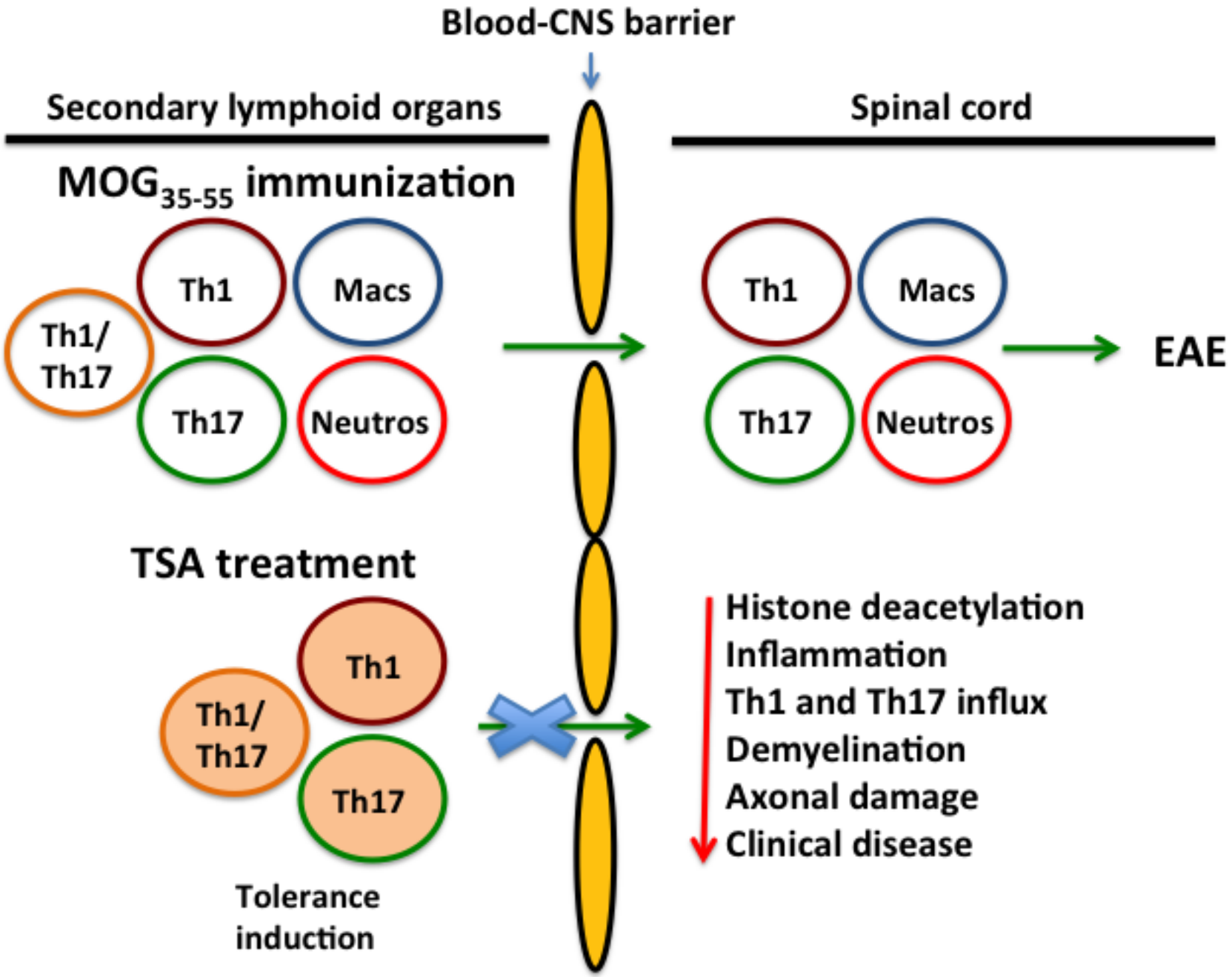
Graphical Abstract



Highlights

- Immunization of NOD mice with MOG_{35-55} peptide induces primary progressive EAE
- Trichostatin A treatment provides irreversible protection against EAE
- Drug treatment induces T cell tolerance and prevents histopathological manifestations
- Generation of T helper cells in secondary lymphoid organs is diminished by drug treatment
- Protection accompanies ablation of T lymphocyte trafficking to the spinal cord

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The epigenetic drug Trichostatin A ameliorates experimental autoimmune encephalomyelitis via T cell tolerance induction and impaired influx of T cells into the spinal cord

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Abbreviations:

CFSE, carboxyfluorescein succinimidyl ester; CNS, central nervous system; ConA, Concanavalin A; DMSO, dimethyl sulfoxide; EAE, experimental autoimmune encephalomyelitis; IFN, interferon; GM-CSF, granulocyte macrophage-colony stimulating factor; HDAC, histone deacetylase; IL-17A, interleukin 17A; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NOD, non-obese diabetic; PMA, phorbol myristic acetate; PP-EAE, primary progressive EAE; TCR, T cell receptor; Th, T helper; Treg, T regulatory; TSA, Trichostatin A

Abstract

Multiple sclerosis is a T cell mediated chronic demyelinating disease of the central nervous system. Although currently available therapies reduce relapses, they do not facilitate tolerization of myelin antigen-specific T lymphocytes to ensure prolonged protection against multiple sclerosis. Here, we show that treatment of NOD mice with the histone deacetylase inhibitor, Trichostatin A affords robust protection against myelin peptide induced experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis. Protection was accompanied by histone hyperacetylation, and reduced inflammation and axonal damage in the spinal cord. Drug treatment diminished the generation of $CD4^+$ memory T cells and induced tolerance in $CD4^+$ T cells recognizing the immunizing myelin peptide. During the early immunization period, $CD4^+$ T cells producing GM-CSF + IFN-γ, GM-CSF + IL-17A, as well as those expressing both IL-17A + IFN-γ (double-producers) were detected in the secondary lymphoid organs followed by the appearance of cells producing IFN-γ and GM-CSF. On the other hand, IFN-γ producing Th1 cells appear first in the spinal cord followed by cells producing IL-17A and GM-CSF. Treatment with Trichostatin A substantially reduced the frequencies of all T cells secreting various lymphokines both in the periphery and in the spinal cord. These data indicate that epigenetic modifications induced by histone hyperacetylation facilitates T cell tolerance induction in the periphery leading to reduced migration of T cells to the spinal cord and mitigation of neuronal damage and improved clinical outcome. These results suggest that epigenetic modulation of the genome may similarly offer benefits to multiple sclerosis patients via abrogating the function of encephalitogenic T lymphocytes without exerting severe side effects associated with currently used disease-modifying therapies.

Key words:

Central nervous system; Experimental autoimmune encephalomyelitis; Histone deacetylase inhibitor; Myelin oligodendrocyte glycoprotein; Neuroinflammation; T helper cells; Trichostatin A

Footnote:

[1]Jayaraman *et al.* 2017. Epigenetic drug selectively targets neutrophils during amelioration of chronic neurodegeneration**.** Manuscript under consideration of publication.

1. Introduction

Multiple Sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the central nervous system (CNS) [1-2]. Several disease-modifying therapies reduce relapses in MS patients but are associated with significant side effects including fatalities [3-4]. Although anti-virals such as interferon (IFN)-β-1a and 1b are well tolerated, they are inefficient in affecting Th17 cells [5], which are found abundantly in the blood and cerebrospinal fluid of MS patients during clinical exacerbations [6]. Natalizumab, a humanized mAb against widely expressed α4 integrin, a part of the VCAM-1 cognate ligand, and Dimethyl fumarate are associated with progressive, fatal multifocal leukoencephalopathy in John Cunningham virus seropositive patients [2-4]. Fingolimod that targets sphingosine -1-phosphate receptor and blocks T cell transmigration into the CNS results in cardiac complications, reactivation of varicella zoster and herpes simplex virus, and exacerbation of MS and experimental autoimmune encephalomyelitis (EAE), the mouse model of MS [7]. Other severe adverse side effects include leukemia and cardiovascular contradictions (Fingolimod), teratogenic risk (Teriflunomide), and secondary autoimmune diseases [2-4]. Although these non-specific immunomodulatory therapies target activation of immune cells in the periphery and their entry into the CNS, they hardly impinge upon the function of encepahalitogenic T lymphocytes or resident inflammatory component in the CNS. Thus, therapies that can induce antigen-specific tolerance in encephalitogenic T lymphocytes are an unmet need for MS treatment.

Many clinical trials had been conducted in attempts to induce T cell tolerance in MS patients [8-9]. These include administration of synthetic peptides corresponding to T cell epitopes mapped within myelin components such as myelin basic protein, proteolipid proteins, and myelin oligodendrocyte glycoprotein (MOG). Bovine myelin, a bacterial plasmid encoding

whole human myelin basic protein and altered ligand peptide, a peptide derivative of myelin basic protein that was modified at the T cell receptor (TCR) contact region were also used for tolerance induction. Other strategies included the TCR vaccination constituting attenuated autologous antigen-specific T cells or peptides from the complementarity determining region 2 or 3 of myelin reactive T cells and autologous peripheral blood mononuclear cells chemically coupled with myelin peptides. None of these maneuvers induced T cell tolerance as assessed by the ability of peripheral blood T cells to proliferate and produce IFN-γ in response to a challenge with the corresponding immunizing peptide *in vitro*. Importantly, they also did not improve the clinical outcome in MS patients. Although transient $CD4^+$ T cell deletion in conjunction with the administration of antigen (spinal cord homogenate) coupled splenocytes late during EAE in Biozzi ABH mice suppressed relapses, they failed to prevent the long-term neurodegeneration caused by axonal damage [10-11]. Thus, an effective antigen-specific T cell tolerance-inducing strategy is required to protect against relapses and minimize long-term neurological deficits in EAE and in MS patients.

Although Genome Wide Association Studies have implicated genes encoding human leukocyte antigens in MS pathogenesis [12], environmental factors including Epstein-Barr virus infection, smoking, and Vitamin D deficiency may have substantial influence by indirectly altering gene expression without changing the DNA sequence, termed epigenetics [13]. However, the field of epigenetics of MS is in its infancy and remains to be fully elucidated [14]. A clear understanding of these mechanisms will pave the way for better manipulation of MS without causing undesirable side effects induced by currently used disease modifying agents. Accumulating evidence indicates that the histone deacetylase (HDAC) inhibitors such as Trichostatin A (TSA), originally developed for cancer treatment [15], and other similar drugs,

Vorinostat and Valproic acid can ameliorate monophasic, self-limiting EAE in C57BL/6 mice [16-18]. This model is frequently used to test the efficacy of various disease manipulations because of the availability of genetically modified strains on the C57BL background. However, the pathology of monophasic EAE studied in C57BL/6 mice does not parallel that of an MS form [19]. Since each variant of EAE recapitulates some but not all features of MS, it is critical to ascertain the efficacy of HDAC inhibitors in a preclinical model that closely resembles MS. Immunization of autoimmune prone female non-obese diabetic (NOD) mice with the immunodominant MOG_{35-55} peptide consistently induces EAE with a high frequency that shares unique features with MS including life long disease, prominent demyelination, axonal loss, and astrogliosis [19-22]. Thus, the NOD mouse model appears ideal for investigating the efficacy of HDAC inhibitor like TSA for MS treatment. In addition, we have recently shown that treatment of female NOD mice with TSA ameliorated spontaneously occurring autoimmune type 1 diabetes associated selective regulation of a set of proinflammatory genes [23-24]. Therefore, we envisaged that the epigenetic modulation of the genome could similarly modify the development of EAE induced by immunization with an immunodominant myelin antigen.

