treg response but is more likely caused by a disturbed naïve T cell priming.

Discussion

Susceptibility to autoimmune encephalomyelitis as well as multiple sclerosis requires activation of myelin-specific T cells that have escaped central tolerance (reviewed in (34)). It is believed that those autoreactive T cells are present in the peripheral naïve T cell pool in a state of ignorance (35). However, under certain circumstances, these cells can become activated and differentiate into effector T cells that then acquire access to the central nervous system. The contribution of the naïve T cell pool size and composition for the development of experimental autoimmune encephalomyelitis (EAE) is currently not well defined. We here show that mice lacking coronin 1, which possess a drastically reduced peripheral naïve T cell pool, are highly resistant to induction of EAE as induced by immunization with myelin-derived antigen (MOG). Resistance to EAE development was T cell intrinsic, since transfer of wild-type $CD4^+$ T cells into coronin 1-deficient mice restored disease susceptibility and furthermore depletion of regulatory T cells had no impact on the resistance to EAE. Strikingly, leukocyte mobilization upon immunization, polyclonal T cell responses and regulatory T cell functions in the absence of coronin 1 were comparable to wild-type mice. Together these results show that coronin 1-deficient mice are resistant to the induction and development of autoimmune encephalomyelitis.

Coronin 1 is a leukocyte specific protein that in T cells has been implicated in a variety of processes such as migration, cell signaling and survival (36). Despite its expression in all leukocyte subsets with the exception of Kupffer cells, the major cell type affected by the absence of coronin 1 *in vivo* are naïve T cells in the periphery; in mice lacking coronin 1 expression, peripheral T cell numbers are greatly diminished (19, 20, 23). Coronin 1 is essential for intracellular calcium mobilization downstream of T cell receptor triggering and the consequent translocation of the NFAT into the nucleus (20). This activation defect of naïve T cells lacking coronin 1 is reflected by impaired proliferation and cytokine expression upon TCR stimulation *in vitro*. However, coronin 1-deficient mice are capable to mount relatively normal antibody responses to T cell independent as well as T cell dependent antigens (24). The most striking effect of coronin 1-depletion is the apparent increased spontaneous apoptosis of naïve T cells *ex vivo* (19, 20, 23). This observation together with the here

described resistance of coronin 1-deficient mice to EAE development is in accordance with the finding that the extent of T cell death is decisive for autoimmunity (37).

The resistance of coronin 1-deficient mice to EAE induction as describe here is consistent with a role for coronin 1 in T cell signaling. Indeed, in the absence of coronin 1, MOG-specific T cell responses such as proliferation and cytokine expression following immunization with MOG_{35-55} peptide are delayed as well as reduced, strongly suggesting a priming defect in the absence of coronin 1. In addition, several lines of evidence exclude a role for coronin 1 in antigen processing and presentation. These include (i) the restoration of EAE upon transfer of naïve, wild-type T cells as shown here; (ii) the absence of any defect in either macrophage, B cell and dendritic cell function (24, 25, 38); (iii) the absence of any gross defects in thymic selection, a process that is heavily dependent on antigen processing and presentation (19-21). Naïve T cell priming, which is the initial step of an immune response, usually takes place the draining lymph nodes where professional antigen presenting cells activate naïve T cells leading to clonal expansion and differentiation into cytokine-expressing effector T cells. While peripheral lymph nodes of coronin 1-deficient mice are smaller compared to wild-type mice, the segregation of T and B cells is normal as well as the formation of germinal centers upon immunization (24). However, lymph node swelling as an indication of an ongoing immune response was reduced in coronin 1-deficient mice (unpublished data), which might be due to impaired lymph node access or defective T cell activation in the lymph node. However, it has to be noted that T cell access to lymph nodes may not be essential for EAE development, since T cell priming can also occur in the spleen or extra-lymphoid structures such as in liver (39-41).

Our results suggest a more general activation defect in coronin 1-deficient mice, since splenocytes isolated at several time points after immunization with MOG_{35-55} peptide proliferated poorly upon *ex vivo* restimulation with the same peptide. This finding implies a diminished *in vivo* expansion of MOG-specific T cells in coronin 1-deficient mice upon immunization; resulting in a low frequency of MOG-specific effector T cells, which can potentially cause disease. In line with the reduced number of MOG-specific effector T cells, cytokine expression upon restimulation with MOG_{35-55} peptide was reduced in the absence of coronin 1. In contrast, proliferation induced by polyclonal

stimulation with anti-CD3 and anti-CD28 antibodies was less affected, especially if splenocytes were analyzed at later time points after immunization. Thus, it appears as if the threshold for polyclonal activation *in vitro* is altered by immunization *in vivo*. Furthermore, coronin 1-deficient $CD4^+$ T cells did not display a general defect to differentiate into effector T cells, since the cytokine-profile of wild-type and coronin 1-deficient $CD4^+$ T cells was comparable after polyclonal stimulation. This is in line with the finding that the memory T cell pool in coronin 1-deficient mice is comparable to wild-type mice (19, 20). However, this does not explain the observed increase of cytokine-expressing $CD4^+$ T cells after immunization *in vivo*. Interestingly, expression of IL-4, which has been described to protect from EAE development (8, 9), was even higher in coronin 1-deficient mice. But since no MOG-specific IL-4 secretion was detected, we rule out that the resistance to EAE is caused by immune deviation to a Th2 response. Another possible explanation for the increased resistance of coronin 1-deficient mice to EAE provides the elevated Treg:naïve T cell ratio that was observed in the absence of coronin 1. However, since the depletion of Tregs, which showed normal suppressive activity *in vitro*, did not restore EAE sensitivity, an important contribution of this T cell subset is unlikely. This finding supports the idea that the resistance of coronin 1-deficient mice to EAE development is primarily a consequence of reduced naïve T cell number and/or impaired activation upon immunization.

