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Early exposure of NOD/ShiLtJ mice to Freund's adjuvant prompts delayed, spontaneous progressive encephalomyelitis

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ABSTRACT

Drugs able to efficiently counteract primary progressive MS (PP-MS) remain an unmet need. The availability of reliable animal models of PP-MS might boost the identification of treatments capable of counteracting disease evolution. Recently, we characterized primary progressive EAE (PP-EAE) in NOD/ShiLtJ mice, showing that it recapitulates several key features of PPMS. However, a fundamental difference between PPMS and PPEAE is that the latter is triggered by loss of tolerance deliberately induced *via* peripheral expansion of myelin-specific effector T cells (Teff). In the present study, we report that NOD/ShiLtJ mice challenged with complete Freund's adjuvant (CFA) to prevent diabetes onset, developed spontaneous PP-EAE (SPP-EAE). Specifically, we report that the sole CFA challenge induced encephalomyelitis with a similar pattern of that prompted by the complete immunization protocol including CFA, pertussis toxin and $MOG_{35–55}$. Mice with SPP-EAE show primary progressive disease evolution, widespread neurodegeneration, and insensitivity to dexamethasone-dependent immunosuppression. Remarkably, however, at variance with the rapid onset of PP-EAE, SPP-EAE manifested after a latency of approximately 4.5 months following CFA injection. This model may represent a valuable experimental tool to study mechanisms underlying spontaneous loss of self-tolerance toward CNS antigens and MS progression, as well as to identify therapies of relevance to treatment of PMS patients.

1. Introduction

In spite of the remarkable achievements obtained in treatment of patients with relapsing-remitting multiple sclerosis, drugs able to efficiently counteract primary progressive MS (PP-MS) remain an unmet need. It is largely acknowledged that the availability of reliable animal models of PP-MS might help the identification of treatments capable of counteracting disease evolution. In this regard, we recently contributed several studies showing that induction of experimental autoimmune encephalomyelitis (EAE) in non-obese diabetic (NOD)/ShiLtJ mice recapitulates various immunopathological and clinical aspect of PP-MS. We defined this progressive MS model as PP-EAE (Buonvicino et al., 2019; Pistolesi et al., 2025a; Buonvicino et al., 2024; Buonvicino et al., 2021a; Buonvicino et al., 2023; Buonvicino et al., 2021b; Pistolesi et al., 2025b; Buonvicino et al., 2020). PP-EAE mice develop a slow but progressive worsening of neurological impairment that inexorably leads to *exitus* within 3–6 months (Buonvicino et al., 2019). Consistently, upon immunization with complete Freund's adjuvant (CFA), pertussis toxin (PTX) and myelin oligodendrocyte glycoprotein (MOG)-$_{35–55}$, NOD/ShiLtJ mice progressively accumulate CD4 and CD8 immune infiltrates in the spinal cord with a caudo-cranial direction. Interestingly, soon after immunization and before development of neurological deficits, mitochondria of spinal cord columns of PP-EAE mice show morphological abnormalities and functional derangement (Buonvicino et al., 2023) that are followed by severe demyelination and neurodegeneration (Buonvicino et al., 2019). Remarkably, we found that various immunosuppressant drugs targeting adaptive and/or innate immunity efficiently counteract the neuroinflammatory response in PP-EAE mice, but do not reduce neurodegeneration or delay/stop disease progression (Pistolesi et al., 2025a; Buonvicino et al., 2024; Buonvicino et al., 2021a). Overall, data from our studies hint that both neurodegeneration and disease evolution in PP-EAE mice, although triggered by autoimmunization, become immune-independent and are sustained by autonomous neuronal/axonal demise. Accordingly, we found that drugs able

Abbreviations: NOD, non-obese diabetic; CFA, complete Freund's adjuvant; PTX, pertussis toxin; PPEAE, primary progressive experimental autoimmune encephalomyelitis; SPPEAE, spontaneous primary progressive experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein.

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to sustain mitochondrial bioenergetics, such as dexpramipexole and rapamycin reduce neurodegeneration as well as disease progression in PP-EAE mice (Buonvicino et al., 2024; Buonvicino et al., 2020).

Understanding the mechanisms leading from immunization to neurodegeneration in PP-EAE mice might be instrumental to decipher the pathogenesis of PMS. Further, the identification of the initial events responsible for the breakdown of tolerance in MOG-immunized NOD/ShiLtJ mice on the one hand could help understanding why immunosuppressants do not halt progression, and on the other may promote development of novel therapeutic strategies for PMS patients.

The NOD/ShiLtJ mouse strain is a well-established rodent model of insulin-dependent diabetes. These mice spontaneously develop insulitis between 3 and 4 weeks of age, leading to β-cell destruction and diabetes onset between 12 and 14 weeks of age. Interestingly, Sadelain and colleagues demonstrated that a single injection of CFA at the prediabetic stage (5 weeks of age) prevents development of hyperglycaemia and significantly extends lifespan of NOD/ShiLtJ mice (Sadelain et al., 1990). The mechanisms through which CFA prevents diabetes in NOD/ShiLtJ mice have been intensively investigated but are still unclear. CFA might suppress NK cells (Lee et al., 2004; Lee et al., 2008), induce T cell apoptosis (Qin et al., 2004), sustain β cell survival (Huszarik et al., 2010), and promote APC-dependent tolerance (Manirarora et al., 2008). Further, several studies suggest that CFA may also prevent diabetes in NOD/ShiLtJ mice by inducing or expanding regulatory lymphocytes (Tregs) (Shevach, 2000; Tian et al., 2009). In keeping with this, the T regulatory response is deficient in NOD/ShiLtJ mice (Salomon et al., 2000; Alard et al., 2006; Wu et al., 2002), probably because of dysfunctional IL-2 signalling (Tang et al., 2008).

In the present study, we report that encephalitogenic T cells spontaneously develop in NOD/ShiLtJ mice 4,5 months after a CFA challenge adopted to prevent diabetes development. We present here the first characterization of this spontaneous model of PP-EAE (SPP-EAE).

2. Methods

2.1. EAE induction and treatment

All animal care and experimental procedures were performed according to the European Community guidelines for animal care (European Communities Council Directive 2010/63/EU) and were approved by the Committee for Animal Care and Experimental Use of the University of Florence. Female NOD/ShiLtj (10 week old), NOD.scid (7 week old) and C57Bl/6 (10 week old) purchased from Charles River (Milan, Italy) were housed in a conventional unit (5–6 per cage) with free access to food (Harlan Global Diet 2018, Harlan Laboratories, Udine, Italy) and water, and maintained on a 12 h light/dark cycle at 21 °C room temperature.

