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# Modulation of MS-like disease by a multi epitope protein is mediated by induction of $CD11c^{+}CD11b^{+}Gr1^{+}$ myeloid-derived dendritic cells☆

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A B S T R A C T

Specific neutralization of the pathogenic autoimmune cells is the ultimate goal in therapy of Multiple Sclerosis (MS). However, the pathogenic autoimmunity in MS, can be directed against several major target antigens, and therefore targeting pathogenic T-cells directed against a single target antigen is unlikely to be effective. To overcome this multiplicity and the potential complexity of pathogenic autoreactivities in MS, we have put forward the concept of concomitant multi-antigen/multi-epitope targeting as, a conceivably more effective approach to immunotherapy of MS. We constructed an (Experimental Autoimmune Encephalomeylitis (EAE)/MS-related synthetic human Target Autoantigen Gene (MS-shMultiTAG) designed to encode in tandem only EAE/MS related epitopes of all known encephalitogenic proteins. The MS–related protein product (designated Y-MSPc) was immunofunctional and upon tolerogenic administration, it effectively suppressed and reversed EAE induced by a single encephalitogenic protein. Furthermore, Y-MSPc also fully abrogated the development of "complex EAE" induced by a mixture of five encephalitogenic T-cell lines, each specific for a different encephalitogenic epitope of MBP, MOG, PLP, MOBP and OSP. Strikingly, Y-MSPc was consistently more effective than treatment with the single disease-specific peptide or with the peptide cocktail, both in suppressing the development of "classical" or "complex" EAE and in ameliorating ongoing disease. Overall, the modulation of EAE by Y-MSPc was associated with anergizing the pathogenic autoreactive T-cells, downregulation of Th1/Th17 cytokine secretion and upregulation of TGF-β secretion. Moreover, we show that both suppression and treatment of ongoing EAE by tolerogenic administration of Y-MSPc is associated also with a remarkable increase in a unique subset of dendritic-cells (DCs), CD11c + CD11b + Gr1 + -myeloid derived DCs in both spleen and CNS of treated mice. These DCs, which are with strong immunoregulatory characteristics and are functional in down-modulation of MS-like-disease displayed increased production of IL-4, IL-10 and TGF-β and low IL-12. Functionally, these myeloid DCs suppress the in-vitro proliferation of myelin-specific T-cells and more importantly, the cells were functional in-vivo, as their adoptive transfer into EAE induced mice resulted in strong suppression of the disease, associated with a remarkable induction of CD4 + FoxP3 + regulatory cells.

These results, which highlight the efficacy of "multi-epitope-targeting" agent in induction of functional regulatory CD11c + CD11b + Gr1 + myeloid DCs, further indicate the potential role of these DCs in maintaining peripheral tolerance and their involvement in downregulation of MS-like-disease.

## 1. Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterized by perivascular infiltration accompanied by primary demyelination (Raine, 1984). The cause of MS is not yet known, and the genetic and immunologic mechanisms that control disease progression are poorly understood. The substantial heterogeneity in clinical course and CNS pathology between MS patients suggests a multifactorial disease cause (t Hart et al., 2001). Although the etiology of the disease is unknown, it is believed that MS results from autoimmune mechanisms, leading to destruction of myelin, presumably initiated by abnormally activated potentially pathogenic autoimmune T cells that recognize components of the myelin sheath in the CNS of MS patients. Identification of potential target antigens in MS and a detailed investigation into the pathogenic T-cell autoimmunity against the primary target antigens is of major importance in eventually considering an antigen-specific therapy for treating the disease, and in better understanding the etiology of MS. Several myelin proteins, e.g., myelin basic protein (MBP), proteolipid protein (PLP), and more recently, myelin oligodendrocyte glycoprotein (MOG), myelin-oligodendrocytic basic protein (MOBP), and oligodendrocye specific protein (OSP) have been regarded as primary candidate target autoantigens for MS (Holz et al., 2000; Kaye et al., 2000; Maatta et al., 1998; Stevens et al., 1999; Zhong et al., 2000). The potential multiplicity of primary target antigens in MS, the possible variability among patients, and the dynamics of autoimmunity ("autoimmune spread") by which specificity of anti-myelin pathogenic autoreactivities may shift or expand to one or more myelin proteins with progression of the disease in the same patient, impose major difficulties in devising antigen-specific immunotherapies for MS. In view of the complex and dynamic anti-myelin

☆ Dedicated in memory of Professor Avraham Ben-Nun, a great human being and a courageous scientist

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autoimmunity in MS, a multi-antigen targeting approach to immune-specific modulation is likely to be more effective than a single antigen/epitope-directed immunomodulation of the disease. We recently showed (Kaushansky et al., 2011) that a concomitant "multi-epitope-targeting" approach using a specifically designed artificial multi-epitope protein (Y-MSPc) is required for effective antigen-based immune-specific therapy of organ-specific autoimmune diseases associated with complex and dynamic pathogenic autoimmunity, such as MS. Y-MSPc was superior to peptide(s) in concomitantly downregulating pathogenic T-cells reactivity against multiple myelin antigens/epitopes, via inducing more effective, longer lasting peripheral regulatory mechanisms (cytokine shift, anergy, and Foxp3+ CTLA4+ regulatory T-cells).

Dendritic cells (DCs) include a heterogeneous family of professional APCs involved in the initiation of both immunity and immunological tolerance (Rutella et al., 2006). T cells peripheral tolerance can be generated and maintained by DCs leading to the promotion of anergy, immune deviation, deletion of self reactive lymphocytes, or generation of regulatory T cells (Rutella et al., 2006). DCs are heterogeneous in their phenotype and their localization in lymphoid tissue (Merad et al., 2013). There are two distinct subsets of DCs in murine spleen (Li et al., 2008): the majority are CD8-CD11b + (~70%), while the others are (~25%) $CD8^+CD11b^-$. $CD8^+CD11b^-$ DCs are of lymphoid lineage and mainly reside in T cell-rich periarteriolar lymphatic sheats. In contrast, $CD8^-CD11b^+$ DCs are of myeloid lineage, and reside in spleen marginal zones (Li et al., 2008). Each spleen DC subset appears to have a distinct lineage and set of functional characteristics. Lymphoid like DCs $CD11c^+CD8^+$ are IL-$10^{low}$IL-$12^{high}$ induce Th1 cytokines INF-γ and IL-2 whereas myeloid –like $CD11c^+CD11b^+$ DCs are IL-$10^{high}$IL-$12^{low}$ induce Th2 cytokines IL-4 and IL-10 in vivo (Li et al., 2008).

Several studies demonstrated the tolerogenic potential of $CD11c^+CD11b^+$ DCs (Florez-Grau et al., 2018; Li et al., 2008; Manicassamy and Pulendran, 2011). The immune tolerance induced by repeated feeding with oral antigen in Experimental Collagen-induced Arthritis was demonstrated to be initiated by an increase in tolerogenic $CD11c^+CD11b^+$ DCs in Peyer's patches. These $CD11c^+CD11b^+$ DCs exhibited immunosuppressive characteristics, such as increased IL-10 production, inhibition of T- cells proliferative responses to type 2 collagen (CII), and CD4 + CD25+ regulatory T- cell induction. Furthermore, $CD11c^+CD11b^+$ DCs have shown to suppress autoimmune diabetes in an in vivo transfer model, supporting that this cell type is responsible for the protection against T- cell-mediated autoimmune destruction of pancreatic islets in the NOD mouse (Saxena et al., 2007). In addition, Li et al. study (Li et al., 2008) indicated that the $CD11c^+CD11b^+$, which are abundant in the CNS of tolerized animals, play a crucial role in i.v tolerance and EAE.

In the present study, we identify a tolerogenic myeloid DC subset in the CNS and spleen of EAE induced mice that plays an important role in the immunoregulation of EAE following treatment with Y-MSPc. Our results demonstrate that immune tolerance induced by Y-MSPc is associated by $CD11c^+CD11b^+GR1^+$ myeloid derived dendritic cells increase in the CNS and spleen. These myeloid DCs exhibited immunoregulatory characteristics, including increased production of IL-4, IL-10 and TGF-β but reduced IL-12. Furthermore, $CD11c^+CD11b^+GR1^+$ DCs were also capable of inhibiting the proliferation of PLP139-151-specific T- cells in vitro and significantly suppressed ongoing EAE upon adoptive transfer. Taken together, these findings suggest that systemic administration of the multi- epitope- protein (Y-MSPc) results in maintaining peripheral tolerance and reduce EAE incidence by an increase in tolerogenic $CD11c^+CD11b^+GR1^+$.

**Table 1**
The myelin peptides used in this study

| Peptide | Amino acid sequence |
|---|---|
| phMOG34-56 | GMEVGWYRPPFSRVVHLYRNGKD |
| phMBP89-104 | FFKNIVTPRTPPPSQG |
| phOSP55-80 | DCVMATGLYHCKPLVDILILPGYVQA |
| phOSP179-201 | AGDAQAFGENRFYYTAGSSSPTH |
| phMOBP15-36 | QKYSEHFSIHCCPPFTFLNSKK |
| phPLP139-151 | HCLGKWLGHPDKF |
| phPLP178-191 | NTWTTCQSIAFPSK |

## 2. Materials and methods

### 2.1. Mice

Female SJL/J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) or obtained from the Weizmann Institute colony. All mice were 2–3 month-old, when used in the experiments. The IACUC of the Weizmann Institute has approved the experiments, permit number: 03530710-3, were performed in accordance to its relevant guidelines and regulations.

### 2.2. Myelin antigens, peptides, and the Y-MSPc multi- epitope- protein

Y-MSPc (Y-MS-relevant multi-epitope Protein; the Y is an arbitrary symbol for the series of multi-epitope proteins designed for several organ-specific autoimmune diseases in our laboratory) is a recombinant artificial protein encompassing multiple human myelin epitopes protein was prepared as described previously (Kaushansky et al., 2011). The myelin peptides used in this study, listed in Table 1 (over 80% purity), were synthesized in the laboratory of Prof. M. Fridkin, Department of Organic Chemistry, The Weizmann Institute of Science.

