PhD Thesis

Delineation of novel immunoregulatory mechanisms in autoimmune diseases: the role of plasmacytoid dendritic cells in the initiation of autoimmune response

Marianna Ioannou

Laboratory of Autoimmunity and Inflammation

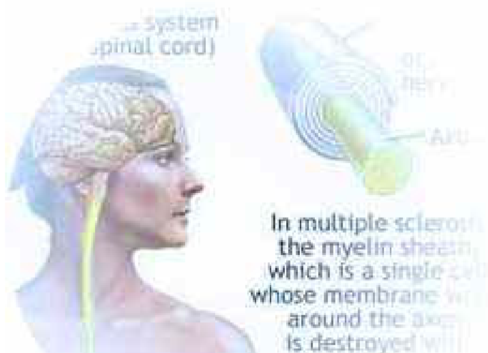
Post Graduate Program of ‘Molecular Biology and Biomedicine’

University of Crete School of Medicine

Heraklion 2011

Διδακτορική Διατριβή

Περιγραφή νέων μηχανισμών ρύθμισης της ανοσολογικής απόκρισης στα αυτοάνοσα νοσήματα: ο ρόλος των πλασματοκυτταροειδών δενδριτικών κυττάρων στην έναρξη της ανοσολογικής απόκρισης



Μαριάννα Ιωάννου

Εργαστήριο Αυτοανοσίας και φλεγμονής

Μεταπτυχιακό πρόγραμμα Μοριακής Βιολογίας και Βιοϊατρικής

Πανεπιστήμιο Κρήτης- Ιατρική Σχολή

Ηράκλειο 2011

PhD Thesis

Delineation of novel immunoregulatory mechanisms in autoimmune diseases: the role of plasmacytoid dendritic cells (pDCs) in the initiation of autoimmune response.

Project Supervisor: Panayotis Verginis, PhD

Supervising Professor: Dimitrios T. Boumpas, MD

Members of advisory committee:

Boumpas D. Professor, University of Crete -Medical school

Tsatsanis C. Assistant Professor, University of Crete –Medical school

Iliopoulos A. Associate Professor, University of Crete -Medical school

Members of examination committee:

Boumpas D. Professor University of Crete -Medical school

Tsatsanis C. Assistant Professor, University of Crete –Medical school

Iliopoulos A. Associate Professor, University of Crete -Medical school

Sidiropoulos P. Lecturer, University of Crete -Medical school

Athanasaki I. Professor, Biology Department

Mamalaki K. Researcher A’ IMBB (FORTH)

Acknowledgements…

This dissertation would not have been possible without the guidance and the help of several individuals who contributed in the preparation and completion of this study.

First and foremost, my utmost gratitude to Panos Verginis, my project supervisor whose sincerity and encouragement I will never forget. Panos has been my inspiration as I hurdle all the obstacles in the completion of this research work. He enabled me to develop an understanding of the subject and to wander in the world of immunology. This project would be nothing without the enthusiasm and imagination from you. You are the best supervisor I could have!

I am also grateful to my Professor, Dimitrios Boumpas. It was an honor to work in your laboratory and have your support. Thank you for the understanding, encouragement, guidance and helpful suggestions.

I would also like to thank my colleagues and friends, especially those who work together in the lab: Irene, Elena, Themis, Andriana, Hlias, Dimitra, Christiana, Melina, Amalia, George B. and Magda. They make me realize the value of working together as a team, especially in this working environment, which challenges us every minute. Not to forget Dimitris, Sofia, George and Marios for their support and friendship. I have great moments to remember!

A very special thanks to Katerina, Panos, Anastasia, and Dafni who were always next to me, giving their support to me in all aspects of life and tolerate me.

I would further like to acknowledge several individuals for their assistance and support throughout my Thesis: Dr. G Chamilos, Dr. A.Gravanis, Dr. V. Mastorodemos, Prof. A Plaitakis, as well as Xaroula, Niki and Takis the sorting facility at IMBB. Financial support to undertake my Ph.D. degree, of which this thesis is an integral part, was provided by Manassaki's and Bodossaki's Foundations.

These acknowledgments would not be complete without thanking my family and in particular my parents for providing everything!

….*Dedicated to my parents Nikolao and Lefki*

Table of Contents

1. ABSTRACT 7

1. ΠΕΡΙΛΗΨΗ 9

2. ABBREVIATIONS 12

3. INTRODUCTION 15

3.1 Tolerance and Autoimmunity 15

3.2 Multiple Sclerosis (MS) 24

3.3 Dendritic cells (DCs) 29

3.4 Myeloid Derived Suppressor Cells (MDSCs) 34

4. OBJECTIVES 44

5. MATERIAL AND METHODS 45

6. RESULTS 53

7. DISCUSSION 85

8. FUTURE DIRECTIONS 92

9. REFERENCES 103

APPENDIX I 117

APPENDIX II 118

1. ABSTRACT

One of the main unresolved issues in autoimmune diseases is the restoration of immune homeostasis and self-tolerance. Although the variety of therapeutic targets is enormous, a large number of patients with autoimmune syndromes fail to either respond to current therapy or to achieve long-lasting remission after its cessation. Thus, despite our increasing knowledge of the cellular and molecular processes involved in the development of autoimmune diseases, the most effective targets for immunotherapy remain unknown. The goal therefore, would be to develop novel therapeutic protocols that could cure and not only palliate autoimmunity by resolving inflammation and establishing lasting tolerance. To achieve this goal it is important to decipher the mechanisms involved in the initiation and perpetuation of autoimmune diseases and in particular to understand how the different cell subsets and molecules participate in such processes.

In this Thesis, we focused on a dendritic cell subset, the plasmacytoid dendritic cells (pDCs), that have been shown to have an instrumental role in the regulation of autoimmune diseases. pDCs represent a unique DC subset, capable of inducing immunity or tolerance. However, the contribution of these cells in the priming of an autoimmune response remains elusive.

Our aims were (a) to investigate the role of pDCs during the priming of the autoimmune response and (b) to explore the mechanism involved in the pDC-mediated regulation of the autoimmune response.

In this study, we demonstrate that pDCs depletion during (MOG)-induced EAE resulted in less severe disease compared to control mice. pDC-depleted/MOG-immunized mice, had reduced cellularity in the draining lymph nodes (dLNs) which associated with decreased frequency of IA^b-MOG^+CD4^+ T cells than in control mice. DLNs MOG-specific T cells, from pDC-depleted/MOG-immunized mice showed impaired proliferation and decreased IFN-γ secretion in recall assays in vitro. Impaired T cell priming in pDC-depleted mice, was not attributed to defective recruitment of conventional DCs in the dLNs or impaired development of $CD4^+$ T regulatory cells. Of interest, pDC-depleted mice had a markedly

increase in the frequency and absolute numbers of an immature population of myeloid cells (myeloid-derived suppressor cells -MDSCs) in the dLNs, spleen and bone marrow, implying that expansion of MDSCs after pDC depletion may account for the amelioration of the autoimmune pathology.

To further explore the roles of MDSCs in the resolution of autoimmune inflammation and reestablishment of self tolerance, we did a series of experiment.

We found that granulocytic MDSCs (G-MDSCs), were abundantly accumulated within the peripheral lymphoid compartments and target organ of mice with EAE, prior to disease remission. *In vivo* transfer of G-MDSCs ameliorated EAE, significantly decreased demyelination and delayed disease onset, through inhibition of encephalitogenic Th1 and Th17 immune responses. Exposure of G-MDSCs to the autoimmune milieu led to up-regulation of the programmed death 1 ligand (PD-L1) that was required for the G-MDSC-mediated suppressive function both *in vitro* and *in vivo.* Importantly, MDSCs were enriched in the periphery of subjects with active multiple sclerosis (MS) and suppressed the activation and proliferation of autologous $CD4^+$ T cells *ex vivo*. Collectively, this study reveals a crucial role of MDSCs in the regulation of EAE and MS.

Overall, our data provide novel insights into the regulatory pathways of MS as well as the mechanisms that limit inflammation during autoimmune diseases. Further understanding on the immunosuppressive mechanisms of MDSCs, may open new avenues for the development of more specific cell-based therapies in patients with autoimmune inflammatory diseases.

Keywords: pDCs, MDSCs, G-MDSCs, EAE, MS, PD-L1, Immunoregulation

1. ΠΕΡΙΛΗΨΗ

Η αποκατάσταση της ομοιόστασης και της αυτοανοχής συνιστά ένα από τα βασικότερα ζητήματα στην αυτοανοσία . Παρά την ποικιλία των μέχρι τώρα διαθέσιμών θεραπευτικών στόχων, ένας μεγάλος αριθμός ασθενών με αυτοάνοσα νοσήματα αποτυγχάνει να ανταποκριθεί σε αυτες. Για το λόγο αυτό κρίνεται αναγκαία η ανάπτυξη νέων ανοσοθεραπειών, οι οποίες θα στοχεύουν πιο εξειδικευμένα στην καταστολή της φλεγμονής και στην αποκατάσταση της ομοιόστασης. Η επίτευξη αυτού του στόχου απαιτεί την καλύτερη κατανόηση των μηχανισμών που εμπλέκονται στην έναρξη, καθώς και στην εξέλιξη των αυτοάνοσων νοσημάτων. Ειδικότερα, η κατανόηση του ρόλου των διαφορετικών κυτταρικών υποπληθυσμών, αλλά και των παραγόντων που εκκρίνονται κατά τη διάρκεια της φλεγμονής, θα μπορούσε να οδηγήσεο στο σχεδιασμό νέων πιο αποτελεσματικών θεραπευτικών στόχων.

Σε αυτή την διατριβή, το ενδιαφέρον εστιάσθηκε σε έναν υποπληθυσμό δενδριτικών κυττάρων, τα αναφερόμενα ως πλασματοκυτταροειδή δενδριτικά κύτταρα (pDCs), τα οποία παίζουν καθοριστικό ρόλο στη ρύθμιση των αυτοάνοσων νοσημάτων. Τα pDCs συνιστούν ένα μοναδικό υποπληθυσμό δενδριτικών κυττάρων, ικανών να προάγουν είτε την αυτοανοσία είτε την ανοχή. Ωστόσο, η συνεισφορά αυτών των κυττάρων στην έναρξη της αυτοάνοσης απόκρισης δεν έχει διερευνηθεί. Οι στόχοι ήταν (α) η διερεύνηση του ρόλου των pDCs κατά την έναρξη της αυτοάνοσης απόκρισης και (β) η ταυτοποίηση του μηχανισμού ρύθμισης της αυτοάνοσης απόκρισης απο τα pDCs.

Η παρούσα μελέτη απέδειξε οτι η απαλοιφή των pDCs κατα την διάρκεια της αυτοάνοσης εγκεφαλίτιδας (επαγόμενης από το αυτοντιγόνο MOG) σε ποντίκια, επέφερε μείωση της ασθένειας συγκριτικά με την ομάδα ελέγχου. Ειδικότερα, απουσία των pDCs κατά την έναρξη της αυτοάνοσης εγκεφαλίτιδας, οδηγησε σε μειωμένη κυτταροβρίθεια στους επιχώριους λεμφαδένες αυτων των ποντικών σε σύγκριση με τα ποντίκια ελέγχου, συνοδευόμενη από την μειωμένη συχνότητα αντιγονοειδικών για το MOG T κυτταρων.In vitro πειράματα πολλαπλασιασμού έδειξαν μειωμένο πολλαπλασιασμό και ενεργοποίηση των αντιγονοειδικών T κυττάρων, στους επιχώριουσ λεμφαδένες των ποντικών που δεν διέθεταν pDCs, όταν αυτά συγκρίθηκαν

με τα ποντίκια που είχαν τον φυσιολογικό πληθυσμό pDCs. Η ανεπαρκής ενεργοποίηση των Τ κυττάρων στα ποντίκια που έγινε η απαλοιφή των pDCs, δεν οφειλόταν σε τυχόν παθολογική ανάπτυξη των Τ ρυθμιστικών κυττάρων (Treg) αλλά ούτε σε ελαττωματική μετανάστευση των δενδριτικών κυττάρων μυελικής σειράς(cDCs) στους παροχετευτικούς λεμφαδένες.

Ωστόσο, τα ποντίκια στα οποία απαλοίφθηκαν τα pDCs, παρουσίασαν ιδιαίτερα σημαντική αύξηση των κατασταλτικών κύτταρων μυελικής προέλευσης (Myeloid derived suppressor cells) στους επιχώριους λεμφαδένες, στον σπλήνα και στον μυελό των οστών. Τα αποτελέσματα αυτά υποδηλώνουν ότι η αύξηση των MDSCs, στα ποντίκια που απαλοίφθηκαν τα pDCs, πιθανών να εξηγεί την πιο ήπια ασθένεια σε αυτά τα ζώα.

Συνολικά, τα δεδομένα αυτά τονίζουν τον σημαντικό ρόλο των pDCs κατά την έναρξη της αυτοανοσίας. Ωστόσο, απαιτούνατι περισσότερα πειράματα, ούτως ώστε να κατανοηθεί πλήρως πως η απουσία των pDCs επηρεάζει την αύξηση των MDSCs.

Συνεχίζοντας την διερεύνηση, δείχθηκε ότι ένας υποπληθυσμός των MDSCs με μορφολογία παρόμοια με αυτη των κοκκιωκυττάρων τα λεγόμενα G-MDSCs, συσσωρευόταν στα περιφερικά λεμφικά όργανα καθώς και στο νωτιάιο μυελό των ποντικών με αυτοάνοση εγκεφαλίτιδα, πρίν αυτά εισέλθουν στην φάση της ύφεσης. Η αυτόλογη μεταφορά των G-MDSCs σε ποντίκια με ΕΑΕ, κατέστειλε τα αυτοδραστικά Th1 καιTh17 κύτταρα και μείωσε σημαντικά τα συμπτώματα της ασθένειας. Περαιτέρω μελέτη του μηχανισμού δράσης των G-MDSCs *in vitro* και *in vivo*, έδειξε ότι για την κατασταλτική τους ικανότητα, είναι απαραίτητη η έκφραση του μορίου PD-L1 στην επιφάνεια τους, ενός αρνητικού ρυθμιστή της ενεργοποίησης των Τ κυττάρων. Μελέτη σε ανθρώπινα δείγματα ασθενών με πολλαπλή σκλήρυνση, έδειξε ότι τα G-MDSCs ήταν αυξημένα στην περιφέρεια των ασθενών με ενεργή νόσο, ενω μειώνονταν σημαντικά όταν οι ασθενείς εισέρχονταν στην φάση της ύφεσης. Μετά την απομόνωση των MDSCs από τους ασθενείς με ενεργή νόσο διαπιστώθηκε ότι αυτά τα κύτταρα μπορούσαν να καταστείλουν την ενεργοποίηση και τον πολλαπλασιασμό των αυτόλογων $CD4^{+}$ κυττάρων ex vivo.

Συλλογικά τα δεδομένα αυτά, αποκαλύπτουν τον κρίσιμο ρόλο των MDSCs στην αυτοάνοση εγκεφαλίτιδα στα ποντίκα καθως και στην πολλαπλή σκλήρυνση στον άνθρωπο. Συνολικά αυτή η μελέτη παρέχει ενδείξεις για την ύπαρξη νέων ρυθμιστικών

μονοπατιών για τον περιορισμό της φλεγμονής στα αυτοάνοσα νοσηµάτα. Η περαιτέρω κατανόηση των ανοσοκατασταλτικών μηχανισμών που χρησιμοποιούν τα MDSCs, θα μπορούσε να υποβοηθήσει στον σχεδιασμό καινοτόμων, πιο ειδικών θεραπειών για την θεραπεία των αυτοάνοσων νοσημάτων.

Λέξεις κλειδιά: pDCs, MDSCs, G-MDSCs, EAE, MS, PD-L1, Ανοσορύθμιση

2. ABBREVIATIONS

IFNγ	Interferon γ
IL-17	Interleukin-17
IL-35	Interleukin-35
IL-10	Interleukin-10
IL-2	Interleukin-2
IL-12	Interleukin-12
IL-6	Interleukin-6
IL-1	Interleukin-1
IL-21	Interleukin-21
IL-23	Interleukin-23
VEGF	Vascular Endothelial Growth Factor
GM-CSF	Granulocyte Macrophage- Colony Stimulating Factor
TNF	Tumor Necrosis Factor
TGFβ	Transforming Growth Factor β
HLA	Human Leukocyte Antigen
MHC	Major Histocompatibility Complex
LAG-3	Lymphocyte-Activation Gene 3
Cox-2	Cycloxygenase-2
Myd88	Myeloid Differentiation Primary Response Gene(88)
NF-kB	Nuclear Factor kB
MAPK	Mitogen-Activated Protein Kinase
CD	Cluster of Differentiation
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
IRF	IFN Regulatory Factor
Tim-3	T cell immunoglobulin mucin-3

Gal-9	Galectin-9
Foxp3	Forkhead box P3
IDO	Indoleamine 2,3-Dioxygenase
TLR	Toll like Receptor
MS	Multiple Sclerosis
SLE	Sustemic Lupus Eryuthomatosus
IPEX	Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked inheritance
EAE	Experimental Autoimmune Encephalomyelitis
RR	Relapsing-Remitting
CNS	Central Nervous System
DLN	Draining Lymph Nodes
PLP	Myelin ProteoLipid Protein
MBP	Myelin Basic Protein,
MOG	Myelin Oligodendrocyte Protein
CSF	Cerebrospinal Fluid
NOD	Nonobese Diabetic (mice)
PGE2	Prostaglandin E2
SCF	Stem Cell Factor
STAT	Signal Transducers and Activators of Transcription
INOs	inducible Nitric Oxide Synthase
NO	Nitric Oxide
ROS	Reactive Oxygen Species
Arg	Arginase
LPS	Lipopolysaccharide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate

TCR	T Cell Receptor
PD-L1	Programmed Death Ligand 1
PD-L2	Programmed Death Ligand 2
PD-1	Programmed Death 1
MDSCs	Myeloid Derived Suppressor Cells
G-MDSCs	Granulocytic MDSCs
M-MDSCs	Monocytic-MDSCs
Brdu	Bromodeoxyuridine
MCP-1	Monocyte Chemoattractant Protein-1 (CCL2)

3. INTRODUCTION

3.1 Tolerance and Autoimmunity

The immune system has evolved mechanisms to recognize and eliminate threats, caused by foreign pathogens, as well as to protect against self-destruction. The immunologic unresponsiveness to self-constituents is called self-tolerance. Self-tolerance is established and maintained through two fundamental mechanisms: (*a*) elimination of self-reactive cells in the thymus during selection (central tolerance) and (*b*) generation of a variety of peripheral regulatory mechanisms to control potentially pathogenic self-reactive cells that escape the thymus (peripheral tolerance). Break-down of the peripheral mechanisms of tolerance could lead to activation of self-reactive cells and development of autoimmune diseases. The mechanisms involved in the induction of self-tolerance are discussed below.

3.1.1 Mechanisms of Central T cell tolerance

Central tolerance of T cells is achieved through deletion of self-reactive T cells in the thymus. Lymphocytes migrate from the bone marrow to the thymus, where they encounter peptides derived from endogenous proteins bound to major-histocompatibility complex (MHC) molecules (Delves and Roitt, 2000). T cells whose receptors do not recognize peptide–MHC complexes, do not receive survival signals and these cells therefore die in the thymus. Moreover, T cells with high-affinity receptors for MHC-peptide complexes undergo apoptosis and die in a process called negative selection. The remaining T cells, which have receptors with an intermediate affinity for such complexes, mature in the thymus and migrate to the periphery, a process referred as positive selection (**Figure 1**). The surviving T lymphocytes form the peripheral T cell repertoire where additional mechanisms operate in order to keep these cells silent.

3.1.2 Mechanisms of Peripheral T cell tolerance

To date four mechanisms of peripheral tolerance have been described: Ignorance, deletion, anergy and suppression.

Ignorance

Immunological ignorance referred to a state in which T cells coexist with antigen without being affected by it. Several mechanisms could promote immunologic ignorance: the level of the antigen may be below the threshold required to induce the activation or deletion of T cells, (Akkaraju et al., 1997; Ferber et al., 1994) or antigens may be physically separated from T cells (**Figure 2**) (Barker and Billingham, 1977). The best example of ignorance mechanism is illustrated at the blood- brain barrier that separates circulating blood and brain extracellular fluid (BECF) in the central nervous system (CNS), preventing therefore cells from the blood entering the brain.

Deletion

Another mechanism of peripheral tolerance is achieved through deletion of T cells. The presentation of antigens in the absence of co-stimulation not only fails to prime T cells but can also delete them (Akkaraju et al., 1997) (Ferber et al., 1994) (Critchfield et al., 1994) The death of T cells is also mediated by the pathway involving Fas (also called CD95) and its ligand. Engagement of the Fas receptor induces apoptosis in Fas-positive cells (Suda et al., 1993) . The importance of this mechanism is highlighted by the fact that Fas-/- mice (MRL) spontaneously develop several autoimmune syndromes likes SLE {Cohen, 1991 #1069} and that patients with defective Fas have a severe lymphoproliferative disease (Rieux-Laucat et al., 1995) (Fisher et al., 1995) .

Anergy

T cell anergy is another mechanism of peripheral tolerance in which the lymphocytes are functionally inactivated following an antigen encounter, but remain alive for an extended period of time in a hyporesponsive state. Anergy is induced when T cells are stimulated in the absence of co-stimulation (e.g decrease co-stimulatory molecules CD80, CD86) or in the presence of high co-inhibition [e.g., (CTLA-4)]. For example cytotoxic T lymphocyte antigen 4 (CTLA4; CD152) is an inhibitory receptor expressed by T cells that recognizes the costimulatory molecules B7-1 (CD80) and B7-2 (CD86), the ligation of which shuts off T-cell responses and promotes long-lived anergy (Salomon and Bluestone, 2001). Knockout of the inhibitory receptor CTLA-4 interferes with the induction of T cell anergy, and thus leads to systemic autoimmunity (Tivol et al., 1995) (Ueda et al., 2003). Moreover anergic T cells are also characterized by IL-10 production which have widespread consequences in the suppression of T cell activation (Buer et al., 1998) .

Suppression (T regulatory cells)

A dominant mechanism by which the pathogenic potential of autoreactive clones are kept in check, is through the dedicated lineage of regulatory T (Treg) cells. T regs develop both in thymus (the natural occurring Tregs) and periphery (the adaptive Tregs), and characterized by the expression of *FOXP3* transcription factor. The importance of this mechanism revealed when knockout or spontaneous mutation of the mouse *Foxp3* gene ('scurfy' mice) led to a systemic autoimmune disease associated with the absence of $CD4^{+}CD25^{+}$ regulatory T cells (Hori et al., 2003) (Fontenot et al., 2003) (Khattri et al., 2003). The crucial role of Tregs in tolerance is best demonstrated with the description of the IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) in humans, which is associated with mutations in *FOXP3* gene. Patients carrying the mutation lack Tregs in their periphery and develop multi-organ autoimmune syndromes leading to death.

Although the precise mechanism involved in the Treg mediated maintenance of peripheral tolerance is unkown, Tregs have been proposed to exert their

immunoregulatory function mainly through secretion of suppressor cytokines (IL-10, TGF-β, IL-35) that can directly inhibit the function of responder T cells and antigen presenting cells. Treg cells also express high CD25 (the IL-2 receptor a chain) and thus might compete with effector T cells for IL-2, resulting in cytokine-mediated deprivation and finally apoptosis of the effector cells. Activated Tregs may also function as cytotoxic cells and induce granzyme-mediated apoptosis, or express molecules on their cell surface (e.g., galectin-1, CTLA4, LAG-3) that can interact with receptors on effector T cells and induce cell cycle arrest or prevent their activation. Finally, other T cells with 'regulatory' role have been described such as Tr1 and Th3 cells which are negative for Foxp3 expression, but also exert a suppressive function by producing the two major immunomodulatory cytokines IL-10 and TGF-β (Curotto de Lafaille and Lafaille, 2009).

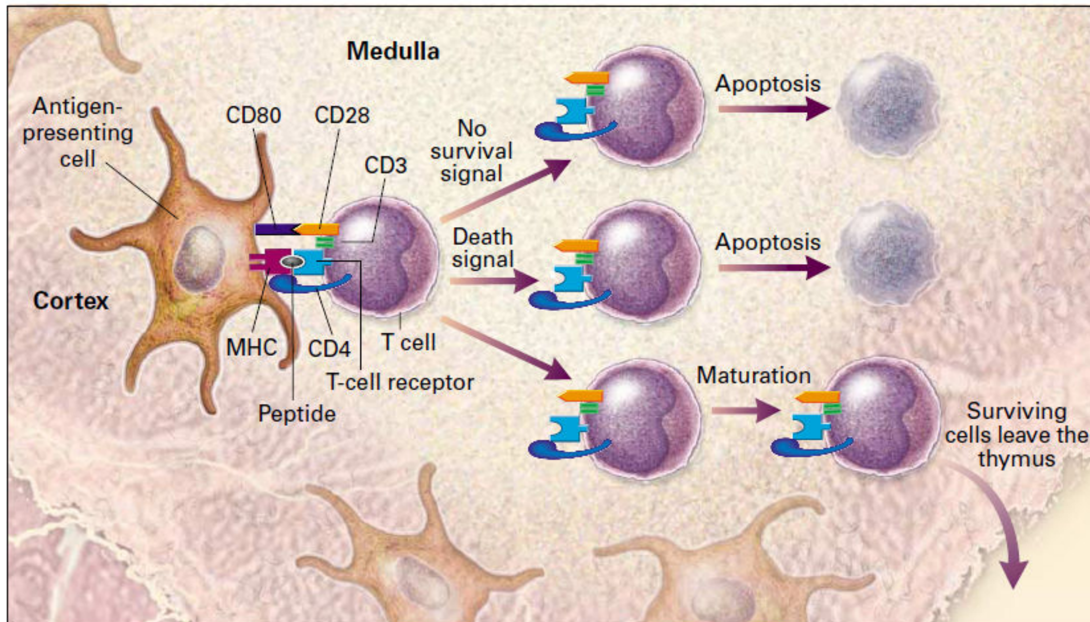


Figure 1: Central Mechanisms of the Induction of Tolerance.