EAE was induced in NOD/ShiLtj mice by subcutaneous immunization, with 250 μg of MOG35–55 peptide (synthesized by EspiKem Srl., University of Florence, Italy), emulsified in complete Freund's adjuvant (CFA) (Sigma, Milan, Italy) containing 4 mg/ml of *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI, USA) in the flanks and at the base of the tail. Immediately thereafter and 48 h later, mice received intraperitoneal injection (i.p.) of 200 ng pertussis toxin (PTX) (Sigma, Milan, Italy) in 100 μl Phosphate-Buffered Saline (PBS). Alternatively, 10-week-old NOD/ShiLtj or C47Bl/6 mice were challenged with CFA (Sigma, Milan, Italy) containing 4 mg/ml of *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI, USA) in the flanks and at the base of the tail. Clinical signs of EAE were examined daily by blinded operators. The following score were assigned: 0, normal; 0.25 or 0.5, splay reflex test (performed by lifting the mouse by its tail and observing the degree of hindlimb splay during 10 s. If both hindlimbs were splayed outward away from the abdomen, a 0 score was assigned. If one or both hindlimbs were partially retracted toward the abdomen without touching it, a score 0.25 or 0.5 was assigned, respectively); 1, weakness of the tail/hind limbs; 2, ataxia and/or difficulty in righting; 3, paralysis of the hind limbs and/or paresis of the forelimbs; 4, tetraplegia. Because of ethical reasons, score 4 mice were euthanized as soon as they reached the score. A disease course characterized by ongoing accumulation of neurological disability and neurodegeneration over time without distinct periods of remission was defined as progressive EAE. Blood glucose levels were measured throughout a glucometer from a drop of blood obtained by the tail. Mice were considered diabetic when plasma glucose was >300 mg/dl for 4 consecutive days. Dexamethasone (Sigma, Milan, Italy) was dissolved in PBS and administrated daily oral at 0.3 mg/kg from disease onset. Immunized vehicle-treated animals daily receive the same amount of PBS.

2.2. Adoptive transfer

Single-cell suspensions were prepared from spleen and lymph node of non-diabetic MOG-immunized NOD/ShiLtj mice at score 2 or score 4. Erythrocytes were removed with hemolytic Gey's buffer, and the nucleated cells were washed in HBSS supplemented with sodium bicarbonate (0.35 g/l), HEPES (2.2 g/l), penicillin (25 U/ml), and streptomycin (25 μg/ml) (Gibco, Grand Island, NY) and counted. Lymphocytes were re-challenged with $MOG_{35–55}$ (20 μg/ml) for 48 h and then 1×10^7 T-cells were injected intravenously into 7-wk-old female NOD.scid mice (100 μl/mouse) as reported (Lublin, 1985; Cravens et al., 2011). Where reported in the text, PTX (200 ng/100 μl) was injected intraperitoneally on the day of lymphocyte transfer and 48 h later.

2.2.1. Immunohistochemistry

For immunohistochemical analysis, spinal cords from NOD.scid mice, either untreated controls or sacrificed 2.5 months after diabetic onset following adoptive transfer, were collected; 10-μm thin sections were blocked with 5 % normal goat serum (Thermo Fisher Scientific, Waltham, MA, USA) containing 0.3 % Triton X-100 (Sigma, Milan, Italy). One hour later, sections were incubated overnight at 4 °C with a rabbit polyclonal anti ionized calcium-binding adapter molecule 1 (IBA1) (1:300, Wako Cat# 013–26,471) for microglia. After washings, sections were incubated with an anti-mouse secondary antibody Cy3-conjugated (1:1000, Jackson ImmunoResearch Labs Cat# 715–165-020). Images were acquired under a LEICA TCS SP5 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) with 20× objective. Quantification of immunofluorescence was performed using ImageJ by blinded operator. Values correspond to the mean of five different microscopic fields of four different mouse spinal cord sections.

2.3. Flow cytometry

Spinal cord infiltrating leukocytes were characterized by flow cytometry. Briefly, CFA/PTX/MOG- or CFA-immunized NOD/ShiLtJ mice were sacrificed at score 4 and perfused with cold saline prior to organ collection, then a single cell suspension for each sample was prepared (and stained with anti-CD45 VioGreen, anti-CD4 FITC, anti-CD8 PE and anti-C45R (B220) APC or isotype-matched control IgG antibodies (all from Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Dead cells were excluded based either on propidium iodide staining and on their forward and side scatter profiles. The cells were then analyzed by blinded operator using a FACSCanto II flow cytometer (BD Biosciences) equipped with FACSDiva software (BD Biosciences), acquiring a total of 10^5 events for each spinal cord extract (Buonvicino et al., 2019).

2.4. Antigen-dependent T-cell proliferation

Cells extracted from lymph nodes of score 4 MOG35–55- or CFA-immunized mice were cultured in complete RPMI in 96 wells plates (2×10^5 cells per well) and stimulated or not with MOG35–55 (20 μg/ml), MOG8–22 (20 μg/ml) PLP 139–151 (20 μg/ml), PLP 141–149 (20

μg/ml) (Cavone et al., 2015). After 72 h, the proliferative response was measured by [3H]thymidine incorporation test. [3H]Thymidine (1 Ci/well; PerkinElmer) was added and pulsed for the last 12 h. Plates were then harvested (Tomtec MacIII) on glass fibre filter (PerkinElmer), and thymidine uptake was measured by liquid scintillation in a MicroBeta 1450 Trimux counter (Wallac).

2.5. Histological analysis

Lumbosacral spinal cord from control (10-week-old), CFA/PTX/MOG- and CFA-immunized NOD/ShiLtJ mice at score 4 were collected, fixed in 4 % paraformaldehyde in 0.1 M PBS, embedded in paraffin and cut into 10 μm thin section. Axonal loss and myelin loss were assessed by Bielschowsky's silver staining and Luxol Fast Blue, respectively. Images were acquired by using an Olympus BX40 microscope (Olympus, Milan, Italy) and a digital camera (Olympus DP50) with NIS-Elements software; sections were analyzed by blinded operator by using ImageJ software (71 - Schneider CA).