### 2.3. Induction of EAE

SJL/J mice were injected subcutaneously at one site in the flank with 200 µl of emulsion containing PLP139-151 (200 µg), in CFA containing 300 µg *Mycobacterium tuberculosis* H37Ra. Mice received 300 ng pertussis toxin in 500 µl PBS in the tail vein immediately and 48 h after immunization. Following the encephalitogenic challenge, mice were observed and scored as previously described (Kaushansky et al., 2011)

### 2.4. T cells proliferative responses and flow cytometry

Mice were immunized with 200 µg of PLP139-151 emulsified in CFA containing 150 µg of Mt. H37Ra (catalog no. 3114-25; Difco Laboratories). On day 21 postimmunization (p.i), spleens, were removed and cultured in- vitro in triplicate in microtiter plates in the presence or absence of relevant Ags as previously described (Zhong et al., 2000) The cultures were incubated for 48 h at 37 °C in humidified air containing 6% CO2. [3H]Thymidine (1 mCi/well) was added for the last 16 h of incubation and the cultures were then harvested and counted using a Matrix 96 direct beta counter (Packard Instruments). The results were expressed as the stimulation index (mean) cpm of Ag containing cultures/mean cpm of cultures without Ag). For flow cytometry, these cells were labeled with anti-murine CD11b, CD11c, I-A, CD80, CD86, Gr1 and CD4 Abs. All Abs were purchased from BioLegend and used according to the manufacturer's protocols. Cells were collected using Cytomics FC 500 system (Beckman Coulter) and analyzed

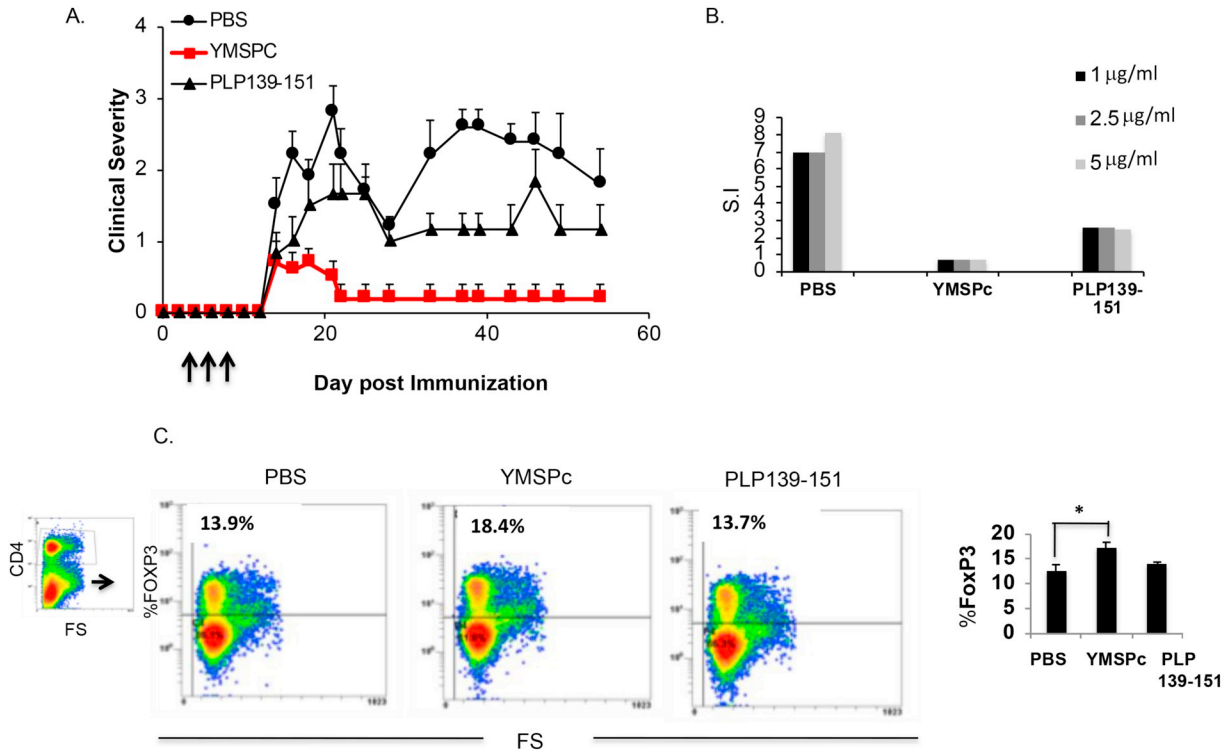



**Fig. 1.** Systemic administration of Y-MSPc results in EAE suppression, down-regulation of pathogenic T cells and increased levels of CD4/FoxP3 regulatory cells in the spleen. (A). Suppression of PLP139-151 induced EAE in SJL/J mice. SJL/J mice were immunized with PLP139-151 in CFA for EAE induction. On days indicated by arrows, the mice ($n = 5$ mice per group) received i.v injection of Y-MSPc (75 μg), or PLP (75 μg), or PBS alone. (B). Suppression of PLP139-151 induced EAE by Y-MSPc was associated with down-regulation of PLP139-151 specific T cells proliferation. On day 21 p.i splenocytes were isolated from the different treated groups and tested for their response to PLP139-151. (C). Flow cytometry of regulatory T-cells induced following systemic administration of Y-MSPc. Splenocytes from the different treatment groups were co-stained with anti-CD4-PE and anti-FoxP3 –FITC. The percentage of the FoxP3 expressing cells on gated CD4 + cells is shown. The FACS plots are from one representative experiment, and the panels at the right end are the mean values +/2SD from four independent experiments. *,$p < .05$.

by Beckman Coulter software.

### 2.5. Isolation of CNS cells and flow cytometry

MNCs from the CNS of PLP139-151-immunized mice were isolated by Percoll gradient centrifugation as previously described (Zilkha-Falb et al., 2016) at the peak of clinical disease (day 21 p.i.). In brief, mice were sacrificed and transcardially perfused with ice-cold GKN solution with 2 U/ml heparin (Sigma-Aldrich). Spinal cords were removed into GKN/0.02% BSA (*w/v*), mechanically dissociated through a 100-μm cell strainer, and enzymatically digested by incubation with 250 μg/ml collagenase/dispase and 250 μg/ml DNase I (Roche) at 37 °C for 20–30 min. The digested CNS preparation were washed with GKN/BSA, and the pellet was fractionated on a 70/37/30% Percoll gradient. MNCs were recovered from the 37/70 interface, washed, and resuspended in RPMI 1640 with 10% FCS. Pooled cells were washed in FACS buffer. Cells were stained with anti-murine CD11b, CD11c, Gr1 and CD4 Abs (all obtained from Biolegend.).

### 2.6. Intracellular cytokines staining

SJL/J mice induced for EAE and treated with different agents were injected with Brefeldin A (250 μg/mouse) in PBS 6h before MNCs isolation. Then, mice were perfused and splenocytes and CNS cells were isolated and stained with fluorescently labeled Abs to CD11b, CD11c. Cells were washed, fixed, and permeabilized using Fix and Perm cell permeabilization reagents (eBioscience). Cells were stained for intracellular cytokines with APC-conjugated rat anti-mouse IL-4, IL-10, IL-2, IL-12, IL-17 and TGF-β Abs. Intracellular Foxp3 (eBioscience) was determined according to the manufacturer's instruction. All flow cytometric analyses were performed using appropriate isotype controls. (All Abs were purchased from BioLegend and used according to the manufacturer's protocols. Cells were analyzed on Cytomics FC 500 system (Beckman Coulter) and analyzed by Beckman Coulter software

### 2.7. Purification of DCs subsets and functional assay

Splenocytes of Y-MSPc, PLP139-151 and PBS treated EAE induced mice were isolated on day 21 p.i and stained with anti-mouse PE conjugated CD11c, FITC conjugated CD11b and APC conjugated Gr1. These cells were sorted to $CD11c^{-}CD11b^{+}GR1^{-}$, $CD11c^{+}CD11b^{-}Gr1^{-}$ and $CD11c^{+}CD11b^{+}Gr1^{+}$cells, (purity > 90%) by using FACSAria. To determine in- vitro function of these DCs subsets, cells were co- cultured ($1.2 \times 10^4$) with PLP139-151 primed LNCs ($0.5 \times 10^6$) in presence of PLP139-151 (2.5 μg/ml). The cultures were incubated for 72 h at 37 °C in humidified air containing 6% CO2. [3H]Thymidine (1 mCi/well) was added for the last 16 h of incubation and the cultures were then harvested and counted using a Matrix 96 direct beta counter (Packard Instruments). The results were expressed as the stimulation index (mean cpm of Ag containing cultures/mean cpm of cultures without Ag).

### 2.8. Adoptive transfer of DC subsets in suppression model of EAE

Splenocytes of Y-MSPc, and PBS treated EAE induced mice were isolated on day 21 p.i and stained with anti-mouse PE conjugated CD11c, FITC conjugated CD11b and APC conjugated Gr1. These cells were sorted to $CD11c^{+}CD11b^{+}Gr1^{+}$cells, by using FACSAria (purity > 90%). $CD11c^{+}$ $CD11b^{+}Gr1^{+}$cells derived from PBS or Y-MSPc treated mice were then ($3 \times 10^4$) transferred into PLP139-151-

induced EAE mice i.v. on day 5 p.i. At the experimental end point (day 32 p.i), splenocytes were isolated and stained with anti -CD4 and intracellular Foxp3 mAbs.

### 2.9. Statistical analysis

All experiments were tested for statistical differences using unpaired, two tailed, Student's *t*-tests. Differences were considered significant if $p < .05$.