Nevertheless, some coronin 1-deficient mice developed EAE symptoms and displayed MOG-specific IL-17A and IFN-γ secretion, which was in some cases as high as observed for wild-type mice. However, in all of these cases the disease onset was delayed, even if in these few cases disease severity was indistinguishable from wild-type mice. Consistent with the delayed disease onset of the few diseased coronin 1-deficient mice MOG-specific IL-17A and IFN-γ secretion was delayed. This observation might be explained by the important role of pioneer cells for immune surveillance and for initiating immune responses in peripheral tissues (42). After priming and differentiation in lymphoid tissues, myelin-specific effector T cells must acquire access to the non-inflamed central nervous system, where they act as pioneer T cells to induce disease. Recently, the impact of $CCR6^+$ Th17 pioneer cells for EAE induction has been highlighted by Reboldi *et al.* (43). Given the strongly reduced naïve T cell pool and the T cell activation defect of coronin 1-deficient mice, the accumulation of

threshold numbers of MOG-specific effector T cells that can function as pioneer cells may be severely disturbed. It is well possible that disease induction in those few diseased coronin 1-deficient animals occurred through the migration of only a few pioneer cells in the central nervous system. Indeed, our results suggest, that coronin 1-deficient MOG-reactive T cells once activated and arrived in the central nervous system, have the same potential than wild-type T cells to activate local tissue cells and mediate the recruitment of further immune cells. In agreement with this idea, we found a clear correlation between EAE symptoms and the extent of immune cell infiltration. The hypothesis of a threshold number of MOG-specific T cells is further supported by our observation that transfer of one and five million of wild-type $CD4^+$ T cells from naïve mice did not increase EAE susceptibility, while ten million had already a slight effect and 20 million transferred $CD4^+$ T cells did break resistance of coronin 1-deficient mice to EAE induction. In line with this, it has been shown in rodent models of EAE that modulation of the $CD4^+$ T cell pool, which is available for activation, by administration of anti-CD4-antibodies, either depleting or inhibitory, prevented development of EAE (44-46).

Taken together, the results presented here demonstrate a hitherto unknown and specific role for the naïve T cell pool 1 in shaping $CD4^+$ T cell responses towards autoantigens and reveal coronin 1 as a crucial molecule in the induction of autoimmune encephalomyelitis.

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References

1. Compston, A., and A. Coles. 2002. Multiple sclerosis. *Lancet* 359:1221-1231.
2. Gold, R., C. Linington, and H. Lassmann. 2006. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* 129:1953-1971.
3. Iglesias, A., J. Bauer, T. Litzenburger, A. Schubart, and C. Linington. 2001. T- and B-cell responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and multiple sclerosis. *Glia* 36:220-234.
4. Kivisakk, P., J. Imitola, S. Rasmussen, W. Elyaman, B. Zhu, R. M. Ransohoff, and S. J. Khoury. 2009. Localizing central nervous system immune surveillance: meningeal antigen-presenting cells activate T cells during experimental autoimmune encephalomyelitis. *Ann Neurol* 65:457-469.
5. Greter, M., F. L. Heppner, M. P. Lemos, B. M. Odermatt, N. Goebels, T. Laufer, R. J. Noelle, and B. Becher. 2005. Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. *Nat Med* 11:328-334.
6. Stromnes, I. M., L. M. Cerretti, D. Liggitt, R. A. Harris, and J. M. Goverman. 2008. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med* 14:337-342.
7. Kroenke, M. A., T. J. Carlson, A. V. Andjelkovic, and B. M. Segal. 2008. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J Exp Med* 205:1535-1541.
8. Cua, D. J., and S. A. Stohlman. 1997. In vivo effects of T helper cell type 2 cytokines on macrophage antigen-presenting cell induction of T helper subsets. *J Immunol* 159:5834-5840.
9. Falcone, M., and B. R. Bloom. 1997. A T helper cell 2 (Th2) immune response against non-self antigens modifies the cytokine profile of autoimmune T cells and protects against experimental allergic encephalomyelitis. *J Exp Med* 185:901-907.
10. Kohm, A. P., P. A. Carpentier, H. A. Anger, and S. D. Miller. 2002. Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J Immunol* 169:4712-4716.
11. Reddy, J., Z. Illes, X. Zhang, J. Encinas, J. Pyrdol, L. Nicholson, R. A. Sobel, K. W. Wucherpfennig, and V. K. Kuchroo. 2004. Myelin proteolipid protein-specific CD4+CD25+ regulatory cells mediate genetic resistance to experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 101:15434-15439.
12. Stephens, L. A., K. H. Malpass, and S. M. Anderton. 2009. Curing CNS autoimmune disease with myelin-reactive Foxp3+ Treg. *Eur J Immunol* 39:1108-1117.
13. McGeachy, M. J., L. A. Stephens, and S. M. Anderton. 2005. Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. *J Immunol* 175:3025-3032.
14. Boyman, O., S. Letourneau, C. Krieg, and J. Sprent. 2009. Homeostatic proliferation and survival of naive and memory T cells. *Eur J Immunol* 39:2088-2094.