2.6. Quantitative PCR

Total RNA from mice spinal cord was isolated using Trizol Reagent (Life Technologies). One μg of RNA was retrotranscribed using iScript (Bio-Rad, Milan, Italy). RealTime PCR was performed using Rotor-Gene 3000 (Qiagen, Milan,Italy) and the Rotor-Gene TM SYBR® Green PCR Kit (Qiagen, Milan,Italy), the reactions were run at 95 °C for 30 s, 95 °C for 5 s and 60 °C for15 s for 45 cycles (Buonvicino et al., 2021c). The following primers were used: T-bet: forward 5′- TGCCTACCAGAACGCAGAGATCACTC -3′ and reverse 5′- GTAGAAACGGCTGGGAACAGGATACTG −3′; RORγt: forward 5′- CCATTCAGTATGTGGTGGAGTTTGCCAA −3′ and reverse 5′-GCAGCC-CAAGGCTCGAAACAGCT -3′; GATA3: forward 5′-TGCCTGTGGGCTGTAC-TACAAGCTTCA-3′ and reverse 5′-GATGTGGCTCAGGGATGACATGTGTC-3′; IFNγ: forward 5′- TCAGGCCATCAGCAACAACATAAGCG −3′ and reverse 5′- TTCCGCTTCCTGAGGCTGGATTCC -3′; IL-17: forward 5'-TCAGCGTGTCCAAACACTGAGGCCAA-3′ and reverse 5'-GGCACT-GAGCTTCCCAGATCACAGA-3′; 18S: forward 5'-AAAACCAACCCGGT-GAGCTCCCTC-3′ and reverse 5'-CTCAGGCTCCCTCTCCGGAATCG-3′. Primers were purchased from Integrated DNA Technologies (Iowa, USA).

2.7. Statistical analysis

Data are expressed as mean ± SEM. Differences among groups were performed using ANOVA followed by Tukey's *W*-test or Student's *t*-test. In order to evaluate the difference in continuous parameters between the two groups, Mann–Whitney test (according to Kolmogorov–Smirnov test for normality and Bartlett's test for variance equality) is used. The differences in survival and disease onset between groups are tested using Kaplan–Meier curve and log-rank test. Differences were considered to be significant when P-value<0.05. Statistical analyses were carried out using GraphPad Prism (version 8).

3. Results

3.1. CFA unmasks delayed development of spontaneous, encephalitogenic lymphocytes in NOD/ShiLtJ mice

To investigate whether dysfunctional Treg-mediated suppression of encephalitogenic Teff cells contributes to PP-EAE pathogenesis, we embarked upon experiments aimed at developing a model of adoptive transfer PP-EAE in NOD.scid mice. Spleen and lymph node lymphocytes were isolated from PP-EAE NOD/ShiLtJ mice at intermediate (score 2) or advanced (score 4) stages of disease progression and re-challenged *in vitro* with $MOG_{35–55}$ before injection in NOD.scid mice (1×10^7 cells per mouse) (Fig. 1A). To our surprise, all recipient mice developed severe diabetes starting from day 20 after the transfer (Fig. 1B). Conversely, as reported (Buonvicino et al., 2019) donor mice never developed diabetes upon immunization (evaluated up to 12 months). Recipient, diabetic mice were monitored up to 2,5 months after diabetes onset but did not develop signs of encephalomyelitis. Although the role of PTX during immunization in EAE induction remains unclear, we attempted to promote transfer of PP-EAE by administering PTX to recipient mice the day of lymphocyte transfer and 48 h later. Again, recipient mice developed diabetes but did not shown signs of encephalomyelitis up to 2,5 months after lymphocyte transfer (Fig. 1C). Accordingly, we found that immune infiltrate markers (Fig. 1D), assessed *via* PCR to enhance sensitivity, as well as microglial activation (Fig. 1E and F), were comparable in the spinal cords of NOD.scid mice sacrificed 2.5 months after diabetes onset following adoptive transfer and in naïve animals. These findings demonstrated inefficacy of adoptive transfer of PP-EAE and also the presence of silent/suppressed diabetogenic clones in PP-EAE mice. This assumption led us to hypothesize that silent, encephalitogenic T cells are also present in naïve NOD/ShiLtJ mice throughout their lifespan. Addressing this question in naïve NOD/ShiLtJ mice is precluded by their spontaneous development of diabetes that, starting from 2,5 months, severely compromises the health status and does not allow prolonged monitoring (Sadelain et al., 1990). To circumvent this problem, naïve NOD/ShiLtJ mice were challenged with CFA at 10-week of age to prevent diabetes (Tian et al., 2009). Because we were unaware of the putative length of time after which CFA-challenged NOD mice might have developed signs of encephalomyelitis, animals were monitored up to 5 months of age. During this time frame, however, no evidence of neurological impairment was found. It is worth noting, however, that the thymus has a key role in the maintenance of Tregs homeostasis, and that it undergoes atrophy at 6 months of age, concomitantly with a reduction in the number of thymic emigrant Tregs (Manley et al., 2011; Owen et al., 2019). We reasoned, therefore, that a dysfunctional Treg response promoting development of encephalitogenic lymphocytes would be more likely to occur after this time point. Strikingly, we found that CFA-challenged mice develop a progressive encephalomyelitis at 7 months of age. We called this delayed, *autoimmunization-independent* EAE as spontaneous PP-EAE (SPP-EAE).

3.2. Characterization of SPP-EAE

We compared the neuro-immunopathological features of SPP-EAE to those of PP-EAE. To this end, 10-week-old female NOD/ShiLtJ mice were immunized with CFA or CFA + PTX + MOG and monitored over time (Fig. 2A). We found that while onset of PP-EAE occurred until 1 month post immunization, onset of SPP-EAE took place ~4.5 months after CFA, with median disease onset values of 30 and 133 days [HR = 5.180 (1.460 to 18.38), $P < 0.0001$), respectively (Fig. 2B).