## 3. Results

### 3.1. Tolerogenic administration of Y-MSPc suppresses EAE and induces regulatory mechanisms

The efficacy of Y-MSPc in suppression of "classical" EAE (induced by a single encephalitogen/peptide) upon tolerogenic administration (soluble, i.v.) have been shown by us in F1(C57BlxSJL/J) mice (Kaushansky et al., 2011). Here we show that administration of Y-MSPc before disease onset almost totally abrogated the development of EAE in SJL/J mice actively induced with PLP139-151 (Fig. 1A). Moreover, upon tolerogenic administration, the Y-MSPc was more effective than PLP139-151 in the suppression of active EAE. The efficacy of the regulatory mechanisms induced by administration of the Y-MSPc is shown in Fig. 1B,C. PLP139-151/CFA immunized mice were treated with i.v. injections of PLP139-151, Y-MSPc, or PBS, on days 3, 5 and 7 p.i. 14 days after last injection (day 21 post immunization), the effects of the various treatments on PLP139-151-reactive T-cells in the draining spleen were analyzed ex-vivo. Fig. 1B. shows that administration of Y-MSPc reduced the recall proliferative response to PLP139-151 by about 95% compare to PBS, whereas the effect of the treatment by PLP139-151 reduced the effect by 65%. The down regulation of the PLP response following Y-MSPc treatment was associated with induction of regulatory T cells as previously was shown (Kaushansky et al., 2011). Indeed, FACS analysis of splenocytes derived from PBS, Y-MSPc or PLP139-151 treated mice, showed that 18.4% of the $CD4^+$ splenocytes from Y-MSPc treated mice were FoxP3+ cells, compared to 13.9% and 13.7% FoxP3+ of total $CD4^+$ T cells from PBS or PLP139-151 treated mice,respectively (Fig. 1C). The increase in Foxp3+ cells following treatment with Y-MSPc was consistent in all the four independent experiments that were carry out (Fig. 1C).

### 3.2. Increased proportion of $CD11c^+CD11b^+GR1^+$ DCs in spleen of Y-MSPc treated mice

Several reports about specific DC subsets that can induce regulatory mechanisms have been reported (Dunne et al., 2009; Florez-Grau et al., 2018; Li et al., 2008; Steimle and Frick, 2016). Based on this, we further investigated the efficacy of Y-MSPc in induction of regulatory DCs. The relative proportions of $CD11c^+CD11b^+$ and $CD11c^+CD8\alpha^+$ DCs in the spleen were measured following administration of different agents. Splenocytes from PLP139-151- EAE induced mice and treated with Y-MSPc, PLP139-151, huPEP mix (detailed in Fig. 2), non-relevant control recombinant Protein ΔConx (detailed in Fig. 2)or PBS were analyzed on day 21 p.i for their $CD11c^+CD11b^+$ and $CD11c^+CD8\alpha^+$ expression. While there was no differences in $CD11c^+CD8\alpha^+$ proportion (data not shown), a significantly higher proportion of $CD11c^+CD11b^+$ was detected only in splenocytes derived from Y-MSPc treated mice (Fig. 2A). Thus, while treatment with Y-MSPc increased the proportion of $CD11c^+CD11b^+$ by ~40% compare to PBS, the treatment with other agents was only marginal. The increase in $CD11c^+CD11b^+$ DCs following treatment with Y-MSPc was consistent in all the four independent experiments that were carry out (Fig. 2B).

Gr-1 is a myeloid differentiation antigen expressed by myeloid cells in a developmentally regulated manner in the bone marrow (Fleming et al., 1993). In mice, several subpopulations of myeloid-derived cells are positive for the marker Gr-1. Here, we tested the expression of Gr1 on $CD11c^+CD11b^+$ DCs following administration of different agents. As shown in Fig. 2c, $CD11c^+CD11b^+$ DCs derived from splenocytes of different treated groups of mice expressed high levels of Gr1. Expression of about 70% of Gr1 was detected in $CD11c^+CD11b^+$ DCs derived from different agents administrated group of mice.

### 3.3. Downregulation of I-A(MHC II) and co-stimulatory molecules expression on $CD11c^+CD11b^+Gr1^+$ DCs following Y-MSPc administration

It has been reported that DC deficiency in costimulatory molecules can induce T cell anergy, generate T regs cells and promote alloantigen-specific tolerance (Janikashvili et al., 2011). To investigate the Ag-presenting capability of spleen freshly isolated $CD11c^+CD11b^+Gr1^+$ DCs, the expression of I-A and costimulatory molecules on these DCs was examined by flow cytometry (Fig. 3). Low level of costimulatory molecules CD80 and CD86 (Fig. 3A,B) and of I-A (Fig. 3C) expression was determined on $CD11c^+CD11b^+Gr1^+$ DCs derived from mice treated with different agents. This immature phenotype is consistent with previous reports regarding DCs freshly isolated from both lymphoid and nonlymphoid tissues (Dubsky et al., 2005; Quah and O'Neill, 2005). The results presented in Fig. 3 shows that only Y-MSPc administration resulted in significantly lower costimulatory molecules CD80 (Fig. 3A) and CD86 (Fig. 3B) expression on $CD11c^+CD11b^+Gr1^+$ DCs compare to PBS administration. Moreover, decrease expression of I-A on $CD11c^+CD11b^+Gr1^+$ DCs following Y-MSPc administration was detected, however to not significant levels compare to PBS administration.

### 3.4. Spleen $CD11c^+CD11b^+Gr1^+$ DCs are characterized by high level of Th2, and low level of Th1 cytokines in Y-MSPc treated mice

We next characterized the intracellular cytokine secretion by $CD11c^+CD11b^+Gr1^+$ DCs. Splenocytes derived $CD11c^+CD11b^+Gr1^+$ DCs from mice that were immunized with PLP139-151/CFA and treated with Y-MSPc, PLP139-151, huPEP mix, ΔConx or PBS were analyzed for intracellular Th1 or Th2 cytokine secretion on day 21 p.i. As a control we also analyzed the cytokine secretion in $CD11c^-CD11b^+Gr1^-$ cells. As shown in Fig. 4A(a-c) significantly elevated levels of Th2 cytokines; IL-4, IL-10 and TGF-β were detected only upon Y-MSPc administration. Neither agent elevated Th2 cytokine secretion in $CD11c^-CD11b^+Gr1^-$ cells. Administration of Y-MSPc was also associated with strong reduction of IL-12 (about 90%; Fig. 4Ad) and IL-2 (about 50%; Fig. 4Ae). Reduction in IL-12 and IL-2 was also detected in $CD11c^-CD11b^+Gr1^-$ cells, however was not specific to any administrated agent. Interestingly, elevation in IL-17 secretion was detected in $CD11c^+CD11b^+Gr1^+$ DCs following Y-MSPc administration (~about 57%; Fig. 4Af). Similar elevation of IL-17 was detected also in $CD11c^-CD11b^+Gr1^-$ cells (Fig. 4Bf).

### 3.5. $CD11c^+CD11b^+Gr1^+$ DCs derived from Y-MSPc treated mice induce suppression of PLP139-151 T cells proliferation

We next assessed the functional properties of $CD11c^+CD11b^+Gr1^+$ DCs from spleen of mice immunized with Y-MSPc, PLP139-151 or PBS. PLP139-151/CFA immunized mice were treated on day 3,5 and 7 with YMSPc, PLP139-151 or PBS. On day 21 p.i spleens were isolated and sorted for $CD11c^+CD11b^+Gr1^+$, $CD11c^-CD11b^+Gr1^-$,$CD11c^+CD11b^-Gr1^-$ cells by using FACs. These sorted cells were co cultured ($1.2 \times 10^4$) with PLP139-151 primed LNCs ($0.5 \times 10^6$) in the presence of the primed Ag. Fig. 5 shows that while co culturing of Y-MSPc, PLP139-51 or PBS derived $CD11c^-CD11b^+Gr1^-$ or $CD11c^+CD11b^-$ $Gr1^-$ sorted cells with PLP139-151 primed LNCs had no effect on the proliferation of LNCs (Fig. 5 A,B), co culturing of PLP139-151 primed LNCs with Y-MSPc $CD11c^+CD11b^+Gr1^+$ DCs inhibited the proliferation of the LNCs by about 40% compare to co culturing of LNCs with PBS or PLP139-151 $CD11c^+CD11b^+Gr1^+$ DCs (Fig. 5C). These finding suggest that Y-MSPc - $CD11c^+CD11b^+Gr1^+$ DCs