We demonstrate herein the utility of the HDAC inhibitor TSA to afford robust protection against primary progressive EAE (PP-EAE) in the autoimmune prone NOD strain of mouse. In addition to preventing inflammation, demyelination and axonal damage, TSA treatment also induced antigen-specific tolerance in both Th1 (T helper 1) and Th17 cells and thus ensuring irreversible inactivation of the pathogenicity of these functionally distinct subsets implicated in MS pathogenesis [1-2, 5-6]. Thus, epigenetic modulation of the genome can prevent experimentally induced demyelinating disease in a preclinical model that closely resembles MS. These data suggest that similar epigenetic regulation using drugs such as TSA may alter the

epigenetic landscape leading to tolerance induction in myelin reactive T lymphocytes and reduction in accrual of disabilities in MS patients.

2. Materials and methods

2. 1. Animals

Six to eight wk old female NOD/ShiLtj mice purchased from the Jackson Laboratory (Bar Harbor, ME) were maintained under specific pathogen-free conditions with standard animal chow and water *ad libitum*. All procedures in studies involving animals were approved by the Office of Animal Care and Institutional Biosafety Committee of the University of Illinois at Chicago and were conducted in accordance with the ethical standards of the institution and the National Institutes of Health guide for the care and use of animals.

2. 2. EAE induction and assessment

Five mice per experimental group were randomly assigned. All mice in each experiment were littermates. Mice were injected s.c with 0.1 ml of phosphate buffered saline containing 100 µg of mouse MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK, Tocris Bioscience) and emulsified with an equal volume of complete Freund's adjuvant with an additional 4 mg/ml of *Mycobacterium tuberculosis* (H37Ra) (Fisher Scientific), as described [20]. Mice were injected i.v with 300 ng of reconstituted lyophilized Pertussis toxin (List Biological Laboratories) on the day of immunization and two days later. The stock solution of TSA (1 mg/ml in DMSO, Sigma) was diluted 20X in phosphate-buffered saline and injected s.c on the flank at a final dose of 500 µg/Kg body weight three times a week, as we described earlier [23-24]. Controls received 0.2 ml of 20X diluted DMSO in phosphate buffered saline. Body weight was recorded every other day and EAE score assigned as follows: 0, normal, 1, limp tail, 2, one hind limb weakness, 3, both hind limbs weakness, 4, one or both fore limb weakness, and 5, paralysis, moribund or death.

2. 3. Histological analysis

At specified time points, mice were anesthetized and perfused intracardiacally with 4% paraformaldehyde. The spinal cord was dissected out, fixed in 10% buffered formalin, embedded in paraffin and cut into 5 μM thin sections. Hematoxylin and eosin stained sections were observed for inflammation. Luxol fast blue staining was performed for assessing demyelination. Silver impregnation method was used for determining axonal integrity. Investigators unaware of treatment conditions examined the spinal cord sections from various regions of individual mice.

2. 4. Western blotting

Nuclear proteins from the spinal cord were isolated by acid extraction and separated on a 15% PAGE gel, blotted onto a PVDF membrane, incubated with rabbit antibodies generated against total histone H3 and acetyl-histone H3 (Upstate) as we described [23]. The fold-increase in acetylation of H3 protein was calculated using the ImageJ software as described [23].

2. 5. T cell proliferation

Pooled splenocytes and lymph node cells (10×10^6/ml) from the same experimental group were labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) for 5 min at 37^o C, diluted in RPMI media substituted with antibiotics and 10% fetal bovine serum, washed and cultured with 20 μg/ml of MOG_{35-55} peptide and/or 20 U/ml of recombinant human IL-2 (eBioscience) for varying periods as indicated in Results. Cells were labeled with anti-CD4 antibody conjugated with Alexa Flour 700 (clone RM4-5, eBioscience) and analyzed for CFSE fluorescence in $CD4^+$ cells on a BD Fortessa flow cytometer.

2. 6. Flow cytometry

A single cell suspension of spleens and draining lymph nodes was stained with anti-CD4 antibody conjugated with Alexa Flour 700 (clone RM4-5), anti-CD8-PE (clone 53-6.7), anti-

CD40-FITC (clone 14-0401), anti-CD44-FITC (clone IM7) or anti-CD62L-APC (clone MEL-14). All antibodies were obtained from eBioscience. Cells stained with anti-CD4-Alexa Fluor 700 were fixed, permeabilized and stained with anti-Foxp3-PE (clone MF23, BD Pharmingen). The spinal cords from mice that were intracardiacally perfused with phosphate-buffered saline were minced, digested with type I collagenase (500 µg/ml, Sigma) and DNase I (10 µg/ml, Sigma) for 30 min at room temperature and leukocytes were separated over a 30-70% percoll gradient (Sigma). For the analysis of intracellular cytokine expression, 5-10 x 10^6 cells were cultured in 1 ml of media containing PMA (phorbol myristic acetate, 100 ng), ionomycin (1µg) and brefeldin A (5 µg) for 4 hr. Reagents were purchased from Sigma. Cells were stained with anti-CD4-Alexa Flour 700, fixed, permeabilized, blocked with 10% goat serum and then stained with anti-IFN-γ-FITC (clone XMG1.2, eBioscience), anti-IL-17A-PE (clone eBio17B7, eBioscience) or anti-GM-CSF-PE (clone MP1-22E9, BioLegend), and analyzed on a flow cytometer. Data were further analyzed using FlowJo software (Treestar).

2. 7. ELISA

Spleens and draining lymph nodes were obtained at specific time points and isolated cells (10 x 10^6/ml) were cultured with 20 µg of MOG_{35-55} or 10 µg of Concanavalin A (Con A, Sigma) for 72 h. Supernatant was collected and kept at -20^{o}C until analysis. Concentrations of IFN-γ, IL-17A, IL-4, and GM-CSF (granulocyte macrophage colony stimulating factor) were determined in 0.1 ml of the supernatant in comparison to standards provided in ELISA Ready-SET Go kits (eBioscience).

2. 8. Statistics

Statistical significance in clinical scores between indicated groups was assessed using the Mann-Whitney U test. Two-way ANOVA analyzed statistical significance in T cell proliferation.

Flow cytometry and ELISA data were analyzed for statistical significance using two-tailed unpaired Student's t test. All statistical analyses were conducted using GraphPad Prism 6.0 software.

3. Results

3.1. Histone hyperacetylation is associated with amelioration of PP-EAE

Immunization of nine wk old prediabetic female NOD mice with MOG_{35-55} peptide resulted in robust and long-lasting EAE in 100% of animals (Fig. 1A). Whereas some studies indicated that immunization of NOD mice with MOG_{35-55} induced relapsing-remitting EAE, characterized by remissions between relapses [20, 22], a clear relapse was not noticed in another study [21]. Consistent with the latter study, we also noted prolonged and severe clinical disease with only mild remissions between the apparent attacks (Fig. 1A). Similar to previous studies in NOD mice [20-22], we recognized three phases of the disease: the acute disease (first attack between 15-20 days; clinical score of 2.8-3.2), the second attack around 44-53 days (clinical score of 3.0-3.2) followed by the protracted disease between 82 and 115 days (~3.0 clinical score). The sustained disease course without pronounced remissions is reminiscent of primary, progressive MS, which lacks clear remission [1-2] and therefore we refer to this form of EAE as primary progressive EAE (PP-EAE). Neither the course nor tempo of PP-EAE was influenced by the administration of the vehicle, diluted DMSO in comparison to untreated, immunized mice (Fig. 1A). Treatment with TSA dissolved in DMSO on alternate days for 8 wk following our previously reported protocol that attenuated spontaneously occurring autoimmune diabetes in female NOD mice [23-24] substantially diminished the clinical severity (1.5-1.8 score) during all three phases of the disease. Of note is the stability of protection seen even after 9 wk of drug cessation (Fig. 1A). Whereas in controls the incidence of EAE (clinical score: 2 and above)

reached 100% between 14 and 16 days, 80% disease incidence was attained only after 35 days of TSA treatment (Supplementary Fig. 1). As noted earlier [20], a small but noticeable loss of body weight preceded the disease onset, which was not influenced by TSA treatment (Supplementary Fig. 2). Further, TSA administration during the first two weeks of immunization was sufficient to curtail the progression of EAE (Fig. 1B). Interestingly, established EAE was also amenable to reversal by TSA treatment in NOD mice initiated after the onset of severe disease (score >2.0) on day 15 and continued up to 45 days (Fig. 1C). The clinical score was reduced as soon as 5 days after the first dose of TSA given therapeutically and protection lasted for at least 5 more wk subsequent to drug termination. Thus, administration on alternate days with a low dose of TSA (15x less than given daily from 4 to 40 days in C57BL/6 mice, Ref. 16) can exert comparable protection against PP-EAE when administered either prophylactically or therapeutically in autoimmune prone NOD mice.

We observed the association between histone H3 hyperacetylation in the spleen and pancreas and amelioration of type 1 diabetes by TSA treatment [23]. Immunoblotting unraveled hyperacetylation of the core histone H3 in the spinal cord of naïve NOD mice within 24 h of a single s.c injection of TSA (Fig. 1D). The augmenting effect of a single injection of TSA on histone acetylation lasted up to 6 days (data not shown). Thus, TSA-mediated hyperacetylation of histones observed in the peripheral lymphoid tissue earlier [23] and in the CNS reported herein could collaboratively contribute to the amelioration of PP-EAE in NOD mice.

3. 2. TSA treatment diminished the pathological manifestations in the CNS

Administration of NOD mice with the HDAC inhibitor TSA alleviated spontaneously occurring autoimmune type 1 diabetes characterized by the lack of inflammatory infiltration of the islets of Langerhans [23]. Interestingly, Vorinostat (SAHA, suberonylanilide hydroxamic

acid), a hydroxamate HDAC inhibitor similar to TSA could also ameliorate monophasic EAE accompanied by reduced inflammation of the white matter, demyelination and T cell influx into the spinal cord at the peak (day 15) of the disease in non-autoimmune C57BL/6 mice [17]. Thus, it was of interest to evaluate the effect of TSA on inflammatory responses during PP-EAE. Although we examined the spinal cord sections from cervical, thoracic and lumbar regions of five mice per group at time points indicated below, we focused on the lumbar region since the clinical disease in NOD mice is restricted to the tail and hind limbs with occasional involvement of forelimbs, as reported earlier [21]. At the end of the first attack (28 days), lumbar regions of immunized mice treated with DMSO (clinical score >2.0) exhibited abundant parenchymal invasion of inflammatory cells forming inflammatory cuffs around dilated blood vessels in the white matter, which penetrated into the grey matter (Fig. 2A). During the second attack or chronic phase (54 days), severe damage of the periphery was seen throughout the spinal cord with numerous vacuoles in the dorsal and dorso-lateral funiculus (Fig. 2C). Inflammatory cells were abundant in both the white and grey matter, and in the subarachnoid space of ventral funiculus.