15. Tanchot, C., F. A. Lemonnier, B. Perarnau, A. A. Freitas, and B. Rocha. 1997. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science* 276:2057-2062.
16. Kirberg, J., A. Berns, and H. von Boehmer. 1997. Peripheral T cell survival requires continual ligation of the T cell receptor to major histocompatibility complex-encoded molecules. *J Exp Med* 186:1269-1275.
17. Martin, B., C. Becourt, B. Bienvenu, and B. Lucas. 2006. Self-recognition is crucial for maintaining the peripheral CD4+ T-cell pool in a nonlymphopenic environment. *Blood* 108:270-277.
18. Polic, B., D. Kunkel, A. Scheffold, and K. Rajewsky. 2001. How alpha beta T cells deal with induced TCR alpha ablation. *Proc Natl Acad Sci U S A* 98:8744-8749.
19. Foger, N., L. Rangell, D. M. Danilenko, and A. C. Chan. 2006. Requirement for coronin 1 in T lymphocyte trafficking and cellular homeostasis. *Science* 313:839-842.
20. Mueller, P., J. Massner, R. Jayachandran, B. Combaluzier, I. Albrecht, J. Gatfield, C. Blum, R. Ceredig, H. R. Rodewald, A. G. Rolink, and J. Pieters. 2008. Regulation of T cell survival through coronin-1-mediated generation of inositol-1,4,5-trisphosphate and calcium mobilization after T cell receptor triggering. *Nat Immunol* 9:424-431.
21. Haraldsson, M. K., C. A. Louis-Dit-Sully, B. R. Lawson, G. Sternik, M. L. Santiago-Raber, N. R. Gascoigne, A. N. Theofilopoulos, and D. H. Kono. 2008. The lupus-related Lmb3 locus contains a disease-suppressing Coronin-1A gene mutation. *Immunity* 28:40-51.
22. Shiow, L. R., D. W. Roadcap, K. Paris, S. R. Watson, I. L. Grigorova, T. Lebet, J. An, Y. Xu, C. N. Jenne, N. Foger, R. U. Sorensen, C. C. Goodnow, J. E. Bear, J. M. Puck, and J. G. Cyster. 2008. The actin regulator coronin 1A is mutant in a thymic egress-deficient mouse strain and in a patient with severe combined immunodeficiency. *Nat Immunol* 9:1307-1315.
23. Mugnier, B., B. Nal, C. Verthuy, C. Boyer, D. Lam, L. Chasson, V. Nieoullon, G. Chazal, X. J. Guo, H. T. He, D. Rueff-Juy, A. Alcover, and P. Ferrier. 2008. Coronin-1A links cytoskeleton dynamics to TCR alpha beta-induced cell signaling. *PLoS One* 3:e3467.
24. Combaluzier, B., P. Mueller, J. Massner, D. Finke, and J. Pieters. 2009. Coronin 1 is essential for IgM-mediated Ca2+ mobilization in B cells but dispensable for the generation of immune responses in vivo. *J Immunol* 182:1954-1961.
25. Jayachandran, R., V. Sundaramurthy, B. Combaluzier, P. Mueller, H. Korf, K. Huygen, T. Miyazaki, I. Albrecht, J. Massner, and J. Pieters. 2007. Survival of mycobacteria in macrophages is mediated by coronin 1-dependent activation of calcineurin. *Cell* 130:37-50.
26. Linker, R. A., M. Maurer, S. Gaupp, R. Martini, B. Holtmann, R. Giess, P. Rieckmann, H. Lassmann, K. V. Toyka, M. Sendtner, and R. Gold. 2002. CNTF is a major protective factor in demyelinating CNS disease: a neurotrophic cytokine as modulator in neuroinflammation. *Nat Med* 8:620-624.
27. Becher, B., B. G. Durell, A. V. Miga, W. F. Hickey, and R. J. Noelle. 2001. The clinical course of experimental autoimmune encephalomyelitis and inflammation is controlled by the expression of CD40 within the central nervous system. *J Exp Med* 193:967-974.
28. Harlow, E. 1989. *Antibodies: a laboratory manual*. Cold Spring Harbour.