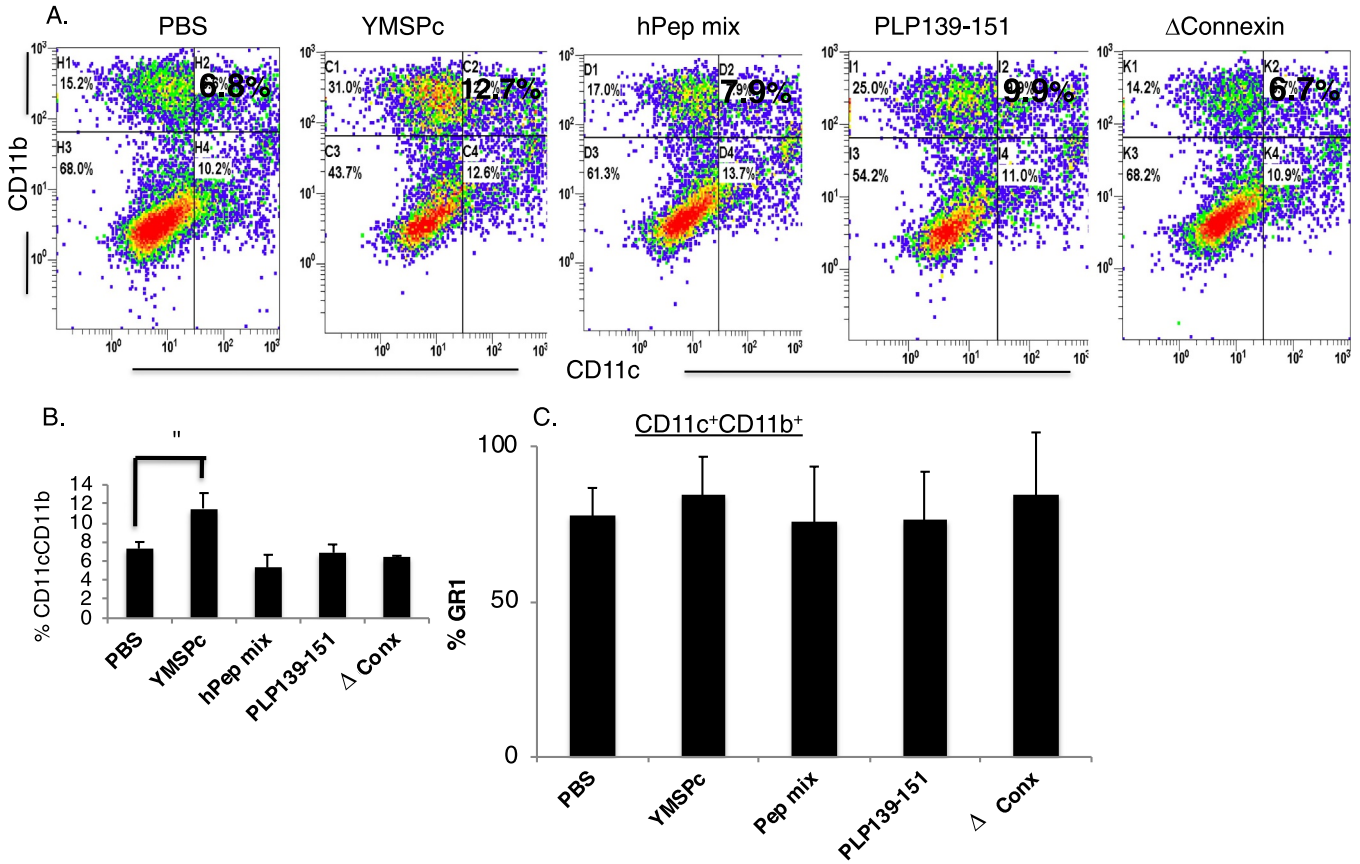



**Fig. 2.** Increased proportion of spleen derived $CD11c^+CD11b^+Gr1^+$ DCs in Y-MSPc treated mice. (A). SJL/J mice were immunized for EAE with PLP139-151. On day 3,5 and 7 post immunization, mice were injected i.v. with Y-MSPc (75 μg/mouse), PLP139-151 (75 μg/mouse), huPEP mix (total 140 μg/mouse), Δ Connexin (ΔConx - Δ refer to deletion of the sequences encompassing the hydrophobic putative transmembrane domains of the Connexin) (75 μg/mouse), or with 0.5 ml PBS. The huPEP mix contained 1:1 ratios of phPLP139-151, phPLP175-194, phMOG34-56, phMBP89-104, phMOBP15-36, phOSP55-80 and phOSP186-205 (20 μg each peptide), representing the major encephalitogenic epitopes of five human myelin proteins. A control treatment with non-relevant recombinant protein (ΔConx) was also included to exclude the possibility that residual bacterial contaminants contributed to the efficacy of Y-MSPc. (Δ-Conx is a neuronal protein and was constructed, expressed and purified similar to Y-MSPc). On day 21 p.i MNCs were isolated from spleen of different treatment groups mice and the proportion of $CD11c^+CD11b^+$ were determined using flow cytometry. The FACS plots are from one representative experiment, and the panels in (B). are the mean values +/2SD from three independent experiments. (C). spleen derived ]$CD11c^+CD11b^+$ DCs express high level of GR1. On day 21 p.i, spleen derived $CD11c^+CD11b^+$ from the different treatment groups of mice were stained with anti Gr1 Ab and analyzed for its expression by Flow Cytometry. Data shown is mean values +/2SD from four independent experiments. *,$p < .05$.

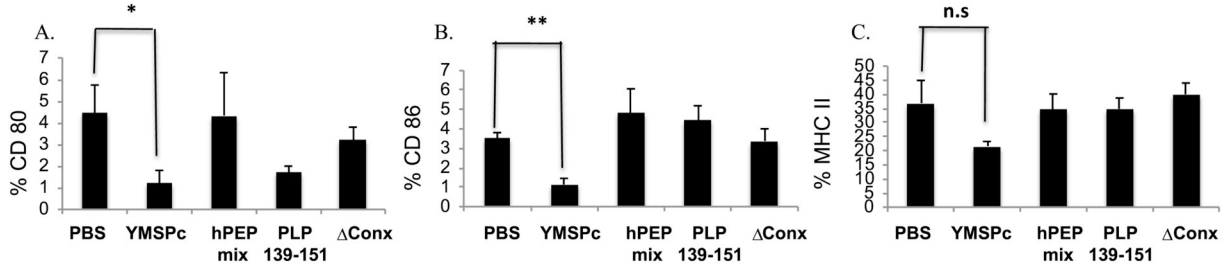



**Fig. 3.** Expression of costimulatory molecules and MHCII (I-A) on ]$CD11c^+CD11b^+Gr1^+$ DCs. Spleen derived $CD11c^+CD11b^+Gr1^+$ DCs isolated (on day 21 p.i) from the different treatment groups of mice, [Y-MSPc (75 μg/mouse), PLP139-151 (75 μg/mouse), huPEP mix (total 140 μg/mouse), ΔConx (75 μg/mouse)] were analyzed by flow cytometry for expression of A. CD80, B. CD86, C. I-A. Data shown is mean values +/2SD from five independent experiments.*,$p < .05$; **, $p < .005$.

are involved in suppression of T cell proliferation.

### *3.6. Adaptive transfer of Y-MSPc-$CD11c^+CD11b^+$DCs result in EAE suppression associated with up-regulation of CD4 + FoxP3+ regulatory cells*

To determine whether Y-MSPc - $CD11c^+CD11b^+$ DCs have suppressive effect in vivo, PLP139-151/CFA immunized mice were treated on day 3,5,and 7 with Y-MSPc, or PBS and on day 21 p.i spleens were isolated and sorted into $CD11c^+CD11b^+$ DCs. Then, these sorted $CD11c^+CD11b^+$ DCs derived from PBS or Y-MSPc treated mice were transferred into recipient mice immunized to develop EAE. As shown in Fig. 6A, while transfer of PBS-$CD11c^+CD11b^+$ DCs had no significant effect on clinical course of the disease, transfer of Y-MSPc $CD11c^+CD11b^+$ DCs was highly effective in suppressing EAE development compare to administration of control PBS. (Moreover, the effect

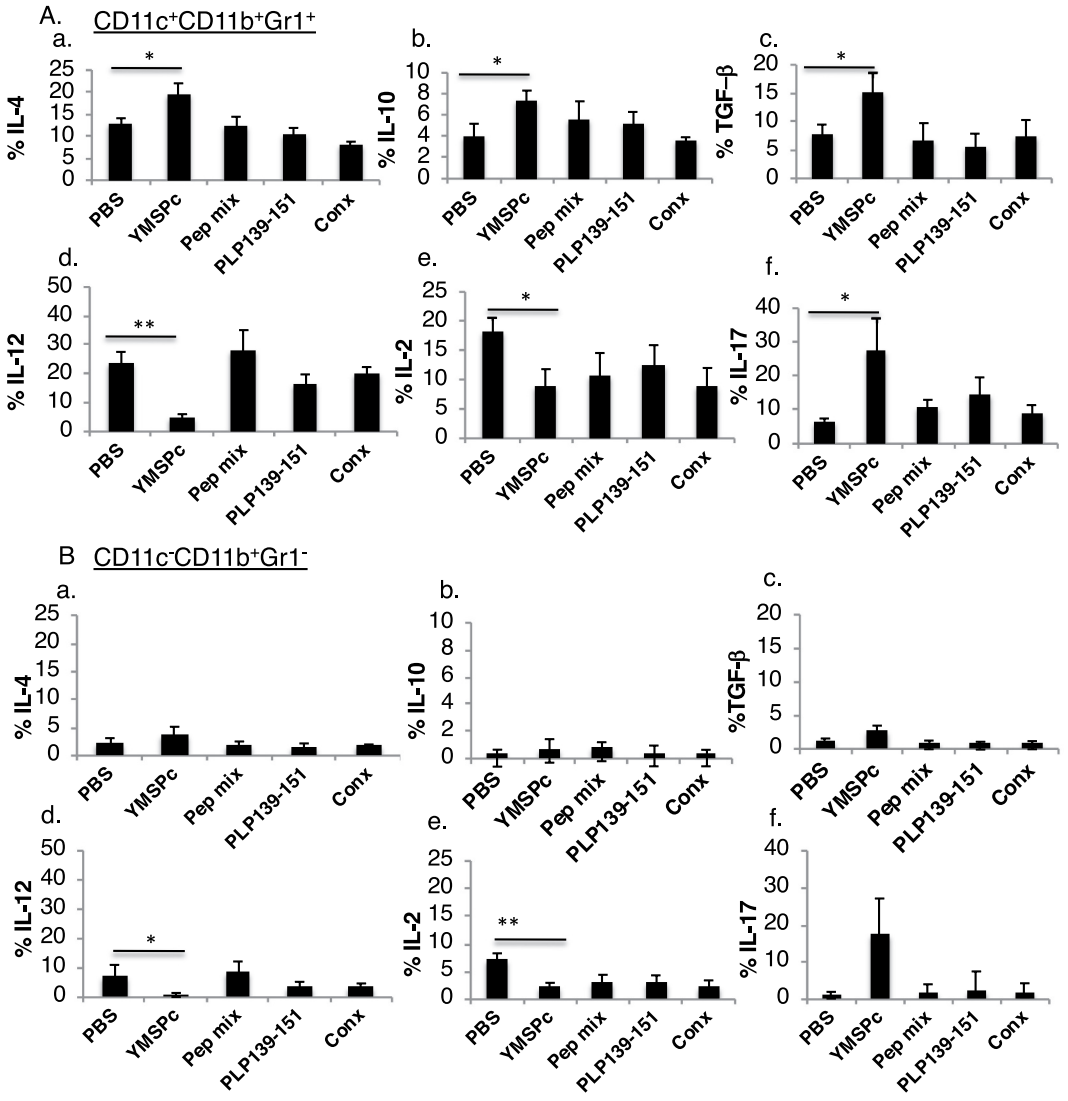



**Fig. 4.** Intracellular of pro/anti inflammatory cytokine expression in spleen derived $CD11c^+CD11b^+Gr1^+$ and $CD11c^-CD11b^+Gr1^-$ DCs. $CD11c^+CD11b^+Gr1^+$(A), and $CD11c^-CD11b^+Gr1^-$ (B), DCs - spleen derived were isolated on day 21 p.i from the different treatment group of mice, and intracellular IL-4, IL-10, TGF-β, IL-12, IL-2 and IL-17 was examined by flow cytometry. Data shown is mean values +/2SD from three independent experiments. *,p < .05; **, p < .005.