Luxol fast blue staining of lumbar sections from DMSO treated mice displaying chronic disease (54 days) unraveled reduction in myelin content of the white matter (Fig. 2E). Bielschowsky's silver impregnation method revealed paucity of neurofilaments in both the white and grey matter of control mice (Fig. 2G), indicative of severe axonal loss during PP-EAE. Significantly, prophylactic treatment with TSA reduced the inflammation of the spinal cord both during the early (28 days, Fig. 2B) and chronic stage (54 days, Fig. 2D) and prevented demyelination and loss of axonal density during the chronic stage of the disease on day 54 (Fig. 2F, H), indicating drug induced inhibition of autoimmunity and neuronal deficit, typical of PP-

MS and secondary progressive MS [1-2]. Thus, TSA treatment reduced the clinical severity throughout the lifetime of the NOD mouse accompanied by diminished inflammation and reduced neuronal deficit. These observations have clinical relevance for the treatment of MS patients using drugs like TSA.

3.3. Immunomodulation in secondary lymphoid organs by TSA treatment

The immunomodulatory effects of TSA on splenocytes and draining lymph node cells were then determined by flow cytometry. A transient increase in $CD4^+$ cells in MOG_{35-55} immunized mice was observed 28 days post-immunization, which was subsided by TSA treatment (Fig. 3A). Importantly, prophylactic treatment with TSA reduced the frequency of $CD4^+CD44^+$ activated/memory cells [25] after 14 and 28 days of immunization (Fig. 3B). However, the frequency of naïve $CD4^+CD62L^+$ cells [25] or $CD4^+CD40^+$ cells, a T regulatory (Treg) subset [26] was not influenced by TSA treatment (Fig. 3C-D). Similarly, the prototypical $CD4^+Foxp3^+$ Treg cells [27] remained unaltered during the chronic or late phase of PP-EAE **(**Fig. 3E-F), indicating the lack of a role for these cells in fine-tuning of neuroinflammatory responses. This is similar to the absence of modulation of $Foxp3^+$ cells in mice that were protected from EAE by Atorvastatin treatment [28]. A nonessential role for $Foxp3^+$ cells was revealed by the failure to ameliorate EAE despite an increase in their numbers and *Foxp3* transcription in the CNS [29-30]. Moreover, in MS patients, $Foxp3^+$ Treg cell number is not drastically altered [31]. Taken together, these data suggest that although $Foxp3^+$ Treg cells are necessary for the maintenance of self-tolerance [27], they play a less prominent role in regulating EAE/MS [29-31] and other mechanisms(s) may contribute to the control of neurodegeneration. We also found a significant depletion of granulocytes between 14 and 28 days postimmunization without affecting monocytes or dendritic cells[1]. Thus, the action of TSA on select adaptive and

innate immune cells rather than on the $Foxp3^+$ or $CD40^+$ Treg cells appears to be critical for the regulation of PP-EAE.

3.4. Induction of T cell tolerance by TSA treatment

Vorinostat (SAHA) suppressed the production of Th1- and Th17-polarizing cytokines by dendritic cells *in vitro* [17]. Although tolerization with antigen-coupled splenocytes in MOG_{8-21} immunized Biozzi ABH mice repressed T cell proliferation and IFN-γ production in response to an *in vitro* challenge with the immunizing peptide [32], it was not clear whether this represented nonspecific immunosuppression as observed in Atorvastatin treated mice [28] or induction of reversible form of tolerance, anergy [33]. To investigate whether TSA administration could induce non-specific suppression or antigen-specific tolerance, draining lymph node cells and splenocytes were restimulated *in vitro* with the immunizing peptide or the T cell mitogen, Concanavalin A (ConA) and the lymphokines released into the supernatant were quantified by ELISA. A previous report indicated the production of IFN-γ by splenocytes during the first attack (11-14 days) but not subsequently in MOG_{35-55} immunized female NOD mice [22]. However, we found sustained production of Th1-specific IFN-γ by peripheral lymphoid cells derived from DMSO treated, MOG_{35-55} immunized NOD mice during the acute (28 days) and chronic (54 days) stage of PP-EAE (Fig. 4A). Importantly, TSA treatment either during the induction (Fig. 4A) or effector phase (Fig. 4B) of the disease completely abolished antigen-induced IFN-γ production. Similarly, antigen-driven IL-17A production was also efficiently repressed by TSA treatment either at the induction (Fig. 4C) or effector phase (Fig. 4B). GM-CSF is crucial for EAE pathogenesis [34] and produced by Th1 cells [35]. Consistently, antigen activated lymphoid cells released GM-CSF during all stages of PP-EAE albeit at lower levels than IL-17A and IFN-γ (Fig. 4B, D). Expression of GM-CSF was reduced regardless of the stage

of the disease at which TSA was administered. Importantly, ConA activation of T lymphocytes obtained 54 days after immunization and treated with TSA revealed the production of comparable levels of IFN-γ, IL-17A and GM-CSF as that of lymphocytes derived from DMSO-treated mice (Fig. 5A, C-D). Thus, TSA treatment resulted in antigen-specific tolerance induction as indicated by the secretion of lymphokines upon polyclonal activation but not by TCR-mediated signaling. Hence, at day 28 time-point, activation of lymphocytes with ConA was omitted. Interestingly, only a small increment in IL-17A and IFN-γ production was observed when lymphocytes were stimulated with ConA on day 115 after immunization, (Fig. 4A and C), indicating the lower frequency of antigen-specific T cells late during the immune response. Although IL-4 was produced by T lymphocytes in response to a challenge with MOG_{35-55}, it was not repressed by TSA treatment (Fig. 4B), indicating the lack of epigenetic regulation of IL-4 under these conditions. Taken together, these data indicate that prophylactic or therapeutic treatment of NOD mice with TSA induced a prolonged unresponsiveness to the TCR-mediated signaling in MOG_{35-55}-specific T cells without affecting their ability to respond to polyclonal stimulation, referred to as T-cell anergy [33].

Engagement of the TCR (signal 1) in the absence of costimulation (signal 2) results in T-cell anergy, as assessed by T cell proliferation *in vitro*, which was partially reversed by the addition of exogenous IL-2 [33]. The T lymphocytes derived from TSA-treated mice failed to proliferate as indicated by the lack of CFSE dilution in response to stimulation with the immunizing peptide *in vitro* (Fig. 5A). Addition of exogenous IL-2 partially restored antigen induced T-cell proliferation in tolerant T cells (Fig. 5B), consistent with the early observation [33]. In data not shown, we observed that addition of IL-2 also restored the ability of tolerized T cells to produce IFN-γ and IL-17A. Activation with ConA induced higher T cell proliferation

than that induced by antigen in splenocytes irrespective of treatment. Our results indicate that administration of TSA during immunization with MOG_{35-55} involves induction of T-cell tolerance (anergy) leading to attenuation of PP-EAE.

3. 5. Impact of tolerance induction on T cell generation in the secondary lymphoid tissues

Although all $CD4^+$ T helper subsets regardless of their lymphokine profiles have been implicated in EAE, disagreement exists as to their relative roles. Some studies suggested that IFN-γ producing Th1 cells are crucial for EAE whereas IL-17A-expressing Th17 cells have a minor role [30, 36-37]. In contrast, Th17 cells [36] and double-producers (IFN-γ + IL-17A) [38] have been found to migrate to the CNS prior to the appearance of Th1 cells. Ex-Th17 cells (that lost IL-17A expression and acquired IFN-γ production) were found in the periphery but not investigated in the CNS of EAE mice [39]. Since the PP-EAE induced in NOD mice shares several similarities with human MS [19-22], it is interesting to evaluate the contribution of differing $CD4^+$ T helper subsets in this model and importantly their susceptibility to epigenetic regulation.

To test this postulate, splenocytes and draining lymph nodes were obtained from mice that were immunized with MOG_{35-55} and treated with DMSO or TSA. Cells were stimulated with PMA and ionomycin *in vitro* and analyzed by flow cytometry. Live cells were electronically gated and $CD4^+$ cells were further analyzed for the expression of intracellular lymphokines. Inasmuch as detectable level of T cell activation was observed between 14 and 28 days of immunization (see Fig. 3), we analyzed the status of Th1 and Th17 cells on day 21. Equal numbers of $CD4^+$ cells at the acute phase (21 days) produced both IL-17A and IFN-γ (double-producers) in DMSO-treated mice (Fig. 6A). $CD4^+$ cells that produced IFN-γ and IL-17A also co-expressed GM-CSF. Although the basal level of these cytokines was higher in lymphocytes

recovered from TSA treated mice, *in vitro* activation resulted in a small increment of cytokine expression, indicating the substantial repressive effect of the drug on putative Th1, Th17 and Th1/Th17 (double-producing) cells. In contrast to these findings during the early phase of EAE, cells producing IFN-γ (Th1) but not IL-17A (Th17) were prominent in secondary lymphoid tissues at the late stage of PP-EAE (Fig. 6B). Treatment with TSA did not repress the frequency of Th1, consistent with the increase in the message level of *Ifng* in TSA treated NOD mice [23]. Neither distinct Th17 cells nor double-producers were apparent in the peripheral lymphoid tissues at the late stage of the disease. Interestingly, a large proportion of $CD4^+$ cells produced GM-CSF and activation resulted in a small increment in GM-CSF expression. Although GM-CSF was expressed in large numbers of T cells late during PP-EAE, its expression was not apparently associated with Th1 or Th17 cells. In contrast to the observation early during PP-EAE, TSA treatment had minimal effect on the expression of GM-CSF at the later stage. Taken together, these data suggest the sequential appearance of double-producers and Th1 cells in the peripheral lymphoid compartment during the course of PP-EAE, similar to that observed in monophasic EAE in non-autoimmune C57BL/6 mice [38]. Importantly, our data indicate the susceptibility of Th17 and double-producers but not IFN-γ-expressing Th1 cells or GM-CSF alone producing cells to the repressive action of the epigenetic drug TSA in secondary lymphoid organs during the evolution of the disease (Fig. 6A).