Immature T cells migrate to the thymus, where they encounter antigen presented by thymic epithelial cells. Cells whose T-cell receptors have a low affinity for the complex of self peptide and a self major-histocompatibility-complex (MHC) molecule do not receive a signal to switch off the process of spontaneous apoptosis and therefore die in the thymus. Cells whose T-cell receptors have a high affinity for such complexes are also eliminated by apoptosis. The remaining T cells have an intermediate affinity for these complexes, and these mature in the thymus and migrate to the periphery, where they can become activated (*source: Kamradt and Mitchison, N Engl J Med, 2001*).

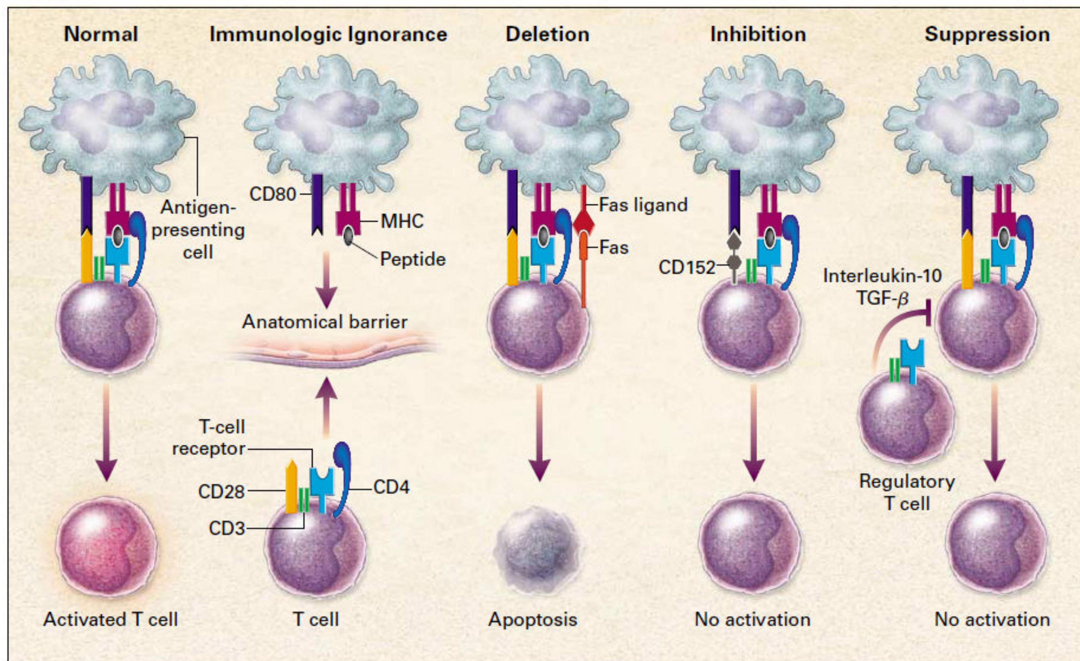


Figure 2: Peripheral Mechanisms of the Induction of Tolerance.

The mechanisms that maintain tolerance in $CD4^+$ T cells are illustrated, and compared with a normal immune response. T cells that are physically separated from their specific antigen, for example by the blood–brain barrier, cannot become activated, a circumstance referred to as immunologic ignorance. T cells that express the Fas molecule on their surface can receive their signals from cells that express Fas ligand and undergo apoptosis, a process known as deletion. Anergy (inhibition) is occurred at least when CD152 (CTLA-4) binds CD80 on antigen-presenting cells, thereby inhibiting the activation of T cells. Regulatory T cells can inhibit, or suppress, other T cells, most likely through the production of inhibitory cytokines such as interleukin-10 and transforming growth factor β (TGF β). (*source: Kamradt and Mitchison, N Engl J Med, 2001*).

3.1.3 Break down of tolerance mechanisms results in development of autoimmune diseases.

Autoimmune diseases comprise a heterogeneous group of poorly understood disorders that affect approximately 5% of the population in Europe and North America (Marrack et al., 2001) (Davidson and Diamond, 2001) . Among the most common autoimmune disorders are rheumatoid arthritis, multiple sclerosis, Chrohn's disease and lupus erythematosus. Autoimmune diseases develop when self-reactive lymphocytes escape from tolerance mechanisms and become activated. In this case, the body's immune responses being directed against its own tissues, causing prolonged inflammation and subsequent tissue destruction. Although the mechanisms by which an autoimmune disorder occurs are not entirely known, autoimmune responses are thought to result from a combination of genetic variants, acquired environmental triggers such as infections, and stochastic events (**Figure 3**). Thus, linkage analysis of human genome has revealed candidate loci for susceptibility to almost all autoimmune syndromes (Forabosco et al., 2009) (Heward and Gough, 1997) (Hafler et al., 2007). The chromosomal regions identified, include genes for MHC as well as non-MHC genes such as for cytokines, cytokine receptors, and other immunoregulatory molecules. For example, two inbred strains of mice have the same MHC haplotype and have T cells that recognize the same epitope on myelin basic protein, yet one of the strains is susceptible to autoimmune encephalitis and the other is not (Scott et al., 1994) (Chang et al., 1999).
Considerable evidence also implicates environmental triggers like infection, in the development of autoimmune diseases, such as multiple sclerosis and type 1 diabetes (Kurtzke, 1993). Mechanisms that could lead from infection to autoimmunity include the release of autoantigens through tissue damage (Miller et al., 1997) the activation of a large fraction of the T-cell population by superantigens (Perron et al., 1997) and the induction of inflammatory cytokines and costimulatory molecules by microbial products (Tough et al., 1997) (Infante-Duarte et al., 2000) (Kamradt et al., 1991) (Klinman et al., 1996) (Cella et al., 1999). In mice, so-called bystander activation of this type can precipitate autoimmune diabetes (Horwitz et al., 1998). Alternatively, a structural similarity between microbial and self-antigens ("molecular mimicry") could have a key role in activating autoreactive T cells (Fujinami et al., 1983) (Albert and Inman, 1999).

Indeed, some T cells can recognize both a microbial peptide and a self-peptide with a similar amino acid sequence (Jahnke et al., 1985) (Fujinami and Oldstone, 1985) (van Eden et al., 1985).

Although various theories have been proposed about the causes of autoimmunity, major questions remain in order to fully understand the precise mechanisms underlying the pathogenesis of autoimmunity.

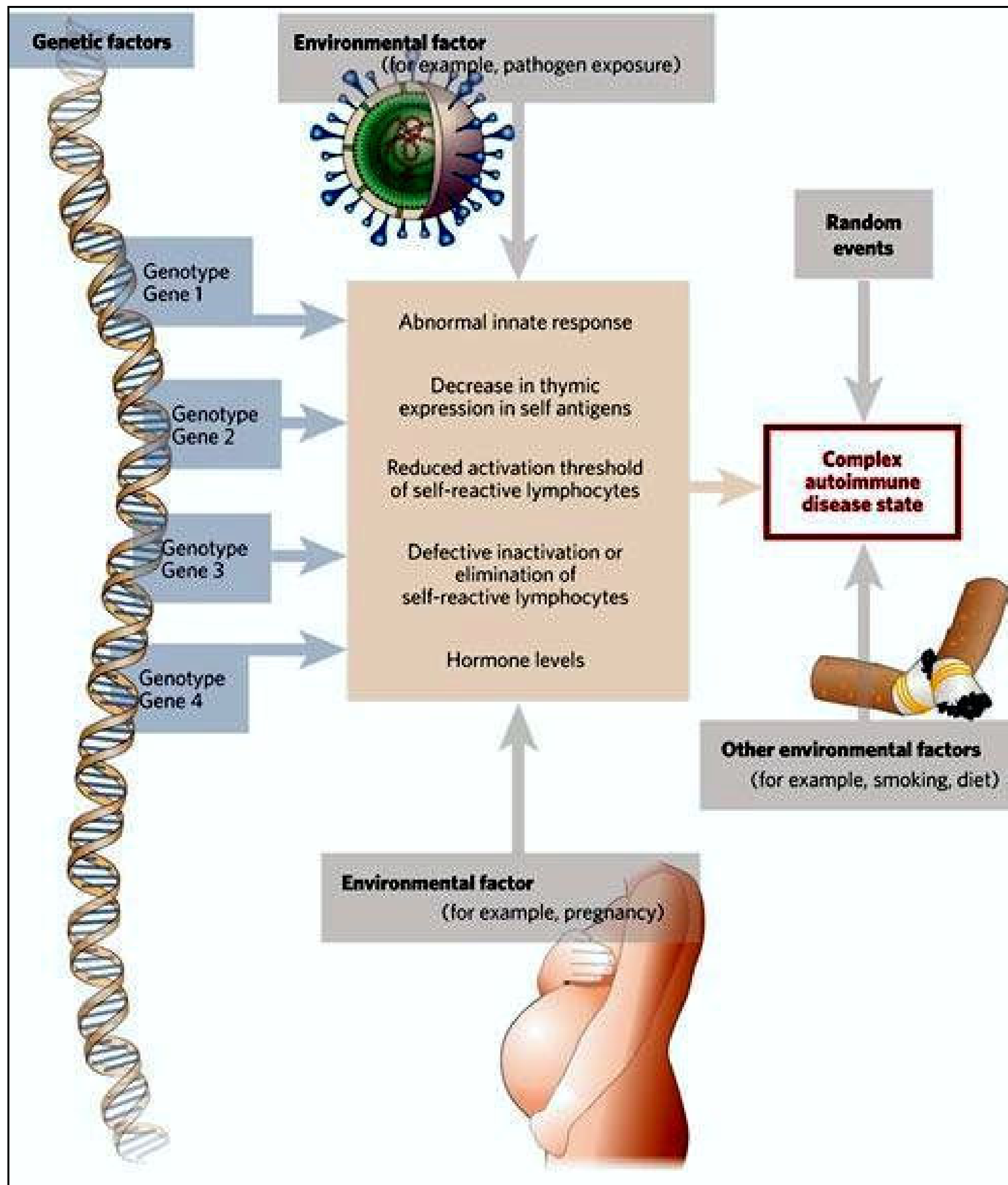


Figure 3: Combination of factors that might be involved in the break down of the mechanisms of tolerance and triggers autoimmunity.
Autoimmune diseases are believed to result from a combination of susceptibility alleles at multiple loci, environmental factors (such as smoking, pathogen exposure and hormone levels), and stochastic events *(source: Rioux and Abbas, Nature Rev. 2005).*

3.2 Multiple Sclerosis (MS)

3.2.1 Multiple sclerosis- An Inflammatory disease of CNS

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS. It usually begins in young adulthood affecting more than one million people worldwide and its etiology remains unclear (Steinman, 1996).

Multiple sclerosis is thought to occur in genetically predisposed individuals following exposure to an environmental trigger that activates myelin specific T cells. Activated T cells travel to the CNS, recognize myelin antigens and initiate an inflammatory cascade resulting in demyelination and axonal damage. Immune cell infiltrates and plaques of demyelination in the brain and spinal cord are the hallmark features of multiple sclerosis. Most patients develop lesions in the brain or in both the brain and spinal cord, which comprise T cells, macrophages, activated microglial cells and other inflammatory cells. The severity, frequency, specific clinical symptoms and CNS pathology vary greatly among patients with this disease, and the basis for this variation is not understood. Approximately 85% of patients with MS have a relapsing-remitting form of the disease, which usually converts over years into a progressive disease that is characterized by severe neurological deterioration whereas 10–15% of patients follow a primary progressive disease. Only a small percentage of patients develop severe disease that leads to extreme disability or death after only months.

The reasons underlying these different disease courses are poorly understood. It is likely that several immune pathways are involved in the initiation and perpetuation of MS (McFarland and Martin, 2007) (Lucchinetti et al., 2000). Understanding of such processes will provide better insights in the development of MS and generate new tools for the design of specialized therapies.

3.2.2 Experimental Autoimmune Encephalomyelitis (EAE)- The animal model of MS

MS has been widely studied using the animal model of experimental autoimmune encephalomyelitis (EAE) (Furlan et al., 2009) (Gold et al., 2006). The inflammatory infiltrates and demyelination seen in EAE have many similarities to the pathology of multiple sclerosis, and represents a vital tool to study the

neuroimmunological events related to the disease as well as to develop therapeutic protocols.

There are two models of EAE in mice, the *acute* and the *relapsing-remitting* model. Acute model characterized by an acute paralytic episode followed by a partial or total recovery, whereas, relapsing-remitting EAE (RR-EAE) model causes a relapsing – remiting course of paralysis **(Figure 4).** RR-EAE offers the advantage that can be used to study the pathogenesis and immunoregulation of T cell–mediated demyelination. Moreover RR-EAE has histopathological and clinical similarities to the relapsing-remitting (RR) form of human multiple sclerosis, and thereby serves as an ideal system in which to examine the molecular basis of disease induction and progression.

The Relapsing-Remitting model of EAE

RR-EAE can be actively induced by immunizartion with synthetic peptides corresponding to the major encephalitogenic regions of PLP (Myelin ProteoLipid Protein), MBP (Myelin Basic Protein), or MOG (myelin oligodendrocyte protein) in susceptible mouse strains. It is important to mention here that induction of EAE with defined peptides of myelin proteins in different mouse strains depends on the ability of the respective peptide to form a stable association with host MHC class II molecules. Alternatively, RR-EAE can be induced by the adoptive transfer of neuroantigen/epitope-specific in vitro–activated $CD4^+$ T cells (McRae et al., 1992; Pettinelli and McFarlin, 1981).

The disease is characterized clinically by transient ascending hind limb paralysis and histologically by perivascular mononuclear-cell infiltration and fibrin deposition in the brain and spinal cord with adjacent areas of acute and chronic demyelination (Brown et al., 1982). CNS damage apparently results from the direct and indirect effects of chemokines (Karpus et al., 1995)) and pro-inflammatory cytokines such as TNF, IFN-γ, IL-17, (Powell et al., 1990) (El-behi et al., 2010) which cause the chemoattraction and activation of monocytes and macrophages.

The mechanisms responsible for the clinical remission that follows the initial acute paralytic episode are poorly defined. However, it has recently been demonstrated that the primary disease relapse is mediated predominantly by T cells specific for endogenous myelin epitopes that are activated as a result of presentation of myelin

debris released during acute disease, a phenomenon known as epitope spreading (Tuohy et al., 1998). The relapsing-remitting clinical disease thus makes it an ideal system for studying the evolution of T cell responses during the chronic disease and for determining the efficacy of various immunoregulatory strategies for treatment of an ongoing autoimmune disease.

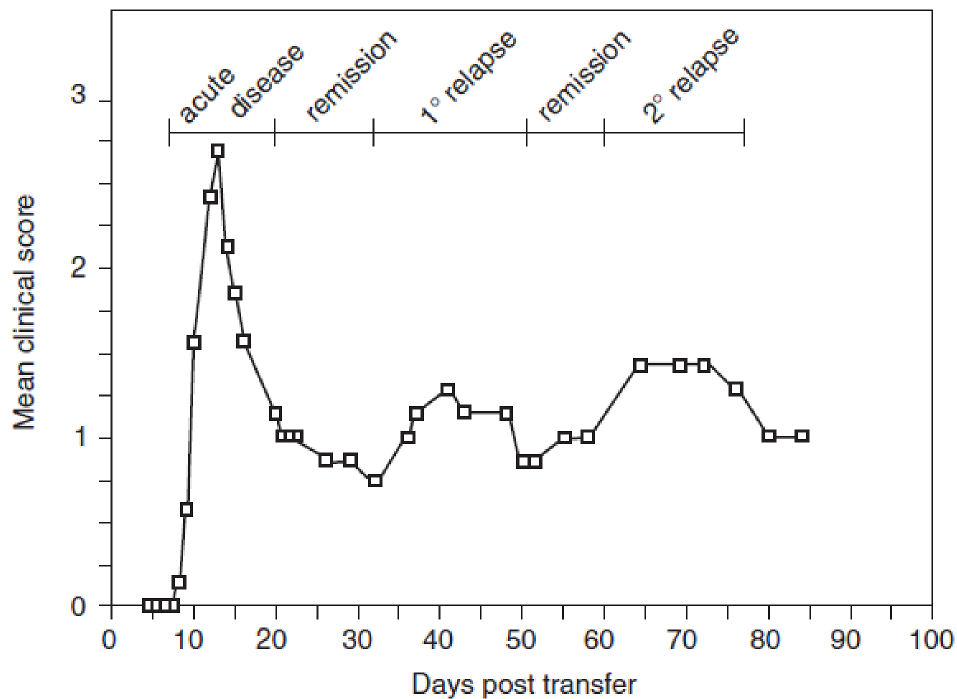


Figure 4: A typical course of relapsing EAE in adoptive transfer of $PLP_{139-152}$ specific T cell in SJL recipients. A relapsing -remitting pattern is seen with disease onset 7-10 days post transfer and peak acute disease at 15 days. The mice then exhibited the first relapse beginning 35 days post transfer and peaking around 40 days. A second remission was followed by a secondary relapse *(source: Current Protocols in Immunology-Chapter 15.1.10).*

3.2.3 Immune responses in EAE and MS

Myelin-specific $CD4^+$ rather than $CD8^+$ T cells are the primary mediators in most models of EAE since adoptive transfer of myelin-specific $CD4^+$ T cells can transfer the disease (Baron et al., 1993; Stromnes and Goverman, 2006). Specifically, CNS autoimmunity can be mediated by two distinct lineages of $CD4^+$ T cells the Th1 and Th17 that are defined by the production of either IFN-γ or IL-17 respectively. The activity of these $CD4^+$ T cell subsets within the CNS influences the pathology and clinical course of disease.

Th1 requires (IL-12) for its differentiation and is characterized (apart from IFNγ), by the secretion IL-2 and TNF. IFNγ has been proposed to induce MHC class II expression in the CNS, trigger the production of chemokines that attract macrophages and monocytes and activate macrophage function. These data are consistent with the idea that Th1 cell-mediated responses establish a pro-inflammatory environment in the CNS.

Over the past decade, the observations that mice deficient in IL-12, IFNγ and TNF develop severe EAE (Steinman, 2007) (Cua et al., 2003), lead to the discovery of the Th17 cell subset. Th17 differentiation and survival depends on TGFβ, IL-6, IL-1, and IL-21 and IL-23 (McGeachy et al., 2009) (Langrish et al., 2005) (Korn et al., 2009). Th17 cells are characterized by the production of IL-17A, IL-17F and IL-22 (Korn et al., 2009). The ability of Th17 cells to produce IL-17A and IL-7F, as well as to activate other celltypes to produce pro-inflammatory mediators (such as Il-6, granulocyte/macrophage colony-stimulating factor (GM-CSF), matrix metalloproteinases and CXC chemokines) places them as an important population during the autoimmune response (Park et al., 2005) (Hofstetter et al., 2005) (Komiyama et al., 2006) (Diveu et al., 2008) (Sospedra and Martin, 2005). Importantly, transfer of Th17 cells, induce more severe EAE compared with transfer of Th1 cells and neutralizing IL-17 activity ameliorated EAE.

In humans, both Th1 and Th17 cells correlate with the pathology of MS. This view stemmed in part from the finding that increased clinical activity in multiple sclerosis associated with the expression of IFNγ and IL-12 in the CNS and CSF (Gutcher and Becher, 2007). In addition, increased numbers of IL-17 trancripts are detected in chronic multiple sclerosis lesions compared with either acute lesions or control tissue from

individuals without CNS pathology (Lock et al., 2002). Finally autoimmune pathology in MS was exacerbated upon administration of IFN-γ (Panitch et al., 1987).

3.2.4 Regulation of autoreactive T cell responses in EAE.

Several mechanisms involving immune cells such as $CD4^+$ regulatory T cells (McGeachy et al., 2005) and type II monocytes (Weber et al., 2007) or neutrophils (Zehntner et al., 2005) as well as immunosuppressive mediators, like IL-10 (Bettelli et al., 1998) (Cua et al., 2001) have been proposed to regulate EAE. **However, how inflammation is resolved and disease remits and in particular, which immune cells are important for naturally terminating the relapsing phase, is poorly understood. Therefore, there is a currently unmet need to delineate such mechanism in order to facilitate the design of more effective protocols for the re-establishment of tolerance and prevention of autoimmune diseases of the CNS.**

3.3 Dendritic cells (DCs)

Dendritic cells (DCs) are professional antigen presenting cells and key coordinators of innate and adaptive immune responses. DCs form a heterogeneous group of cells based on phenotype, location, function, and migratory capabilities (Shortman and Naik, 2007) (Shortman and Liu, 2002) (Liu et al., 2001) . Based on their lineage origin, DCs can be divided in two major subsets: conventional DCs (cDCs) that comes from myeloid progenitors and plasmacytoid DCs (pDCs) that originate from lymphoid progenitors. cDCs are the most abundant DC population characterized by an extraordinary capacity to stimulate naïve T cells and initiate primary immune responses. Whereas, pDCs constitute a smaller subset of DCs and were first described as the most effector cells in antiviral immunity by secreting large amount of type I IFN after virus infection. Intriguingly, depending on the microenvironment and their activation status, both subsets have demonstrated to induce either immunity or tolerance.

3.3.1 Plasmacytoid Dendritic cells (pDCs)

pDCs constitute the 0.3–0.5% of the human peripheral blood as well as of murine lymphoid organs. They are called plasmacytoid because of their round plasma cell-like morphological appearance and their lymphoid origin (**Figure 5**). pDCs, develop in the bone marrow and in the steady state they reside primarily in the lymphoid organs, where they enter through blood (Sozzani et al., 2010) (Randolph et al., 2008). They are characterized by being $CD11c^{int}B220^{+}PDCA\text{-}1^{+}$ and unlike conventional DCs (cDCs) they express low or intermediate levels of MHC-II. Although, in a steady state, pDCs are thought to be poor inducers of T cell activation, upon activation, pDCs upregulates cell surface expression of MHC II and co-stimulatory molecules (e.g CD80, CD40) increasing therefore their T cell stimulatory ability (Grouard et al., 1997) (Cella et al., 2000) (Asselin-Paturel et al., 2001) (**Figure 6**)

The hallmark function of pDCs, is the production of high amounts of type I interferons (IFNs) following viral infection or TLR7 /TLR9 triggering by specific agonists. Type I IFN secretion by pDCs can be up to 1,000-fold higher than in other cell types. For that

reason, pDCs were first described as natural interferon-producing cells (IPCs) (Liu, 2005) . In fact, IFN-α secretion in response to CpG challenge in vivo is mediated exclusively by pDCs, as suggested by antibody mediated (Asselin-Paturel et al., 2003), genetic ablation (Cisse et al., 2008) and IFN report strain analysis (Kumagai et al., 2007). In addition to their direct antiviral activity, type I IFNs have potent immunomodulatory functions and act as powerful DC maturation stimuli and may therefore contribute to a local milieu that is conducive of T-cell priming (Santini et al., 2000) (**Figure 6**).

Other consequences of TLR-induced pDCs activation include the secretion of cytokines such as TNF-α and (in the mouse) IL-12, and the acquisition of antigen presentation ability through upregulation of costimulatory molecules. Transcriptional regulation of type I IFN genes is controlled mainly by IRF7 (Taniguchi et al., 2001) (Coccia et al., 2004) (Izaguirre et al., 2003) (Dai et al., 2004) whereas upregulation of pro-inflammatory cytokines/ chemokines, and costimulatory molecules (CD80, CD86, CD40) are induced by other signal mediators such as NF-Kb or MAPKs (Osawa et al., 2006).

Altogether, these powerful immunostimulatory functions of pDCs contribute to the recruitment and/or activation of nearly all immune cell types [e.g.,cDCs, natural killer (NK) cells (Krug et al., 2004)and plasma cells (Jego et al., 2003) , establishing pDCs as a key link between innate and adaptive immunity (Villadangos and Young, 2008) (Colonna et al., 2004) (Cao and Liu, 2007) (Reizis et al., 2011) (Gilliet et al., 2008).