of Y-MSPc - $CD11c^+CD11b^+$ DCs transfer was superior to PBS-$CD11c^+CD11b^+$ DCs transfer in suppressing EAE development). Thus, on day 32 (experiment end point) the mean clinical score of PLP139-151/CFA immunized mice transferred with Y-MSPc - $CD11c^+CD11b^+$ DCs was 1.5 compare to mean clinical score of 4 of PLP139-151/CFA immunized mice transferred with PBS - $CD11c^+CD11b^+$ DCs. The suppression of EAE by adoptive transfer of Y-MSPc - $CD11c^+CD11b^+$ DCs was associated with upregulation of regulatory T cells. On day 32, day of experiment termination, splenocytes derived from PBS-$CD11c^+CD11b^+$, Y-MSPc - $CD11c^+CD11b^+$ DCs transferred mice or only PBS administered mice (non transferred mice) were analyzed for CD4 + FoxP3+ expression. As shown in Fig. 6B, increase proportion of CD4 + FoxP3+ regulatory cells were detected in Y-MSPc - $CD11c^+CD11b^+$ DCs transferred mice (5.1%) in comparison to.

PBS-$CD11c^+CD11b^+$ DCs (0.9%) or non transferred, PBS administered mice (0.7%).The increase in CD4 + FoxP3+ regulatory cells following transfer of Y-MSPc - $CD11c^+CD11b^+$ DCs was consistent in all the three independent experiments that were carry out (Fig. 6B).

### *3.7. The suppression of EAE by Y-MSPc was associated in increased proportion of $CD11c^+CD11b^+Gr1^+$ DCs that secret high level of Th2 but not Th1 and with up-regulation of CD4 + FoxP3 regulatory cells in CNS of Y-MSPc treated mice*

It was interesting to test whether Y-MSPC injection also influence the proportion of $CD11c^+CD11b^+Gr1^+$ in CNS. PLP139-151/CFA immunized mice were treated on day 3, 5, and 7 with Y-MSPc, PLP139-151 or PBS and on day 21 p.i, MNCs were isolated from CNS. Fig. 8Aa, 8Ba show that the proportions of $CD11c^+CD11b^+Gr1^+$ were higher in Brain (Fig. 7A) and in spinal cord (Fig. 7B) of mice administration with Y-MSPc than in mice administered with PLP139-151 or PBS. As was previously shown in the spleen (Fig. 4A), CNS derived $CD11c^+CD11b^+Gr1^+$ DCs following administration of Y-MSPc were characterized by increase of Th2 and decrease in Th1 cytokines secretion. Administration of Y-MSPc was associated with strong reduction in IL-12 (about 72%, 75%; Fig. 7Ab, 7Bb) and IL-2 (about 84%, 86%;Fig. 7Ac,Fig. 7Bc) in the brain and spinal cord respectively compared to PBS treated mice. While increase in IL-17 was detected in brain of Y-MSPc $CD11c^+CD11b^+Gr1^+$ mice (45%, Fig. 7Ad), reduction in secretion of this cytokine was detected in spinal cord of Y-MSPc

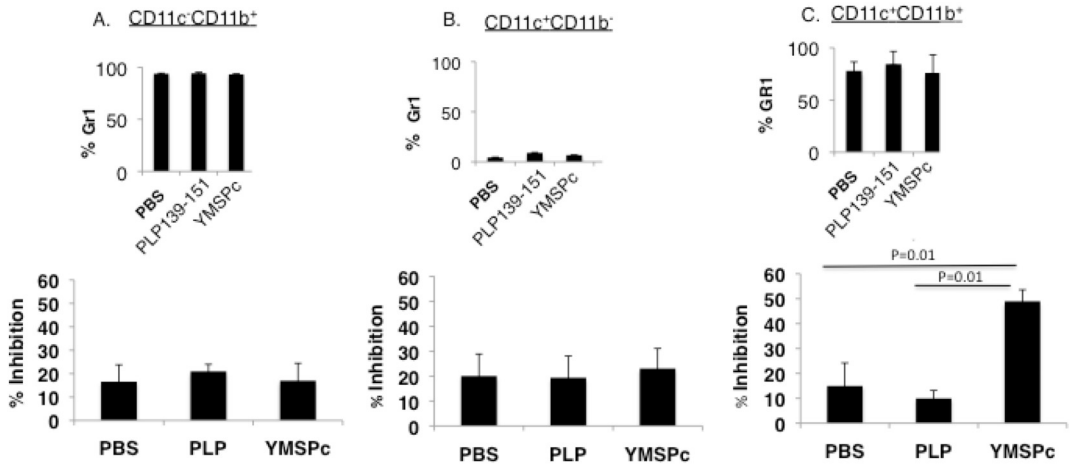



**Fig. 5.** $CD11c^+CD11b^+Gr1^+$ DCs downregulate the proliferation of PLP139-151 specific T cells in vitro. SJl/J mice were immunized s.c with PLP139-151 (100 μg) in CFA. On day 10 p.i, draining LNCs were isolated and co-cultured ($0.5 \times 10^6$/ml) with sorted $CD11c^-CD11b^+$(A), $^-CD11c^+CD11b^-$(B), or $CD11c^+CD11b^+$(C) DCs ($1.2 \times 10^4$/ml). The different subtypes of DCs were isolated on day 21 p.i from spleen of Y-MSPc (75 μg/mouse), PLP139-151 (75 μg/mouse) or PBS treated mice. On day 21 p.i, spleen DC's derived from different treatment groups were stained with anti Gr1 Ab and analyzed for its expression by Flow Cytometry (upper panel). LNCs were co- cultured with different DC's subtypes for 72 h in microtiter wells in triplicates in the absence or presence of PLP139-151 (5 μg/ml). [H3]Thymidine was added for the last 18 h.

$CD11c^+CD11b^+Gr1^+$ mice (40%;Fig. 7Bd) compare to CNS derived PBS $CD11c^+CD11b^+Gr1^+$ DCs. As shown in Fig. 7A(e-g), 7B(e-g) significantly elevated levels of Th2 cytokines; IL-4, IL-10 and TGF-β were detected upon Y-MSPc administration. Moreover, elevated Th2 cytokines secretion were detected in CNS $CD11c^+CD11b^+Gr1^+$ following Y-MSPc administration. As shown in Fig. 7A(e-f), 7B(e-f) higher levels of IL-4, IL-10 and TGF-β were secreted by $CD11c^+CD11b^+Gr1^+$ upon Y-MSPc treatment both in brain and spinal cord compared to PBS treatment. This pattern of cytokine secretion was consistent in the three independent experiments that were carried out. As compared to Y-MSPc administration, administration of PLP139-151 increased only the secretion of TGF-β in CNS derived $CD11c^+CD11b^+Gr1^+$.