29. Storch, M. K., A. Stefferl, U. Brehm, R. Weissert, E. Wallstrom, M. Kerschensteiner, T. Olsson, C. Linington, and H. Lassmann. 1998. Autoimmunity to myelin oligodendrocyte glycoprotein in rats mimics the spectrum of multiple sclerosis pathology. *Brain Pathol* 8:681-694.
30. Weissert, R., E. Wallstrom, M. K. Storch, A. Stefferl, J. Lorentzen, H. Lassmann, C. Linington, and T. Olsson. 1998. MHC haplotype-dependent regulation of MOG-induced EAE in rats. *J Clin Invest* 102:1265-1273.
31. Austyn, J. M., and S. Gordon. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol* 11:805-815.
32. Hirsch, S., J. M. Austyn, and S. Gordon. 1981. Expression of the macrophage-specific antigen F4/80 during differentiation of mouse bone marrow cells in culture. *J Exp Med* 154:713-725.
33. O'Connor, R. A., and S. M. Anderton. 2008. Foxp3+ regulatory T cells in the control of experimental CNS autoimmune disease. *J Neuroimmunol* 193:1-11.
34. Goverman, J. 2009. Autoimmune T cell responses in the central nervous system. *Nat Rev Immunol* 9:393-407.
35. Seamons, A., A. Perchellet, and J. Goverman. 2003. Immune tolerance to myelin proteins. *Immunol Res* 28:201-221.
36. Hogquist, K. A. 2008. Immunodeficiency: when T cells are stuck at home. *Nat Immunol* 9:1207-1208.
37. Shi, Y., Y. Feng, J. Kang, C. Liu, Z. Li, D. Li, W. Cao, J. Qiu, Z. Guo, E. Bi, L. Zang, C. Lu, J. Z. Zhang, and G. Pei. 2007. Critical regulation of CD4+ T cell survival and autoimmunity by beta-arrestin 1. *Nat Immunol* 8:817-824.
38. Jayachandran, R., J. Gatfield, J. Massner, I. Albrecht, B. Zanolari, and J. Pieters. 2008. RNA Interference in J774 Macrophages Reveals a Role for Coronin 1 in Mycobacterial Trafficking but Not in Actin-dependent Processes. *Mol Biol Cell* 19:1241-1251.
39. Davalos-Misslitz, A. C., J. Rieckenberg, S. Willenzon, T. Worbs, E. Kremmer, G. Bernhardt, and R. Forster. 2007. Generalized multi-organ autoimmunity in CCR7-deficient mice. *Eur J Immunol* 37:613-622.
40. Greter, M., J. Hofmann, and B. Becher. 2009. Neo-lymphoid aggregates in the adult liver can initiate potent cell-mediated immunity. *PLoS Biol* 7:e1000109.
41. Uboldi, C., A. Doring, C. Alt, P. Estess, M. Siegelman, and B. Engelhardt. 2008. L-Selectin-deficient SJL and C57BL/6 mice are not resistant to experimental autoimmune encephalomyelitis. *Eur J Immunol* 38:2156-2167.
42. Carrithers, M. D., I. Visintin, S. J. Kang, and C. A. Janeway, Jr. 2000. Differential adhesion molecule requirements for immune surveillance and inflammatory recruitment. *Brain* 123 (Pt 6):1092-1101.
43. Reboldi, A., C. Coisne, D. Baumjohann, F. Benvenuto, D. Bottinelli, S. Lira, A. Uccelli, A. Lanzavecchia, B. Engelhardt, and F. Sallusto. 2009. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol* 10:514-523.
44. Brostoff, S. W., and D. W. Mason. 1984. Experimental allergic encephalomyelitis: successful treatment in vivo with a monoclonal antibody that recognizes T helper cells. *J Immunol* 133:1938-1942.
45. Waldor, M. K., S. Sriram, R. Hardy, L. A. Herzenberg, L. Lanier, M. Lim, and L. Steinman. 1985. Reversal of experimental allergic encephalomyelitis with monoclonal antibody to a T-cell subset marker. *Science* 227:415-417.

46. Waldor, M. K., D. Mitchell, T. J. Kipps, L. A. Herzenberg, and L. Steinman. 1987. Importance of immunoglobulin isotype in therapy of experimental autoimmune encephalomyelitis with monoclonal anti-CD4 antibody. *J Immunol* 139:3660-3664.

Figure legends

Figure 1. EAE pathology in the presence and absence of coronin 1. Coronin 1-deficient ($coro1^{-/-}$) and wild-type (WT) mice were immunized with MOG_{35-55} peptide in CFA to induce active EAE. A-E, Disease development and progression were monitored over time using the scoring system described in *Material and Methods*. A, Mean clinical score ± S.E.M of 3 independent experiments (n = 20). B, Mean body weight ± S.E.M of 2 independent experiments (n = 10). C, D, Maximal clinical score and day of disease onset are displayed for every single mouse together with the mean. The numbers included in the graphs represent the mean ± SD calculated only for animals showing signs of EAE (5 independent experiments, n = 33 and 32). E, Average disease incidence, results of 5 independent experiments are shown. F, Immunohistological analyses of spinal cord sections (lumbar) at end of the experiment and quantification of lesion sizes (% of area of the whole spinal cord cross section) of the individual animals ($n_{Coro1-/-}$ = 15, n_{WT} = 14). Representative F4/80 staining of wild-type and coronin 1-deficient mice are shown scale bar: 500 µm. The corresponding maximal clinical score is displayed.

Figure 2. Immune response of wild-type and coronin 1-deficient mice upon EAE induction. A-C, The frequency of leukocyte subsets in blood of $coro1^{-/-}$ and WT mice was analyzed by flow cytometry at the indicated time points before and after immunization with MOG_{35-55} peptide in CFA. Total leukocyte (A), granulocyte (B), lymphocyte (B) and $CD4^+$ T cell (C) counts per ml blood and fold change upon immunization are shown in the graphs. Representative FACS dot plots depicting forward versus sideward scatter (B) and CD4 versus GFP (C, $coro1^{-/-}$: coronin 1 gene is disrupted by insertion of GFP) of blood samples of $coro1^{-/-}$ and WT mice before and at day six after immunization are shown. The graphs summarize the results of two independent experiments, presented as mean ± SD (n = 10). D, The frequency of IFN-γ, IL-17A and IL-4-expressing cells among $CD4^+$ T cells was analyzed by flow cytometry. Splenocytes were re-stimulated *ex vivo* with anti-CD3 and anti-CD28 in the presence of brefeldin A and the cytokine expression was analyzed by intracellular staining and flow cytometry. Representative FACS dot blots (gated on $CD4^+$ T cells) are shown for MOG-immunized coronin 1-deficient and wild-type mice. The graphs

show cell count of IFN-γ^+, IL-17A$^+$ and IL-4$^+$ CD4$^+$ T cells of naïve mice and mice that have been immunized to induce active EAE 40 days before the analysis (naïve mice: n = 7; immunized mice n=18 for wt and n=20 for coro1$^{-/-}$). Results of individual mice and the mean are shown. ns, not significant