3. 6. Modulation of T cell trafficking to the CNS by TSA treatment

We then analyzed whether TSA treatment could impact the infiltration of various T helper subsets into the CNS of PP-EAE mice, accounting for the protective effect of drug treatment. The lymphocytes recovered from the spinal cord were activated with PMA and ionomycin *in vitro*. Live cells were electronically gated and $CD4^+$ cells were analyzed for

intracellular expression of lymphokines by flow cytometry. Data shown in Fig. 7A indicate that early during PP-EAE (28 days), small numbers of IFN-γ-producing Th1 but not IL-17A expressing Th17 cells or double-producers were found in the CNS of DMSO treated PP-EAE mice. Expression of GM-CSF was not discernible in CNS invading lymphocytes at this stage. Similar data were also obtained on day 21 after immunization (not shown). These data are similar to the lack of IL-17A or GM-CSF in the CNS of mice during the initiation of naturally progressing EAE in mice that transgenically express a myelin basic protein specific TCR and human DR15 Ab^o [30]. In contrast to our model and the transgenic humanized mice that develop long-lasting disease [30], Murphy et al. [38] reported the sequential appearance of Th17, double-producers and Th1 cells in the spinal cord of monophasic, self-resolving EAE in C57BL/6 mice within 14 days of immunization. Although the reasons for this discrepancy are not clear, inherent differences between the monophasic [38] and chronic EAE [Ref. 30 and the current study] might account for this inconsistency. Treatment of mice with TSA treatment diminished the infiltration of Th1 cells in NOD mice. Interestingly, during the late phase of the disease (97 days), in addition to Th1 cells, Th17 cells as well as those producing GM-CSF appeared in the spinal cord (Fig. 7B). Again, TSA treatment reduced the frequency of the T cells infiltrating the CNS regardless of their lymphokine profiles. Thus, our data unravel for the first time that the expansion and homing of functionally distinct T helper subsets respectively in the peripheral lymphoid system and the CNS are under epigenetic control, which can be exploited to provide protection against the demyelinating disease.

4. Discussion

Although current disease modifying therapies reduce relapses, they do not eradicate the root cause of MS-encephalitogenic T lymphocytes [3-4]. They exert nonspecific immunosuppression and hence are associated with a variety of serious side effects including fatality. To circumvent these adversities, concerted efforts were made to induce antigen-specific T cell tolerance by administering whole or peptide derivatives of myelin proteins but they all failed to produce favorable clinical outcome [8-9]. Although a lipid-lowering drug has been shown to ameliorate EAE via exerting nonspecific immunosuppression, the possibility exists that it may lead to undesirable consequences in MS patients [28]. We demonstrate herein that prophylactic or therapeutic treatment with TSA can induce a stable and long lasting protection against clinical and pathologic manifestations including axonal loss in autoimmune prone NOD mice immunized with the immunodominant MOG_{35-55} peptide (Fig. 1, 2). The epigenetic modulation of the genome repressed the induction of $CD4^+CD44^+$ memory T cells (Fig. 3) and induced antigen-specific T cell tolerance, as indicated by diminished production of IFN-γ, IL-17A and GM-CSF when subsequently stimulated through the TCR without affecting their ability to respond to polyclonal activation (Fig. 4). Interestingly, immunization under the influence of TSA also abolished the ability of T cells to proliferate in response to TCR-mediated activation subsequently, which was partially recovered by the addition of exogenous IL-2 (Fig. 5). This is consistent with the induction of anergy, functional unresponsiveness to TCR-mediated signaling without physical deletion [33]. Of note is the diminished ability of T lymphocytes derived from mice immunized with MOG_{35-55} + DMSO to produce IL-17A and IFN-γ without affecting GM-CSF production when challenged with the antigen during the late phase (days 97 and 115) (Fig. 4). Polyclonal activation of these cells also resulted in minimal release of IL-17A and IFN-γ late

during the immune response. These results could be explained by exhaustion of antigen-specific T lymphocytes over time after a single injection of the antigen. Further work is needed to elucidate this phenomenon, which may provide significant insights into the immunobiology of encephalitogenic T cells in MS patients.

The unique feature of the system described herein is that the induction of a tolerance program in T cells specific to the immunizing antigen *in vivo* did not require reintroduction of encephalitogenic peptide(s) unlike other previously attempted maneuvers that provided only partial protection in MS patients [3, 8-9]. The failure of antigen-coupled splenocytes to induce complete tolerance administered late during the disease course could be attributed to the fact that they actually serve as an antigenic re-challenge, leading to exacerbation of long term neuronal deficits. Similar reasoning could explain the failure of myelin peptides and altered peptide ligands that enhanced myelin basic protein reactive Th1 responses and development of new inflammatory lesions in a subgroup of MS patients [40]. Our results underscore the utility of epigenetic modulation of the genome to ameliorate PP-EAE, similar to the reversal of type 1 diabetes in autoimmune prone NOD mice observed earlier [23-24] in the absence of additional manipulation.

Genetic studies unraveled the link between susceptibility to develop MS and the expression of genes encoding certain immune response related molecules including human leukocyte antigens, implying a role for T cell immune responses in disease induction [12]. However, the immune mechanisms involved in MS pathogenesis remain poorly understood [1-2]. Our data show that prophylactic treatment with TSA can repress antigen-activated memory T cell formation and induce anergy leading to repression of PP-EAE and hence have clinical relevance. The success of treatment of EAE at the induction phase with drugs including TSA is

related to the ability of the drug to quell the activation of a small number of antigen-specific T cells. On the other hand, effective therapeutic treatment of chronic autoimmune diseases including type 1 diabetes and MS requires repression of a large number of already 'activated' autoantigen responsive T lymphocytes. Treatment with TSA led to a substantial attrition of PP-EAE in NOD mice. In contrast, injection of splenocytes coupled with the whole spinal cord homogenate (encephalitogens) along with depleting anti-CD4 antibody reduced relapses without abrogating disease progression as indicated by continued deterioration in mobility and development of spasticity, axonal damage, astrogliosis, and neuronal loss late during the clinical course in Biozzi ABH mice [10-11]. Although we did not observe limb stiffness and axonal loss in NOD mice treated with TSA late during the disease course, its effect on astrogliosis and microgliosis remains undetermined. The reversal of PP-EAE by TSA administration after the onset of clinical disease (day 15) is a feature not addressed in previous studies using the HDAC inhibitors TSA, Vorinostat and Valproic acid in monophasic EAE [16-18]. Our unpublished data indicate that TSA administration could also reduce established EAE as TSA administration even after 45 days of immunization. Thus, TSA is capable of producing comparable beneficial effects on PP-EAE when administered either prophylactically or therapeutically. By extrapolation, it is possible that TSA can similarly inhibit the expansion of activated T cells responding to a plethora of myelin autoantigens and render them tolerant leading to mitigation of MS symptoms and accrual of disabilities. Many HDAC inhibitors have been in clinical use with high safety profile for a number of cancers [15], schizophrenia and epilepsy (Valproic acid) and are promising for correcting Frataxin deficiency in Friedreich ataxia [41], a genetic condition that affects the nervous system and causes movement problems. Our data obtained in an experimental

model that closely resembles MS [19-22] offer promise for the treatment of this neurodegenerative disease using the HDAC inhibitor TSA.

Whereas the Th1-specific IFN-γ [42], and IL-17A [43] and IL-22 [44] produced by Th17 cells do not directly contribute to EAE pathogenesis, GM-CSF expressed by both Th1 and Th17 cells is considered critical during the effector phase of monophasic EAE [34-35, 45-46]. Consistently, we also found GM-CSF co-expression in IFN-γ-producing Th1 and IL-17A-expresing Th17 cells in splenocytes and not in Th1 cells in the CNS during the early phase (21 to 28 days) of PP-EAE. In contrast, at the later stage (97 days) of the disease, secondary lymphoid organs expressed abundant GM-CSF apparently not associated with Th1 or Th17 cells whereas in the CNS, its expression was minimal. Interestingly, TSA treatment repressed the expression of GM-CSF in both the peripheral lymphoid compartment and the CNS invading lymphocytes throughout the course of PP-EAE. The minimal expression of GM-CSF in the CNS in comparison to peripheral lymphoid compartment early during PP-EAE suggests that a small amount of this cytokine may be sufficient to produce profound neuronal damage. Alternatively, it is possible that GM-CSF is not the only cytotoxic molecule involved in neurodegeneration and other cytokines collaborate to produce neuronal damage. Our unpublished data indicate a role for cytokines including iNOS and migration inhibitory factor, implicated in MS and EAE [47-48]. Importantly, all of these are amenable to regulation by TSA treatment in NOD mice. These data are consistent with a model in which various inflammatory cytokines play differential roles in the manifestation of EAE, which are amenable for epigenetic regulation.

Controversy exists as to the identity of T helper subsets involved in EAE/MS manifestation [36-39, 45-46, 49-52]. Purified IFN-γ producing Th1 cells have been shown to be crucial early during EAE whereas IL-17A-expressing Th17 cells participate at a later stage [30,

37]. On the contrary, Th17 cells and double-producers (IFN-γ + IL-17A) migrate to the CNS prior to the arrival of Th1 cells [38] or ex-Th17 cells (that lost IL-17A expression and acquired IFN-γ production [39] during the development of clinical disease. Others demonstrated that Th1 [50-52] or Th17 cells alone [45-46, 49-51], could mediate EAE. However, contamination of various levels of IFN-γ producing cells included in Th17 preparations raised questions about the singular ability of Th17 cells to induce EAE (discussed in references 37 and 38). Although there is no question about the importance of $CD4^+$ T lymphocytes in the manifestation of EAE, the relative roles of Th1, Th17 and Th1/Th7 double-producers in EAE remain contentious. Inasmuch as none of the characteristic cytokines associated with these T cell subsets is directly involved in EAE manifestation [42-44], it is difficult to associate encephalitogenicity with any particular T cell subset defined based on the expression of theses lymphokines alone. Moreover, the CD4+ T cells are highly plastic and are capable of transforming into other T cell subsets *in vivo*. Further investigation of the molecular nature of encephalitogenic T cells may provide important biomarkers for the identification of pathogenic T cells that cause EAE and MS.

In the PP-EAE model, we observed the predominance of double-producers in the peripheral lymphoid compartment but not in the CNS during the initial stage of the disease (Fig. 6 and 7). On the contrary, a previous report indicated the sequential appearance of Th17, double-producers in the spinal cord, which disappeared by day 14 [38]. Since we and others [30] have analyzed the CNS for infiltrating Th17 cells and double-producers after the putative disappearance of these cells by 14 days postimmunization, the presence of these cells in the spinal cord of chronic EAE cannot be ruled out. It was shown that the double-producers were abundant in the CNS and could mediate monophasic EAE in C57BL/6 mice independently of the Th1 cells [52]. Although we found abundant double-producers in the spleen and none in the CNS

at the time of analysis, it is possible that the double-producers may differentiate into Th1 cells and enter first into the CNS that can subsequently recruit Th17 cells to manifest PP-EAE. It is noteworthy that double-producers preferentially expand in the blood of MS patients during a relapse, and have a greater capacity to infiltrate the CNS and their prominence in postmortem MS brain tissues [53]. These results indicate a pivotal role for double-producers in EAE/MS [54] regardless of whether they represent an intermediary subset or a distinct cell type. The fact that Th1/Th17 cells are as susceptible (in the peripheral lymphoid organs) to epigenetic modulation as the Th1 and Th17 cells bolsters the utility of TSA to regulate various types of pathogenic T cells. This characteristic bodes well for the application of epigenetic drug treatment for MS in which Th1/Th17 cells appear to contribute to the disease pathogenesis [54].