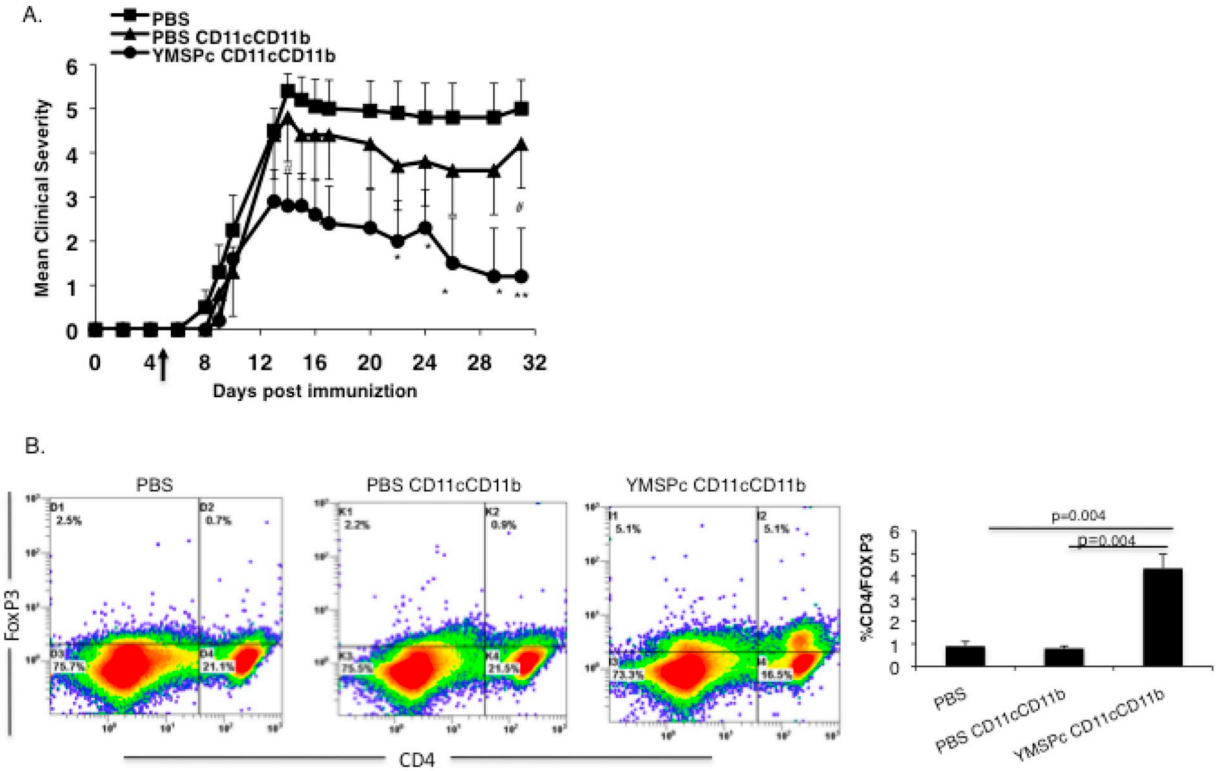
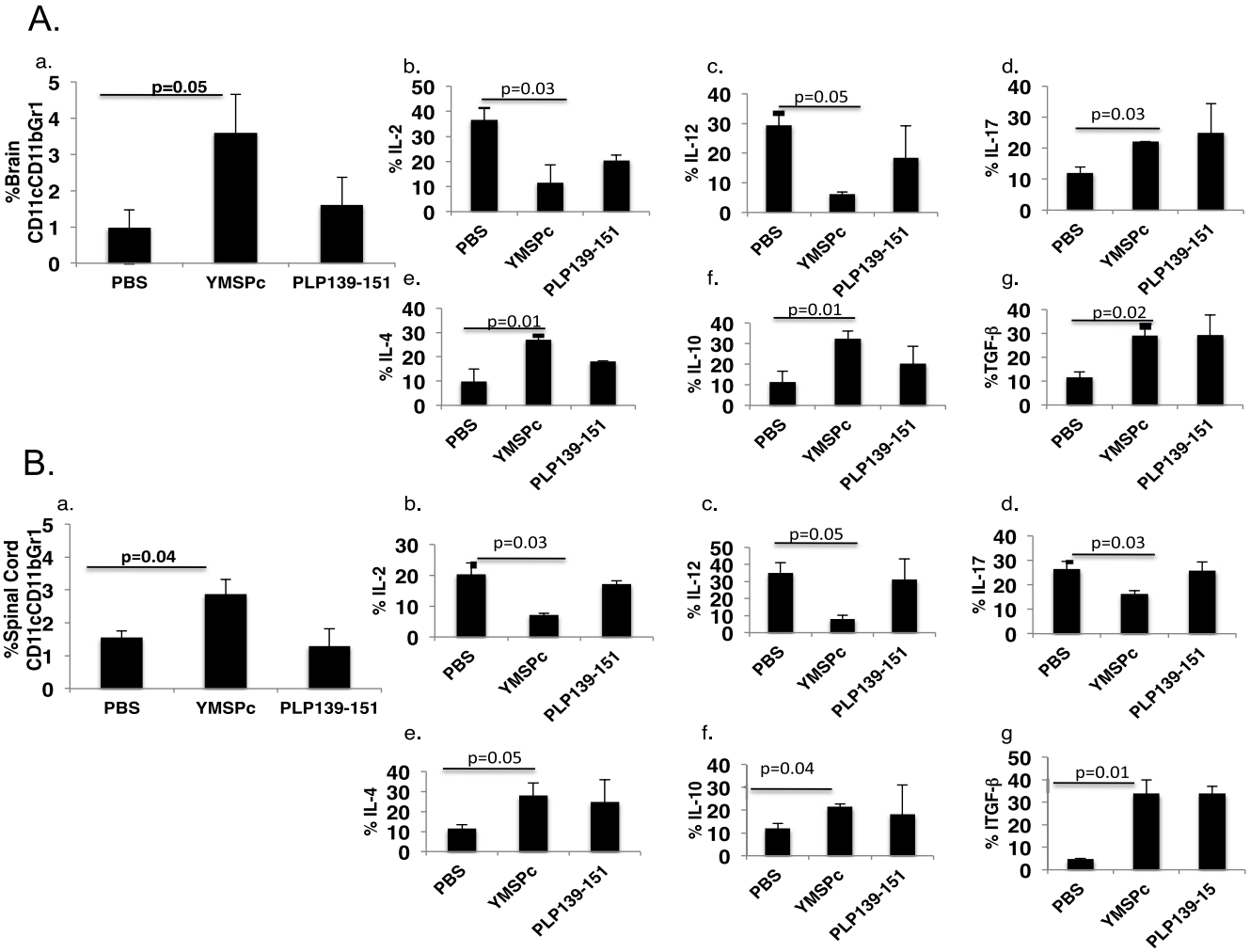
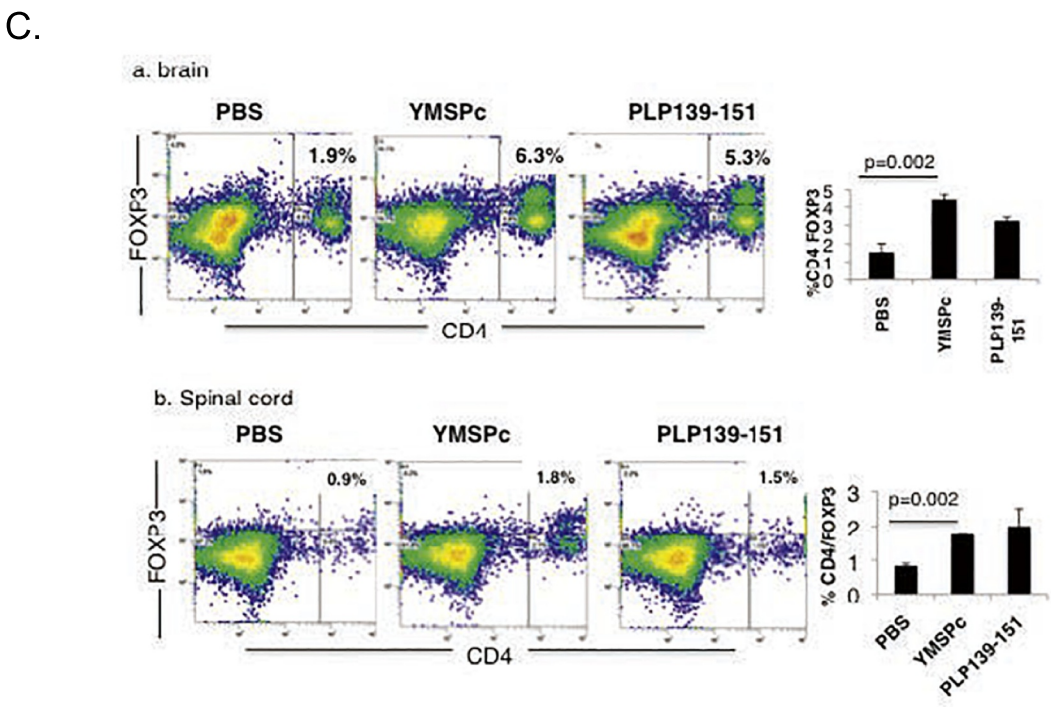



**Fig. 6.** The Suppression of EAE by transfer of $CD11c^+CD11b^+Gr1^+$ DCs spleen derived Y-MSPc treated mice was associated with upregulation of CD4/FoxP3 regulatory T cells in vivo. (A). splenocytes derived from Y-MSPc, or PBS treated mice on day 21 p.i were sorted into $CD11c^+CD11b^+Gr1^+$ DCs using a FACSAria. Sorted $CD11c^+CD11b^+Gr1^+$ DCs from Y-MSPc and PBS treated mice were i.v transferred into EAE mice ($3 \times 10^4$/mouse; $n = 5$/group) on day 5 p.i. One representative experiment of three is shown. *,$p < .05$; **, $p < .005$B; *, values refer to comparisons between Y-MSPc-$CD11c^+CD11b^+$ and PBS. #$p < .05$; # values refer to comparisons between Y-MSPc-$CD11c^+CD11b^+$ and PBS-$CD11c^+CD11b^+$ DCs. (B) On day 32 (termination of the experiment) splenocytes of DCs transferred or EAE control mice were isolated and analyzed for CD4/FoxP3 regulatory T cells by Flow Cytometry. The FACS plots are from one representative experiment, and the panels at the right end are the mean values +/2SD from four independent experiments.




*(caption on next page)*

**Fig. 7.** Increased proportions of $CD11c^+CD11b^+Gr1^+$ are characterized by Th2 cytokines secretion and upregulation of CD4 + FoxP3 regulatory cells in CNS of Y-MSPc treated mice. SJL/J mice were immunized for EAE with PLP139-151. On day 3,5 and 7 post immunization, mice were injected i.v. with Y-MSPc (75 μg/mouse), PLP139-151 (75 μg/mouse) or PBS. On day 21 p.i, MNCs were isolated from (Aa) brain (Ba) spinal cord and proportion of $CD11c^+CD11b^+Gr1^+$ DCs were determined using flow cytometry. (Ab-g, Bb-g) Increased expression of anti inflammatory cytokines in $CD11c^+CD11b^+Gr1^+$ CNS derived DCs from YMSPc treated mice. (Ab-g) Brain, (Bb-g) Spinal cord, $CD11c^+CD11b^+GR1^+$ - DCs - CNS derived were isolated on day 21 p.i from the different treatment group of mice, and intracellular IL-4, IL-10, TGF-β, IL-12, IL-2 and IL-17 was examined by flow cytometry. Data shown is mean values +/2SD from three independent experiments.(Ca, Cb) proportion of CD4 FoxP3 regulatory T in freshly isolated CNS were determined by using flow cytometry. The FACS plots are from one representative experiment, and the panel at the right end are the mean values +/2SD from four independent experiments.

As presented in Fig. 7C(a,b) administration of Y-MSPc was also associated with up-regulation of CD4 + FoxP3 + regulatory T cells in CNS. As demonstrated in Fig. 7C significantly increased proportion of CD4 + FoxP3 + was observed in brain (6.3%; Fig. 7Ca) and in spinal cord (1.8%; Fig. 7Cb) of Y-MSPc treated mice.