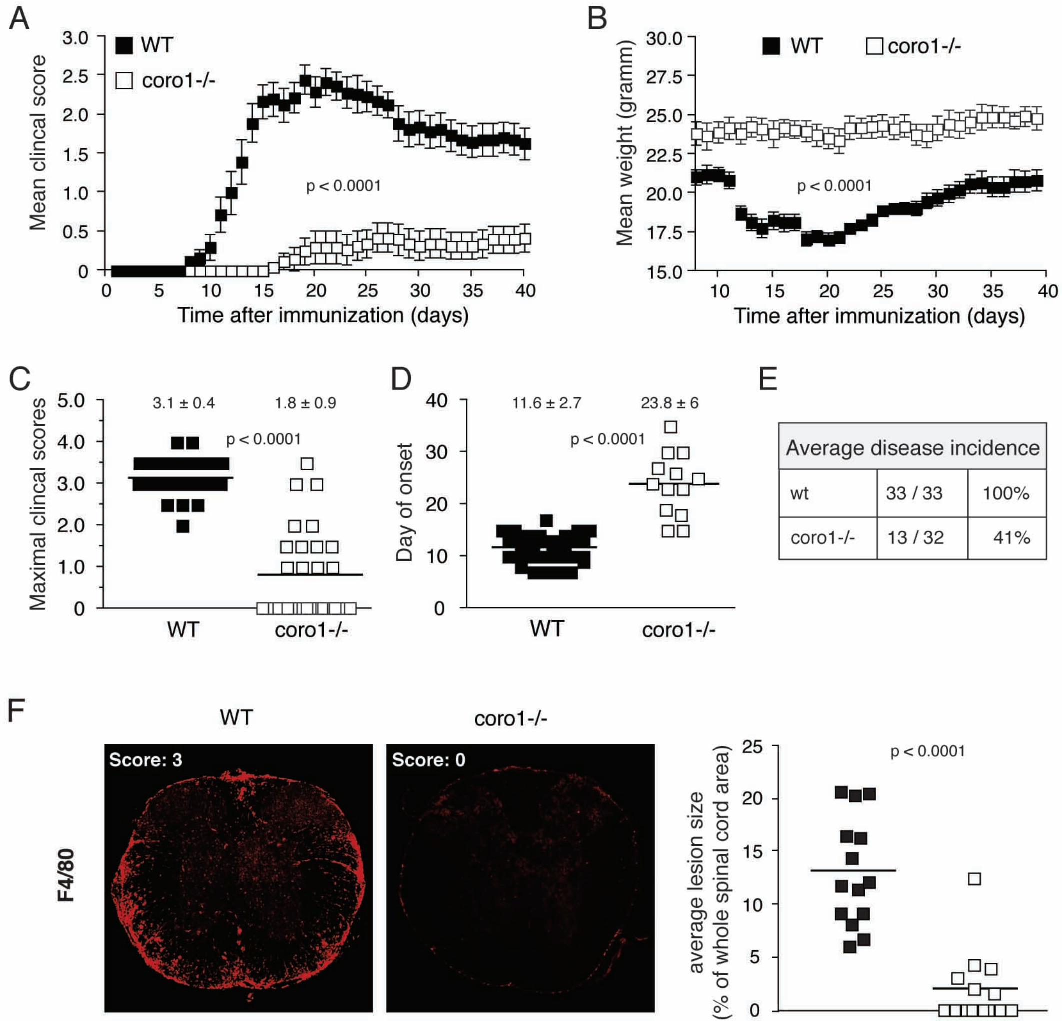
Figure 3. MOG-specific immune responses of wild-type and coronin 1-deficient mice. Active EAE was induced in coronin 1-deficient (coro1$^{-/-}$) and wild-type (WT) mice and MOG-specific responses were analyzed *ex vivo* during EAE development and at peak of the disease (corresponding to day 6, 12 and 16) in one experiment and during chronic disease state at day 40 in two other experiments. A, IFN-γ and IL-17A concentration in the supernatant of MOG_{35-55} peptide-stimulated splenocytes (50µg/ml for 48 hours) were analyzed by ELISA. Each symbol represents data from one mouse. Animal that developed signs of EAE are labeled with *. B, Splenocytes of individual mice were stimulated *in vitro* with increasing concentrations of MOG_{35-55} peptide or anti-CD3/anti-CD28 antibodies. After 72 hours of culture the cells were pulsed with [^{3}H]-thymidin and harvested 16 hours later for measurement of radioisotope incorporation. Mean counts per minute (cpm) ± SD or cpm of individual mice are depicted. n.d., not detectable; ns, not significant; n = 5 (day 6, 12, 16), n = 10 (day 40)