5. Conclusions

Our results demonstrate that treatment of autoimmune prone NOD mice with the HDAC inhibitor TSA during immunization with an immunodominant myelin peptide can induce a tolerance program in encephalitogenic T lymphocytes leading to stable protection against the demyelinating disease. Drug mediated protection involves diminished trafficking of Th1 and Th17 cells into the CNS which may prove to be an effective therapeutic maneuver for MS. Taken together, the data generated in NOD mice that display symptoms similar to MS patients [19-22, 55] open new possibilities for MS treatment using epigenetic drugs like TSA that can modify T lymphocytes without exerting global immunosuppression or fatal side effects.

Author contributions

SJ conceived the project, designed and executed experiments, supervised others, analyzed the data, and wrote the paper; AJ performed animal experiments, performed histological

examination, and edited the manuscript; AS performed experiments; BP and MH supported the work and read the paper.

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Legends to figures

Fig. 1. Amelioration of PP-EAE by TSA administration is associated with histone hyperacetylation.

Female NOD mice were immunized with MOG_{35-55} and treated with nothing (open circle), DMSO (open squares) or TSA (filled circles) on alternate days from 0 to 45 (A), 0 to 15 (B) or 15 to 45 days (C). The clinical scores are depicted as mean +/- SEM. The numbers of animals tested are given in parentheses. Statistical significance of clinical scores between indicated groups was determined by Mann-Whitney U test using GraphPad Prism. Histone hyperacetylation following TSA injection into individual naïve NOD mice is shown (D) and its quantification on the right. Statistical significance between control and TSA-treated mice was calculated using two-tailed unpaired Student's t-test.

Fig. 2. Histopathological manifestations in the spinal cord are prevented by TSA treatment.

NOD mice were immunized with MOG_{35-55} and treated with DMSO or TSA on alternate days from day 0 to 15 (A, B) or from day 0 to 45 as in Fig. 1A (C-H). Spinal cords were harvested on day 28 (A-B) or on day 54 (C-H). The lumbar region was stained with H & E (A-D), Luxol fast blue (E-F) or Silver impregnation method (G-H). Original magnifications: 200X, A-B and 100X, C-H. Five mice per group were analyzed at each time point and representative data are presented.

Fig. 3. Effect of TSA treatment on peripheral T cells.

NOD mice immunized with MOG_{35-55} were treated on alternate days with DMSO (blank bars) or TSA (filled bars) starting on the day of immunization for 13 days and analyzed on days 14 and 28. Mice treated with DMSO/TSA for 45 days as in Fig. 1A were analyzed on day 54 or 115. Mice treated at the effector phase during days 15 and 45 were analyzed on day 97. The

frequencies of $CD4^+$ T cells (A), $CD4^+CD44^+$ cells (B), $CD4^+CD62L^+$ cells (C), $CD4^+CD40^+$ cells (D) and $CD4^+Foxp3^+$ cells (E) were determined at the indicated time points after immunization by flow cytometry. Representative dot blots of lymphoid cells obtained 54 days after immunization are shown (F). n= 5 per group. All data are shown as arithmatic mean +/- SE. *Indicates $P<0.05$ as determined between indicated groups using two-tailed unpaired Student's t-test. NS, not significant.

Fig. 4. Modulation of lymphokine production by peripheral T lymphocytes by TSA treatment.

Mice were immunized with MOG_{35-55} and treated during the induction phase from day 0 to 15 with DMSO or TSA and sacrificed on day 28 or treated between day 0 and 45 and sacrificed on day 54 or 115 (A, C, D). Mice were also immunized and treated at the effector phase with DMSO or TSA between 15 and 45 days and sacrificed on day 97 (B). Splenocytes and lymph node cells from individual mice (n=5/group) were stimulated with antigen or ConA and the supernatant was collected 3 days later and assayed using ELISA kits. Mean +/- SD is shown. *Indicates $P<0.05$ as assessed by two-tailed unpaired Student's t-test. All comparisons were made between media control and those stimulated with antigen or ConA as indicated. NS, not significant.

Fig. 5. Induction of T cell tolerance by TSA treatment.

Mice were immunized with MOG_{35-55} and treated with DMSO or TSA on alternate days starting from the day of immunization for 13 days and sacrificed on day 28 (A). Spleen and lymph node cells were stained with CFSE and cultured with antigen. After 8 days of culture, $CD4^+$ T cells were stained and analyzed for CFSE fluorescence by flow cytometry. Cultures were also stimulated with antigen or ConA and supplemented with or without recombinant human IL-2 and analyzed 6 days later (B). The numbers of dividing cells that were cultured in the presence of antigen with and without IL-2 were determined. Representative data from 3 independent experiments with 5 mice per

group are shown. *Indicates statistical significance of P<0.05 between indicated samples as assessed by two-tailed unpaired Student's t-test. Differences between other data points were not significant.

Fig. 6. Repression of cytokine-producing cells in secondary lymphoid organs by TSA treatment.

Mice were immunized with MOG_{35-55} and treated with DMSO/TSA from day 0 to 13 and splenocytes were analyzed on day 21 by flow cytometry for cytokine expression after culturing with or without PMA + ionomycin and brefeldin A for 4 hr. Cells were stained with anti-CD4 antibody, fixed, permeabilized and stained with anti-cytokine antibodies. Live cells were electronically gated based on forward and side scatter properties and $CD4^+$ cells were gated and analyzed for the intracellular expression of indicated cytokines. Representative contour plots from 5 mice per group are shown (A). Immunized mice were treated with DMSO/TSA between 15 to 45 days and analyzed on day 97 for cytokine expression in $CD4^+$ cells by flow cytometry (B). Mean +/-SE is shown. n=5 mice per group. *Statistically significant, P<0.05 as assessed by two-tailed unpaired Student's t-test between indicated samples. NS, not significant.

Fig. 7. Regulation of the influx of cytokine producing CD4+ cells into the spinal cord by drug treatment.

Mice were immunized with MOG_{35-55} and treated with DMSO/TSA on alternative days between day 0 and 13, sacrificed on day 21 or 28. Leukocytes were isolated from the spinal cord and activated with PMA + ionomycin and brefeldin A for 4 hr. Cells were stained with anti-CD4 antibody, fixed and stained with anti-cytokine antibodies. Live cells were electronically gated based on forward and side scatter properties and then $CD4^+$ cells were selected and analyzed for the intracellular expression of indicated cytokines. Representative cytograms from 5 mice per group are shown from two independent experiments (A). Mice were also immunized and treated with DMSO/TSA from day 15 to 45 on alternate days, sacrificed on day 97, and analyzed for cytokine

expression (B). Mean +/-SEM is shown. *Indicates P<0.05 between the indicated groups as assessed by two-tailed unpaired Student's t-test. NS, not significant.

Supplementary Fig. 1. Treatment with TSA delayed the onset of EAE.

Disease incidence was calculated from the data presented in Fig. 1A. The box and whisker plot with median (middle line), first quartile (lower line) and the third quartile (upper line) are shown to indicate the distribution of the data. Statistical significance was calculated by two-way ANOVA.

Supplementary Fig. 2. Changes in body weight regardless of treatment.

Body weight was recorded for mice shown in Fig. 1A.

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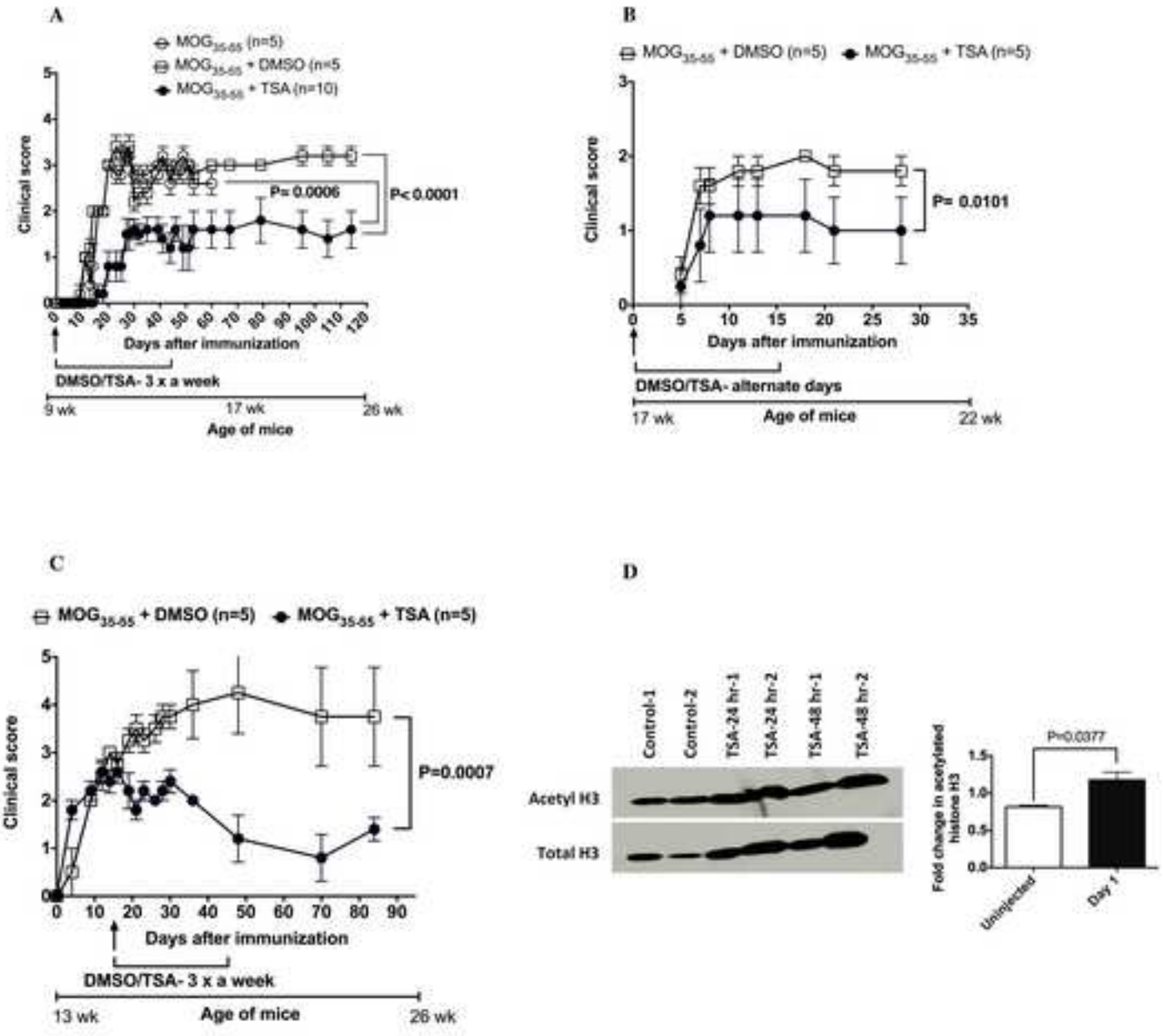