Remarkably, we found that 100 % of CFA-challenged NOD/ShiLtJ mice developed SPP-EAE that progressed up to the maximal neurological impairment (score 4). It is also worth noting that, despite the delayed onset, SPP-EAE mice exhibited temporal kinetics of disease evolution (including pattern of progression and disease duration) and survival identical to animals with PP-EAE, with median survival values of 82 and 85 days [HR = 0.8114 (0.3297 to 1.997), $P = 0.617$), in PP-EAE and SPP-EAE, respectively (Fig. 2C and D). However, SPP-EAE mice did not develop diabetes throughout disease evolution (Fig. 2E). We also found that akin to PP-EAE, neurological impairment of SPP-EAE mice progressed in a caudo-cranial direction, concomitant with severe spinal cord demyelination and neurodegeneration (Fig. 2F and G). We also evaluated spinal cord infiltrates of $CD8^+$ and $CD4^+$ T cell, as well as $CD45R^+$ B lymphocytes in SPP-EAE mice. Flow cytometry analysis revealed profiles of immune infiltrates identical in SPP- and PP-EAE score 4 mice (Fig. 3A and B). We also analyzed the transcription levels of two cytokines, IL-17 and IFN-γ, which play an essential role in EAE pathogenesis, to evaluate whether, despite a similar number of CD4+ cells, these cells might differ in their functionality. Analysis of spinal cord infiltrates showed that the expression levels of these two cytokines were similar in SPP-EAE and PP-EAE mice (Fig. 3C). Interestingly, a key

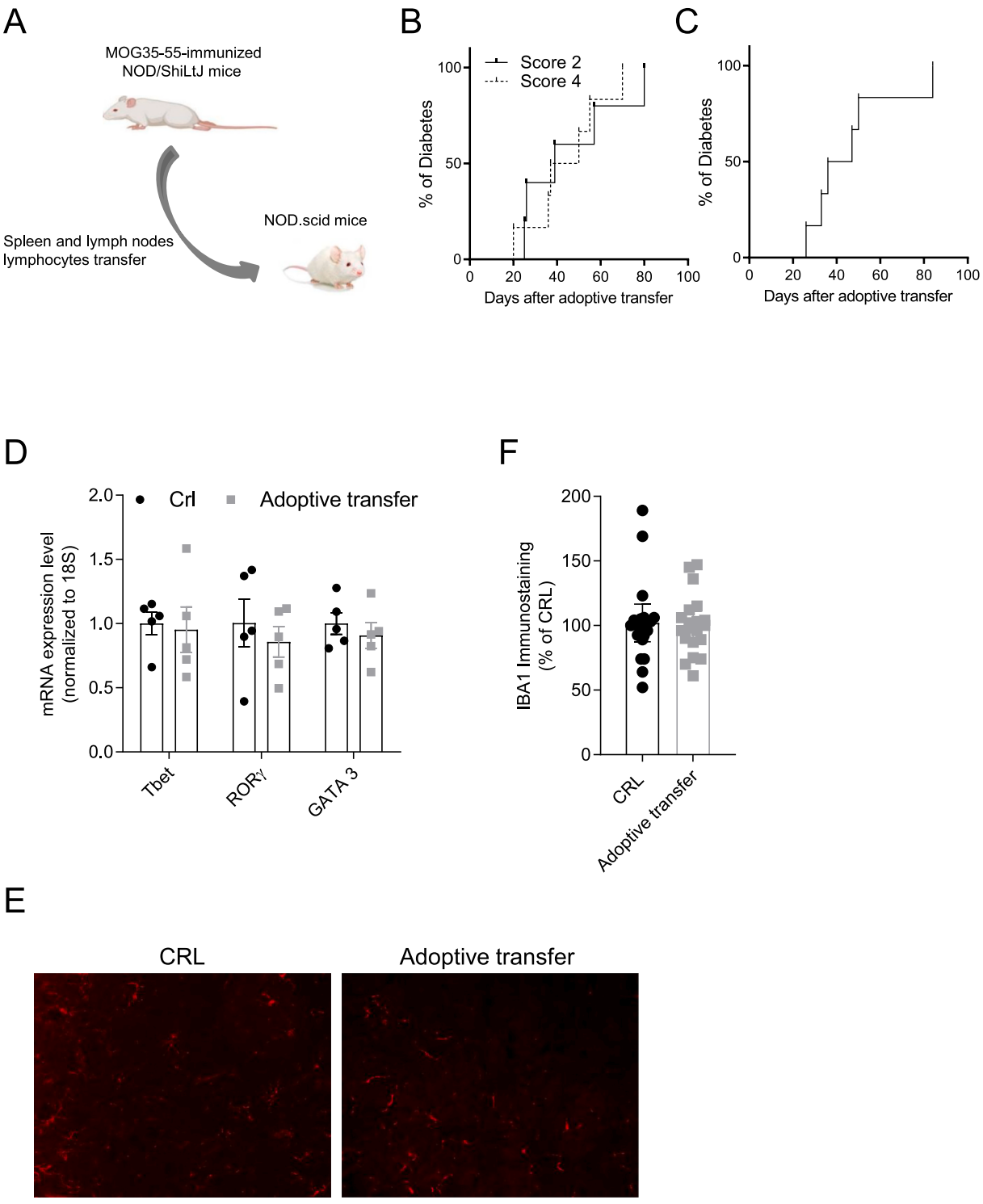

Fig. 1. Adoptive transfer of lymphocytes from non-diabetic PP-EAE NOD/ShiLtJ mice induces diabetes in NOD.scid mice. Experimental scheme reproducing the adoptive transfer of lymphocytes harvested from MOG_{35-55} immunized NOD/ShiLtJ mice in NOD.scid mice (A). Effects of adoptive transfer of 1×10^7 lymphocytes from score 2 or score 4 MOG-immunized NOD mice on diabetes onset in NOD.scid mice (B). Effects of 1×10^7 lymphocytes from score 4 MOG-immunized NOD/ShiLtJ mice plus PTX on diabetes onset in NOD.scid mice (C). Expression levels of T-bet$^+$ Th1, RORγt$^+$ Th17, and GATA3$^+$ Th2 cells in the spinal cord of NOD.scid sacrificed 2.5 months after diabetes onset following adoptive transfer compared to naïve animals (D). Representative confocal images (E) and quantification of immunostaining (F) of microglia (IBA1) in spinal cord sections of NOD.scid mice sacrificed 2.5 months after diabetes onset following adoptive transfer compared to naïve animals. In (B—D) at least five mice per group. In (F) each column is the mean ± SEM of four animals per group, five sections per animal. In (B) and (C) Kaplan-Meier curve analyzed by log-rank test. In (D) and (F) Student's *t*-test.