Figure 4. Immune cell infiltration in the spinal cords of wild-type and coronin 1-deficient mice analyzed by immunofluorescence. Spinal cord sections obtained from wild-type and coronin 1-deficient mice at the indicated time points after immunization were processed for immunofluorescence staining using antibodies for CD4, coronin 1 and F4/80. Note that no infiltration of immune cells was observed before disease onset (day 6), but at day 12 and 16, a massive infiltration of CD4$^+$ T cells, F4/80 positive macrophages and coronin 1 positive cells was detected in wild-type mice (arrowheads). In contrast, no infiltration was visible in coronin 1-deficient mice on neither time point. Representative stainings are shown. Rectangles mark the regions of which day 12 inlets were taken. (Scale bar d6, d12 and d16 = 500µm, Scale bar d12 inlet = 100µm; n=5 per time point (day 6, 12, 16).

Figure 5. Active EAE analyzed following transfer of wild-type $CD4^+$ T cells into coronin 1-deficient mice. Twenty million MACS-sorted $CD4^+$ T cells isolated from wild-type mice were transferred i.v. one day prior immunization with MOG_{35-55} peptide. Wild-type and coronin 1-deficient mice injected with PBS served as controls. The clinical score over time is depicted for each individual mouse (n = 7 or 8).

Figure 6. Importance of regulatory T cells in wild-type and coronin 1-deficient mice with and without EAE induction. The frequency and suppressive activity of regulatory T cells (Tregs) was analyzed *ex vivo* as well as *in vivo*. A, The frequency and number of CD25 and Foxp3-expressing $CD4^+$ splenocytes was analyzed by flow cytometry. Representative FACS dot plots gated for CD4 are shown. Each symbol represents data from one mouse (n = 8 or 9 / 2 independent experiments). B, The suppressive capacity of $CD25^+CD4^+$ T cells from coronin 1-deficient and wild-type mice was analyzed with a *in vitro* suppression assay. $CD25^-CD4^+$ responders were isolated from coronin 1-deficient and wild-type mice. The ratio of responders to Tregs is depicted. Results are expressed as the mean ± SD. [^{3}H]-thymidine incorporation from triplicate cultures. One out of two experiments is shown. C, $CD25^+$ cells were depleted *in vivo* by i.p. administration of anti-CD25 antibody (PC61) three and five days prior immunization with MOG_{35-55} peptide. The control groups were immunized with MOG_{35-55} peptide but did not receive antibodies. Mean clinical score and mean body weight ± S.E.M., maximal clinical disease score and the day of disease onset for individual mice are displayed together with the mean and the EAE incidence. (n = 7 or 6, for non-PC61-treated wt); ns, not significant