### 3.8. Reversal by Y-MSPc of ongoing EAE result in upregulation of $CD11c^+CD11b^+Gr1^+$ DCs in CNS

We previously assessed the effect of the treatment by Y-MSPc on the clinical course of ongoing EAE. We showed that tolerogenic administration of Y-MSPc resulted in an immediate disease amelioration that progressed to almost a full recovery that lasted until the experiment was terminated. In contrast, mice treated with PBS showed a persistent chronic clinical EAE until the experiment was terminated results not shown). Moreover, Administration of PLP139-151 (75 μg/injection) resulted in high frequency of acute hypersensitivity reaction. Here, we investigated whether the potential clinical utility, of Y-MSPc administration in reversal of ongoing EAE is associated with the regulatory function of $CD11c^+CD11b^+Gr1^+$ DCs. SJL/J mice with established EAE induced by active immunization with PLP139-151 were treated (10–12 days after disease onset) every 2–3 days with Y-MSPc or PBS. Tolerogenic administration of Y-MSPc resulted in upregulation of $CD11c^+CD11b^+Gr1^+$ DCs in spleen, however not statistically significant (data no shown). Nevertheless, results in Fig. 8A, B show a significant increase of CNS derived $CD11c^+CD11b^+Gr1^+$ DCs population following administration of Y-MSPc compare to PBS. Increase of about 10% and 12% was detected in brain and spinal cord (respectively) of YMSPc -$CD11c^+CD11b^+Gr1^+$ DCs (Fig. 8A,B) compare to PBS administration. The upregualtion of $CD11c^+CD11b^+Gr1^+$ DCs in Brain and spinal cord following Y-MSPc administration was associated by increase of Th2 and decrease in Th1 cytokines secretion. As shown in Fig. 8A(f-h) and 8B(f-h) higher levels of IL-4, IL-10 and TGF-β were secreted by $CD11c^+CD11b^+Gr1^+$ upon Y-MSPc treatment both in brain and spinal cord compared to PBS treatment. Administration of Y-MSPc was associated with strong reduction in IL-2 (about 50%, 84%; Fig. 8Ab, 8Bb) and INF-γ(about 67%, 78%;Fig. 8Ae,Fig. 8Be) in the brain and spinal cord respectively compared to PBS treated mice. While increase in IL-12 and IL-17 was detected in brain of Y-MSPc $CD11c^+CD11b^+Gr1^+$ mice (40%, 45%Fig. 8Ac,d), reduction in secretion of these cytokines was detected in spinal cord of Y-MSPc $CD11c^+CD11b^+Gr1^+$ mice (73%, 60%;Fig. 8Bc,d) compare to CNS derived PBS $CD11c^+CD11b^+Gr1^+$ DCs. Whether the IL-17 secreted by YMSPc-$CD11c^+CD11b^+Gr1^+$ DCs may function as regulatory rather than pro-inflammatory cytokine is currently under investigation. Elevated Th2 cytokines secretion were detected in CNS

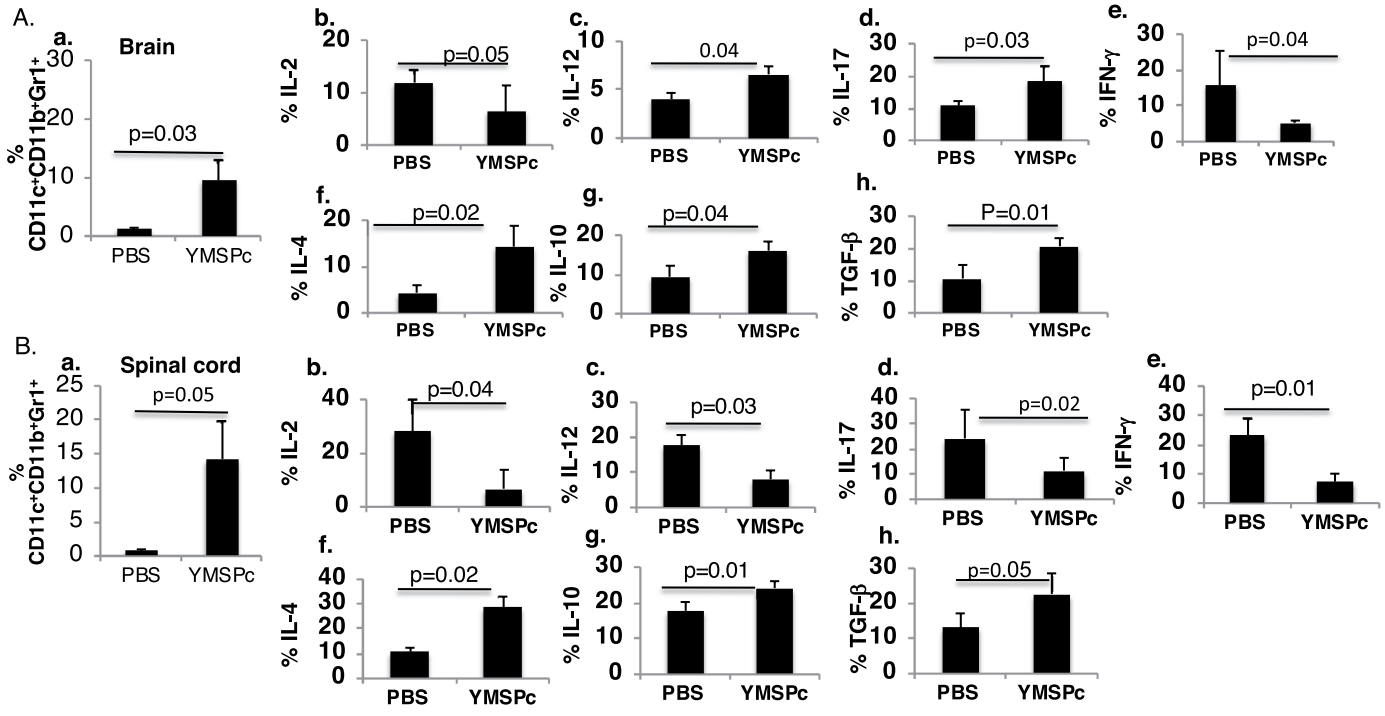



**Fig. 8.** Reversal of ongoing EAE by Y-MSPc ends in upregulation of $CD11c^+CD11b^+Gr1^+$ DCs characterized by Th2 cytokines secretion. SJL/J mice were immunized with PLP139-151 in CFA for EAE induction. On day 15 after immunization, mice with ongoing EAE were grouped (with equal mean clinical score of,2/group; n = 5 mice/group) and injected i.v. with Y-MSPc (75 μg/mouse), or PBS on days 16,18,20,and 22. On day 28, MNCs were isolated from (Aa) brain (Ba) spinal cord and proportion of $CD11c^+CD11b^+Gr1^+$ DCs were determined using flow cytometry. (Ab-g) Brain, (Bb-g) Spinal cord, $CD11c^+CD11b^+Gr1^+$ - DCs - CNS derived were isolated on day 28 p.i from the different treatment group of mice, and intracellular IL-4, IL-10, TGF-β, IL-12, IL-2 and IL-17 was examined by flow cytometry. Data shown is mean values +/2SD from three independent experiments.(Ba, Bb).

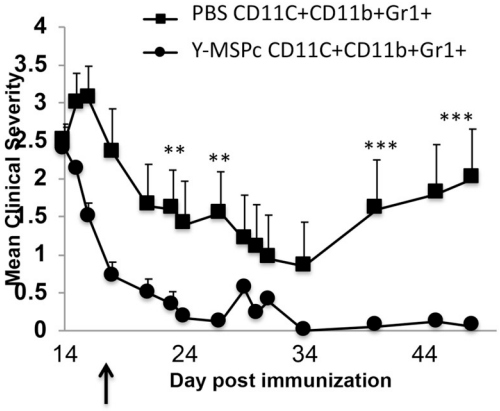



**Fig. 9.** Reversal of ongoing EAE by transfer Y-MSPc - CNS $CD11c^+CD11b^+Gr1+$ derived DCs. SJL/J mice were immunized with PLP139-151 in CFA for EAE induction. On day 15 after immunization, mice with ongoing EAE were grouped (with equal mean clinical score of,2/group; n = 5 mice/group) and injected i.v. with Y-MSPc (75 μg/mouse), or PBS on days 16,18,20 and 22. On day 28, $CD11c^+CD11b^+Gr1+$ were isolated from the CNS by flow cytometry and transferred into EAE mice ($3 \times 10^5$/ mouse, $n = 10$ per group). **,$p < .005$; ***, $p < .0005$.

$CD11c^+CD11b^+Gr1^+$ following YMSPc administration. As shown in Fig. 8A(f-h), 8B(f-h) significantly elevated levels of Th2 cytokines; IL-4, IL-10 and TGF-b were detected upon Y-MSPc administration, suggesting that CNS -Y-MSPc $CD11c^+CD11b^+Gr1+$ exhibit regulatory function, which may result in induction of immune tolerance. Thus, we determine the functionality of CNS- Y-MSPc-$CD11c^+CD11b^+Gr1$ DCs in reversing of reverse ongoing EAE. SJL/J mice with established chronic EAE induced by active immunization with PLP139-151 were transferred with CNS $CD11c^+CD11b^+Gr1+$ DCs derived from Y-MSPc or PBS treated mice. As shown in Fig. 9, transfer of CNS- Y-MSPc-$CD11c^+CD11b^+Gr1$ DCs resulted in an immediate disease amelioration that progressed to almost a full recovery that lasted until the experiment was terminated. In contrast, transfer of CNS-PBS $CD11c^+CD11b^+Gr1$ DCs resulted in persistent chronic clinical EAE.