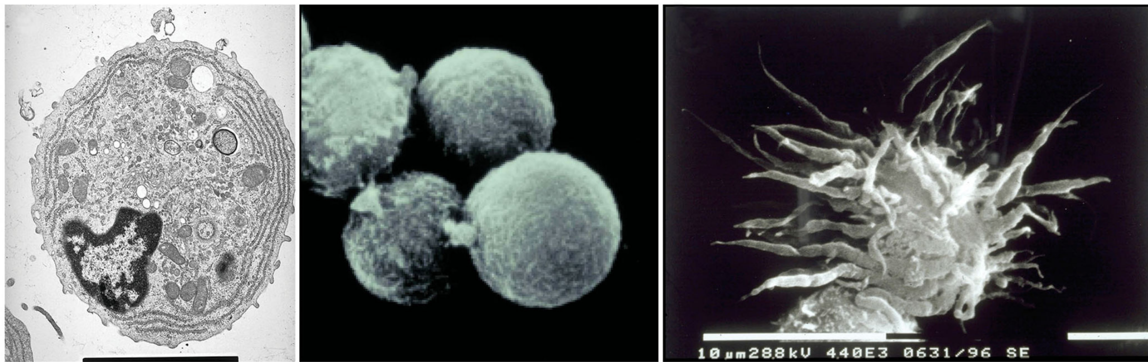


Figure 5: Morphology of pDCs. (**a**) pDCs appear as lymphoblasts with a medium-to-large diameter, a slightly eccentric, indented, round or oval nucleus, lightly stained perinuclear areas and well developed rough endoplasmic reticulum. (**b**,**c**) By scanning electron microscopy, resting pDCs have a spherical shape (**b**), whereas activated pDCs have a dendritic cell–like morphology (**c**). Reproduced from ref. 74.

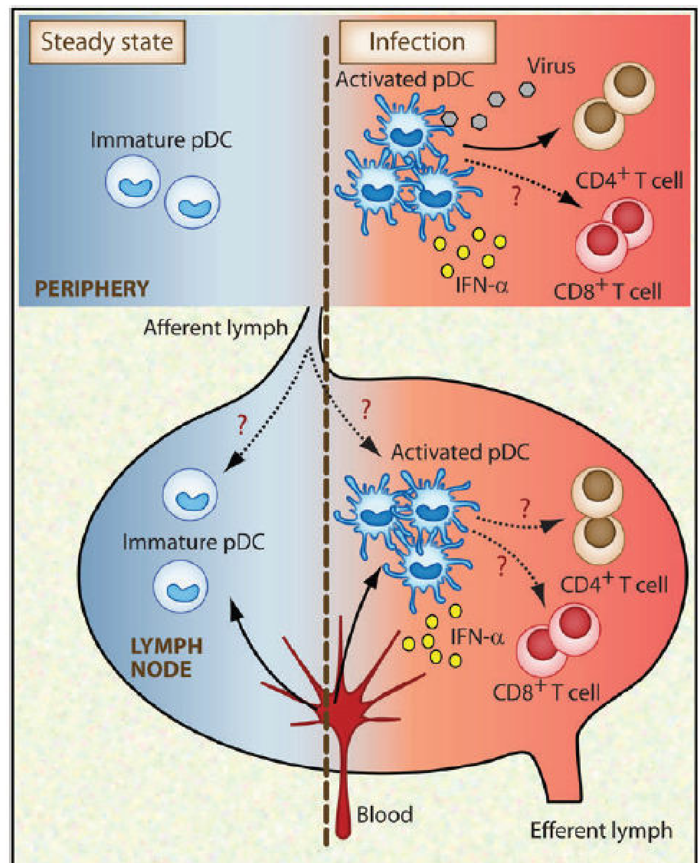


Figure 6: Activated pDCs prime $CD4^+$, $CD8^+$ T cells in the dLNs.

In the steady state, pDCs are produced in the bone marrow and disseminate via the blood to the thymus and the secondary lymphoid organs (spleen, not shown, and the lymph nodes [LNs]). Their primary route of entry into the LNs is through the blood via the high endothelial venules and not by means of the lymph. Detection of activating signals (e.g. viruses) induces pDCs accumulation at the infection site and associated draining LNs. Activated pDCs secrete large amounts of IFN I and undergo maturation. Mature pDCs acquire dendritic morphology and upregulate MHCII and costimulatory molecules, so they have the potential to present antigens via MHC I and II to $CD8^+$ and $CD4^+$ T cells.

3.3.2 The Role of pDCs in T Cell Responses: Activation or Tolerance?

As a major effector cell type in immunity, pDCs have been implicated in nearly all normal and pathological immune responses. For example, important roles of pDCs have been suggested in allergy and asthma (Kool et al., 2009), antitumor immunity (Liu et al., 2008)and responses to nonviral pathogens (Pepper et al., 2008) (Ang et al., 2010).

It has been shown that, the capacity of pDCs to prime productive T cell responses after infection or immunization prime $CD4^+$ T cell responses in the lymph nodes (Sapoznikov et al., 2007), induce Th1 polarization (Cella et al., 2000) (Boonstra et al., 2003) and prime (Salio et al., 2004) and cross-prime (Mouries et al., 2008) (Di Pucchio et al., 2008) (Hoeffel et al., 2007) (Moseman et al., 2004) CD8+ T cell responses. These results are consistent with the postulated role of pDCs as key sensors and immune system activators during viral infections.

Intriguingly, the tolerogenic role has been proposed for pDCs in several systems, mostly associated with the induction of regulatory T cells (Tregs). Thus, human pDCs induce T cell differentiation into IL-10-producing Tregs in vitro (Moseman et al., 2004) (Ito et al., 2007) (Kavousanaki et al., 2010) or promote Treg differentiation in the human thymus (Martin-Gayo et al., 2010). In mice, pDCs also induce Treg-mediated tolerance in different mouse models. For instance, pDCs have been shown to activate mature Tregs via IDO -indoleamine 2,3-dioxygenase in tumor-bearing mice (Sharma et al., 2007), induce Treg development and mediate tolerance to cardiac allografts (Ochando et al., 2006)-and induce T regulatory function in a model of graft versus- host disease (Hadeiba et al., 2008). Moreover an interesting recent study suggested an important role for liver pDCs in oral tolerance induction, largely through T cell clonal deletion (Goubier et al., 2008).

Taking together, these results suggest that pDCs are considerably flexible in directing T cell responses depending on the microenvironment, maturation stage, nature and concentration of the antigen (Boonstra et al., 2003) (Liu et al., 2001). However, there are still several questions remain to be answered regarding the immunogenic or tolerogenic role of pDCs during immune responses.

3.3.3 The Role of pDCs in Human Autoimmune Diseases

The discovery of elevated IFN levels in several autoimmune diseases, has led to the appreciation of an important role of pDCs in autoimmunity (Ronnblom and Alm, 2001). To date, the strongest evidence for pDCs involvement has been accumulated from the study of two autoimmune diseases: psoriasis and systemic lupus erythematosus (Gilliet et al., 2008) (Ronnblom et al., 2009).

In psoriasis, early skin lesions are highly infiltrated by activated pDCs, corresponding with decreased numbers of circulating pDCs in the blood (Nestle et al., 2005). Blocking IFN production inhibited the development of skin lesions in a xenograft model, providing proof of pDC function in the disease (Nestle et al., 2005). Subsequently, Lande et al. (Lande et al., 2007) identified the activating stimulus for pDCs, as complexes of self-DNA with antimicrobial peptides. Thus, aggregation of released cellular DNA and RNA into large complexes can efficiently activate pDCs in psoriasis (Lande et al., 2007) (Ganguly et al., 2009).

Similarly, lupus patients show a decrease in circulating pDCs and the accumulation of activated, IFN-producing pDCs in affected tissues such as the skin(Farkas et al., 2001) . The hallmark of lupus is the production of antinuclear antibodies, and immune complexes of such antibodies with endogenous nucleic acids were shown to activate pDCs through TLR7/9 (Bave et al., 2003) (Barrat et al., 2005). The direct relationship between pDC derived IFN and lupus progression is hard to establish in the human system and should await elucidation in animal models. Nevertheless, the likely connection between the formation of nucleic acid–containing immune complexes, pDC activation, and IFN secretion and the pronounced IFN signature of the disease make a strong case for the pDC as a major player in lupus pathogenesis(Ronnblom et al., 2009) .

Overall, the aberrant conversion of self-nucleic acids into ligands for TLR7/9 on pDCs (via immune complex formation, antimicrobial peptide binding, and other mechanisms to be discovered) may represent a common pathogenesis step in psoriasis, lupus, and possibly other autoimmune diseases (Bave et al., 2005). However, further investigation in animal models are required in order to shed light about the role of pDCs in the development and progression of autoimmunity.

3.3.4 The Role of pDCs in Experimental Autoimmune Mouse Models

Studies in animal models have yielded conflicting results. For instance, antibody-mediated pDC ablation before or during the onset of disease results in exacerbation of autoimmune responses in a model of experimental autoimmune arthritis (Jongbloed et al., 2009) and experimental autoimmune encephalomyelitis (Bailey-Bucktrout et al., 2008) respectively.

In contrast, pDC depletion performed during the priming of the autoimmune response, caused amelioration of EAE and attenuation of Th17 responses (Isaksson et al., 2009). Similarly, results of pDC ablation in the NOD model of type I diabetes (Saxena et al., 2007) (Tisch and Wang, 2009) show a regulatory role of pDCs in T cell responses.

These conflicting data argue for a different role of pDCs in the initiation and the effector phase of autoimmunity. The reasons underlining these differences remains elusive and the mechanism through which pDCs shape the autoimmune response remain to be addressed.

3.4. Myeloid Derived Suppressor Cells (MDSCs)

Accumulating data indicates that adaptive immunity is initiated, programmed and constantly regulated by innate immune cells. Recently, a population of myeloid cells, responsible for regulating immune responses has been described that termed Myeloid-Derived Suppressor Cells (MDSCs). MDSCs, have been shown to accumulate during tumor progression, autoimmunity, chronic infection and other pathological conditions, and can potently suppress T cell function (Gabrilovich and Nagaraj, 2009)

3.4.1 Origin, subsets, phenotype and localization of MDSCs

MDSCs represent a heterogenic population of immature myeloid cells (IMCs) that consists of myeloid progenitors and precursors of macrophages, granulocytes and dendritic cells (DCs).

In healthy individuals, the immature cells (IMCs) generated in the bone-marrow, quickly differentiate into mature granulocytes, macrophages or dendritic cells (DCs). However, in some pathological conditions, such as cancer, various infectious diseases, sepsis, trauma, bone marrow transplantation and some autoimmune diseases, a partial block in the differentiation of IMCs into mature myeloid cells results in the expansion of the MDSC population (**Figure 7**).

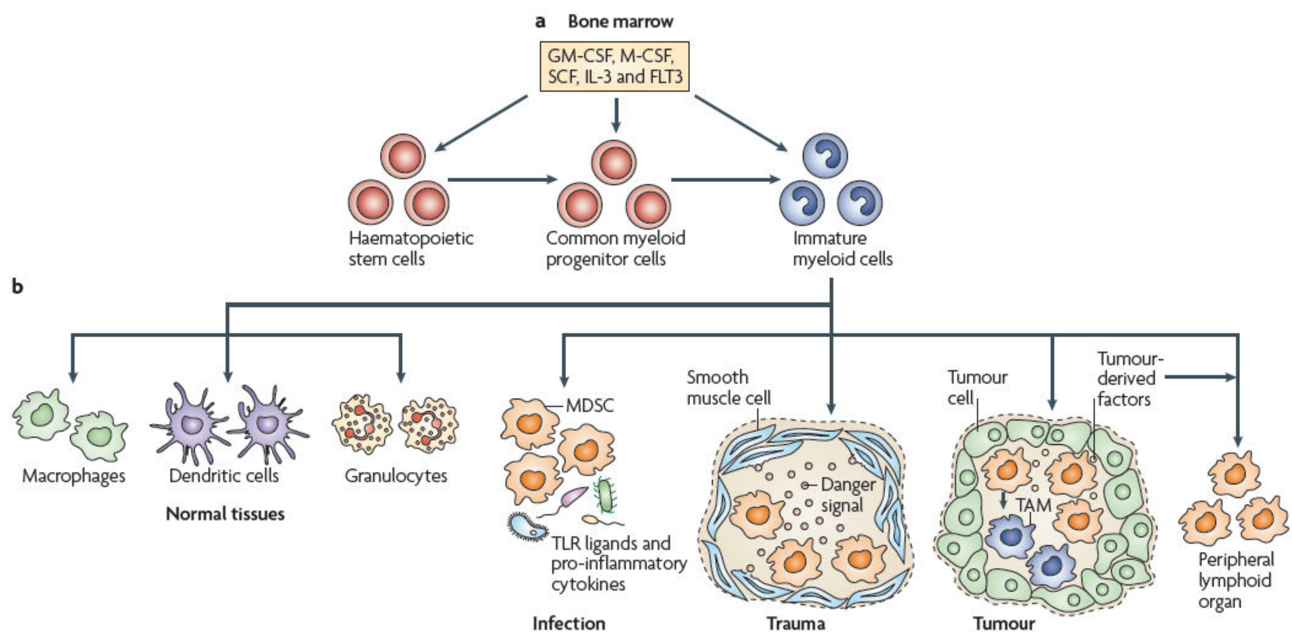


Figure 7. The origin of MDSCs. a) Immature myeloid cells (IMCs) are part of the normal process of myelopoiesis, which takes place in the bone marrow and is controlled by a complex network of soluble factors, including cytokines (such as granulocyte/macrophage colony-stimulating factor (GM-CSF), macrophage CSF (M-CSF), stem-cell factor (SCF), Interleukin-3 (IL-3) and FMS-related tyrosine kinase 3 (FLT3). Haematopoietic stem cells differentiate into common myeloid progenitor cells and then into IMCs. b) Normally, IMCs migrate to different peripheral organs, where they differentiate into macrophages, dendritic cells or granulocytes. However, factors that are produced during acute or chronic infections, trauma or sepsis, and in the tumour microenvironment promote the accumulation of IMCs at these sites, prevent their differentiation and induce their activation. These cells exhibit immunosuppressive functions and are therefore known as myeloid-derived suppressor cells (MDSCs). MDSCs also accumulate in peripheral lymphoid organs in response to tumour-derived factors *(source: Dmitry I. Gabrilovich, Nature Rev. Immunol., 2009).*

In mice, MDSCs are identified as cells that simultaneously express the two markers CD11b and Gr-1 (Gabrilovich and Nagaraj, 2009) . More recently, MDSCs were subdivided into two different subsets based on their morphology and the expression of the two molecules Ly-6C and Ly-6G, which can be detected by specific antibodies (Hestdal et al., 1991; Youn et al., 2008). $CD11b^{+}$ $Ly\text{-}6G^{-}$ $Ly\text{-}6C^{high}$ cells have monocytic-like morphology and are termed monocytic-MDSCs (M-MDSCs). $CD11b^{+}$ $Ly\text{-}6G^{+}$ $Ly\text{-}6C^{low}$ cells have granulocyte-like morphology and are termed granulocytic-MDSCs (G-MDSCs), (**Table 2, Figure 8**). Several surface molecules have been used to identify additional subsets of suppressive MDSCs, including CD80 (B7.1) (Yang et al., 2006) ,CD115 (macrophage colony-stimulating factor receptor (M-CSF) and CSF1 (Huang et al., 2006) (Gallina et al., 2006) and CD124 (interleukin-4 receptor α-chain (IL-4Rα) (Gallina et al., 2006). In naive mice, MDSCs comprise the 20-30% of bone marrow, 2-4% of spleen , whereas are almost absent from lymph nodes. In contrast, in several mouse tumor models MDSCs expand and reach the 20-40% of nucleated splenocytes. In addition MDSCs are found in tumor sites and in the lymph nodes of tumour bearing mice.

In humans, MDSCs are defined as cells that express the common myeloid marker CD33 but lack expression of markers of mature myeloid and lymphoid cells (Almand et al., 2001). In healthy individuals MDSCs constitute ~0,5% of peripheral blood mononuclear cells (Almand et al., 2001). In contrast, up to tenfold increase in MDSC numbers was detected in the blood of patients with different types of cancer (Almand et al., 2001) (Ochoa et al., 2007) (Mirza et al., 2006) (Diaz-Montero et al., 2009). For example, monocytic MDSCs, with the phenotype $CD14^{+}CD11b^{+}HLA\text{-}DR^{low/neg}$, have been detected in melanoma patients (Filipazzi et al., 2007) (Poschke et al., 2010). MDSCs are also defined as $CD11b^{+}CD14^{-}CD15^{+}CD33^{+}$ cells in patients with advanced non-small cell lung cancer (Liu et al., 2010) (Srivastava et al., 2008). MDSCs from patients with renal cell cancer, express markers of activated granulocytes, including high levels of CD66b and low levels of CD62L, CD16 and vascular endothelial growth factor (VEGF) receptor 1 (Rodriguez et al., 2009) (Peranzoni et al., 2010) (Ribechini et al., 2010) (Nagaraj and Gabrilovich, 2010).

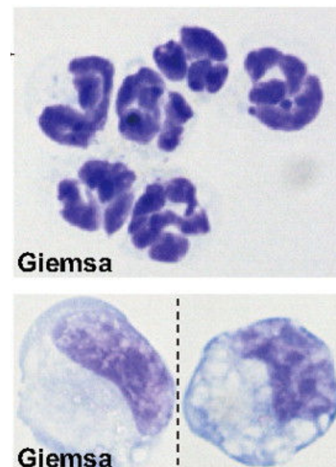
Table 2

Phenotype of MDSC

	Mouse	Human
Membrane markers (common)	Gr-1, CD11b	CD33,CD11b, CD34, low MHC class II
Granulocyte-like subset	$CD11b^{+}$ $Ly6G^{+}$ $Ly6C^{low}$ $CD11b^{+}$ $Gr\text{-}1^{high}$ $CD49d^{-}$	$CD33^{+}$, $CD15^{+}$, HLA-DR low
Monocyte-like subset	$CD11b^{+}$ $Ly6G^{-}$ $Ly6C^{high}$ $CD11b^{+}$ $Gr\text{-}1^{int}$ $CD49d^{+}$	$CD33^{+}$ $CD14^{+}$ HLA-Dr low

Source: Boros et al, Human Immunology, 2010

Figure 8 : Granulocytic and Monocytic subsets of MDSCs. MDSCs isolated from tumor bearing mice. Giemsa staining of (a) granulocytic ($CD11b^{+}$ $Ly\text{-}6G^{+}$ $Ly\text{-}6C^{low}$) and (b) monocytic ($CD11b^{+}$ $Ly\text{-}6G^{-}$ $Ly\text{-}6C^{high}$) - like subsets of MDSCs sorted from tumor infiltrates *(source: Sawanobori Y et al, Blood, 2008).*



3.4.2 Expansion of MDSCs is associated with diverse pathologic conditions.

Although initial observations and most of the current information on the role of MDSCs in immune responses has originated from studies in the field of cancer research**,** there is also a marked increase of MDSCs, in a variety of pathologic conditions: during bacterial and parasitic infections, acute and chronic inflammation, traumatic stress, sepsis and transplantation. Indeed, a systemic expansion of both the granulocytic and monocytic subsets of MDSCs was observed in mice primed with *Mycobacterium tuberculosis* in the form of complete Freund's adjuvant (CFA). Furthermore, acute *Trypanosoma cruzi* infection, which induces T-cell activation and increased the production of interferon-γ (IFNγ), also leads to the expansion of MDSCs (Goni et al., 2002) (Giordanengo et al., 2002). A similar expansion of MDSCs has been reported during acute toxoplasmosis (Voisin et al., 2004), polymicrobial sepsis (Delano et al.,

2007), acute infection with *Listeria monocytogenes*, chronic infection with *Leishmania major* (Sunderkotter et al., 2004) and infection with helminthes (Terrazas et al., 2001) (Gomez-Garcia et al., 2005) (Brys et al., 2005) , *Candida albicans* (Mencacci et al., 2002)or *Porphyromonas gingivalis* (Ezernitchi et al., 2006). MDSCs were also found to infiltrate the spleen and to suppress T-cell function in a model of traumatic stress (Makarenkova et al., 2006) . In addition, a significant transient increase in MDSC numbers was also observed in normal mice following immunization with different antigens, including ovalbumin or peptide together with CFA, recombinant vaccinia virus expressing IL-2, or staphylococcal enterotoxin A8 (Bronte et al., 1998) (Cauley et al., 2000). Finally, MDSC expansion is also associated with autoimmunity and inflammation as discussed in paragraph 3.4.5.

3.4.3 Expansion and activation of MDSCs

The expansion and activation of MDSCs have been shown to be regulated by factors produced by tumor cells, activated T cells, and stromal cells. Thus, expansion inducing factors include macophage–colony stimulating factor (M-CSF), granulocyte-macophage- colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), stem cell factor (SCF), interleukin (IL)–6, and cyclooxygenase 2 also known as PTGS2 (Pan et al., 2008) (Sinha et al., 2007) (Serafini et al., 2004) (Gabrilovich et al., 1998). These factors are primarily produced by tumour cells and exert their effects by stimulating myelopoiesis and by inhibiting differentiation of mature myeloid cells. They do so by triggering the JAK1 and STAT3 signaling pathways involved in cell survival, proliferation, and differentiation (Nefedova et al., 2005) (Kortylewski et al., 2005) (**Table 3**). STAT3 activation is associated with increased survival and expansion of myeloid progenitor cells. Selective STAT3 inhibitors reduced the expansion of MDSC, while increasing T-cell responses in tumor bearing mice, suggesting a central role for this signaling pathway in MDSCs expansion (Foell et al., 2007).

MDSCs require not only factors that promote their expansion but also factors that induce their activation in order to exert their suppresive activity. These factors are produced mainly by activated T cells or tumour stromal cells and their expression is induced by different bacterial and viral products, or as a result of tumor cell death

(Delano et al., 2007). These factors include IFNγ, cyclooxygenase 2, PGE2 (Prostaglandin E2), TLR ligands (e.g LPS, TLRs :Toll-like receptors), IL-4, IL-13, IL-1β and transforming growth factor-β (TGFβ). Several different signaling pathways in MDSCs are activated by these factors that involve STAT6, STAT1 and nuclear factor-κB. Stat1 is the main transcription factor activated by IFNγ or IL-1β signaling and is implicated in the regulation of inducible nitric oxide synthase (iNOS) and arginase activity. Stat6 activation in MDSCs occurs in response to binding of IL-4 or IL-13 to the receptor CD124. This receptor is also described as a MDSC marker and is responsible for upregulation of arginase activity (Sinha et al., 2005b) (Sinha et al., 2005a) (Bronte et al., 2003) and increased TGFβ production by MDSCs (Serafini et al., 2008) (Terabe et al., 2003). NF-Kb transcription factor has also a critical role in MDSC accumulation and immune suppressive function. TLRs play a prominent role of NF-kB activation via activation of MyD88. This is consistent with MDSCs accumulation and activation during microbial and viral infections as well as trauma and sepsis (Delano et al., 2007) (Bunt et al., 2009) (Greifenberg et al., 2009).

Table 3: Factors implicated in the expansion and activation of MDSCs in cancer

Factor	Tumour model (mice)	Type of cancer (humans)
VEGF	Breast cancer, sarcoma, melanoma, lymphoma and lung carcinoma	Breast cancer, renal-cell cancer and pancreatic cancer
GM-CSF	Lewis lung carcinoma, colon carcinoma, mammary adenocarcinoma and Ts/a tumour	Melanoma
G-CSF	Lewis lung carcinoma, methA sarcoma and melanoma	ND
M-CSF	Sarcoma and mammary carcinoma	Human renal carcinoma cell lines
Gangliosides	Neuroblastoma and glioma	ND
Prostaglandins	Mammary carcinoma, lung cancer, renal cancer and colon cancer	ND
IFNγ	Mammary adenocarcinoma, fibrosarcoma, colon carcinoma and lymphoma	ND
C5a	Cervical cancer and lung cancer	ND
SCF	Colon carcinoma	ND
S100A8 and S100A9	Colon carcinoma, lymphoma, fibrosarcoma and mammary carcinoma	ND
TGFβ	Colon carcinoma, fibrosarcoma and mammary adenocarcinoma	Head and neck cancer
IL-1β	Fibrosarcoma and mammary carcinoma	ND
IL-6	Mammary carcinoma	ND
IL-10	Colon cancer, melanoma and mammary carcinoma	ND
IL-12	Colon cancer	ND
IL-13	Colon carcinoma, fibrosarcoma, mammary adenocarcinoma and lymphoma	ND
MMP9	Colon carcinoma, Lewis lung carcinoma and mammary carcinoma	ND
CCL2	Lewis lung carcinoma, methA sarcoma, melanoma and lymphoma	ND
CXCL5 and CXCL12	Mammary adenocarcinoma	ND

Source: Gabrolovich, Nature Rev Immunol., 2009

3.4.4 Mechanisms implicated in MDSC-mediated suppression

Several mechanisms have been attributed to the MDSC-mediated suppression including arginase activity, nitric oxide (NO) and reactive oxygen species (ROS).

L-Arginine metabolism has been associated with MDSC suppression (Bronte and Zanovello, 2005) (Rodriguez and Ochoa, 2008). Both arginase 1 (Arg1) and iNOS use L-arginine as a substrate to produce urea and L-ornithine or NO, respectively. Upregulation of Arg1 activity leads to depletion of L-arginine from the microenvironment, which induces the loss of the CD3ζ chain (Rodriguez et al., 2004) and prevents upregulation of the cell-cycle regulator in T cells (Rodriguez et al., 2007) resulting in an inhibition of T cell proliferation. Upregulation of iNOS activity leads to increased NO production that has also been shown to suppress T cell function. NO is able to inhibit the downstream pathway of the IL-2 receptor by blocking the phosphorylation of signaling proteins (like Jak3 or Stat5) (Bingisser et al., 1998) or to induce T cell apoptosis directly (Mannick et al., 1999). These mechanisms are mostly associated with M-MDSC. In contrast, ROS produced by the NADPH oxidase complex is the main mediator responsible for G-MDSC suppression (Youn et al., 2008) (Movahedi et al., 2008) (**Figure 9**). The major effect is mediated by peroxynitrite, which is the product of a chemical reaction between NO and the superoxide anion (Nagaraj et al., 2007). Peroxynitrite is capable of inhibiting $CD8^+$ Tcells by inducing the nitration of their T cell receptors (TCR), resulting in an altered TCR/MHC-peptide recognition.