Fig. 1

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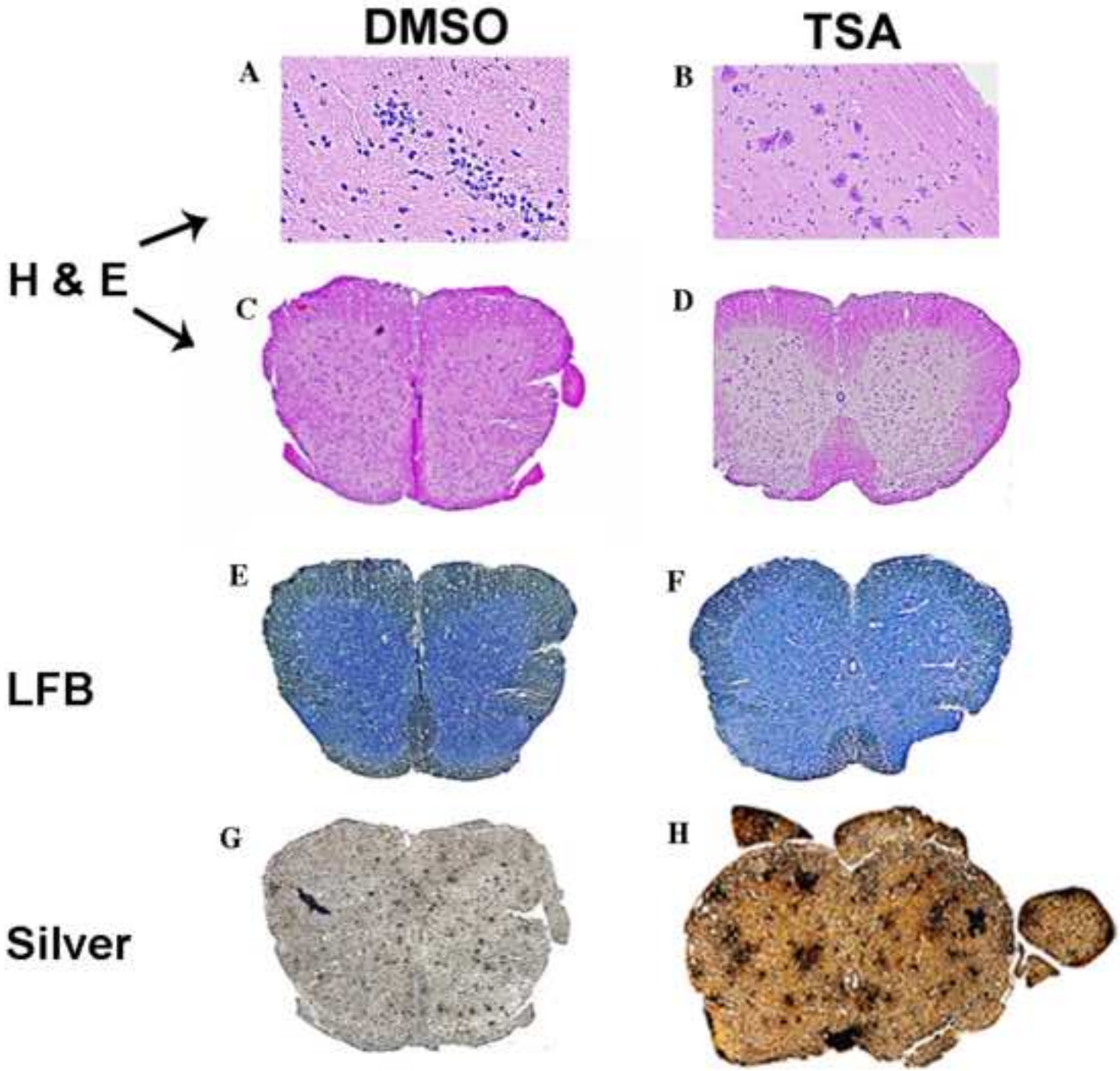

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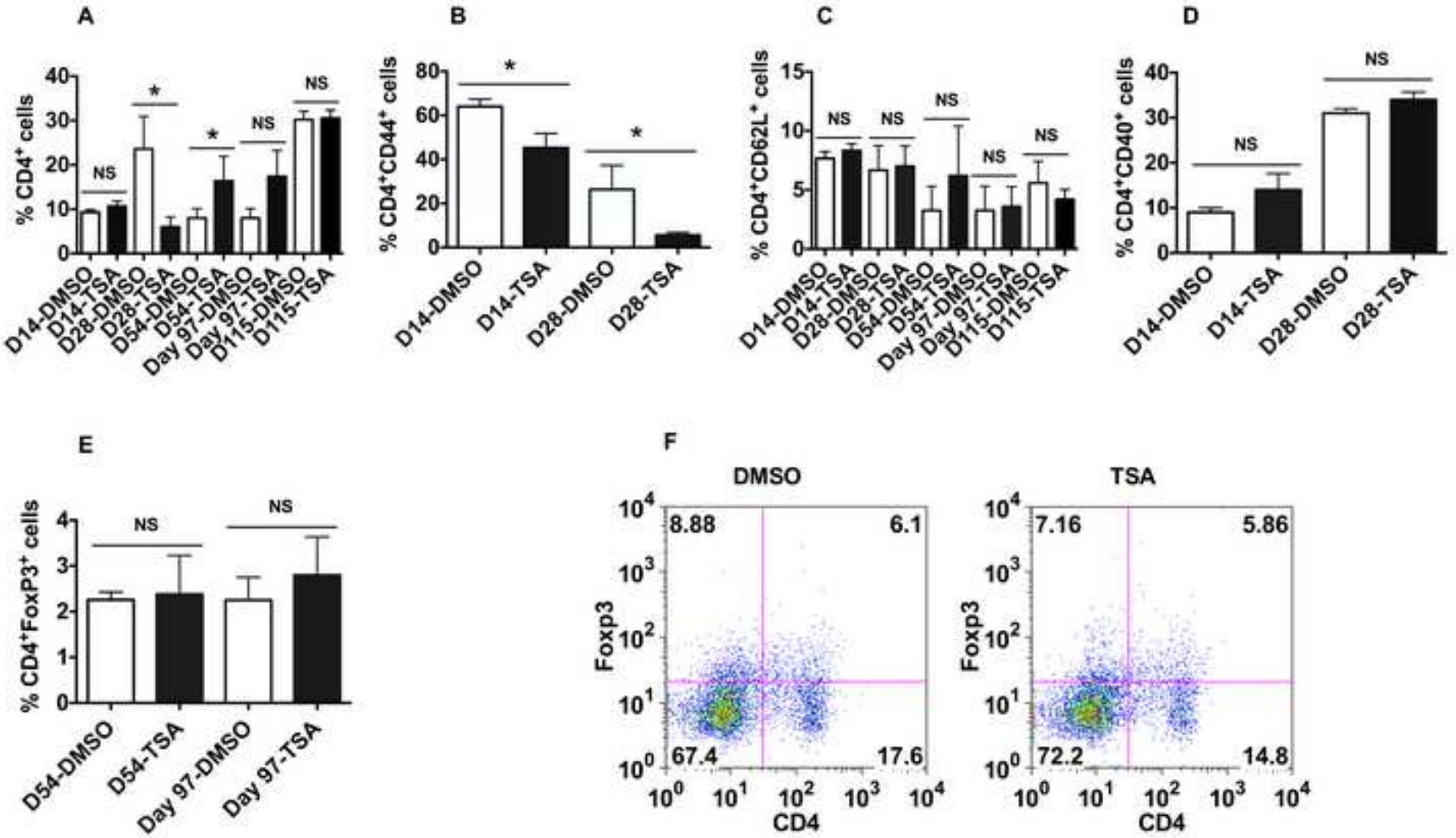

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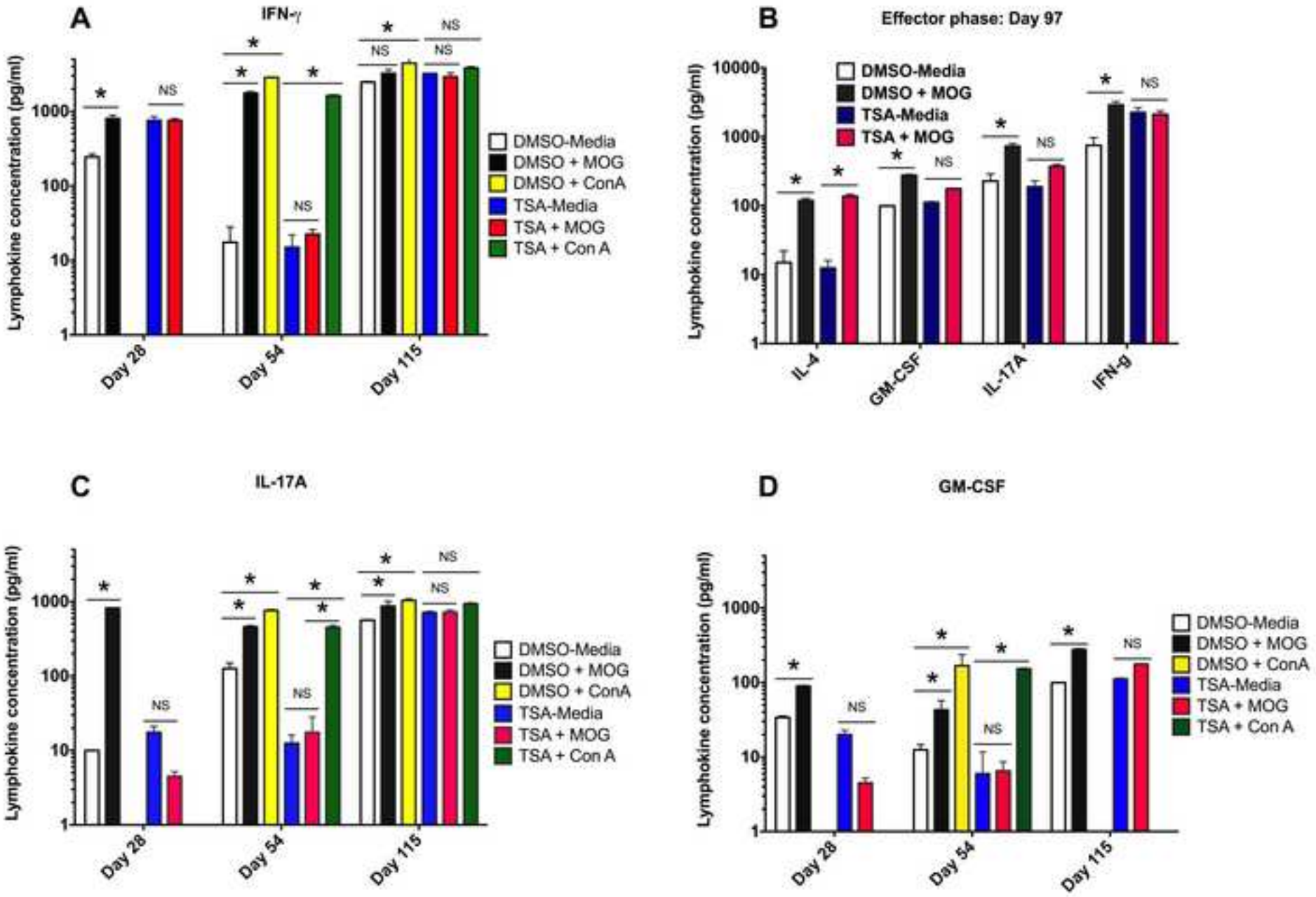