## 4. Discussion

The present work was undertaken to better understand the peripheral regulatory mechanisms that develop during treatment with 'multi-epitope targeting" agent. Such a "multi-epitope-targeted" approach to immune-specific therapy for MS-like disease was investigated using the artificial multi-epitope protein, Y-MSPc, and as control a cocktail of human myelin peptides (huPEP mix), as "multi-epitope-targeting" agents. As was previously shown (Kaushansky et al., 2011), treatment with Y-MSPc was consistently more effective than treatment with relevant peptide cocktail, both in suppressing the development of "complex EAE" and in ameliorating ongoing disease, via the induction of more efficacious and longer lasting peripheral regulatory mechanisms which include cytokine shift, anergy, and induction of CD4 + Foxp3 + CTLA4+ regulatory T-cells (Kaushansky et al., 2011); and, of most significance for its potential clinical utility, the Y-MSPc was also more effective in the reversal of ongoing "complex EAE" associated with multiple pathogenic anti-myelin autoimmunity (Kaushansky et al., 2011). In the present study, we identify a unique subset of dendritic-cells (DCs), $CD11c+CD11b+Gr1+$-myeloid derived DCs, that play an important role in EAE suppression and treatment following tolerogenic administration of Y-MSPc.

Regulatory DCs are promising tools for clinical application in transplantation, autoimmunity, or allergies (Florez-Grau et al., 2018). Tolerogenic DCs are characterized by low constitutive expression of positive co-stimulatory molecules, as well as by their ability to suppress a broad range of effector T cell responses (Audiger et al., 2017). Recently, Li et al., demonstrated that systemic administration of auto-antigen induced CD11c + CD11b + tolerogenic DCs population (Li et al., 2008) that can suppress EAE in vivo. The present investigation demonstrates up-regulation of CD11c + CD11b + following systemic administration of multi epitope protein and not following administration of individual peptide or peptides mix. In contrast to Li et al. study (Li et al., 2008)in which administration of MOG 200 μg/mouse to MOG EAE induced mice resulted in up-regulation of CD11c + CD11b + cells, in this study administration of single peptide, PLP139-151, did not result in any increase in CD11c + CD11b + cells, it might be derived from the use of a low peptide concentration (75 μg/mouse). Nevertheless, in our hands, several mice died from anaphylactic shock following treatment with higher concentration of an individual peptide or peptide mixture.

The function of each distinct DC subset is determined by its anatomic microenvironment in combination with its surface phenotype (Li et al., 2008). In the current study, we characterized a unique regulatory sub population of DCs which highly express $CD11c+CD11b+Gr1+$. Interestingly, all $CD11c+CD11b+$DCs, following the different treatments (Fig. 2C) expressed high levels of Gr1 +. In mice, several subpopulations of myeloid-derived cells are positive for the marker Gr-1, including plasmacytoid DC, CCR2+ inflammatory monocytes, and myeloid suppressor monocytes (Egan et al., 2008). Recently, a specific myeloid immunoregulatory cell population, named natural suppressive cells, exhibiting a $GR1^+$ $CD11b^+$ phenotype, was described in tumor-bearing rodents and was recruited during Chagas' disease. This population has been actively involved in acute hypo-responsiveness of T and B cells throughout the synthesis of a large amount of nitric oxide (NO) at the place of infection (Voisin et al., 2004). Here, we report for the first time (on) Gr1 marker expression on subpopulation of DCs, $CD11c+CD11b+Gr1+$. Theses unique subset of DCs,$CD11c+CD11b+Gr1+$ cells, but not CD11c-$CD11b+Gr1+$, showed a regulatory activity only following Y-MSPc administration. Administration of Y-MSPc to EAE induced mice resulted in up-regulation of $CD11c+CD11b+Gr1+$ cells that secreted significantly more IL-10, TGF-β and IL-4 anti-inflammatory cytokines and less Th1- pro-inflammatory cytokines like, IL-12 and IL-2, than treatment with individual peptide or peptide mix, both in the spleen and in the CNS. The expression of co-stimulatory molecules, I-A, CD80 and CD86 was down-regulated following Y-MSPc administration, indicating (on) immature phenotype of DCs, which are thought to be involved in the induction and maintenance of peripheral tolerance (Yu et al., 1998; Zhong et al., 2002). Furthermore these DCs, following Y-MSPc administration inhibited proliferation of pathogenic CD4+ cells in-vitro that were stimulated with encephalitogenic myelin peptide. These findings suggest that $CD11c^+CD11b^+Gr1^+$ DCs, following treatment with Y-MSPc, may be a unique DC subset that mediates T-cell tolerance in EAE in-vivo.

Three types of regulatory CD4+ T cells have been reported: $CD4^+(CD25^+)FoxP3^+$ Tregs, IL-10 producing Tr1 cells and TGF-β-producing Th3 cells (Gaur et al., 1992). We demonstrated that, following systemic administration of Y-MSPc and not individual peptide or PBS to PLP139-151 EAE induced mice, resulted in increased proportion of $CD4^+FoxP3^+$ in spleen and CNS. Here we suggest that this up-regulation of $CD4^+FoxP3^+$ regulatory cells following administration of Y-MSPc is associated with up regulation of regulatory $CD11c^+CD11b^+Gr1^+$ DCs. We confirmed that $CD11c^+CD11b^+Gr1^+$ DCs have tolerogenic activity by showing their capacity to suppress and reverse EAE in-vivo:adoptive transfer of Y-MSPc – spleen derived -$CD11c^+CD11b^+Gr1^+$ DCs ended with decrease in clinical manifestation of PLP139-151 induced EAE mice, and with significant increase of $CD4^+FoxP3^+$ regulatory cells in the termination of the experiment, compare to adoptive transfer of PBS derived $CD11c^+CD11b^+Gr1^+$ DCs, suggesting that Y-MSPc- $CD11c^+CD11b^+Gr1^+$ DCs may play a crucial role in generating and increasing the proportion of regulatory T-cells. Several well-designed studies have demonstrated that immature DCs, semi-immature DCs, fully mature DCs, or granulocyte-macrophage

colony-stimulating factor-pulsed DCs could cause T cells to differentiate into T regulatory cells in the periphery (Akbari et al., 2001; Dhodapkar et al., 2001; Vasu et al., 2003; Verhasselt et al., 2004). Furthermore, adaptive transfer of Y-MSPc- $CD11c^+CD11b^+Gr1^+$ DCs resulted in reversing of ongoing EAE, supporting that this subset of DCs is responsible for the protection against T cell mediated autoimmune response.

One of possible mechanisms in which Y-MSPc-$CD11c^+CD11b^+Gr1^+$ DCs may induce regulatory T cells is by secretion of high levels of IL-10. The presence of IL-10 has been identified in numerous settings of tolerance. Secretion of IL-10 by DCs is necessary for tolerance in a variety of models of Treg differentiation (Akbari et al., 2001; McGuirk et al., 2002; Wakkach et al., 2003). Svensson et al. proved that spleen derived stromal cells promote selective development of $CD11c^{low}CD11b^{high}CD45^+$ IL-10 producing regulatory DCs from lineage-negative c-kit+ progenitor cells (Svensson and Kaye, 2006). These DC have the capacity to suppress T cells responses and induce IL-10 producing T reg in vitro and to induce Ag-specific tolerance in vivo (Svensson and Kaye, 2006; Zhang et al., 2004). Alternative mechanism by which Y-MSPc- $CD11c^+CD11b^+Gr1^+$ DCs may induce regulatory T cells is by secretion of high levels of TGF-β. TGF-β is unique among cytokines in that it can induce Foxp3 expression and a Treg differentiation. A strong argument for the importance of TGF-β production by DCs has come from animals with a DC-restricted deletion of the TGF-β-activating integrin, $\alpha_v\beta_8$. These mutant mice develop autoimmunity similar to animals in which DCs are chronically depleted or TGF-β-Receptor? signaling is dysfunctional in T cells, suggesting that DCs are important to ensure the bioavailability of active TGF-β (Birnberg et al., 2008; Gorelik and Flavell, 2000; Kim et al., 2006; Ohnmacht et al., 2009; Travis et al., 2007).

Interestingly, high levels of IL-17 were detected following Y-MSPc administration in $CD11c^+CD11b^+Gr1^+$ DCs in spleen and brain. IL-17–producing $CD4^+$ T helper (Th17) cells have recently been defined as a unique subset of proinflammatory helper cells whose development depends on signaling initiated by IL-6 and TGF-β, autocrine activity of IL-21, activation of STAT3, and induction of the orphan nuclear receptor RORγt (Voo et al., 2009). However, IL-17 can also be produced by several other innate immune cell types, such as lymphoid tissue inducer cells, natural killer and natural killer T cells, macrophages and Paneth cells. The functional importance of the IL-17 produced by these cell types during inflammation is not very well characterized (Jin and Dong, 2013).Voo et al. study (Voo et al., 2009)reported that human peripheral blood and lymphoid tissue contain a significant number of $CD4^+FOXP3^+$ T cells that express CCR6 and have the capacity to produce IL-17 upon activation. These cells co-express FoxP3 and RORγt transcription factors. The $CD4^+FoxP3^+CCR6^+$ IL-17–producing cells strongly inhibit the proliferation of $CD4^+$ responder T cells. $CD4^+CD25^{high}$-derived T-cell clones express FoxP3, RORγt, and IL-17 and maintain their suppressive function via a cell-cell contact mechanism. Whether the IL-17 secreted by Y-MSPc-$CD11c^+CD11b^+Gr1^+$ DCs may function as regulatory rather than pro-inflammatory cytokine is currently under investigation.

Among other tolerogenic mechanisms associated with immunospecific therapy by administration of multi epitope targeting (Y-MSPc), which include cytokine shift, anergy, and induction of CD4 + Foxp3 + CTLA4+ regulatory T-cells, these findings suggest that the immune tolerance induced by Y-MSPc administration is associated also by an increase in tolerogenic $CD11c^+CD11b^+Gr1^+$ DCs. These DCs in the CNS and spleen generated as a part of systemic tolerance dramatically suppress the development of EAE and moreover reverse ongoing EAE by secretion of anti inflammatory cytokines and induction of regulatory T cells.

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