MDSCs have also been shown to regulate immune responses through induction of $Foxp3^+$ Treg cells. Thus, expression of the receptor B7.1 (CD80) by MDSCs was required for the induction of Treg in mice with ovarian carcinoma (Yang et al., 2006). This was through binding of CD80 to CTLA4 (CD152) since, injection of anti–CTLA-4 antibodies, blocked the MDSC-mediated Treg induction. MDSCs have also been demonstrated to express the co-inhibitory molecules PD-L1, PD-L2 in tumor bearing mice, however the level of expression of these molecules by MDSCs did not interfere with their suppressive activity (Youn et al., 2008) . In another study, interaction between MDSCs and macrophages resulted in a shift toward a type 2 macrophage responses, with reduced IL-12 release by macrophages and increased IL-10 production by MDSCs, promoting tumor immune evasion (Gallina et al., 2006).

Based on these observations we propose that depending on the microenvironment, MDSCs might acquire a unique suppressive phenotype with the ultimate goal the suppression of the excessive immune response.

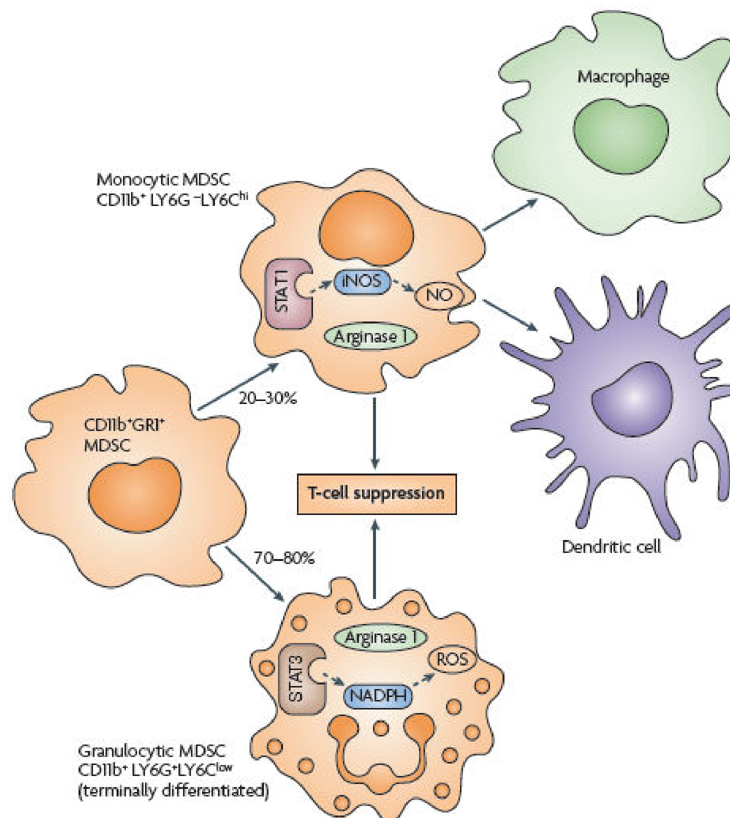


Figure 9: Suppresive mechanisms of MDSCs subsets. The granulocytic and monocytic subsets of MDSC have differences in their functional pathways. Although both subsets express Arg-1, granulocytic MDSC express high levels of ROS and low levels of NO, whereas low levels of ROS and high levels of NO characterize the monocytic subset *(source: Gabrilovich, Nature Rev Immunol., 2009).*

3.4.5 MDSCs in autoimmunity

MDSCs were originally described in the context of murine tumor models and cancer patients. Data on their role in the regulation of autoimmune pathology is just beginning to emerge.

Thus, MDSCs have been shown to prevent murine type 1 diabetes (Yin et al., 2010) by inducing anergy in autoreactive T cells and development of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs. In addition, MDSCs found to suppress inflammatory responses in the gut (Haile et al., 2008), retina (Kerr et al., 2008), and skin (Marhaba et al., 2007) through production of suppressing molecules such as NO or Arginase I and thus suppression of effector T cell responses. In the field of EAE, the role of MDSCs remains controversial. For instance, circulating $Ly6C^{+}$ myeloid precursors have been show to perpetuate EAE upon migration to the CNS (Mildner et al., 2009) (King et al., 2009) and disease severity and onset was associated with an enrichment of these cells in the blood of diseased mice (King et al., 2009). In contrast, other reports demonstrate a regulatory role of MDSCs in EAE (Dardalhon et al., 2010) (Zhu et al., 2007). Thus, it has been shown that MDSCs suppress Th1 and Th17 responses through production of NO and Arginase I (Zhu et al., 2007) or through activation of the Tim-3/-Gal-9 pathway (Dardalhon et al., 2010). Further investigation on the precise role of MDSCs in the autoimmune diseases is required.

Collectively, although the increased immunosuppressive properties of MDSCs could place them as a potential target for therapeutic intervention of autoimmunity, still several questions remain to be addressed. For example the subset of MDSCs as well as the precise mechanism exploited by these cells in the modulation of an autoimmune response is unclear. In addition, the specialized microenvironment that may facilitate the MDSCs function needs to be determined. Finally, whether MDSCs have any role in human autoimmune diseases remains elusive.

4. OBJECTIVES

The present study sought to delineate the role of pDCs was in the initiation of autoimmune diseases. More specifically, the objectives of the study were :

1. **To dissect the contribution of pDCs in the priming of autoreactive $CD4^+$ T cell**
2. **To investigate how ablation of pDCs shape the autoimmune disease**
3. **To decipher the molecular mechanism(s) involved in the development of autoimmune response in the absence of pDCs**

Results obtained from these objectives, indicated the MDSCs as a possible candidate population, participating in resolution of inflammation. Therefore a new series of questions were raised:

1. **What is the role of MDSCs during the different phases of an autoimmune response?**
2. **How MDSCs mediate their suppressive function?**
3. **Do MDSCs contribute to the resolution of autoimmune inflammation?**
4. **Could MDSCs exploited therapeutically in autoimmune diseases?**

5. MATERIAL AND METHODS

4.1 Mice

Female C57BL/6 (B6) mice (6-10 weeks) were obtained from the SPF facility of the Institute of Molecular Biology and Biotechnology (IMBB Heraklion Crete, Greece). PD-1$^{-/-}$ mice bred on B6 background were a kind gift of Dr. Zhang (Department of Orthopedic Surgery, University of Chigaco, IL, USA). PD-L1$^{-/-}$ mice bred on B6 background (Latchman et al., 2004), provided by Prof. A. Sharpe (Department of Pathology, Harvard Medical School, Brigham & Women's Hospital, Boston, MA, USA). Foxp3-GFP reporter mice bred on B6 background, were provided from Dr.Alexander Rudensky. (Department of Immunology Memorial Sloan-Kettering Cancer Center, New York, USA). All procedures were in accordance to institutional guidelines and were approved by the Greek Federal Veterinary Office.

4.2 Reagents

For analysis of mouse cells the following fluorescent-conjugated monoclonal antibodies were used: Ly6C (1G7.G10), PDCA-1 (JF05-1C2.4.1) from Miltenyi Biotec, Gr-1 (RB6-8C5), CD80 (16-10A1), CD86 (PO3.1), CD40 (1C10), CD273 (PD-L2, 122), Foxp3 (FJK-16s), IgG1k isotype control (P3) all from eBioscience. CD11b (M1/70), CD3e (145-2C11), CD19 (1D3), CD274 (PD-L1, M1H5), CD45R/B220 (RA3-6B2), CD4 (RM4-5), Ly6G (1A8), CD44 (Pgp-1, Ly24), I-A^{b} (AF6-120.1), CD25 (PC61), CD45R/B220 (RA3-6B2) all from BD Pharmingen.

For human cell phenotypes, the following monoclonal antibodies were used: CD15 (8OH5), CD33 (D3HL60.251), CD14 (RM052), CD25 (B1.49.9) from Beckman Coulter, HLA-DR (L243, G46-6), CD4 (RPA-T4) from BD Pharmingen. PE-conjucated MOG$_{38-49}$/IA^{b} (MHC class II) specific tetramer were obtained from NIH tetramer facility.

Cell cultures were performed in DMEM or RPMI, supplemented with 10% fetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100μg/ml), 2-mercaptoethanol (5x10^{-5} M) all from Gibco, Carlsbad, CA; recombinant mouse IFN-γ and IL-4 were

purchased from Peprotech, E.coli LPS from InvivoGen, PMA, Complete Freund's adjuvant (CFA) and Pertussis toxin from Sigma-Aldrich.

$MOG_{35–55}$ peptide [MEVGWYRSPFSRVVHLYRNGK] was synthesized by the department of Chemistry, University of Patras (Patras Greece) and was purified to over 95% by HPLC. 120G8 depleting mAb (rat IgG1k) was provided by Louis Boon (Bioceros, B.V., Utrect, The Netherlands).

4.3 EAE induction

EAE was induced in mice by immunization with 100μg MOG_{35-55} peptide emulsified in complete Freund's adjuvant (CFA 1:1) subcutaneously (s.c.) at the base of the tail. Mice also received intraperitoneal (i.p.) injections of 200ng pertussis toxin at the time of immunization and 48h later. Mice were monitored daily for clinical signs of disease. Clinical scores of EAE were assessed as follows: Clinical symptoms of EAE were scored as follows: grade 1,paralyzed tail; grade 2,paralyzed tail and weakness of hind legs; grade 3,paralyzed tail and complete paralysis of hind legs; grade 4,paralyzed tail, complete hind leg and partial front leg paralysis; grade 5,complete hind and complete front leg paralysis, no movement around the cage.

4.4 In vivo Depletion of pDCs

T o deplete pDC fraction mice were injected (i.p) with 400μg of 120G8 depleting mAb on day -1 and day 1 (day 0 : antigenic challenge) and 200 μg of 120G8 on day 3. The efficiency of depletion was controlled by staining and subsequent flow cytometric analysis. The percentage of pDCs in spleen and LNs (Gated on $7AAD^-CD3^-CD19^-CD11b^-$ $CD11c^{low}$ $PDCA\text{-}1^+$ fraction), was measured 24h after the last injection.

4.5 Adoptive transfer experiments

For adoptive transfer experiments, $7AAD^-CD3^-CD19^-$ $CD11b^{hi}Ly6G^+$ (G-MDSCs) or $Ly6G^-$ $CD11b^+$ ($7AAD^-CD3^-CD19^-$) cells were sorted (purity >95%) from spleens of MOG/CFA-immunized mice at day 9 of immunization and transferred (2 x 10^6 cells) intravenously (i.v.) into syngeneic hosts (2 donors/1 recipient) as indicated. In some experiments, sorted G-MDSCs from $PD\text{-}L1^{-/-}$ MOG/CFA-immunized mice were used.

4.6 Immunofluoresence

At the day of collection mice were deeply anesthetized with sodium pentobarbital (Dolethal 0,7 ml/kg i.p) and were perfused transcardially with heparinised saline solution for about 15min and then undergone perfusion in 4% PFA, 15% Picric Acid, 0.05% Glutaraldehyd in PB 0.1M, for another 15min. After the perfusion the spinal cords were collected and maintained in the same fixative over night at 4°C. Spinal cords were then washed in 0.1M PB, embedded in 2.5% agarose and stored at 4°C in 0.1M PB. The samples were sectioned (45µm) in a vibratom and free floating sections was processed for immunostaining.

Sections were washed in PB 0.1 M then in TBS and incubated for 45 min with 10% horse serum in TBS-T 0.1%. For MBP staining sections were prefixed for 15 minutes in cold methanol. The normal serum was drained off and the primary antibodies [anti-MBP 1:500 (chemicon, ab980), anti-CD11b 1:100 (BD 55330), anti-Ly-6G 1:100 (BD 551459)], diluted in TBS-T 0.1% with 1% horse serum, were added. Sections were incubated for 4h at RT and overnight at 4°C; they were then washed in TBS-T 0.1% and the secondary antibodies (Alexa Fluor 488, 546 and 633, 1:1000 in TBS-T 0.1%) were added for 6h at RT. Sections washed in TBS-T, TBS and in PB 0.1 M counterstained with Hoechst for 5min ant RT then coverslipped with Vectashield (Vector, H-1400) and visualized in a confocal microscope.

4.7 Histological analysis

To assess the degree of CNS inflammation, mice were perfused by intracardiac infusion of 4% paraformaldehyde. The spinal cords were dissected out and incubated in the same fixative overnight at 4°C followed by PBS wash (6h at room temperature). Fixed tissues were then immersed in 0,86% saline(6h) and dehydrated through a graded ethanol series(30% (6h), 50% (6h), 70% (6h), 80% (6h), 90% (6h) and 100%(6h) RT). Tissues were cleared with chloroform (12h) at room temperature and then embedded in melted paraffin wax at 56-58°C for 12-24 hrs, with 3 changes of wax. Transverse sections (6-7µm) from cervical, upper thoracic, lower thoracic, and lumbar regions of the spinal cord were stained with Hematoxylin & Eosin, and analyzed in a blinded fashion for infiltrating cells using Nikon Eclipse E800 microscope.

4.8 Flow cytometry and cell sorting

Single-cell suspensions were prepared from tissues and cells were stained for extracellular markers for 20 min at 4°C in PBS/5% FCS. Intracellular Foxp3 staining was performed using the anti-mouse Foxp3 staining set according to manufacturer's protocol (eBioscience). Dead cells were identified and excluded from all analyses by 7-aminoactinomycin D (7AAD, BD-Pharmingen). For tetramer staining, LN cells ($2x10^6$cells) were incubated for 5 min with 10% mouse and rat sera (Jackson ImmunoResearch Laboratories) followed by 45min staining with 10 μg/ml of tetramer at room temperature. Mabs and viability dyes were added thereafter for 20 min on ice. Cells were acquired on a FACSCalibur (BD Biosciences) and analyzed using the FlowJo software (Tree Star Inc.). Cell sorting was performed using the high speed cell sorter MoFlo (Dako).

4.9 T cell proliferation assays and cytokine assessment

Draining inguinal LNs were harvested 9-10 days after immunization and were cultured (6 x 10^5cells/200μl/well) at the presence or absence of MOG_{35-55} peptide for 72h. Cells were then pulsed with 1μCi ^{3}[H] thymidine (TRK120; Amersham Biosciences) for 18h and incorporated radioactivity was measured using a Beckman beta counter. Results are expressed as Stimulation index (S.I.) which is defined as cpm in the presence of antigen/cpm in the absence of antigen. Cytokines were assessed in culture supernatants, collected after 48h of stimulation. Detection of IL-2, IFN-γ (BD OptEIA^{tm}, BD Biosciences) and IL-17 (R&D systems) was performed by ELISA following the manufacturer's recommendations. Light absorbance at 450nm was measured using a Vmax plate reader (Biorad). In other experiments, inguinal lymph nodes were dissected and analyzed by flow cytometry as indicated in the figure legends.

4.10 *In vitro* suppression assay

Naive mouse $CD4^+CD25^-$ T cells (from B6 or $PD1^{-/-}$ mice) were sorted (purity >99%) and were stimulated with 10μg/ml plate bound anti-CD3 (145-2C11, BD Pharmingen) and 1μg/ml anti-CD28 (37.51, BD Pharmingen). Purified $CD11b^+Ly6G^+$ MDSCs (purity >95%) were activated with rmIFN-γ for 24h and then added to the culture at 1:1 ratio.

Proliferation was assessed by $[^3H]$ thymidine uptake. Activation of T cells were determined based on CD44 expression by flow cytometric analysis.

4.11 Phenotypic analysis

Sorted G-MDSCs or $Ly6G^- CD11b^+$ ($7AAD^-CD3^-CD19^-$) cells were cultured ($1,5x10^6$ cells/ml) in the presence of IFN-γ (20ng/ml), LPS (1μg/ml) or IL-4 (20ng/ml) for 18-20h. Cell surface markers were assessed by flow cytometry. Nitrite quantification was assayed by using the Greiss reagent system (Promega). BMDMs generated in the presence of L929 cell-conditioned medium containing M-CSF, activated with LPS (1μg/ml) for 12h, and used as a positive control. Cultured supernatants assessed for production of IL-10 and IL-12 by ELISA (BD OptEIA^{tm} , BD Biosciences). Arginase I production was determined by western blot analysis. ROS production was determined using the oxidation-sensitive dye Aminophenyl fluorescein (APF, Cell Technology Inc), (see also M&M chapters 4.12, 4.13, 4.14)

4.12 Western Blot analysis for Arginase I detection

Whole cell lysates (40 μg protein) were subjected to SDS-PAGE electrophoresis on 10% gels and then transferred to nitrocellulose membranes (Protran, Whatman). Membranes were blocked with 5% milk in TBST and then incubated with: anti-Arginase I antibody (1:1000; BD Biosciences) and anti- β- tubulin (1:2000; Sigma) as a loading control. Detection was performed by using HRP-conjugated anti –immunoglobulin (Sigma) and chemoluminescent reagents (Supersignal Substrate; Pierce).

4.13 Quantification of $NO2^-$

Nitrite quantification was assayed by using the Greiss reagent system (Promega) according to the manufacturer's instructions. Griess Reagents were added to samples in order to convert nitrite into a magenta-colored compound. The absorbance at 550 nm was examined with a microplate reader (Bio-Rad) and the concentration of nitrite was determined by comparing with standards.

4.14 Reactive Oxygen Species (ROS) detection

Sorted G-MDSCs (7AAD$^-$CD3$^-$CD19$^-$) were cultured in the presence of appropriate stimulators (as indicated in chapter 4.13) and for the last 30min the oxidation-sensitive dye Aminophenyl fluorescein was added in a final concentration of 10 μM. Then, cells collected , washed with PBS and intracellular ROS production detected by fluorescuorecene measurement (excitation : 488nm, emission: 515nm) using Flow Cytometry.

4.15 Morphologic analysis

G-MDSCs sorted from spleens of MOG/CFA-immunized mice or HLA-DR$^{-/low}$CD14$^-$ CD15$^+$CD33$^+$ cells sorted from PBMCs of active MS patient, were collected on precoated (poly-L-lysine) coverslips, fixed with methanol and then stained with May-Grunwald and Giemsa dye for 5 and 10 min, respectively. Images were obtained using the Nikon Eclipse E800 microscope. with lenses at 100x/ magnification and with immersion oil.

4.16 Preparation of CNS mononuclear cells.

Mice perfused with 10 to 15 ml PBS and spinal cords dissected and manually homogenized in PBS by passing the the tissue through a cell strainer (70 μm) using the back of a syringe plunger. Single cell suspensions collected and centrifuged for 10 min at 390 g. Cells resuspended in 30% Percoll, overlaid onto 70% Percoll and centrifuged at 390-500 g for 20 min at room temperature. Mononuclear cells collected from the 30/70% interface, washed twice with PBS and resuspended in culture medium for further analysis.

4.17 In vivo Brdu incorporation

5-Bromodeoxyuridine (BrdU) was injected intraperitoneally (1 mg/mouse) into MOG/CFA-immunized mice. Spleens and thymi were isolated 24h later and cells were stained and analyzed by flow cytometry for Brdu incorporation using the BrdU flow kit (BD Biosciences) according to the manufacturer’s instructions.

4.18 In vivo Ag Uptake by DCs (immunization with FITC-conjucated ovalbumin)

Alexa 488-conjucated OVA emulsified in CFA(1:1) and injected subcutaneously in C57/BL6 mice (100µg/mouse). Nine days after immunization draining LN cells collected and stained with DCs specific markers and Ag uptake(Alexa 488-conjucated OVA) was measured using flow cytometric analysis.

4.19 Multiple sclerosis subjects

MS patients were recruited through the Neurology Department of University Hospital of Heraklion. The disease's diagnosis and classification was established by the clinical and MRI criteria of the International Panel on MS. Other autoimmune and/or immune-mediated or infectious diseases of the central nervous system were sought out and excluded by appropriate clinical and diagnostic evaluations. MS subjects with active disease were those with acute or sub acute neurological symptoms either due to the initial MS episode (disease onset) or to a subsequent relapse, followed by improvement. On the other hand, patients with relapsing-remitting MS who were clinically stable and who had experienced no clinical exacerbation for at least six months prior to the time of the study were considered to be in remission. The Clinical Research Ethics Board at the University Hospital of Crete (Heraklion, Crete), approved this study. Informed consent was obtained from all patients prior to sample collection.

4.20 Human cell isolation from peripheral blood

Heparinized blood was collected from healthy subjects and MS patients and peripheral blood mononuclear cells (PBMCs) were isolated on Histopaque-1077 (Sigma) density gradient. MDSCs and $CD4^{+}$ T cells were analyzed by flow cytometry and sorted as described. The Clinical Research Ethics Board at the University Hospital of Crete (Heraklion, Crete), approved this study.

4.21. T cell *in vitro* suppression assay from MS subjects

Human $CD4^{+}CD25^{-}$ T cells were sorted (purity >99%) from PBMCs, labelled with CFSE (1µM for 10min at 37°C in labelling buffer-PBS/0.1% BSA) and co-cultured with autologous sorted $HLADR^{-/int}CD14^{-}CD15^{+}CD33^{+}$ cells (purity >95%) in the presence of 2

μg/ml plate bound anti-CD3 (OKT3, e-Bioscience) and 1 μg/ml anti-CD28 (CD28.2, e-Bioscience). Proliferation and activation of T cells was determined based on CFSE dilution and CD25 upregulation by flow cytometry. The levels of IL-2 in culture supernatants were measured after 48h by using human cytokine ELISA kit (eBioscience).

4.22 Statistics

P values were derived using two-tailed Student's *t* tests when indicated. Mann-Whitney *U* test was used for the statistical analysis of human MDSCs frequency. Non-parametric Wilcoxon signed rank test was used in the longitudinal analysis of MDSCs in MS patients. All analysis were performed using Prism (GraphPad Software Inc.).

6. RESULTS

5.1 In vivo depletion of pDCs ameliorates the severity and onset of EAE.

In order to investigate the role of pDCs during the priming of EAE, C57BL/6 mice were treated with 120G8 mAb one day before, and two days after MOG immunization (day 0) as shown in **Figure 1A**. Injection of 120G8 mAb efficiently depleted pDCs (gates were set on $7AAD^-CD3^-CD19^-CD11b^-$) in spleen and LNs as compare to untreated mice (**Figure 1B**). Interestingly, depletion of pDCs during MOG/CFA immunization resulted in delayed onset (15 ± 1.5 days vs 10 ± 1.9 days) and less severe course (max severity 1.07 ± 0.6 vs 2,93 ± 1.2 ** p= 0,016) of EAE compared with MOG/CFA immunized controls (**Figure 2A**). Histopathological analysis of the spinal cords demonstrated markedly reduced inflammation and demyelination in pDC depleted-MOG/CFA compared to MOG/CFA-immunized mice (control group) (**Figure 2B**). Together these data indicate that depletion of pDCs during the priming of the autoimmune response regulate the development of EAE.

5.2 Absence of pDCs leads to impaired priming of autoreactive MOG-specific T cells in the draining LNs.

Since myelin-specific Th1 cells have a crucial role in EAE development, we examined whether pDC depletion affects the activation and expansion of these cell subsets. Thus, single cell suspensions were prepared from draining LNs (dLNs) isolated from pDC depleted-MOG/CFA or MOG/CFA-immunized mice 9 days after antigenic challenge. The total LN cell numbers were significantly decreased in pDC-depleted mice as compared to untreated group (41,29 ±4,848 vs71,61±4,118 x10^6 cells, *** p=0,0005 **Figure 3A**). Furthermore, *ex vivo* staining of $CD4^+$ T cells with MOG $_{38\text{-}49}$/IA_b tetramer revealed decreased frequency, as well as reduced absolute numbers of MOG-specific T cells in LNs of pDC-depleted mice compared to control group (909±60,95 vs 1186±35,53 /10^5 $CD4^+$ T cells **p=0,0044) **Figure 3B, 3C**). Moreover, upon in vitro antigenic stimulation, LNs from pDC depleted-MOG/CFA immunized mice showed an impaired proliferation compared to control group (S.I: 20,28 ± 2,116vs 78,94 ± 6,932, *p=0,0149 **Figure 4A**) that was

accompanied by significantly reduced levels of IFN-γ in culture supernatants (1932 ± 1553vs 10200 ± 2103**,** *p= 0,0321 **Figure 4B**). Collectively, these data indicate that depletion of pDCs suppresses the priming of autoreactive pathogenic Th1 cells in the draining LNs.