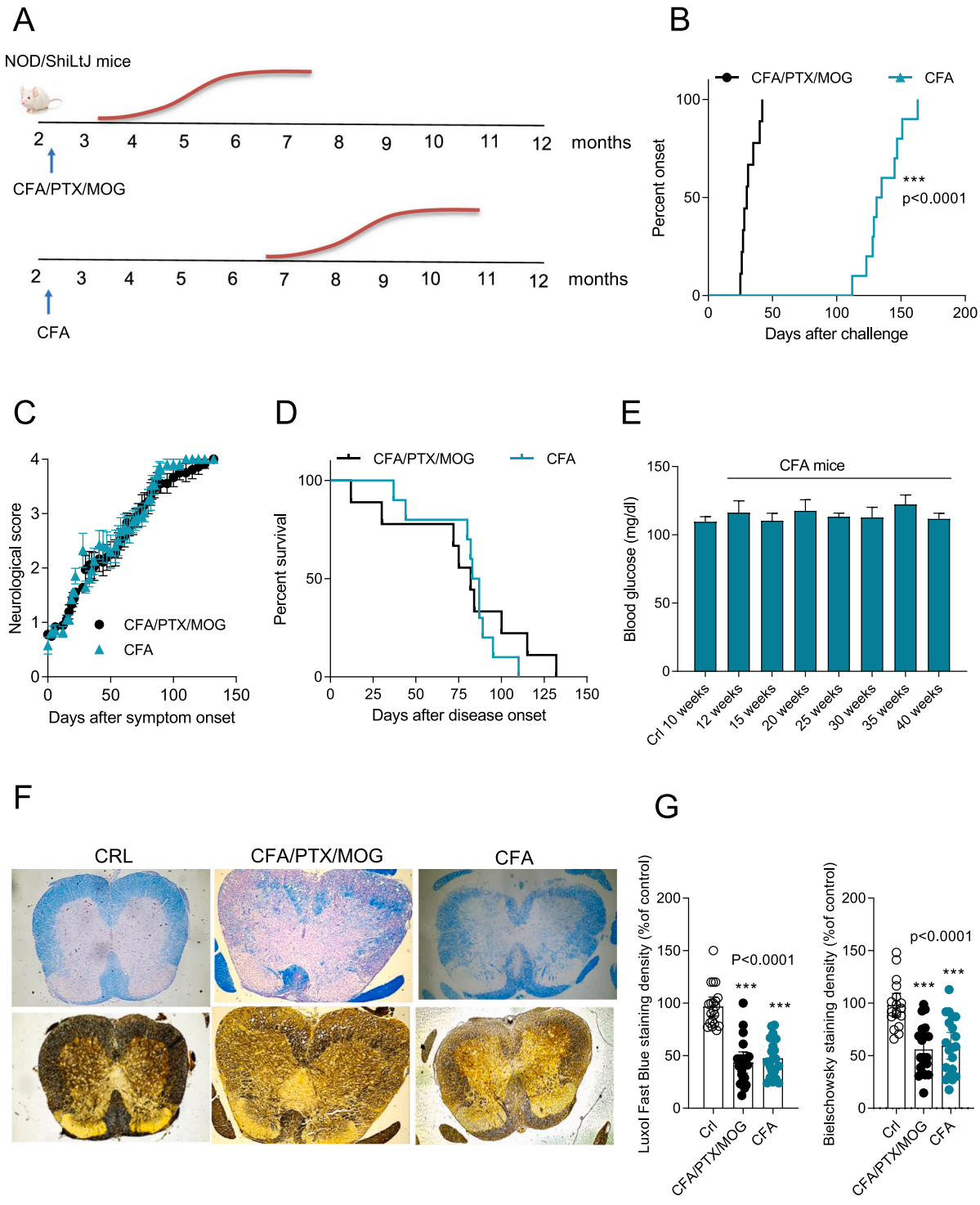

Fig. 2. Characterization of spontaneous EAE progression in NOD/ShiLtJ mice challenged with CFA. Temporal line of EAE progression in 10-week-old NOD/ShiLtJ mice challenged with CFA/PTX/MOG (upper) or CFA (below) (A). In (B) is shown the disease onset of mice in the experiment described in (A). Effects of CFA/PTX/MOG or CFA on neurological score (C), survival analysis (D) and blood glucose (E) in 10-week-old NOD/ShiLtJ mice. Representative (F) and quantification (G) of Luxol Fast Blue or Bielschowsky stained spinal cord sections of CFA/PTX/MOG or CFA challenged NOD/ShiLtJ mice at score 4. In (B—D) each point represents the mean ± SEM of 9 (CFA/PTX/MOG) and 10 (CFA) animals per group. In (E) each column is the mean ± SEM of 6 animals. In (G) each column is the mean ± SEM of four animals per group, five sections per animal. In (B) and (D) Kaplan-Meier curve analyzed by log-rank test. In (C) Mann–Whitney test. In (G) ANOVA plus Tukey's test. ***$p < 0.001$ *vs* CFA/PTX/MOG or Crl. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