Figure 1



Average disease incidence		
wt	33 / 33	100%
coro1-/-	13 / 32	41%

Figure 2

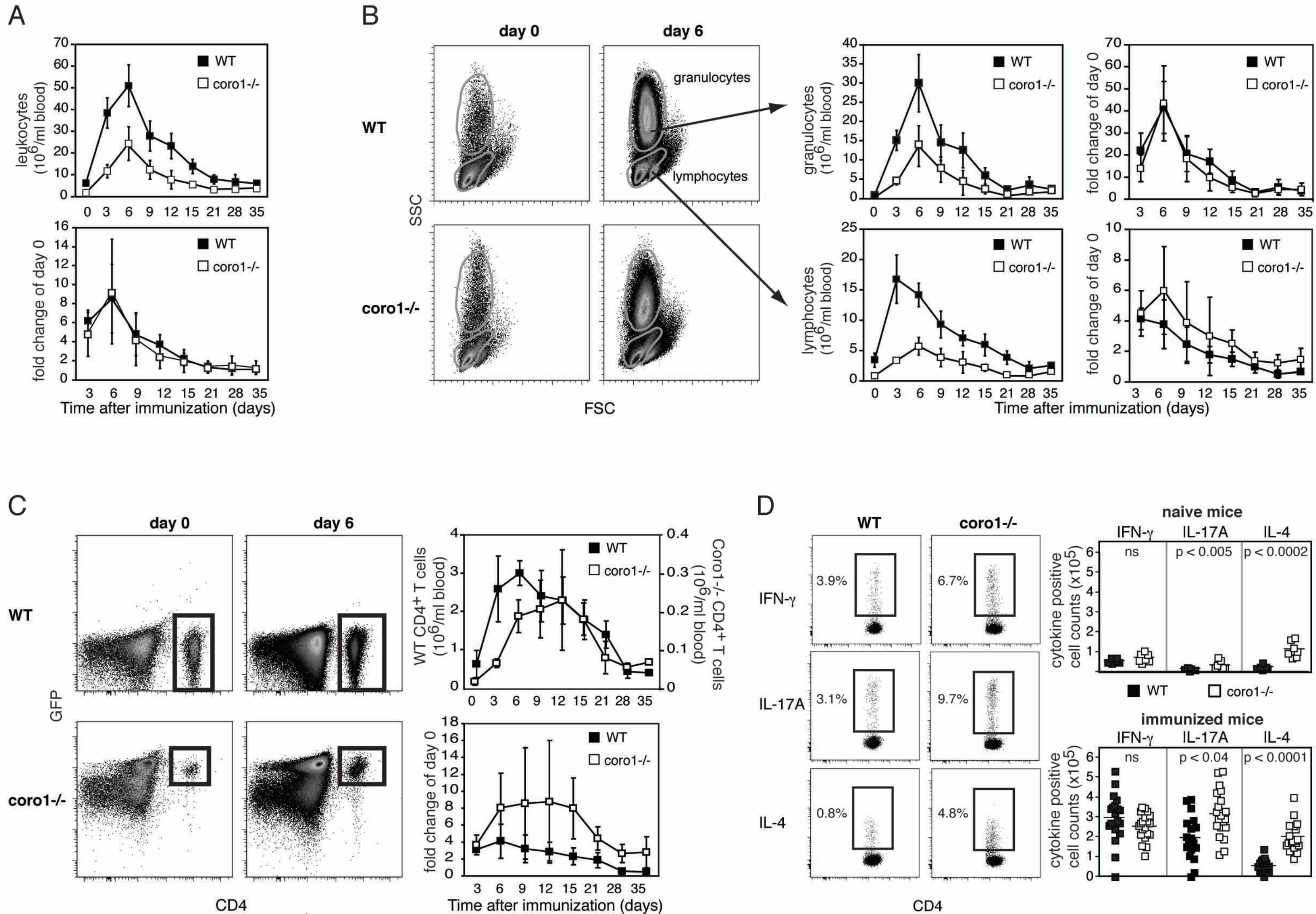


Figure 3