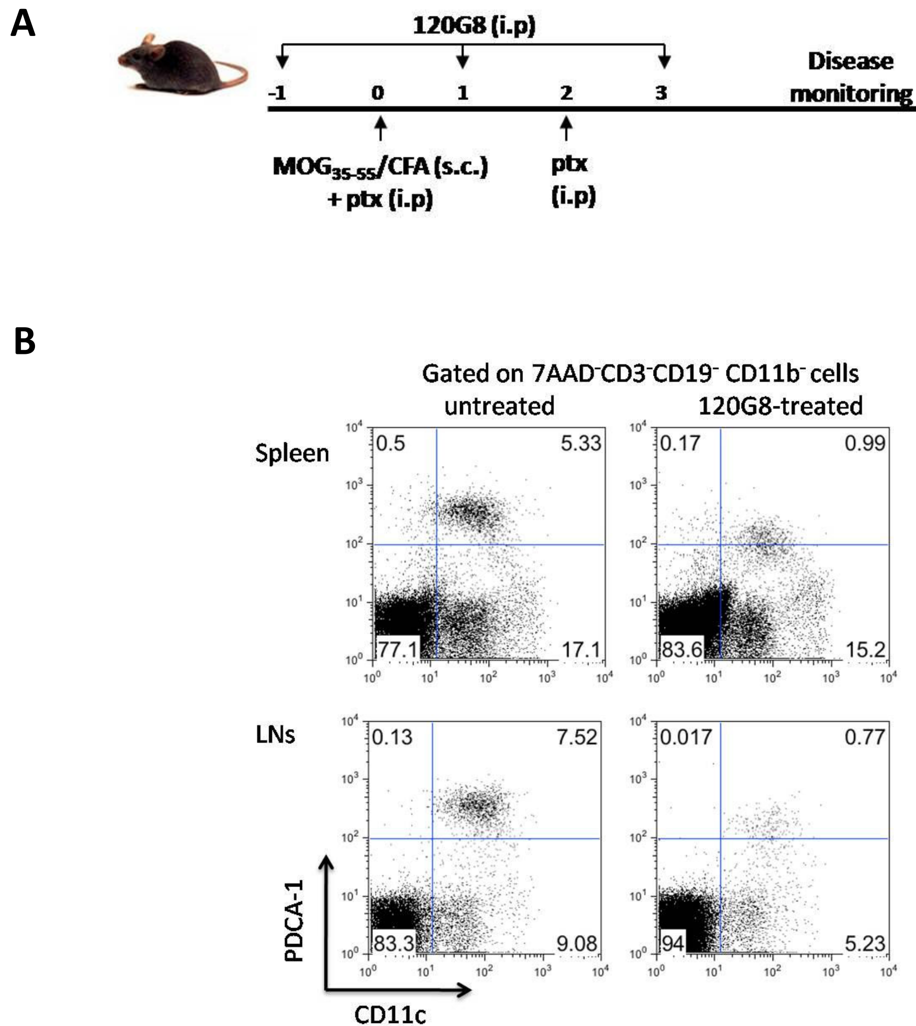


Figure 1: Depletion of pDCs with 120G8 mAb. A. Experimental protocol of in vivo pDC depletion in EAE mice. C57/BL6 female mice were immunized with MOG/CFA at day (0) and received i.p. injections of the 120G8 mAb at day (-1), (1), (3). **B**. Spleens and LNs were collected from 120G8-treated or untreated –non immunized C57/BL6 mice, 4 days after the antigenic challenge and analyzed by flow cytometry for the presence of pDCs ($PDCA1^{+}CD11c^{low}$), based on the expression of PDCA-1 and CD11c markers. Percentages indicate frequency, and gates were set on $7AAD^{-}CD3^{-}CD19^{-}CD11b^{-}$ cells.

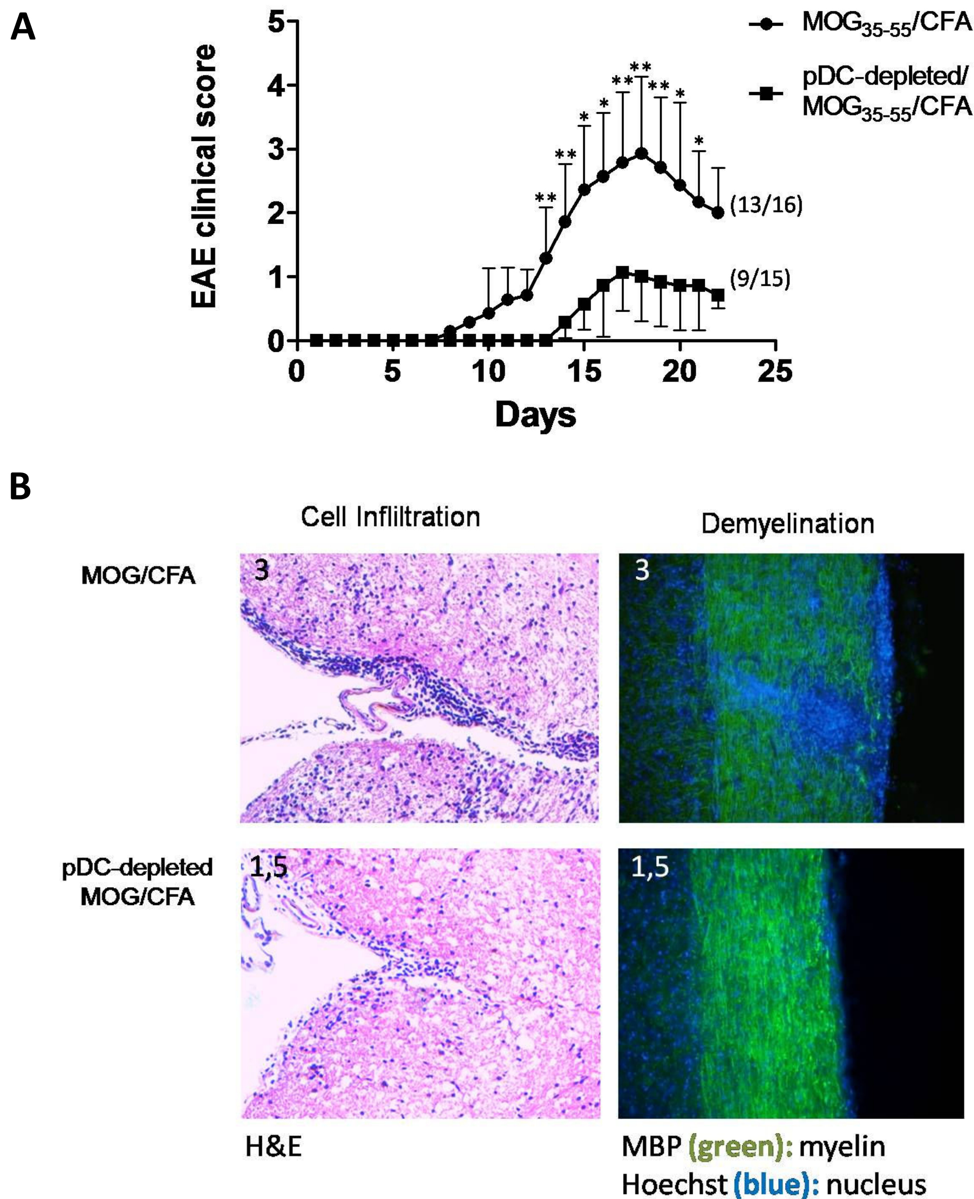


Figure 2: In vivo depletion of pDCs ameliorates immunopathology of EAE. A. Mean clinical score of EAE in pDC-depleted and control mice. Numbers in the parenthesis represent affected /total mice (n=15-16/group). Data are representative of two independent experiments (* p≤0,05 , ** p≤0,02). **B.** Immunohistological analysis of spinal cords isolated from the indicated groups of mice, 14 days after the antigenic challenge. Inflammatory cell infiltration is indicated by H&E staining (Left panel) and demyelination is shown by MBP (green) and Hoechst (blue) staining, right panel; scale bar 100μm. Results are representative of two-three independent experiments.

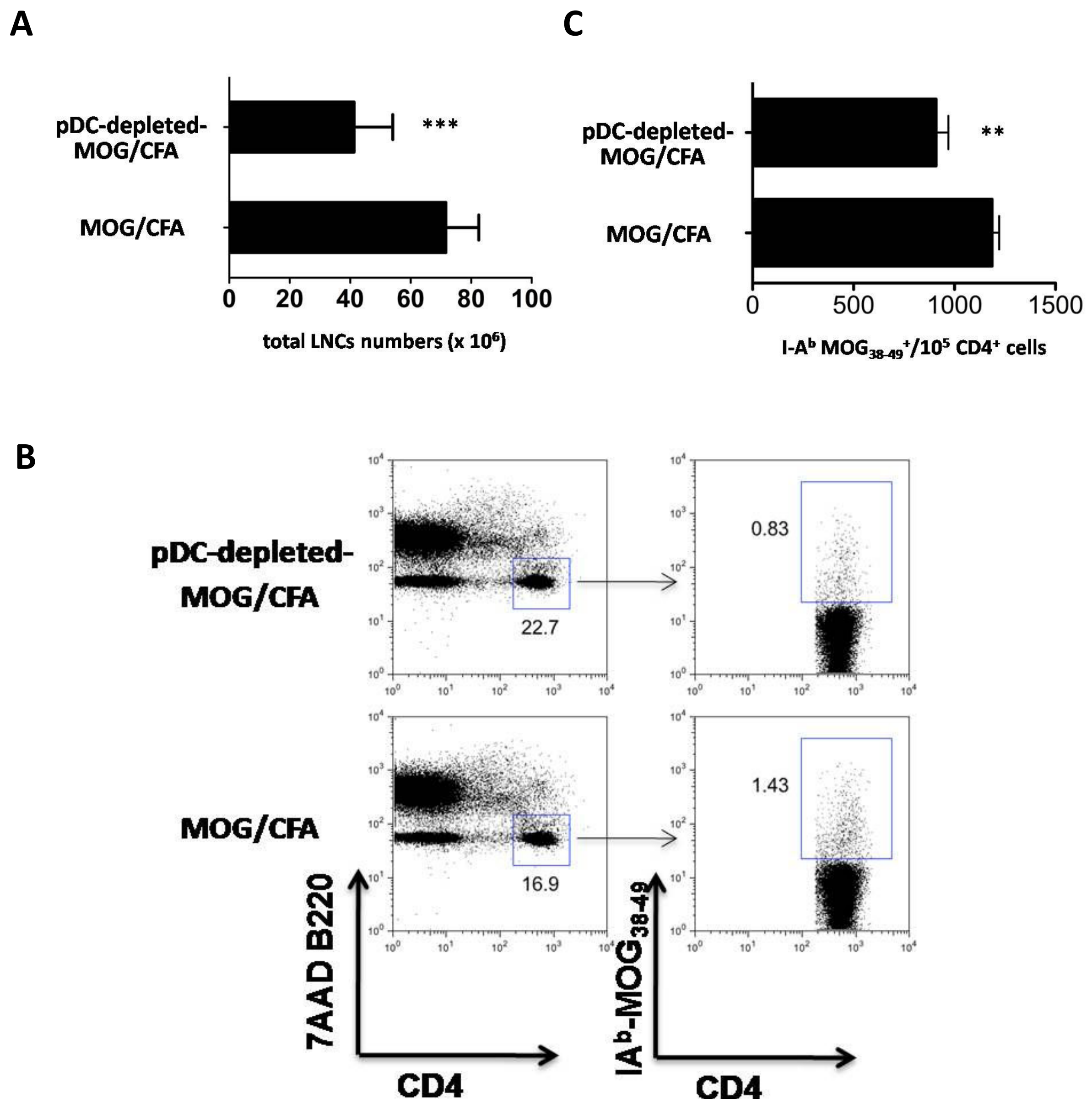
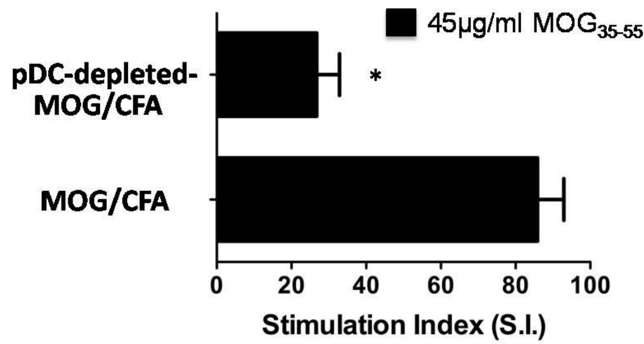


Figure 3: Decreased frequency and absolute numbers of autoantigen–specific CD4$^+$ T cells in the dLNs of pDC-depleted mice. Mice were sacrificed 9 days after immunization and draining lymph nodes (LNs) were excised. **A.** Bars represent the total LNC numbers in control and pDC-depleted MOG/CFA immunized mice. **B** and **C** Frequency and absolute numbers of MOG-specific CD4$^+$ T cells in pDC-depleted MOG/CFA and control immunized mice was determined upon staining of LNCs with IA^b-MOG$_{38-49}$ tetramer. Dot plots depict percentages of tetramer$^+$7AAD$^-$B220$^-$CD8$^-$CD4$^+$ T cells.

A



B

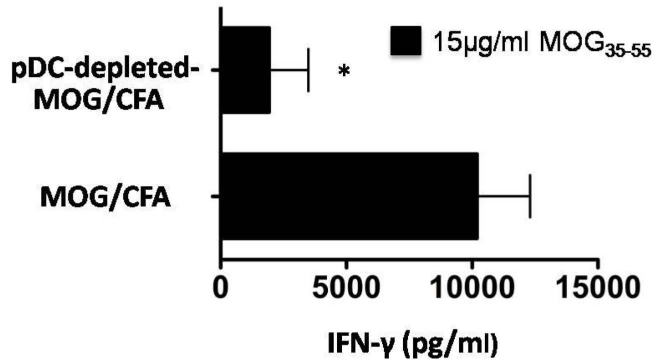


Figure 4: Depletion of pDCs inhibits the priming of autoantigen specific T cells. A. Draining LNs were isolated at day 9 after immunization from control and pDC depleted group and LNCs cultured in the presence or absence of $MOG_{35\text{-}55}$ peptide (45µg/ml). 18 hours before harvesting, 1 µCi of [^{3}H] was added and incorporated radioactivity was measured. Results are expressed as stimulation index (S.I.= cpm in the presence of antigen/cpm in the absence of antigen). **B.** Detection of IFN-γ in culture supernatants harvested after 48h stimulation of LNCs with $MOG_{35\text{-}55}$ peptide (15µg/ml) was performed by sandwich ELISA.

5.3 Depletion of pDCs does not alter the frequency of Foxp3$^+$ Treg in peripheral lymphoid organs.

Since pDCs have been implicated in the regulation of Treg induction (Kavousanaki et al., 2010), we therefore examined whether pDC depletion altered the frequency of CD4$^+$ Foxp3$^+$ T cells in the peripheral lymphoid organs of pDC depleted mice. To address that, Foxp3-GFP mice were immunized with MOG/CFA and treated or not with pDC-depleting mAb as described in Figure 1A. Then single cell suspensions from dLNs were analysed for the frequency of CD4$^+$ Foxp3$^+$ T cells by FACs analysis at day 9 after immunization. As shown in **Figure 5**, no significant alterations in the frequency of CD4$^+$ Foxp3$^+$ Tregs were observed between pDC depleted and control immunized group. These results suggest that impaired priming of MOG-specific T cells in pDC-depleted MOG/CFA immunized mice, might not be attributed to differences in the frequency of Foxp3$^+$ Treg cells.

5.4 pDC depletion does not affect recruitment and antigen uptake ability of conventional DCs (cDCs) in the dLNs.

Priming of antigen-specific T cells takes place in the dLNs upon recognition of antigen presented by dendritic cells (Shortman and Naik, 2007). Since it has been demonstrated that both conventional (cDCs) and pDCs can efficiently prime naïve T cells, we asked whether pDC-depletion affects the recruitment of cDCs in the draining LNs. To this end, we examined the frequency of cDCs (7AAD$^-$CD3$^-$CD19$^-$PDCA-1$^-$CD11c$^+$) at day 0 and day 9 after immunization and we observed similar accumulation of cDCs between control and pDC-depleted mice (**Figure 6A**). To investigate if cDCs in pDC-depleted mice can efficiently uptake antigen, fluorochrome-conjucated OVA emulsified in CFA was injected and after 9 days we followed the fate of cDCs in the dLNs. As shown in **Figure 6B**, similar frequencies of OVA-loaded cDCs were observed in both groups of mice. Collectively, our data suggest that the ability of cDCs to uptake antigen and migrate to dLNs was not affected by the absence of pDCs.

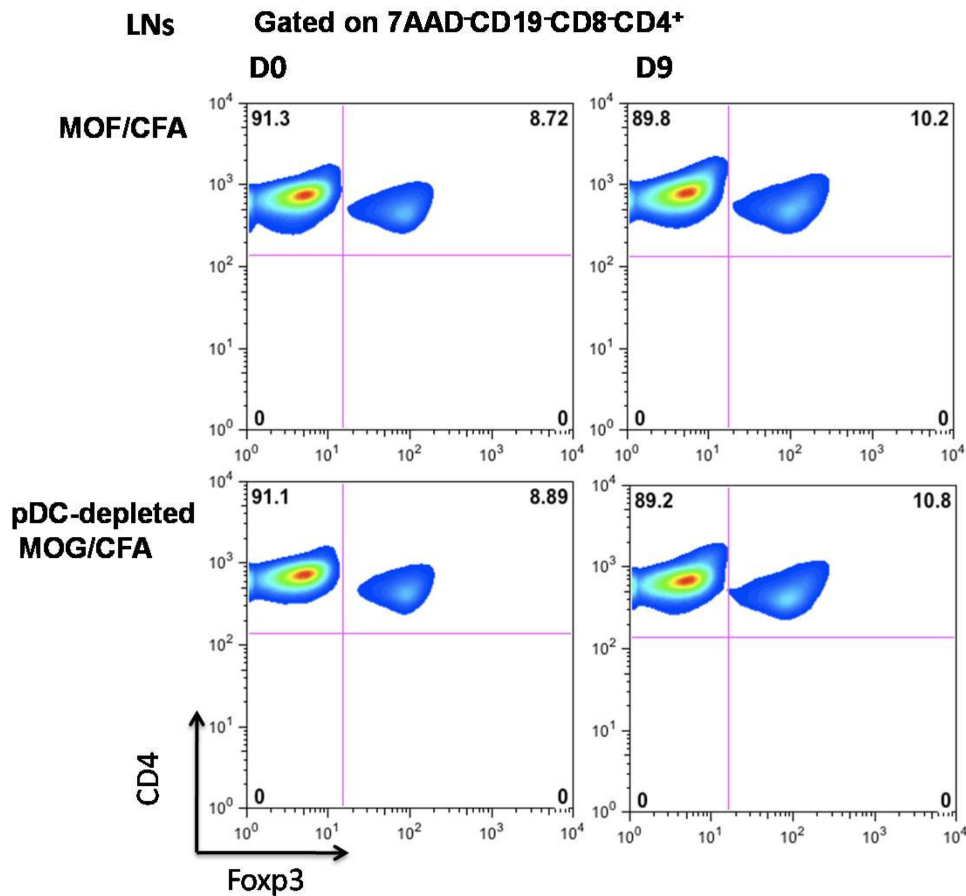
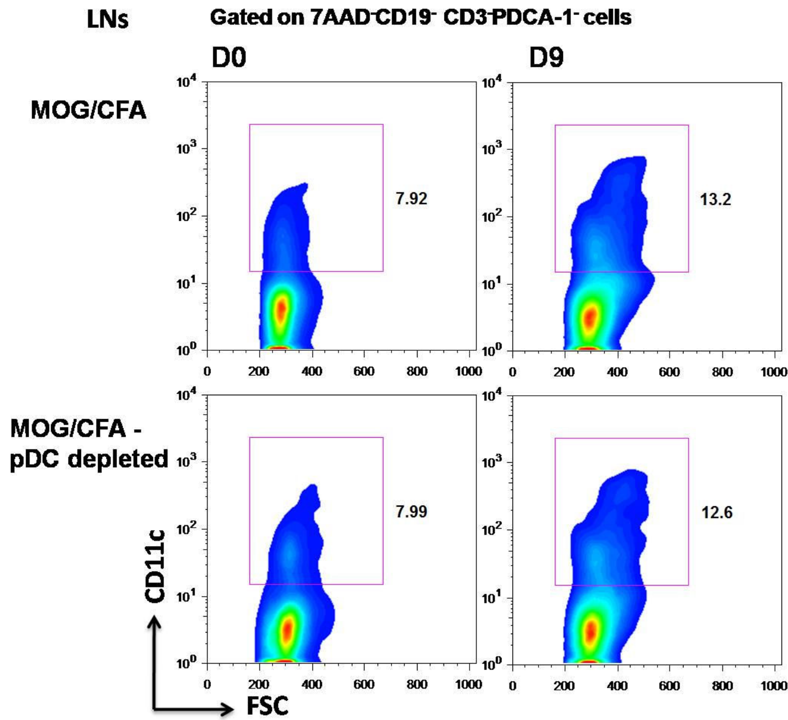


Figure 5: pDC depletion does not affect the frequency of Foxp3⁺ Tregs in the periphery.

Draining LNs from MOG/CFA and pDC-depleted MOG/CFA-immunized mice (Foxp3GFP-C57/BL6) , were isolated at day 0 and day 9 after immunization and analyzed for the frequency of $CD4^+$ $Foxp3^+$(GFP^+) T cells by FACs analysis. Gates were set on $7AAD^-CD19^-$ CD8-$CD4^+$ cells.

A



B

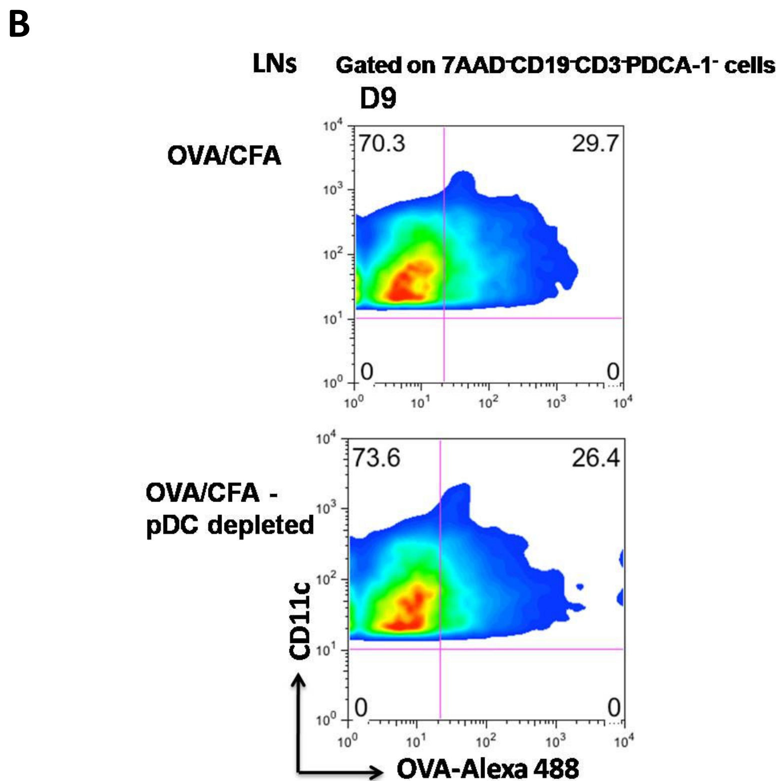
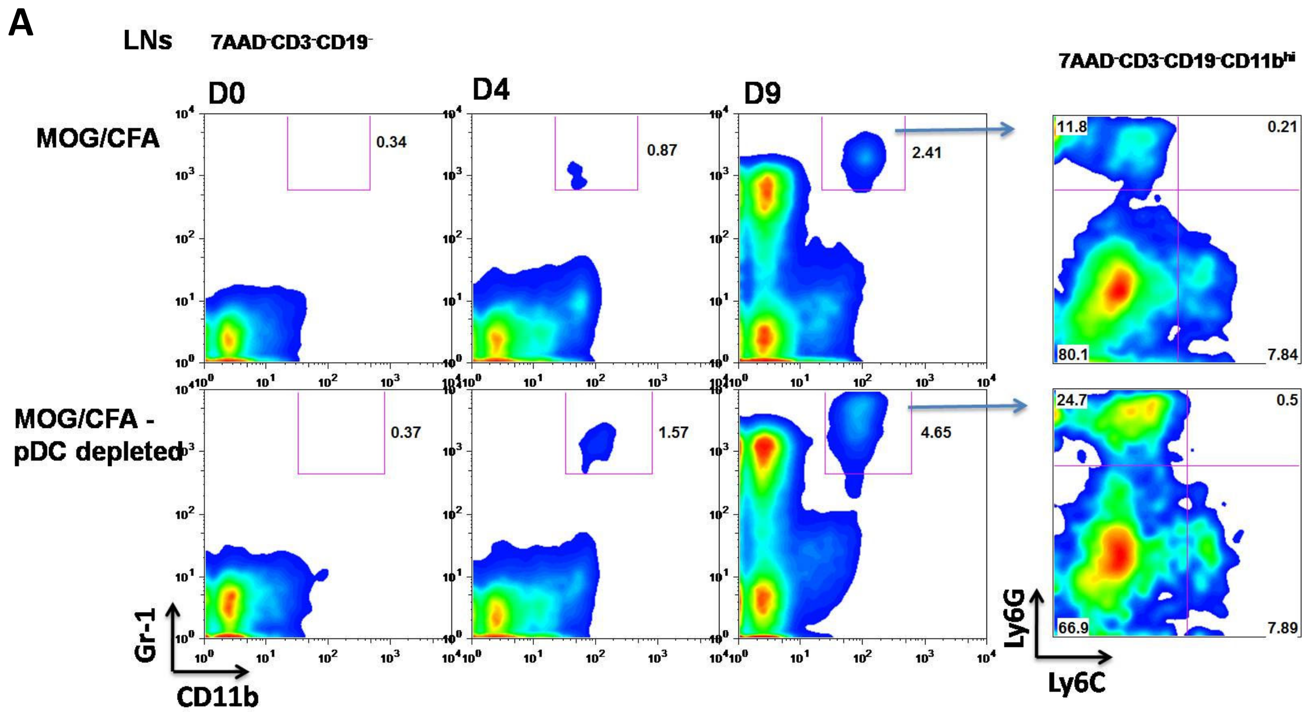
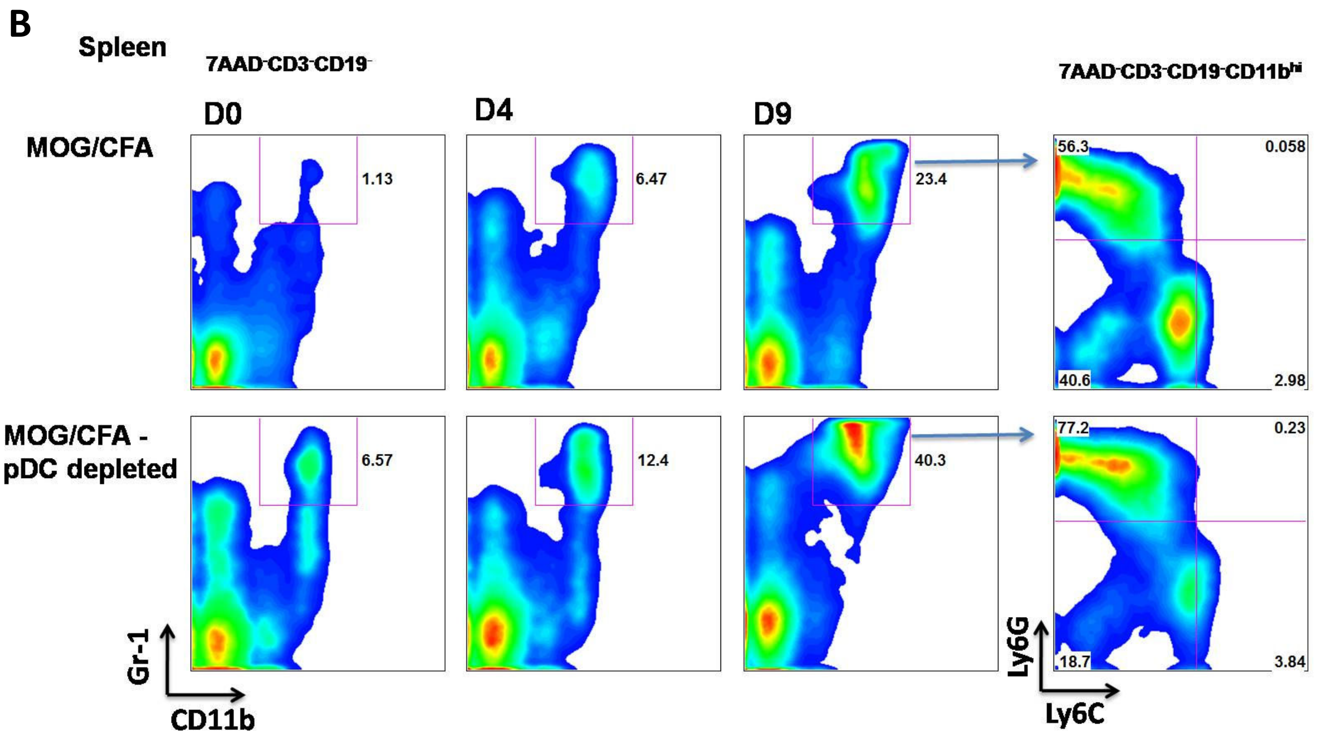