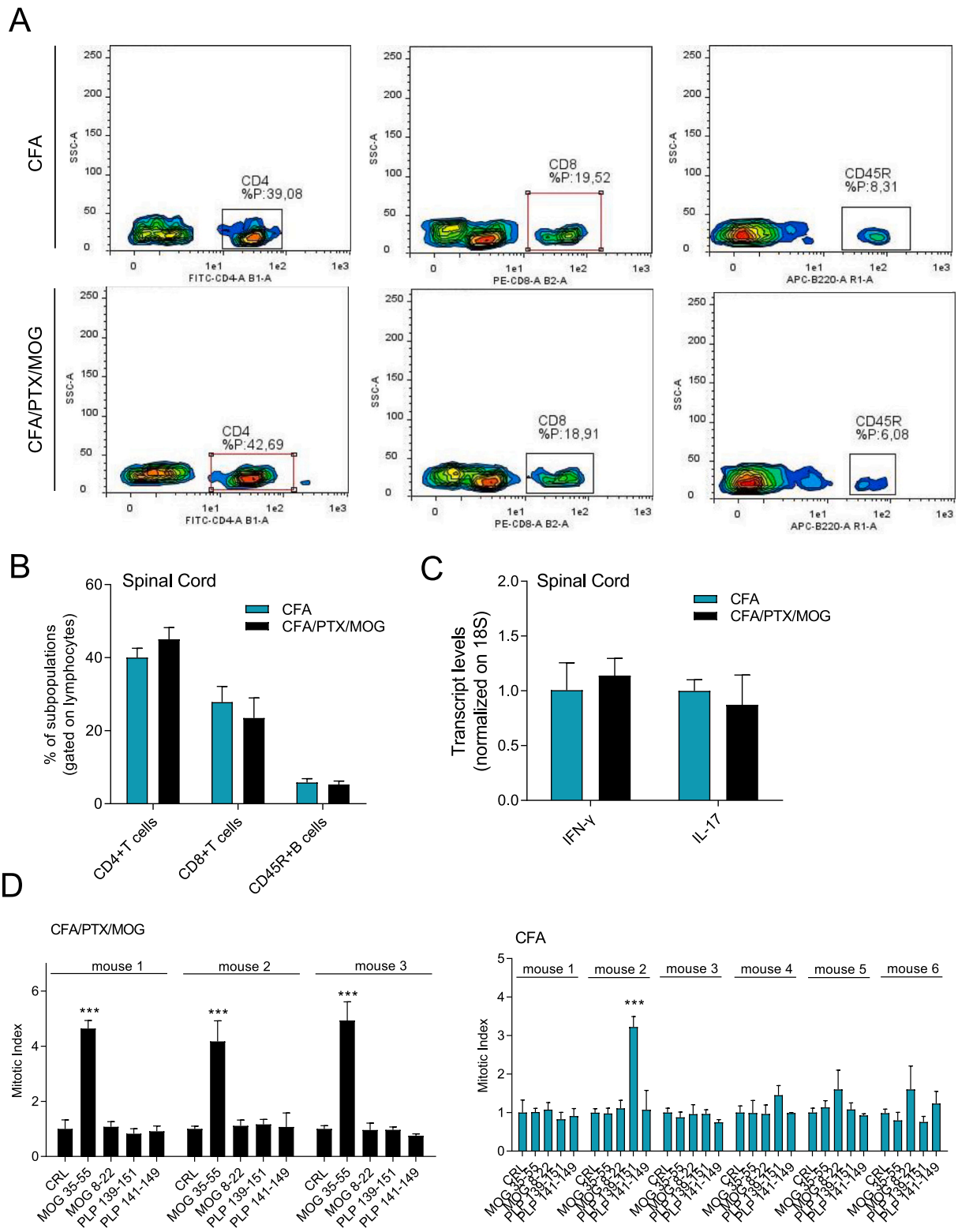

Fig. 3. Characterization of inflammatory infiltrates in CFA-challenged NOD/ShiLtJ mice. Representative plots (A) and quantification (B) of FACS analysis of CD8, CD4 and CD45R (B220) positive cells harvested from spinal cord of CFA/PTX/MOG or CFA challenged NOD/ShiLtJ mice at score 4. Spinal cord transcript levels of IL-17 and IFN-γ of CFA/PTX/MOG or CFA challenged NOD/ShiLtJ mice at score 4 (C). Lymphocytes from lymph nodes of score 4 CFA/PTX/MOG or CFA challenged NOD/ShiLtJ mice were challenged *in vitro* with $MOG_{35–55}$, $MOG_{8–22}$, $PLP_{139–151}$ and $PLP_{141–149}$ (each 20 μg/ml), and proliferation was evaluated (D). In (B—D) each column is the mean ± SEM of 4 animals per group. In (B and C) Student's *t*-test *vs* CFA was performed. In (D) ANOVA plus Tukey's test. ***$p < 0.001$ *vs* Crl.

distinction between the two experimental models occurred in the specificity of the encephalitogenic response. Indeed, splenocytes from score 4 SPP-EAE animals showed no specificity toward any of the myelin antigen tested ($MOG_{35–55}$, $MOG_{8–22}$, $PLP_{139–151}$, $PLP_{141–149}$), in contrast to splenocytes from PP-EAE mice which were specific for the $MOG_{35–55}$ epitope (Fig. 3D). Indeed, a single SPP-EAE mouse out of four tested had splenocytes specific for the $PLP_{139–151}$ antigen (Fig. 3D).

Given that NOD/ShiLtJ mice are prone to autoimmune diseases, we then asked whether S(PP)-EAE is restricted to this strain or also develops in C57Bl/6 mice, a mouse strain developing chronic EAE upon CFA/PTX/$MOG_{35–55}$ immunization. 10-week-old C57Bl/6 female mice were therefore challenged with CFA and monitored for one year. We found that none of the animals showed signs of neurological impairment. Accordingly, analysis of spinal cord shown the absence of demyelination and neurodegeneration in C57Bl/6 mice sacrificed one year after CFA challenge (Fig. 4A and B). In keeping with this data, by means of PCR evaluation adopted to improve sensitivity, we found that markers of immune infiltrates in the spinal cord of CFA treated mice were comparable to those of naïve animals (Fig. 4C).

3.3. *Effects of dexamethasone on CFA-induced autoimmune encephalomyelitis in NOD/ShiLtJ mice*

Several studies from our group show that PP-EAE evolution is insensitive to immunosuppressive therapies [1–4], thereby recapitulating a prototypical feature of PMS (Baldassari and Fox, 2018). On this basis, we therefore evaluated whether also SPP-EAE cannot be counteracted by steroid-dependent immunosuppression. We adopted a chronic treatment schedule consisting of daily oral doses of dexamethasone (0.3 mg/kg) starting at SPP-EAE onset, a dose regimen previously shown to ameliorate neurological impairment in various EAE models (Donia et al., 2010). We found that progression of neurological impairment of SPP-EAE mice receiving the steroid treatment was identical to that of SPP-EAE mice treated with vehicle (Fig. 5A). Remarkably, however, spinal cord immune infiltrates were almost halved in mice receiving a 30-day steroid treatment compared to those present in animals treated with vehicle (Fig. 5B).

4. Discussion

In the present study, we report that NOD/ShiLtJ mice in which spontaneous, early diabetes onset is prevented by CFA injection, develop