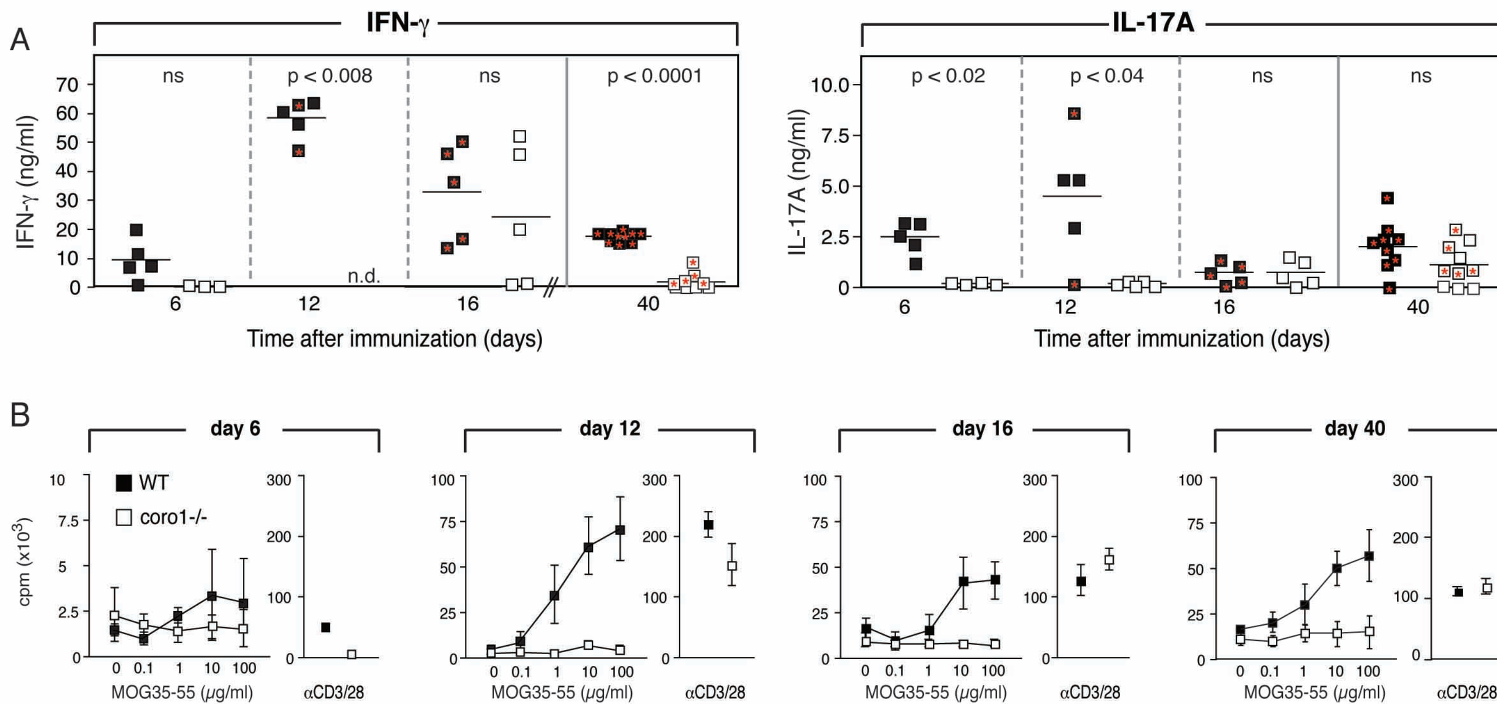


Figure 4

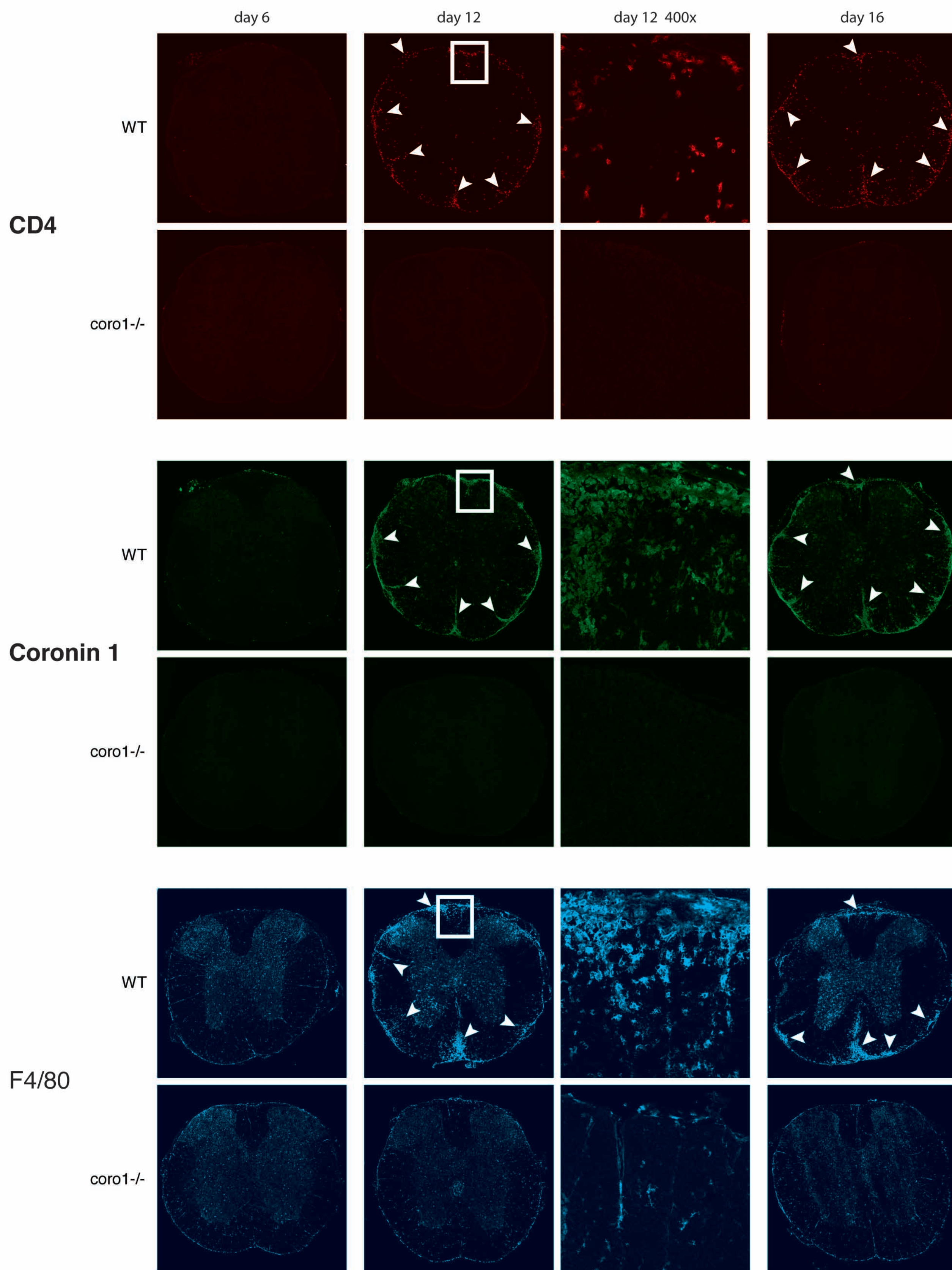


Figure 5

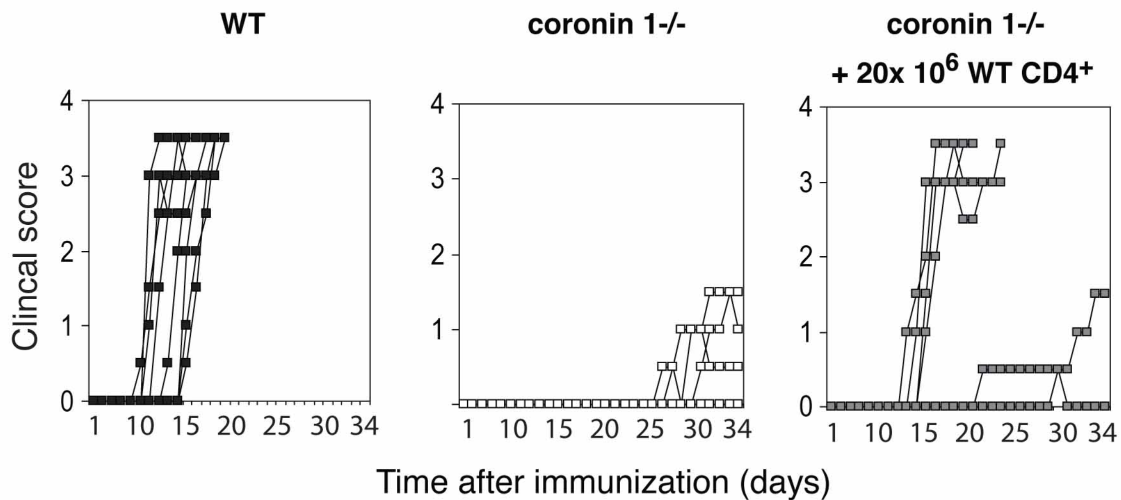


Figure 6