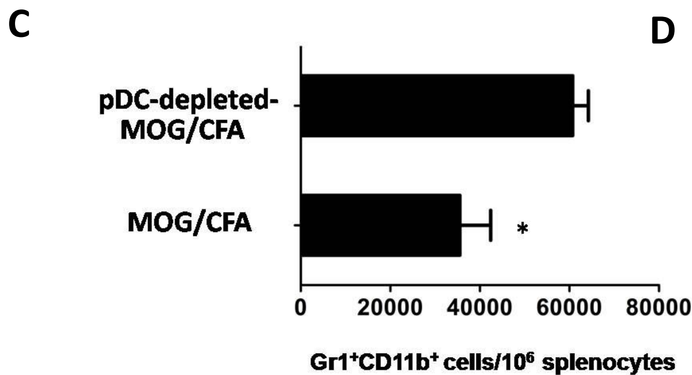
Figure 6: Absence of pDCs does not affect the antigen uptake ability and migration of cDCs to the dLNs. **A.** Draining LNs isolated from pDC-depleted and control mice 9 days after antigenic challenge (Day 0) and analyzed for the frequency of cDCs based on the expression of CD11c. Numbers indicate percentages and gates were set on 7AADCD19CD3PDCA-1$^-$ cells. **B.** Control and pDC-depleted mice were immunized with Alexa 488-conjucated OVA (100μg/mouse). Nine days after immunization draining LN cells stained with DCs specific markers and Ag uptake (Alexa 488-conjucated OVA) was measured using flow cytometric analysis. Plots show the frequency of cDCs that uptake OVA which are the CD11c$^+$OVA$^+$ cells. Gates were set on 7AADCD19CD3PDCA-1$^-$ cells.

5.5 Increased accumulation of myeloid derived suppressor cells (MDSCs) in the peripheral lymphoid organs and spinal cord of pDC-depleted mice.

In order to delineate the mechanisms involved in the inhibition of T cell responses and amelioration of EAE in pDC-depleted mice, we investigated the effect of pDC depletion in the accumulation and expansion of myeloid cells in the peripheral lymphoid compartments. Interestingly we observed that the frequency of $CD11b^{+}Gr1^{+}$ ($7AAD^{-}$ $CD13^{-}CD19^{-}$) cells was increased in LNs of pDC-depleted mice as compared to MOG/CFA-immunized control group (**Figure 7A**). Notably, a significant increase in frequency (**Figure 7B**) as well as absolute numbers (60750± 3511vs35520± 6883/10^{6} cells *p=0.017, **Figure 7C**) of $CD11b^{+}Gr1^{+}$ cells was observed in the spleen of pDC-depleted mice. The phenotype of this cell subset is consistent with the myeloid-derived suppressor cells (MDSCs) that have demonstrated to potently suppress ongoing T cell responses (Youn et al., 2008). MDSCs are further divided into cells with granulocytic morphology ($CD11b^{+}Gr1^{+}Ly6G^{+}Ly6C^{-}$) and cells with monocytic morphology ($CD11b^{+}Gr1^{+}Ly6C^{+}Ly6G^{-}$) (Gabrilovich and Nagaraj, 2009). Further analysis of MDSCs in LNs and spleens from the two groups of mice, revealed a significant expansion of frequency (**Figure 7A and 7B** right column) and numbers (48110± 5061vs25950± 1124 /10^{6} cells **p=0.0067, **Figure 7D)** of granulocytic $Ly6G^{+}$ MDSCs (G-MDSCs) in pDC-depleted mice as compared to control group. Collectively these data suggest that pDC depletion results in an increased accumulation of MDSCs in the peripheral lymphoid compartments that might involved in the suppression of activation and expansion of autoreactive T cells. We next asked whether, MDSCs are also accumulated in the target organ of mice during EAE. To this end, mononuclear cells were isolated from the spinal cord of the two groups of mice at the peak of the disease (d19) and were analysed by flow cytometry for the presence of MDSCs. We found 2.5-fold increased frequency of the G-MDSCs ($CD11b^{+}Gr1^{+}Ly6C^{-}$) in the spinal cord infiltrates of pDC-depleted mice compared to control mice (**Figure 8**). Together our data suggest that pDC depletion impairs the priming of autoreactive T cells and the development of autoimmunity through the accumulation of G-MDSCs in the peripheral lymphoid organs as well as target tissue.


A
LNs
7AAD-CD3-CD19-
7AAD-CD3-CD19-CD11bhi
D0
D4
D9
MOG/CFA
0.34
0.87
2.41
11.8
0.21
80.1
7.84
MOG/CFA -
pDC depleted
0.37
1.57
4.65
24.7
0.5
66.9
7.89
Gr-1
CD11b
Ly6G
Ly6C


B
Spleen
7AAD-CD3-CD19-
7AAD-CD3-CD19-CD11bhi
D0
D4
D9
MOG/CFA
1.13
6.47
23.4
56.3
0.058
40.6
2.98
MOG/CFA -
pDC depleted
6.57
12.4
40.3
77.2
0.23
18.7
3.84
Gr-1
CD11b
Ly6G
Ly6C


C
pDC-depleted-
MOG/CFA
MOG/CFA
*
0
20000
40000
60000
80000
Gr1+CD11b+ cells/10^6 splenocytes

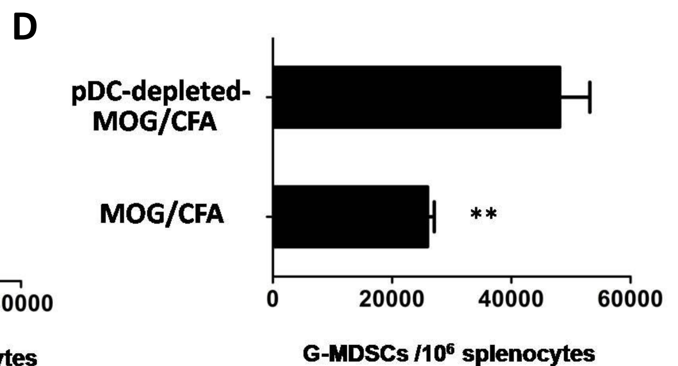

D
pDC-depleted-
MOG/CFA
MOG/CFA
**
0
20000
40000
60000
G-MDSCs /10^6 splenocytes

Figure 7: pDC depletion leads to accumulation of MDSCs in the peripheral lymphoid organs. A, B. Lymph node cells **(A)** and splenocytes **(B)** from pDC-depleted and control MOG/CFA immunized mice were analyzed for the presence of $CD11b^+Gr1^+$ (MDSCs) as well as their subsets: G-MDSCs ($CD11b^+Ly6G^+ Ly6C^-$) and M-MDSCs ($CD11b^+ Ly6G^-Ly6C^+$) at different time points (d4 and d9 after immunization respectively). Numbers show percentage of cells. Gates were set as indicated. **C, D.** Absolute numbers of MDSCs **(C)** or G-MDSCs **(D)** in the spleens of control and pDC-depleted mice 9 days after immunization.

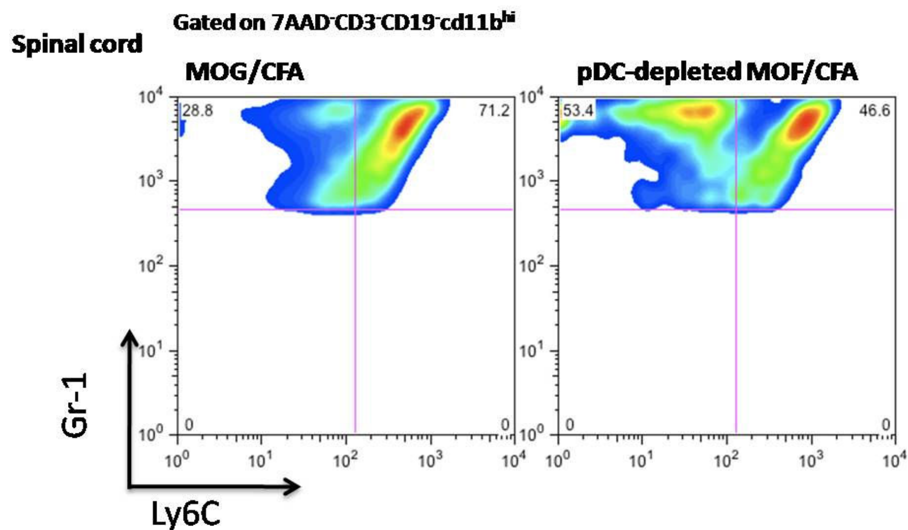


Figure 8: pDC depletion leads to accumulation of G-MDSCs in the spinal cord. Mononuclear cells isolated from the spinal cords of MOG/CFA and pDC-depleted MOG/CFA immunized mice at the peak of the disease (d19) and analysed by flow cytometry for the presence of G-MDSCs based on the expression of CD11b,Gr1 and Ly6C antigens. $CD11b^+Gr1^+Ly6C^-$ cells represent the G-MDSC subset (upper left quadrant). Numbers represents percentages. Gates were set as indicated.

5.6 Increased expansion of MDSCs in the bone marrow of pDC-depleted mice.

Ablation of cDCs in mice results in myeloid-proliferative disorder characterized by increased generation of myeloid cells in the bone marrow (BM) of cDC-deplete mice (Birnberg et al., 2008). To examine whether the increased accumulation of MDSCs in the periphery of pDC-depleted-MOG/CFA-immunized mice is a result of increased myelopoesis in the BM, we performed flow cytometric analysis of BM isolated from pDC-depleted and control mice. Interestingly, depletion of pDCs, significantly increased the frequency (**Figure 9A**) and absolute numbers (**Figure 9B**) of MDSCs (301.0 ± 4.163 vs 254.3 ± 9.871, * p=0,0121) in the BM suggesting that pDC depletion, during an inflammatory response, increases myelopoesis in the BM.

We next determined whether the increased frequency of MDSCs in the peripheral lymphoid organs is due to continued recruitment from the BM or to local expansion in the spleen. To this end, MOG/CFA immunized mice were injected with Brdu and 24h later, Brdu incorporation by MDSCs was determined. As shown in **Figure 9C**, no DNA synthesis by either G-MDSCs ($Ly6G^+CD11b^+$) or $Gr1^+CD11b^+$ MDSCs was observed in the spleen of immunized animals whereas Brdu incorporation was detected in $CD4^+$ thymocytes (positive control). These data indicate that the increased accumulation of MDSCs ($Ly6G^+$ MDSCs) at the peripheral lymphoid organs is due to their continuous release from BM rather than expansion in the site of inflammation, as no proliferation of these cells was observed in MDSCs isolated from the periphery.

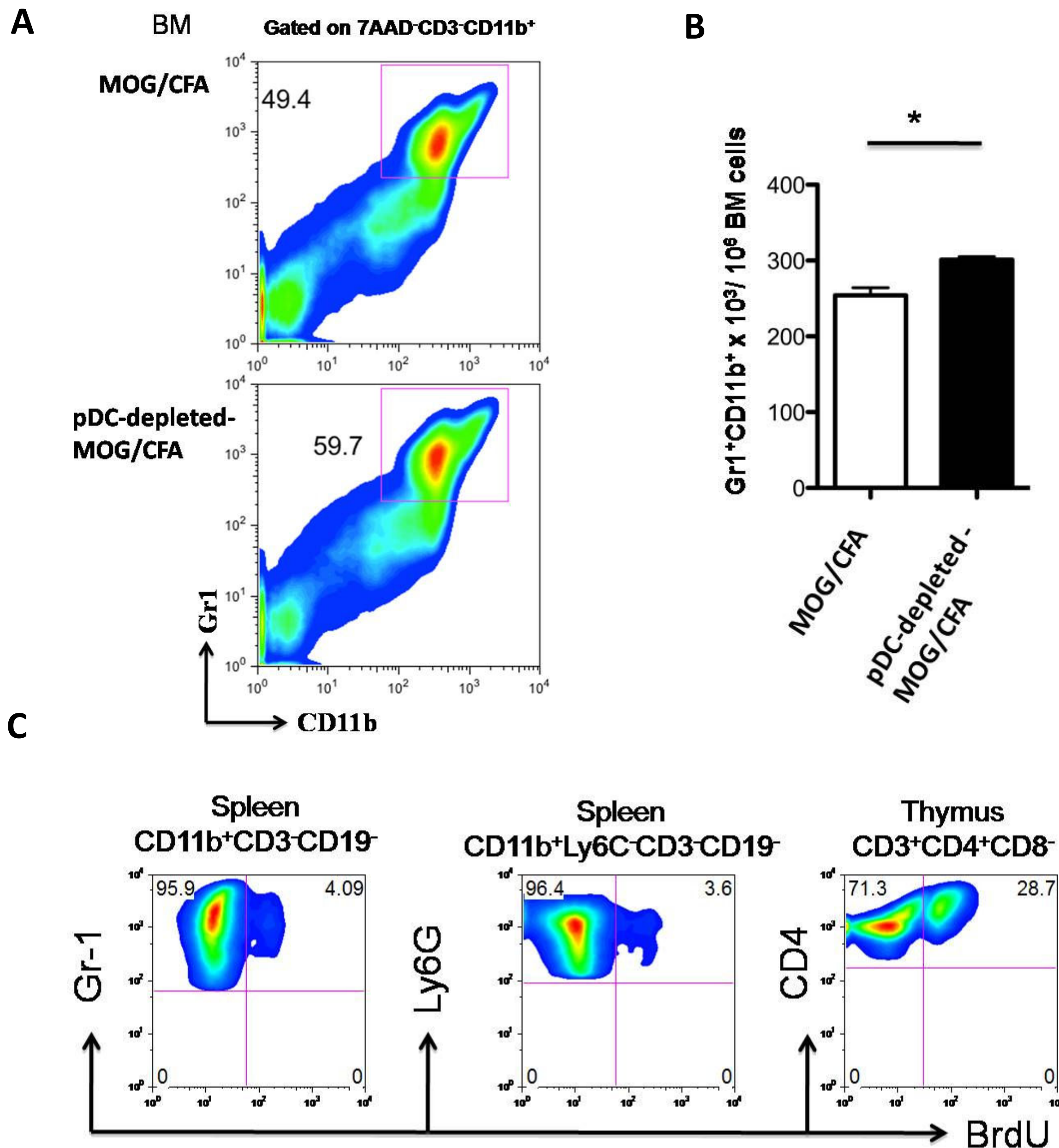


Figure 9: pDC depletion promotes the expansion of MDSCs in the BM and their release in the peripheral lymphoid organs. A, B. Frequency **(A)** and absolute numbers **(B)** of MDSCs in BM cells isolated from MOG/CFA and pDC-depleted MOG/CFA mice. Gates were set on 7AAD$^-$CD3$^-$CD19$^-$ cells. **C.** BrdU incorporation by CD11b$^+$Gr1$^+$and Ly6G$^+$ MDSCs in the spleen of MOF/CFA immunized mice 9 days after immunization. For the last 24h, mice were intraperitoneally injected (1mg/mouse) with BrdU. Incorporation of BrdU by CD4$^+$ thymocytes is shown as a positive control. Representative flow cytometric analyses indicate percentages of BrdU$^-$ or BrdU$^+$ cells in the corresponding gates. Gates were set as indicated. Results are representative of two independent experiments with 2 mice per group.

The results presented in the previous paragraphs demonstrated that ablation of pDCs during the priming of the autoimmune response resulted in (a) amelioration of disease (b) suppression of autoantigen-specific T cell responses and (c) increased accumulation of MDSCs in the peripheral lymphoid organs. Based on these data several questions have been raised that are discussed in the future direction part of this Thesis. Among them, we decided to obtain direct evidence for the role of MDSCs in the suppression of autoimmune response and resolution of inflammation as this has not been previously addressed in the field.

5.7 Increased accumulation of G-MDSC cells at the peripheral lymphoid compartments of mice with EAE.

We first determined the kinetics of $CD11b^+Gr1^+$ MDSCs expansion and/or accumulation in the peripheral lymphoid organs during the different phases of MOG_{35-55}-induced EAE (**Figure 10**). The frequency of $CD11b^+Gr1^+$ MDSCs ($7AAD^-CD3^-CD19^-$) increased during the asymptomatic phase and even more so at the onset of disease, reaching the maximum at the peak of EAE, prior to disease remission, and contracted upon resolution of disease (**Figure 11A**). Among the two subsets of MDSCs, only granulocytic-like cells ($7AAD^-CD3^-CD19^-CD11b^{hi}Ly6G^+Ly6C^-$ cells) closely followed the kinetics of EAE, since their frequency (**Figure 11B**) and absolute numbers (**Figure 11C**) gradually increased until disease peak and declined at the recovery phase, reaching basal level at disease resolution. Morphological analysis of sorted $7AAD^-CD3^-CD19^-CD11b^{hi}Ly6G^+$ MDSCs (denoted as G-MDSCs throughout this report) from spleens of MOG/CFA-immunized mice revealed ring-shaped multi-lobed nuclei, consistent with the granulocytic origin of this MDSC subset (**Figure 11D**). Notably, flow cytometry analysis revealed increased frequency of G-MDSCs in the spinal cord infiltrates isolated from mice during the peak of EAE (**Figure 12A**). Consistent with the flow cytometry data, immunofluorescent analysis of spinal cords demonstrated specific localization of $CD11b^+Ly6G^+$ MDSCs among the inflammatory infiltrates (**Figure 12B**). Collectively, our data show an increased accumulation of G-MDSCs in CNS and enhanced recruitment of this subset at lymphoid organs peaking prior to resolution of EAE. Together these findings raise the possibility of a MDSC-mediate role in the resolution of the autoimmune response.

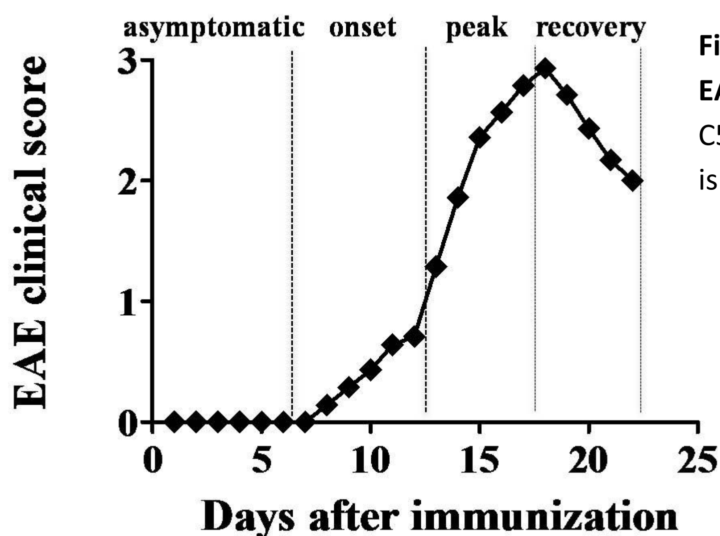


Figure 10. The four phases of EAE. MOG_{35-55}-induced EAE in C57BL/6 mice. Mean clinical score is shown (n=7).

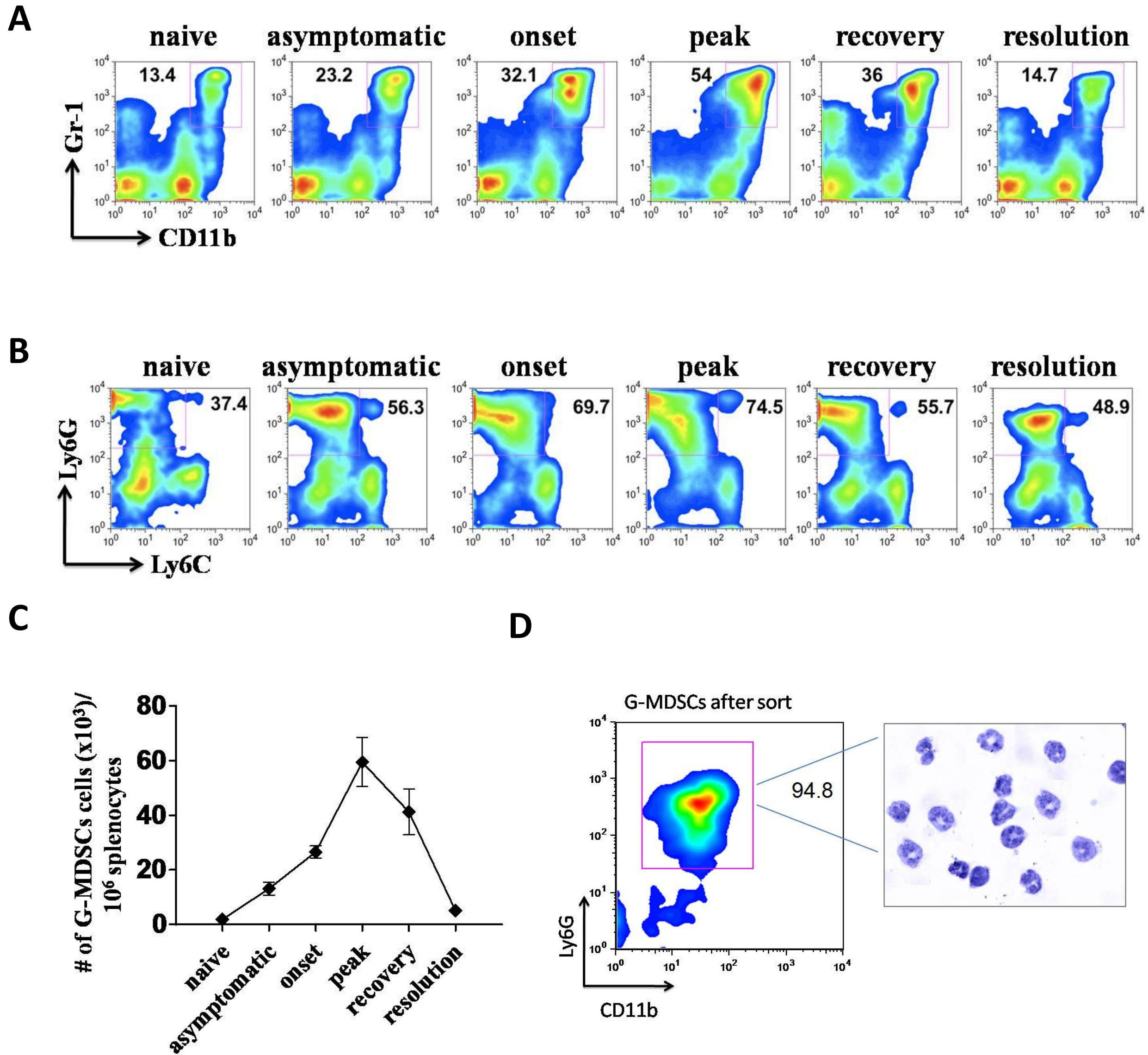


Figure 11. Recruitment of granulocytic Ly6G^{+} MDSCs in the peripheral lymphoid organs correlates with disease activity in EAE. A, B. Kinetics of CD11b^{+}Gr1^{+} MDSCs (top) and G-MDSCs (bottom) accumulation in the spleen of mice during the different phases of EAE. Representative flow cytometric analysis indicates percentages of MDSCs. Gates were set on 7AAD^{-} CD3^{-} CD19^{-} or 7AAD^{-}CD3^{-}CD19^{-}CD11b^{hi} cells for **(A)** and **(B)** respectively. **C**. Relative numbers of Ly6G^{+} MDSCs/10^{6} splenocytes during the different phases of EAE (mean ± s.d. of four mice per time point). **D**. Morphological analysis of G-MDSCs, sorted from spleens of MOG/CFA-primed mice, using May-Grunwald-Giemsa staining.