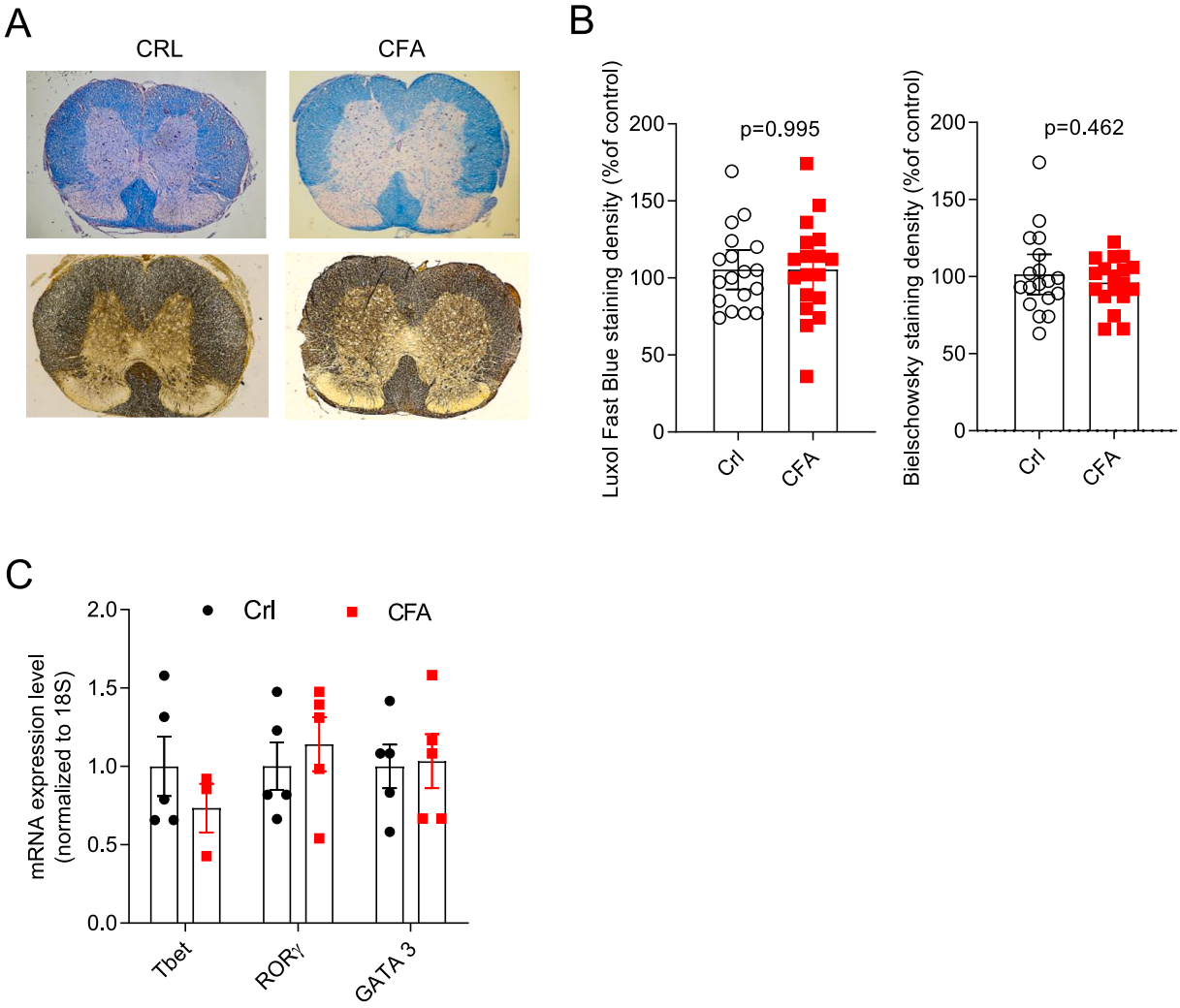

Fig. 4. Evaluation of neurodegeneration and inflammatory infiltrates in CFA-challenged C57Bl/6 mice. Representative images (A) and quantification (B) of Luxol Fast Blue and Bielschowsky-stained spinal cord sections from control or CFA-challenged C57BL/6 mice, analyzed one year post-challenge. Expression levels of T-bet$^+$ Th1, RORγt$^+$ Th17, and GATA3$^+$ Th2 cells in the spinal cord one year after CFA challenge in 10-week-old C57Bl/6 mice (C). In (B) each column is the mean ± SEM of three animals per group, six sections per animal. In (C) each column represents the mean ± SEM of five mice per group. In (B and C) Student's t-test *vs* Crl was performed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

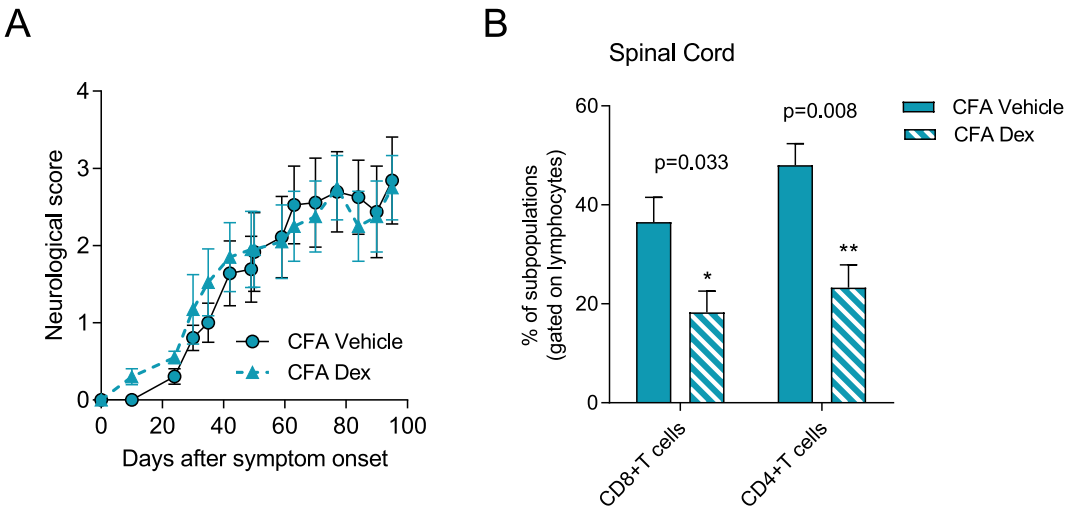

Fig. 5. Effects of dexamethasone on disease progression of CFA-challenged NOD/ShiLtJ mice. Effects of daily oral (0.3 mg/kg) dexamethasone (Dex) treatments starting from disease onset on disease evolution of CFA-EAE mice (A). Quantification by FACS analysis of CD8 and CD4 positive cells from spinal cord of vehicle- or dexamethasone (0.3 mg/kg/30 days) treated mice (B). In (A) each point represents the mean ± SEM of 10 (Vehicle) and 9 (DEX) animals per group. In (B) each column is the mean ± SEM of 4 animals per group. In (A) Mann–Whitney test, In (B) Student's t-test. *$p < 0.05$, **$p < 0.01$ *vs* Vehicle.

a delayed (~4,5 months) SPP-EAE. To the best of our knowledge, this is the first report of an animal model of spontaneous PMS.

Our findings confirm the inherent autoimmune susceptibility of the NOD/ShiLtJ mouse strain, demonstrating the spontaneous development of both diabetogenic and encephalitogenic autoreactive lymphocytes in naïve mice. In this regard, the lethality of diabetes in NOD/ShiLtJ mice precludes to study the occurrence of encephalomyelitis in the absence of a CFA challenge. Interestingly, although more than 150 interventions capable of preventing diabetes have been identified in this strain (Bresson and von Herrath, 2009; Shoda et al., 2005), there are no reports that these treatments prompt delayed occurrence of SPP-EAE. Conceivably, this is due to the fact that none of these studies monitored the mice up to 7 months of age. Apparently, a limited exception is the study by Salomon and colleagues reporting that selective disruption of CD28/B7–2 costimulatory pathway prevents diabetes in NOD/ShiLtJ mice and triggers peripheral autoimmune neuropathy with temporal kinetics similar to those of SPP-EAE (Salomon et al., 2000). However, the authors report that no central lesions occur in these mice that show demyelination restricted to the peripheral nervous system.