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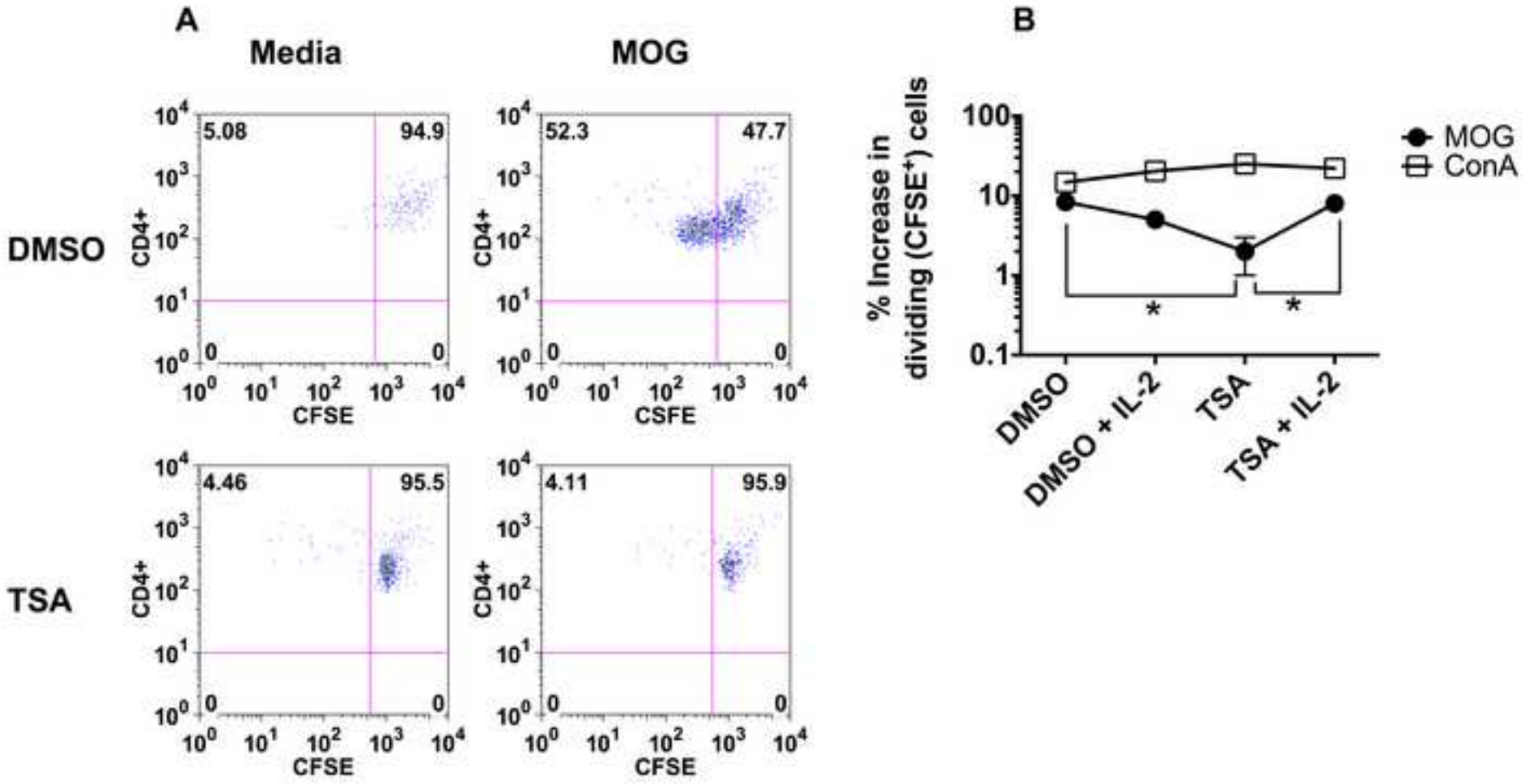

Fig. 5

Figure 6

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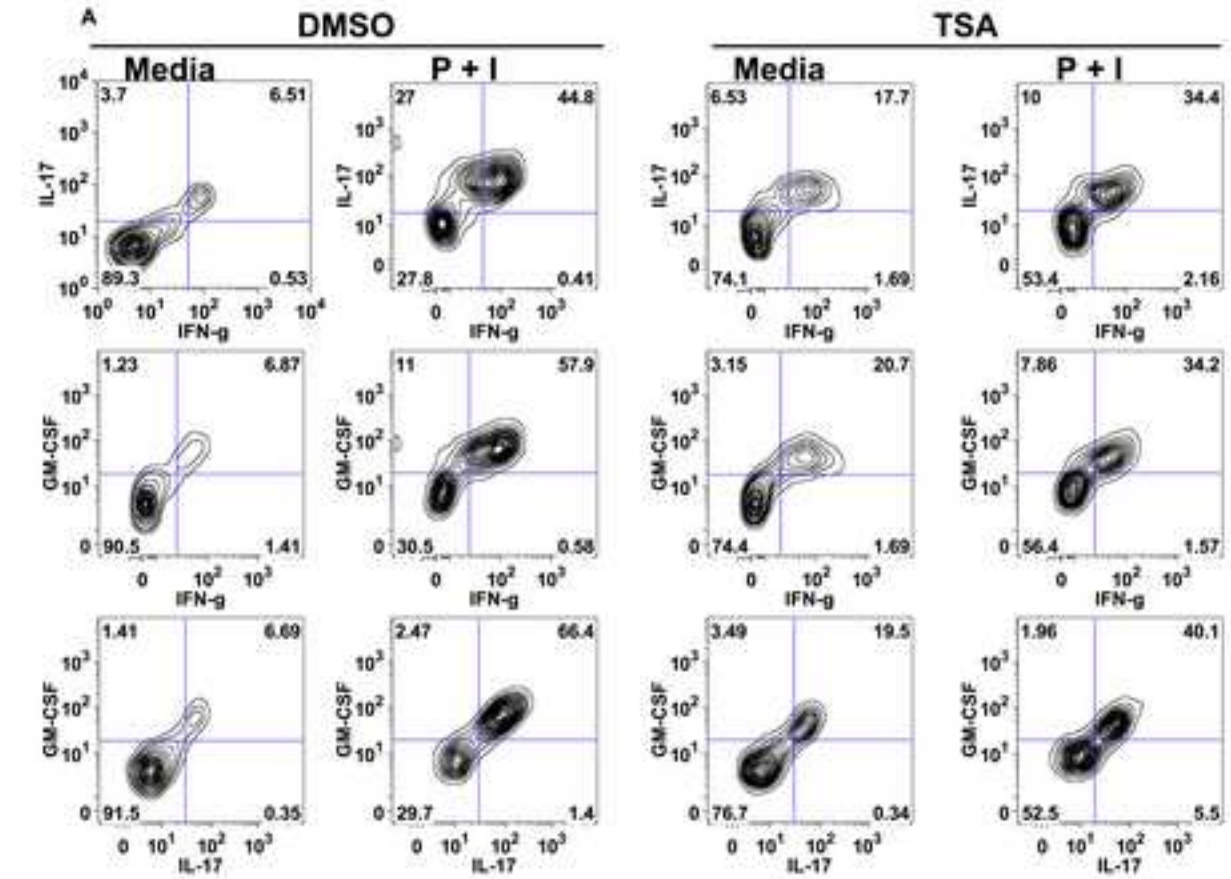