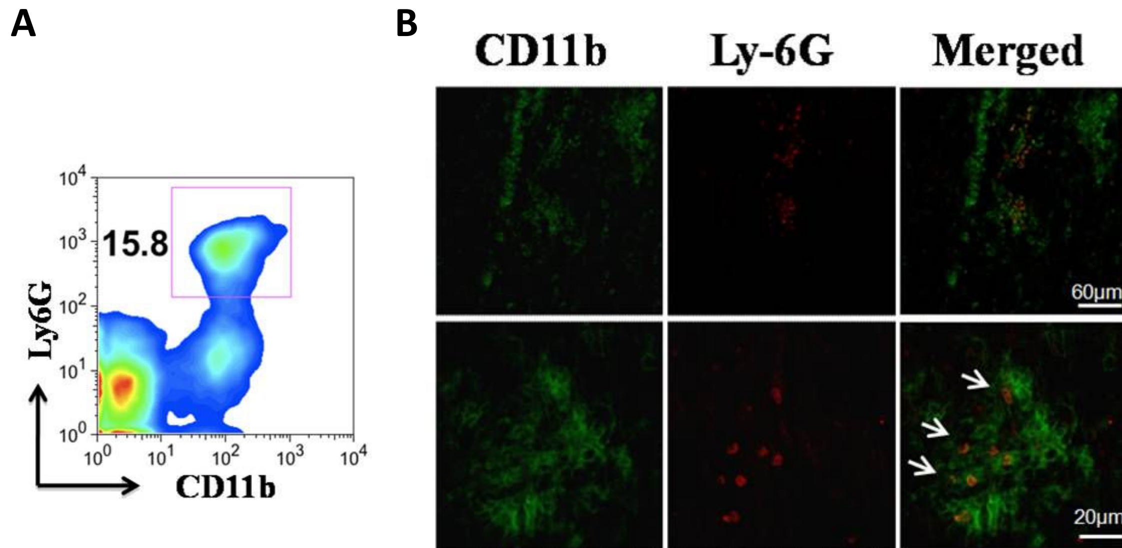


Figure 12. Recruitment of granulocytic Ly6G$^+$ MDSCs in the spinal cord of EAE mice. A. Frequency of G-MDSCs in the spinal cords of mice at the peak of EAE. Gates were set on 7AAD$^-$CD3$^-$CD19$^-$. Data are representative of 4-5 separate experiments. **B.** Immunofluorescence staining for CD11b (green) and Ly6G (red) in spinal cord sections at disease peak (score 3,5). Arrowheads show CD11b$^+$Ly6G$^+$ infiltrating cells. Magnification 40x and 60x1,65, Scale bar 60μm and 20μm, upper and lower panel respectively. Sections are representative of three mice analyzed individually.

5.8 Adoptive transfer of G-MDSCs ameliorates MOG_{35-55}-induced EAE.

To examine the ability of G-MDSCs to mediate disease remission, we adoptively transferred purified G-MDSCs isolated from spleens of MOG/CFA immunized mice into recipient mice during the course of EAE (**Figure 13A**). As control, $Ly6G^{-}CD11b^{+}$myeloid cells isolated from the same mice were used. Adoptive transfer of G-MDSCs decreased the severity of EAE (**Figure 13B**) compared to either untreated group (**p=0.0056 at d14 after the antigenic challenge) or $Ly6G^{-}CD11b^{+}$- treated mice (**p=0.0047 at d14 after the antigenic challenge) as well as significantly delayed disease onset (**Figure 13B, 13C**). Disease amelioration in G-MDSC-treated mice was accompanied with reduced inflammatory lesions in the spinal cords (**Figure 14A**) and diminished demyelination (**Figure 14B**) as compared to control groups. The few inflammatory foci detected in G-MDSC-treated mice were mostly located at the meningeal regions with little or absent parenchymal infiltration. Importantly, immunofluorescent analysis showed increased accumulation of $CD11b^{+}Ly6G^{+}$ MDSCs at the inflammatory lesion in the meningeal area of spinal cord, of G-MDSC-treated mice, whereas this was not the case in the other two groups of mice (**Figure 14C**). Collectively, these results indicate that G-MDSCs potently suppress the clinical and pathologic features of EAE.

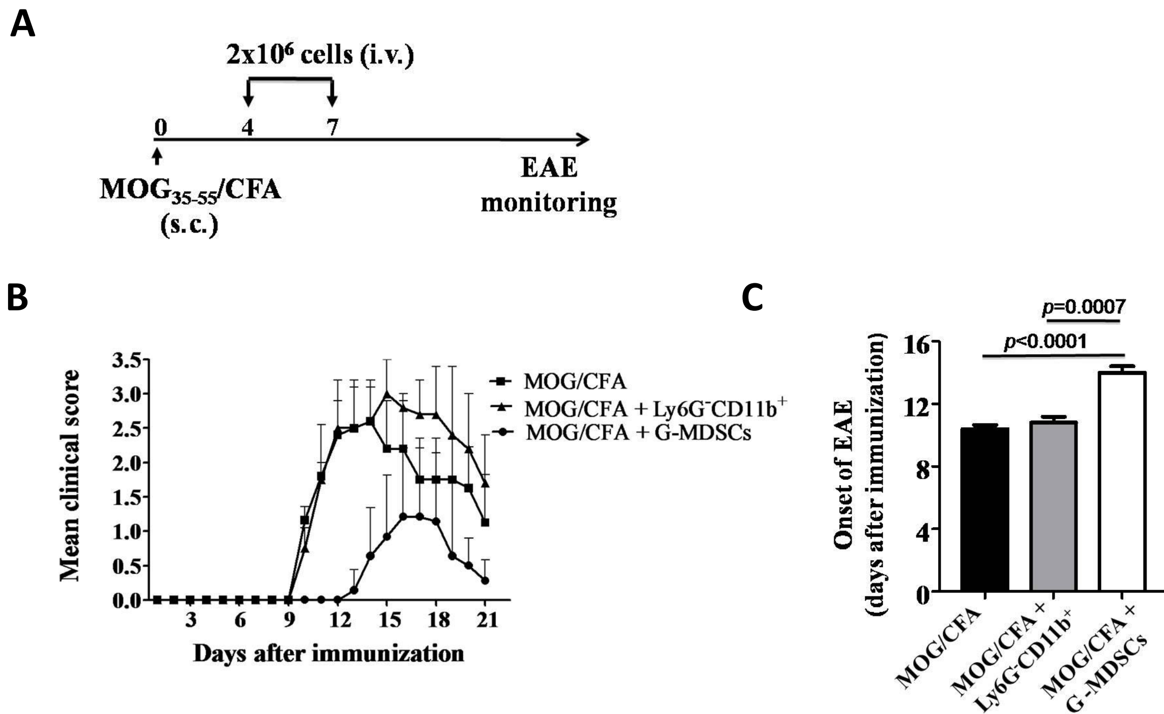
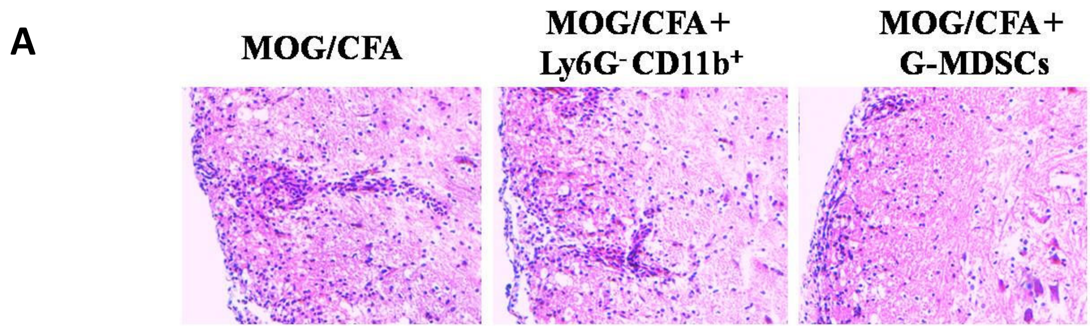
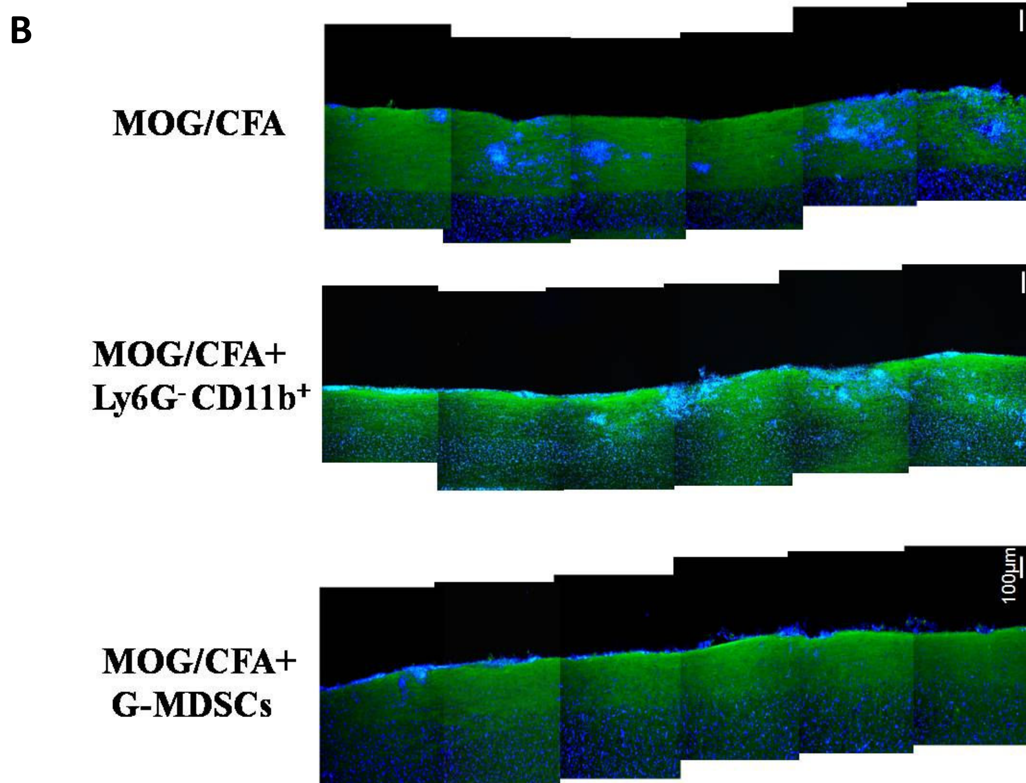


Figure 13. Adoptive transfer of G-MDSCs suppresses the clinical course of EAE. A. G-MDSCs or Ly6G$^-$CD11b$^+$ cells were sorted from spleens of MOG/CFA-immunized mice (purity >95%) and adoptively transferred (2 x 10^6/mouse) into syngeneic MOG/CFA-treated recipients on day 4 and 7 after antigenic challenge. **B.** Mean clinical score of EAE in treated or control mice (n=7 mice per group, mean ± s.d. is shown). ***C.*** EAE onset in indicated groups of mice (mean ± s.d. *p* values, *t*-test).

A



B



C

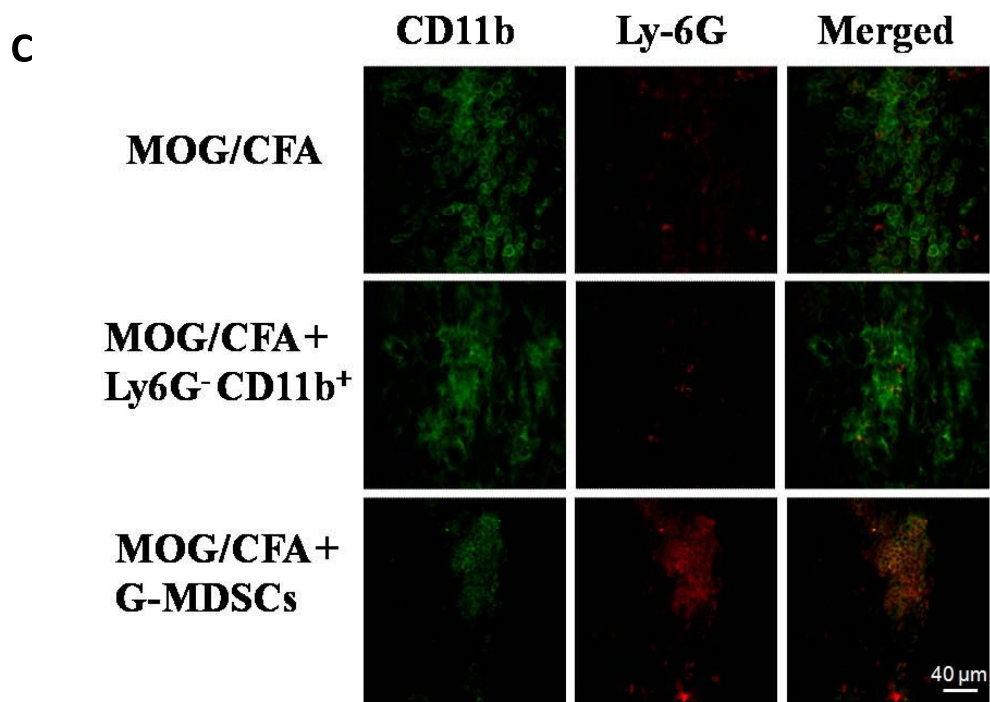


Figure 14. Immunohistological analysis of EAE mice after adoptive transfer of G-MDSCs. A, B, C. Immunohistological analysis of spinal cords isolated from the indicated groups of mice collected 12-14 days after antigenic challenge. **(A)** H&E staining, **(B)** MBP (green) and Hoechst (blue); scale bar 100µm, **(C)** CD11b (green), Ly6G (red) and two-colour overlay; magnification x60, scale bar 40µm. Results are representative of three independent experiments.

5.9 G-MDSCs suppress the priming of MOG_{35-55}-specific Th1 and Th17 cells *in vivo*.

Since EAE is initiated and perpetuated by autoreactive Th1 and Th17 cells, we further assessed whether *in vivo* transfer of G-MDSCs could influence the priming of MOG-specific T cells in the peripheral lymph nodes (LNs). To address this, mice were adoptively transferred with G-MDSCs as described in Fig 13A, and 9 days after the antigenic challenge inguinal draining LNs were assessed for MOG_{35-55}-specific T cell responses. We noted that G-MDSC transfer resulted in decreased frequency (**Figure 15A**) and significantly reduced numbers (**Figure 15B**) of $MOG_{38-49}/IA^{b+}CD3^{+}CD4^{+}$ T cells as compared to control mice, indicating that G-MDSCs suppress the expansion of autoreactive T cells. This was confirmed upon *ex vivo* stimulation of dLNs with MOG peptide where LNs from G-MDSC-treated mice showed markedly reduced cell proliferation and significant suppression of Th1- and Th-17 secreting cytokines as compared to untreated control group (**Figure 15C**). Suppression of MOG-specific Th1 and Th17 responses was accompanied by increased accumulation of G-MDSCs in the dLNs of G-MDSC-injected mice compared to control group (**Figure 15D**). In contrast, no significant difference in the frequency of $CD4^{+}Foxp3^{+}$ regulatory T cells was observed (**Figure 15E**). Collectively these results demonstrate the suppressogenic potential of G-MDSCs against MOG-specific autoreactive T cells *in vivo*.

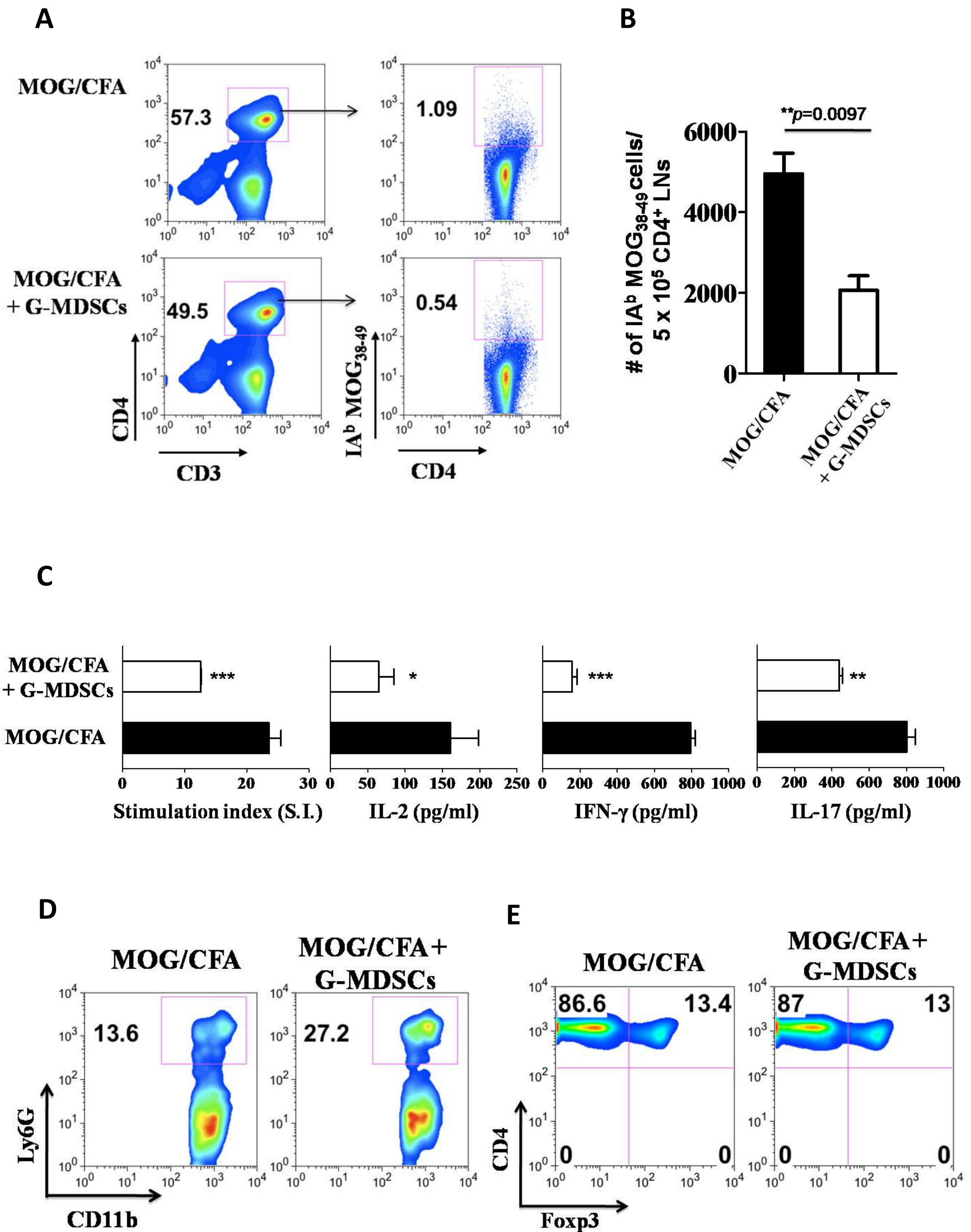

A
B
C
D
E
MOG/CFA
MOG/CFA
+ G-MDSCs
57.3
1.09
49.5
0.54
CD4
CD3
IA^b MOG38-49
CD4
**p=0.0097
# of IA^b MOG38-49 cells/
5 x 10^5 CD4+ LNs
6000
4000
2000
0
MOG/CFA
MOG/CFA
+ G-MDSCs
***
*
***
**
Stimulation index (S.I.)
IL-2 (pg/ml)
IFN-γ (pg/ml)
IL-17 (pg/ml)
MOG/CFA+
G-MDSCs
13.6
27.2
Ly6G
CD11b
86.6
13.4
87
13
0
0
0
0
CD4
Foxp3

Figure 15. G-MDSCs suppress myelin-specific Th1 and Th17-mediated immune responses in the draining LNs. A. MOG/CFA-immunized mice were adoptively transferred with G-MDSCs as in Fig. 13A. Inguinal LNs were isolated 9 days after the antigenic challenge, and assayed for tetramer binding on $7AAD^{-}B220^{-}CD8^{-}CD3^{+}CD4^{+}$ cells. Numbers denotes frequency of $CD4^{+}CD3^{+}$ cells (left panel) and $MOG_{38-49}/IA^{b+}/CD4^{+}$ cells (right panel). **B.** Relative numbers of MOG_{38-49}/IA^{b+} cells/ 5 x 10^{5} $CD4^{+}$ LNs (mean ± s.d. *p* values, *t*-test). **C.** LNs were re-stimulated in vitro with MOG_{35-55} (15 μg/ml) for 72h followed by [^{3}H] thymidine pulsing for 18h. Incorporated thymidine was measured and cell proliferation is expressed as stimulation index (mean ± s.d. of triplicate wells, ***p= 0.0002 *t*-test). Culture supernatants were collected after 48h of culture and were assessed for the presence of IL-2, IFN-γ and IL-17 by ELISA (*p=0.01, **p=0.002, ***p=0.0003, *t*-test). **D.** Frequency of G-MDSCs in the draining LNs of treated and control mice. Gates were set as in Fig. 1*C* and numbers represent percentages. **E.** Dot plots show percentages of $CD4^{+}Foxp3^{+}$ ($7AAD^{-}B220^{-}CD3^{+}$) cells in the LNs of the two groups of mice. Data are derived from 2 independent experiments with 3-4 mice per group. Each mouse was analyzed individually.

5.10 Phenotypic characterization of G-MDSCs in MOG/CFA-immunized mice.

Several mechanisms of suppression have been assigned to MDSCs during cancer and infection (Gabrilovich and Nagaraj, 2009; Nagaraj et al., 2010). However it remains to be determined how G-MDSCs exert their function in an autoimmune setting. To address this, G-MDSCs were sorted from MOG/CFA immunized mice and treated for 24h with LPS or IFN-γ, two well-known pro-inflammatory stimuli. Assessment of cytokines in culture supernatants showed increased secretion of IL-10 upon LPS or LPS/IFN-γ stimulation (**Figure 16A**) whereas IL-12 was not detected (**Figure 16B**). In addition, neither NO (**Figure 16C**) nor Arg-1 (**Figure 16D**) could be detected in stimulated G-MDSCs whereas intracellular ROS accumulation was only observed upon PMA-treatment (**Figure 16E**). In contrast, detailed cell surface phenotypic analysis of untreated G-MDSCs, revealed an increased expression of the inhibitory molecule PD-L1 (**Figure 16F**). Interestingly, treatment with LPS caused a significant up-regulation of PD-L1 expression, which was even more robust in IFN-γ-treated G-MDSCs (**Figure 16F**). Enhanced expression of PD-L1 on G-MDSCs upon treatment with IFN-γ was specific, since expression of other co-stimulatory/inhibitory markers, such as CD80, CD86, CD40 and PD-L2, was not altered (**Figure 16F**).