PP-EAE only occurs when mice are immunized with CFA-PTX-$MOG_{35–55}$ before onset of diabetes, so that the latter is prevented and encephalomyelitis starts within about 15 days and neurological impairment appears after two additional weeks. Notably, we found that both PP-EAE and SPP-EAE mice remain diabetes-free (monitored for at least 1 year). This suggests that CFA-PTX-$MOG_{35–55}$ or CFA alone redirects the autoimmune response from β cells to oligodendrocytes. However, our findings showing development of adoptive diabetes in NOD.scid mice receiving splenocytes from PP-EAE animals reveal that donors prevent diabetes by constant suppression of spontaneously occurring diabetogenic clones. The immunological events underling CFA-dependent suppression of diabetogenic clones are still unknown, as well as why the CFA challenge does not also suppress the encephalitogenic lymphocyte populations.

Interestingly, SPP-EAE shows a pattern of progression identical to that of PP-EAE. Even the kinetics of the caudo-cranial evolution of demyelination and neurodegeneration in the spinal cord appear identical in the two EAE forms. The two clear distinctions of SPP-EAE are delayed onset and lack of $MOG_{35–55}$-specific autoreactive clones. As for the 4,5-month delayed onset, we are currently unaware of the processes that lead from the CFA challenge to proliferation of autoreactive lymphocytes. In this regard, we emphasize that onset of SPP-EAE is relatively time restricted, with mice showing the first signs of neurological impairment from 112 to 163 days after the CFA-challenge (almost 2 months). We reason therefore, that if proliferation of encephalitogenic clones would be a stochastic event, then SPP-EAE onset would be randomly distributed in the post-CFA period of observation (almost 5 months). On the contrary, evidence of the above mentioned, relatively concomitant onset of SPP-EAE hints that specific, life-span related events need to occur to allow proliferation of encephalitogenic clones and attack to the CNS. In this light, and also considering the intrinsic defect of Tregs in the NOD/ShiLtJ strain, we hypothesize that SPP-EAE is triggered by a failure of regulatory activity toward encephalitogenic cells. Given that the thymus is required to maintain Treg homeostasis and their number in peripheral organs (Manley et al., 2011; Owen et al., 2019), we reason that thymus atrophy may represent a key event in SPP-EAE pathogenesis. In keeping with this interpretation, SPP-EAE onset occurs around 7 months of age, *i.e.* few weeks after thymic atrophy (6 months of age). As for the absence of $MOG_{35–55}$-specific clones in SPP-EAE mice, it is consistent with lack of myelin peptide-specific immunization in these animals. It also suggests that epitopes of the $MOG_{35–55}$ peptide are not spontaneously immunogenic in SPP-EAE NOD/ShiLtJ mice. Thus, further work is needed to identify the CNS antigens toward which the spontaneous autoimmune response is directed, as well as the possible occurrence of epitope spreading. It is worth noting that also the adaptive, autoimmune response occurring during SPP-EAE becomes irrelevant to disease progression. Indeed, dexamethasone treatment halved CD8- and CD4-lymphocyte infiltrates in the spinal cord of SPP-EAE mice, without affecting neurological impairment. Similar findings have been obtained in PP-EAE animals exposed to dexamethasone (Buonvicino et al., 2024), thereby strengthening our hypothesis that SPP-EAE and PP-EAE share several features of their neuroimmune pathogenesis. Given the involvement of mitochondrial derangement in the progression of PP-EAE (Buonvicino et al., 2023) and the therapeutic effects of mitochondrial boosting drugs such as dexpramipexole (Buonvicino et al., 2020) or rapamycin (Buonvicino et al., 2024), it will be mandatory in the next future to evaluate the effect of these drugs on the development of SPP-EAE as well.

Inducing and monitoring SPP-EAE is clearly time demanding (almost 10-month duration), being necessary ~4,5 months for its onset after CFA injection and additional 4–6 months for complete disease progression. We therefore wondered whether a spontaneous form of EAE could be obtained in the C57Bl/6 mouse, typically adopted to induce a chronic form of EAE. However, we did not find signs of neurological impairment or of immune infiltration in C57Bl/6 mice up to 12 months from the CFA

challenge. This corroborates the hypothesis that the spontaneous development of EAE, as well as its progressive evolution, require specific immunopathological phenotypes such as those predisposing NOD/ShiLtJ mice to autoimmunity. In light of the present findings further studies should carefully evaluate the occurrence of autoimmune responses triggering dermatitis, thyroiditis as well as peripheral neuropathy in this mouse strain.

The role of the CFA challenge in SPP-EAE remains unclear. It might merely prevent diabetes, or it might also play a role in development of the encephalitogenic response. In this regard, we emphasize that the ~4,5 months from CFA injection to SPP-EAE onset tend to rule out an active role of the adjuvant in the delayed autoimmune attack to the mouse CNS. Prior work in diabetes, indeed, demonstrates that CFA prevents the autoimmune response by means of acute, short-acting mechanisms, losing any protective effect if administered 1–3 days *before* the autoimmune attack (Ulaeto et al., 1992). On this basis, evidence that CFA injection occurs 4,5 months *before* disease onset in our SPP-EAE model suggests that the adjuvant does not play an active role in the spontaneous occurrence of encephalitogenic clones. To shed light on the role of CFA in SPP-EAE, we are now evaluating whether treatments different from CFA (*i.e.* immune unrelated) but known to prevent or treat diabetes in NOD/ShiLtJ mice such as zymosan, BCG or insulin allow delayed development of encephalomyelitis.

Overall, we report the development of a spontaneous model of PP-EAE that closely reproduces the neuropathological and immunological features of the progressive encephalomyelitis driven by the prototypical induction of CNS autoimmunity *via* peripheral expansion of myelin-reactive T cells. As such, SPP-EAE may be harnessed to disclose those events responsible for MS progression and compounds of therapeutic relevance to PMS treatment.

CRediT authorship contribution statement

Giuseppe Ranieri: Methodology, Investigation, Formal analysis, Data curation. **Alberto Chiarugi:** Supervision, Project administration, Funding acquisition, Conceptualization. **Daniela Buonvicino:** Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

Authors declare no conflict of interest.

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Data availability

Data will be made available on request.

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