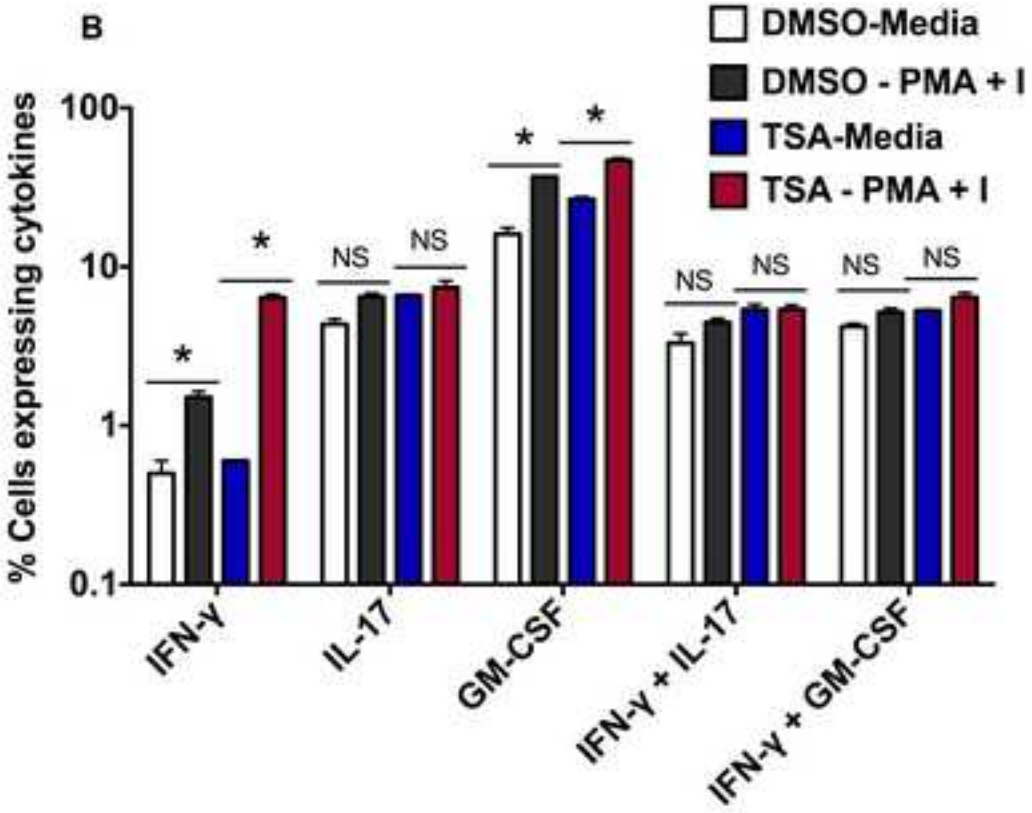

Fig. 6

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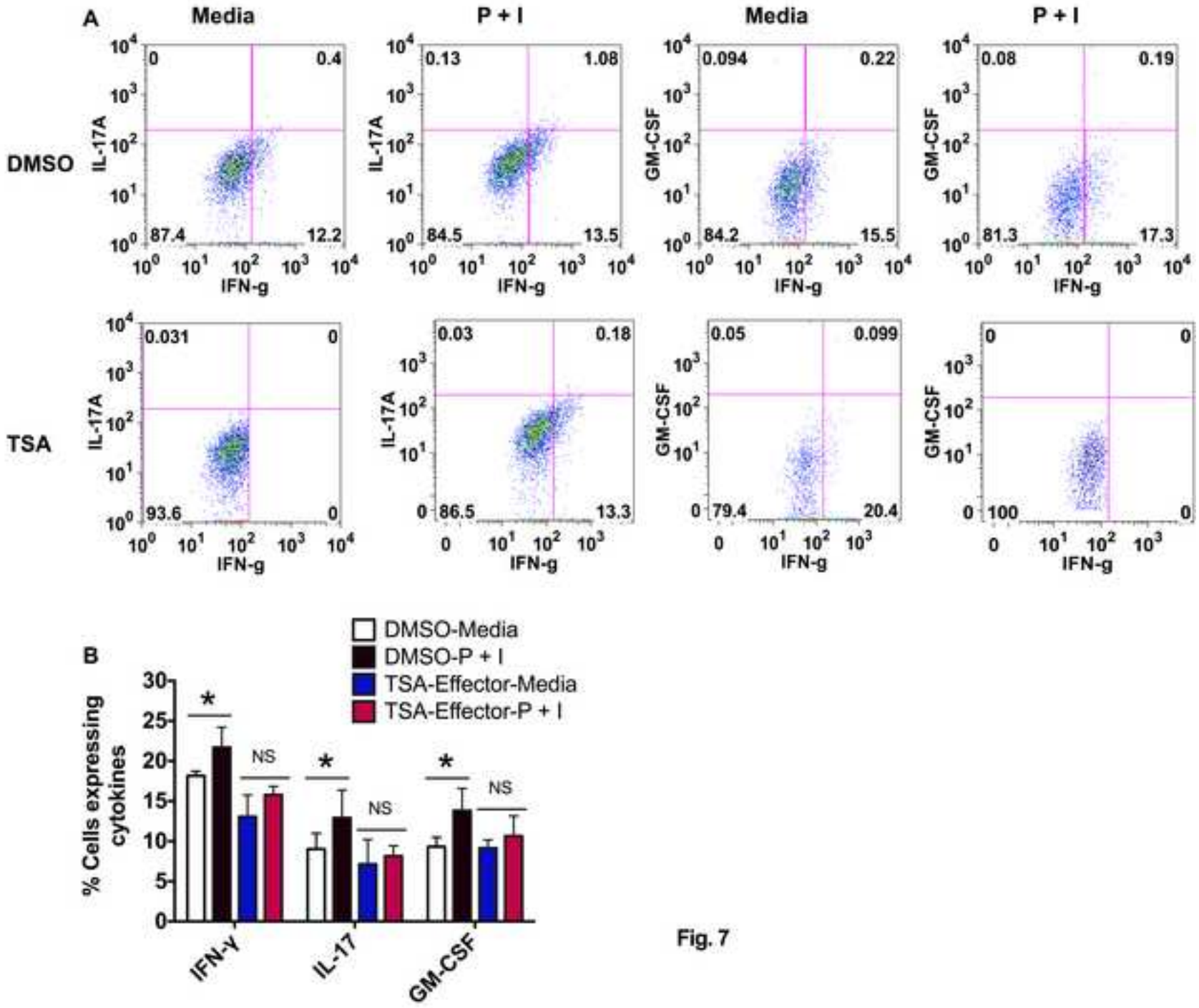
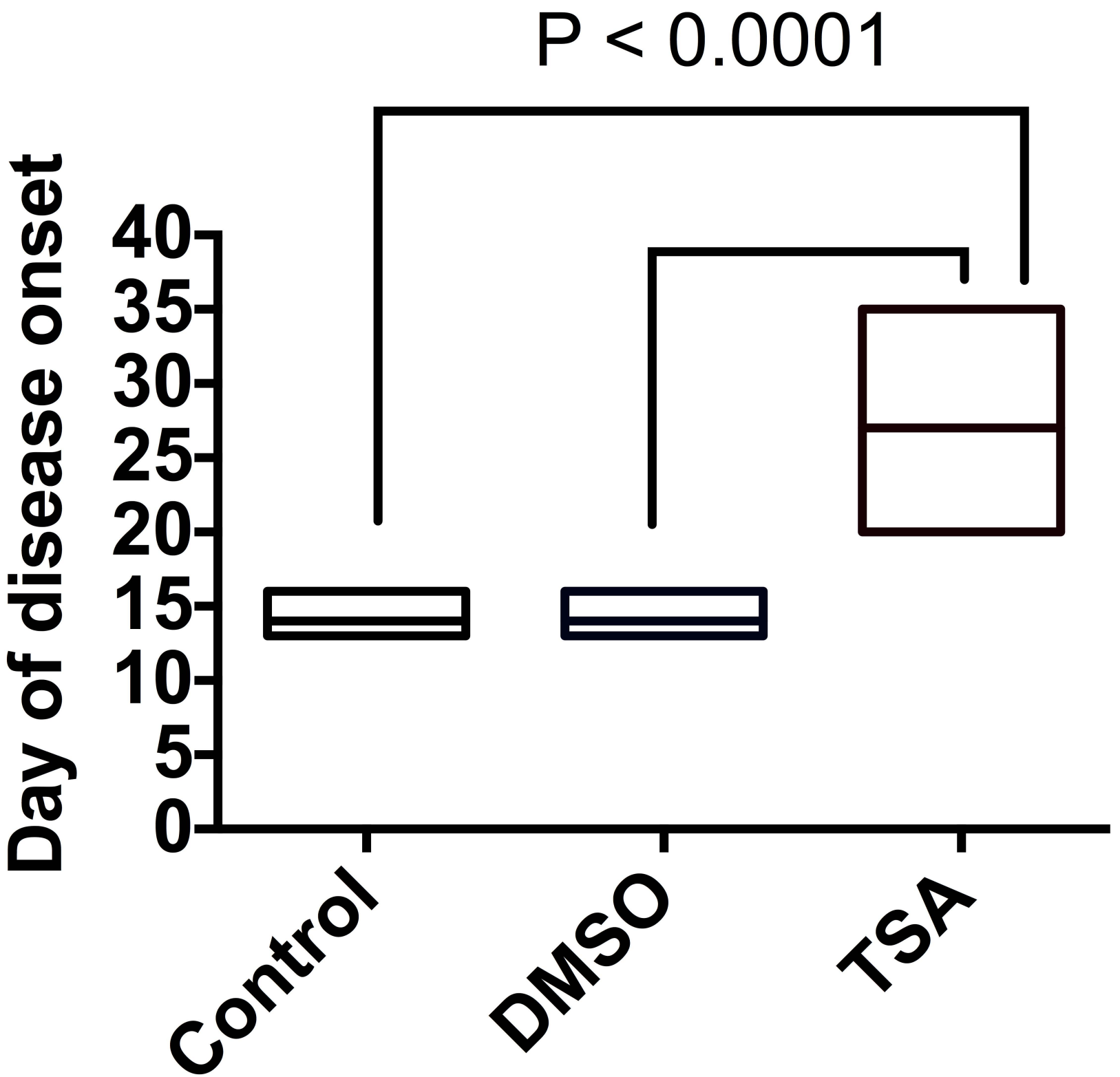


Fig. 7



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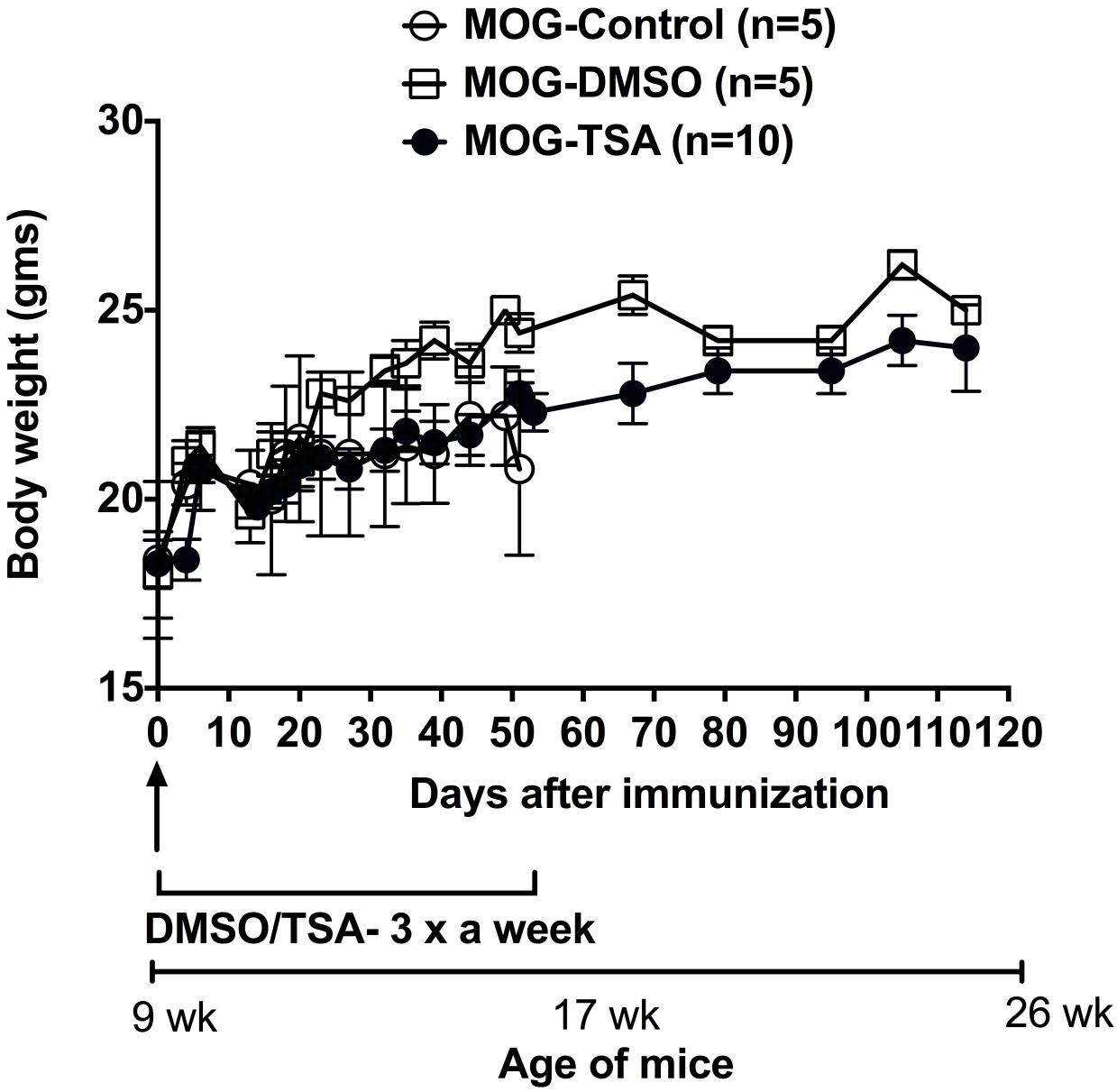

Supplementary Fig. 2.