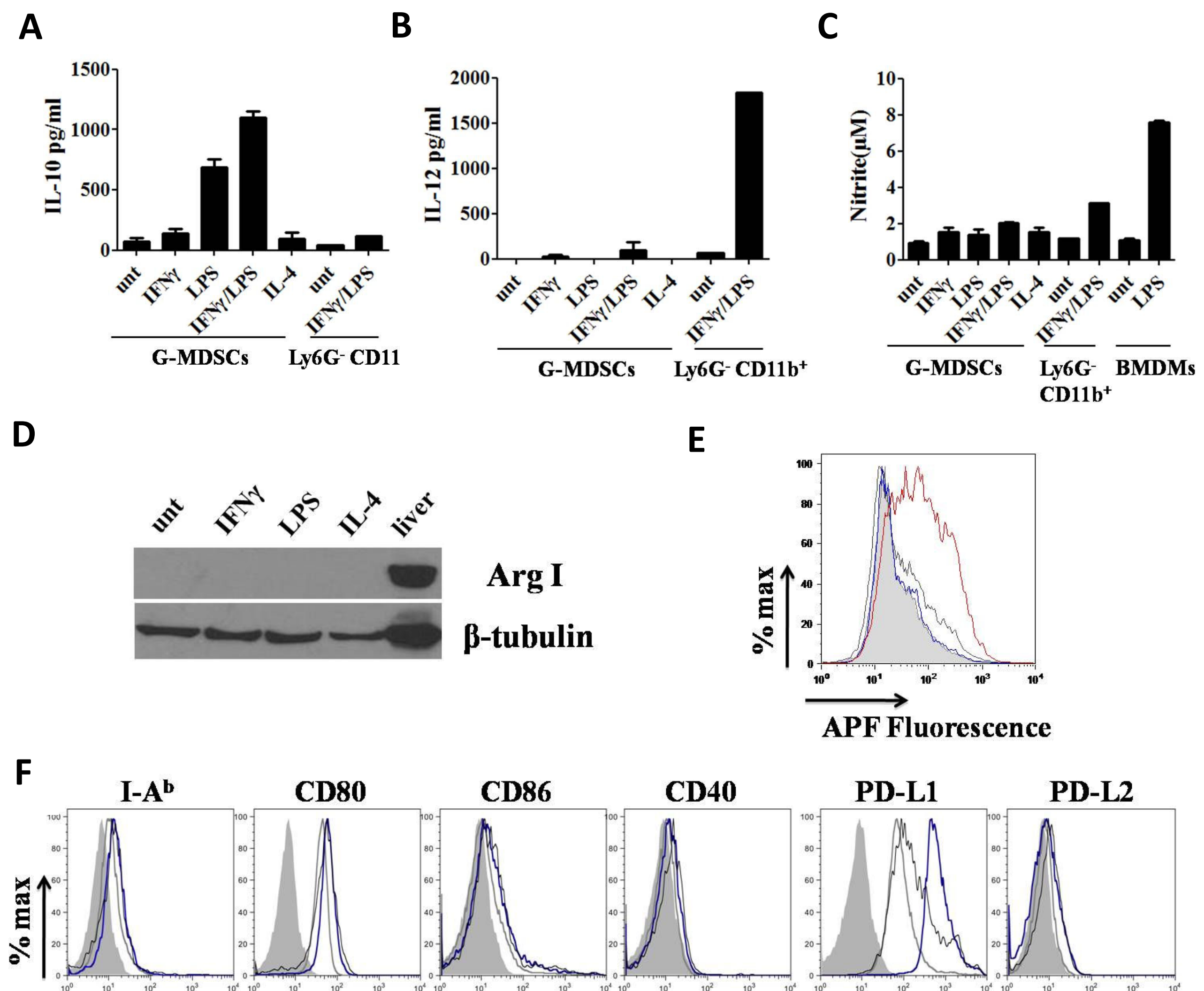


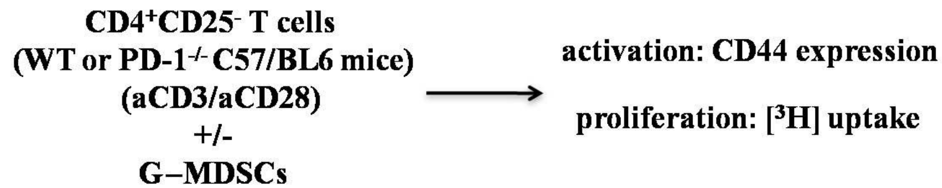
Figure 16. Phenotypic characterization of G-MDSCs isolated from MOG/CFA immunized mice. A, B, C. Sorted G-MDSCs or Ly6G$^-$ CD11b$^+$ cells from spleens of MOG/CFA-primed mice, were cultured in the presence of IFN-γ (20ng/ml), LPS (1μg/ml) or IL-4 (20ng/ml) for 18-20h and supernatants were assessed for IL-10 (**A**), IL-12 (**B**) and NO (**C**) production. When indicated BM-derived macrophages were used as positive control. Mean ± s.d is shown. **D.** Western-blot analysis of arginase-1 expression in G-MDSCs isolated and treated as described above. Whole cell lysate from liver was used as a positive control. **E.** G-MDSCs cells isolated as above and stimulated for 1h in the presence of IFN-γ (20ng/ml), LPS (1μg/ml) or PMA (5ng/ml) followed by addition of 10μM APF. Detection of intracellular ROS accumulation was determined by flow cytometry. Shaded, blue, black or red histograms represents untreated, IFN-γ, LPS, or

PMA-treated cells respectively. Representative experiment from a total of three is shown. **F.** Expression of the indicated cell surface molecules on G-MDSCs (7AAD$^-$CD3$^-$ CD19$^-$CD11b^{hi}, >95% purity) was performed by flow cytometry. G-MDSCs were cultured for 24h in the presence of IFN-γ (20ng/ml, blue line) or LPS (1μg/ml, black line). Shaded histograms show isotype-matched mAb-stained cells and open histograms represent untreated cells. Data are representative of four independent experiments.

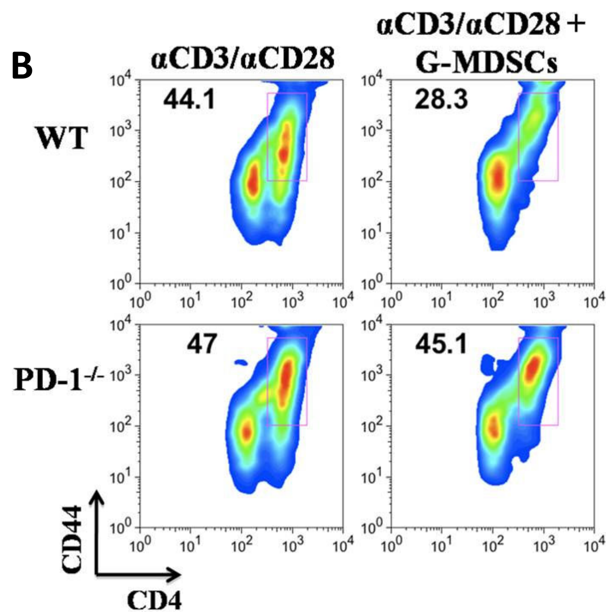
5.11 PD-L1 is required for the G-MDSC-mediated suppression of EAE.

The PD-L1/PD-1 interactions have been reported to deliver co-inhibitory signals leading to attenuation of T cell responses both *in vitro* and *in vivo* (Keir et al., 2008; Latchman et al., 2004). In order to test the functional significance of the increased PD-L1 expression observed in G-MDSCs, we first compared the activation and proliferation of naïve CD4$^+$CD25$^-$ T cells isolated from wild-type (WT) versus PD-1 knockout (PD-1$^{-/-}$) mice in the presence or absence of IFN-γ-treated G-MDSCs (**Figure 17A**). To this end, presence of G-MDSCs significantly reduced the frequency of CD4$^+$CD44^{hi} activated WT T cells whereas the activation of PD-1$^{-/-}$ T cells was not affected (**Figure 17B**). Moreover, G-MDSCs significantly inhibited WT T cell proliferation whereas PD-1$^{-/-}$ T cells were resistant to G-MDSC-mediated suppression (**Figure 17C**). Overall, these data indicate that IFN-γ-exposed G-MDSCs suppress T cell responses via the PD-1-PD-L1 inhibitory pathway *in vitro*. To examine whether PD-L1 expression by G-MDSCs confers a dominant mechanism of EAE suppression, we adoptively transferred G-MDSCs isolated from MOG/CFA-immunized PD-L1$^{-/-}$ mice into syngeneic recipients during the course of EAE as described in Fig. 13A. Deficiency of PD-L1 on G-MDSCs abrogated their suppressive ability since the disease onset and severity were not significantly different between treated and control mice (**Figure 18A**). This finding correlated with immunohistological analysis of the spinal cords of the two groups of mice, where comparable degree of demyelination was observed (**Figure 18B**). Moreover, analysis of the dLNs of PD-L1$^{-/-}$ G-MDSC-treated mice showed comparable frequency of MOG_{38-49}/IA^{b+}CD3$^+$CD4$^+$ effector T cells to untreated mice (**Figure 18C**). Collectively these data provide direct evidence or a PD-L1-dependent G-MDSCs-mediated suppression of the autoimmune response.

A



B



C

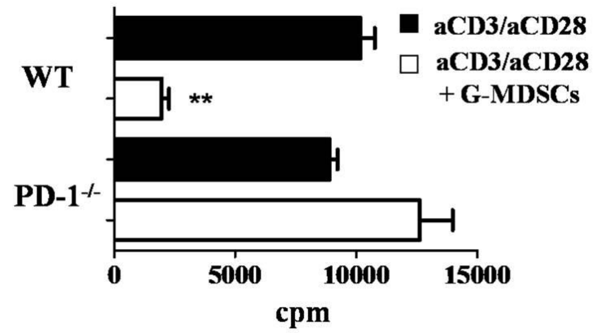
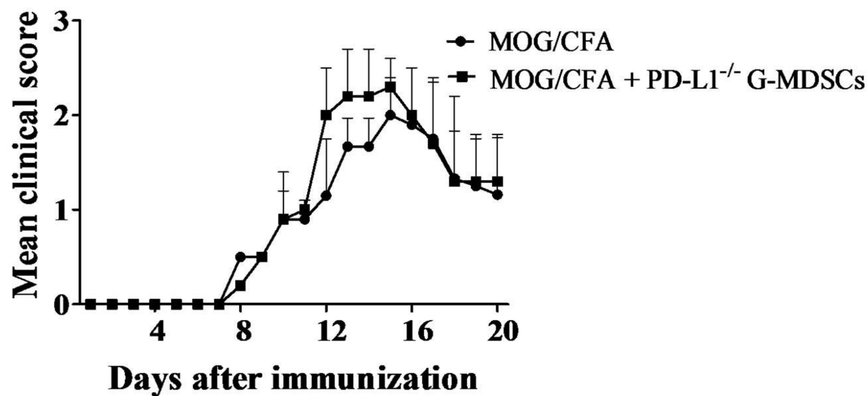
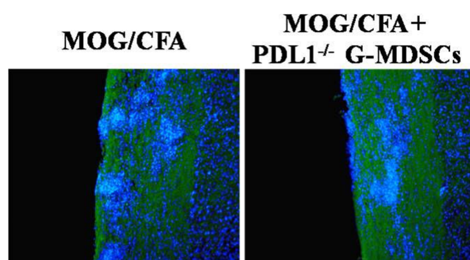


Figure 17. G-MDSCs suppress EAE in a PD-L1-dependent fashion both in vitro. A. Schematic representation of the in vitro G-MDSC-mediated suppression assay. $CD4^+CD25^-$ T cells were sorted (purity >99%) from spleens of naïve WT or PD-$1^{-/-}$ mice, and were cultured ($4x10^5$/well) in the presence of plate-bound αCD3 (10 µg/ml) and αCD28 (1µg/ml). Sorted G-MDSCs (purity >95%) from spleens of MOG/CFA-immunized mice were treated with IFN-γ for 24h and added in the cultures in 1:1 ratio. **B.** Dot plots show CD44 expression on $CD4^+$ T cells from the indicated groups. Cells were analyzed 72h after culture, and gates show percentages of $CD4^+CD44^+$ cells ($7AAD^-Ly6G^-CD4^+$). **C.** Cells were pulsed with 1µCi ^{3}[H]thymidine after 72h of culture and incorporated thymine was measured 18h later. Cell proliferation is expressed in cpm (mean ± s.d. of triplicate wells, **p= 0.003 *t*-test). Data in (B and C) are representative of three independent experiments

A



B



C

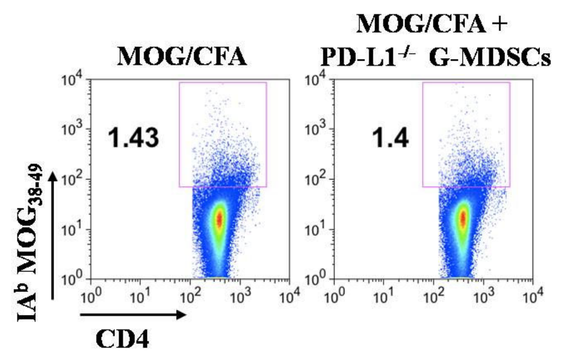


Figure 18. G-MDSCs suppress EAE in a PD-L1-dependent fashion in vivo. A. Mean clinical score of EAE in mice that adoptively transferred with PD-L1$^{-/-}$ G-MDSCs or untreated control mice (n=6 mice/group, mean ± s.d. is shown). Cell transfer was performed as indicated in Fig. 13A. **B.** Representative spinal cord sections from PD-L1$^{-/-}$ G-MDSC-treated or control mice stained with MBP (green) and Hoechst (blue). **F.** LNs from PD-L1$^{-/-}$ G-MDSC-treated and control mice were isolated as described in Fig. 15A and analyzed by flow cytometry for MOG$_{38\text{-}49}$/IA^{b} tetramer binding. Numbers show frequency of MOG$_{38\text{-}49}$/IA^{b+}CD4^{+} cells. Gates were set as in Fig. 15A. Data are representative of two independent experiments.

5.12 Human granulocytic MDSCs from MS patients, potently suppress the activation and proliferation of autologous T cells *in vitro*.

We next examined the presence of MDSCs in MS subjects in the active phase of disease with or during remission. Human MDSCs have mainly been studied in cancer patients and are characterized as HLA-DR$^{-/low}$CD14^{-}CD33^{+}CD15^{+} (Almand et al., 2001; Schmielau and Finn, 2001). Flow cytometry analysis revealed significantly increased frequency (**Figure 19A**) and numbers (**Figure 19B**) of HLA-DR$^{-/low}$CD14^{-}CD33^{+}CD15^{+} MDSCs in the peripheral blood of patients with active MS compared to patients that have achieved remission or healthy controls. A significant decline of MDSC numbers was also observed upon longitudinal analysis of CD33^{+}CD15^{+} cells in seven active MS patients who achieved remission (**Figure 19C**). Similar to mouse G-MDSCs, morphologic analysis of sorted HLA-DR$^{-/low}$CD14^{-}CD33^{+}CD15^{+} cells from active MS patients showed cells with multi-lobed nucleus consistent with a granulocytic phenotype (**Figure 20**). We next assessed the ability of CD33^{+}CD15^{+} MDSCs to suppress the activation and proliferation of stimulated autologous CD4^{+}CD25^{-} cells. To this end, sorted highly pure human MDSCs (CD33^{+}CD15^{+}HLA-DR$^{-/low}$CD14^{-}, purity >95%) from active MS patients were co-cultured with autologous sorted CFSE-labeled CD4^{+}CD25^{-} responder T cells (purity >98%) stimulated with anti-CD3/CD28. After 5 days of co-culture, human MDSCs potently inhibited the activation of responder T cells as indicated by CD25 expression (**Figure 21A**). Importantly, while responder T cells underwent at least 3-4 cell divisions (1st 24.4%, 2nd 7.52% 3rd 3.06), as traced by the dilution of CFSE, presence of MDSCs in the culture caused a proliferation arrest of responder T cells to only 1-2 cell divisions (1st 15.4%, 2nd 5.82%) after 5 days of culture (**Figure 21B**). MDSC-mediated suppression of T cell proliferation was significant as extrapolated by enumeration of the CD4^{+} T cells following the 5-day co-culture (**Figure 21C**). This was further supported by a significant reduction of IL-2 secretion in supernatants of MDSCs/CD4^{+}CD25^{-} co-cultures compared to control cultures (**Figure 21D**). Overall our results demonstrate a significant enrichment of granulocytic MDSCs in active MS patients with a potent ability to suppress the activation and expansion of autologous T cells.

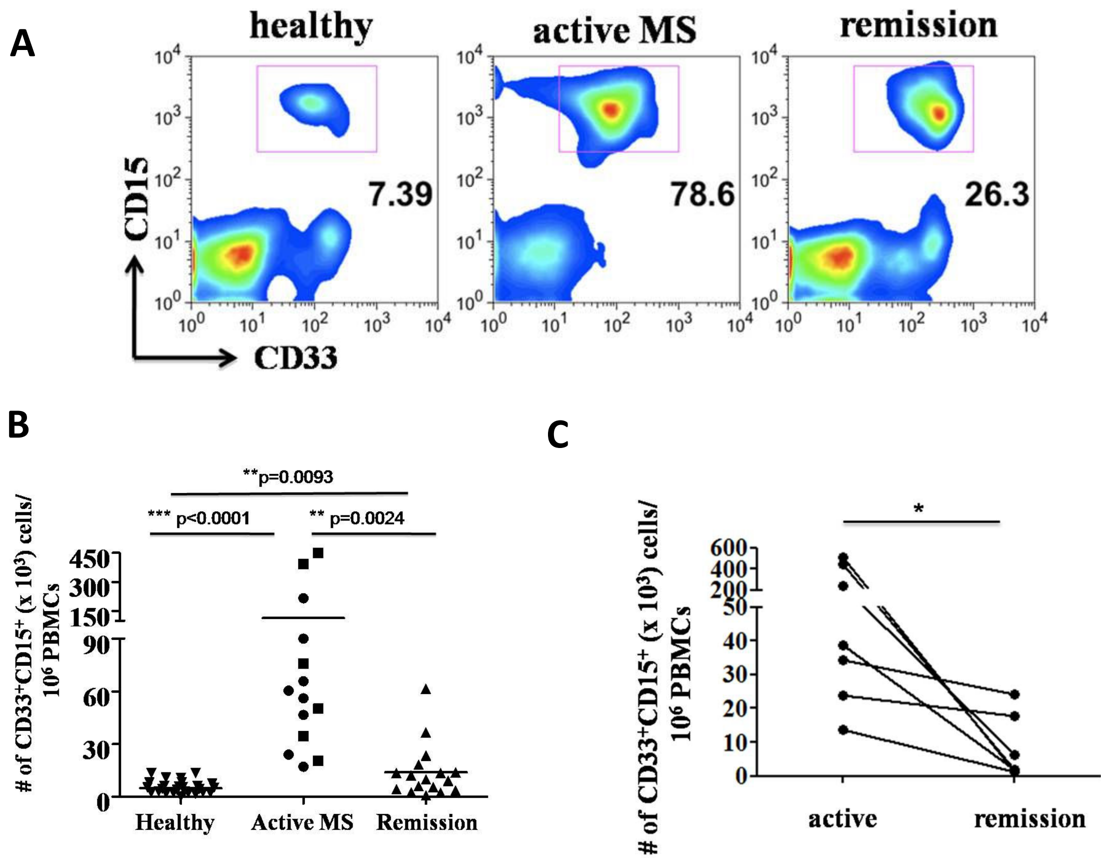


Figure 19. MDSCs are enriched in the periphery of active MS patients, and suppress the proliferation of autologous T cells *in vitro*. A, B. Frequency (**A**) and relative numbers (**B**) of CD33$^+$CD15$^+$ MDSCs in the peripheral blood of MS patients with active disease (n=14), or remission (n=17) and healthy individuals (n=26). Gates were set on HLA-DR$^{-/low}$CD14$^-$ CD33$^+$CD15$^+$ cells. In the active MS group square symbol represents patients with first episode whereas circle symbol those in relapse. **C.** Longitudinal course of CD33$^+$CD15$^+$ MDSCs in MS patients during active disease and 6 months after the last relapse (remission). Each line represents an individual patient (*p=0,0156, Wilcoxon signed rank test).

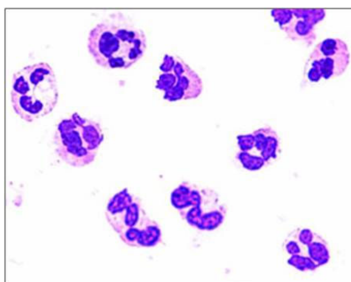


Figure 20: May-Grunwald-Giemsa staining of sorted CD33$^+$CD15$^+$ MDSCs. Cells sorted from PBMCs of active MS patients and gates were set on HLA-DR$^-$CD14$^-$ cells.

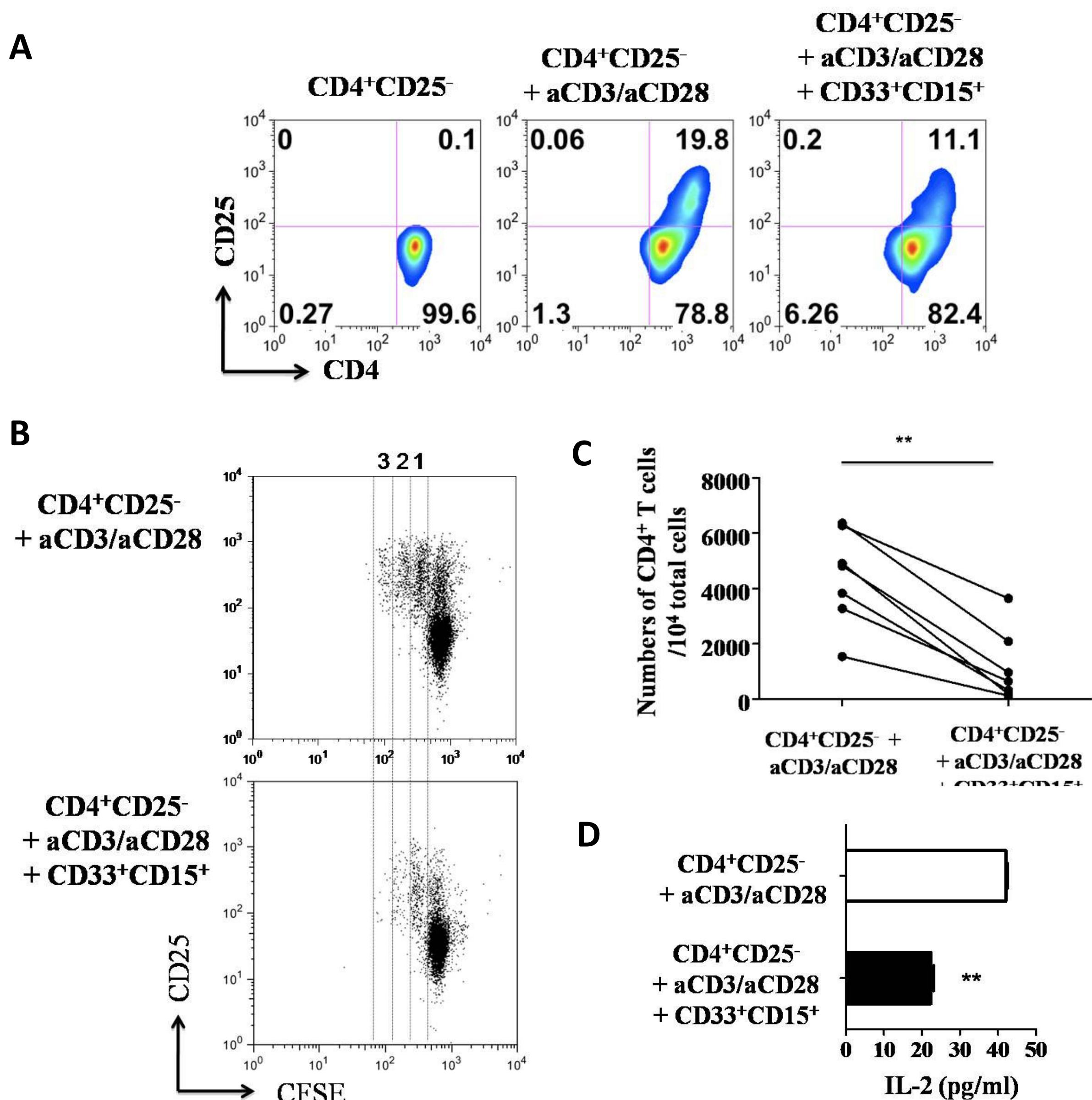


Figure 21: MDSCs from active MS patients, and suppress the proliferation of autologous T cells *in vitro*. A. Sorted CD33^{+}CD15^{+} MDSCs from active MS patients (purity >95%) were co-cultured with autologous CD4^{+}CD25^{-} T cells (purity >98%) with plate bound αCD3 (1μg/ml) and αCD28 (1μg/ml) for 72h. Dot plots show CD4 vs CD25 on gated viable CD4^{+} T cells. Representative results of at least 3 independent experiments. **B.** CFSE dilution of CD4^{+} T cells, (cultured as in (**A**), in the absence (top panel) or presence (bottom panel) of MDSCs is shown after 5 days of co-culture. Numbers indicate cell divisions. **C.** CD4^{+} T cell counts after 5 days stimulation in the presence or absence of MDSCs (**p=0.0016, *t-test*). **D.** Culture supernatants were collected after 48h and assessed for the presence of IL-2 by ELISA. Mean ± s.d. is shown. (**p=0.0018, *t-test*).

7. DISCUSSION

Plasmacytoid dendritic cells (pDCs) are BM-derived leucocytes that have been implicated in the control of both innate and adaptive immune responses (Colonna et al., 2004) . Although, the role of pDCs in antiviral immune responses is well documented (Gilliet et al., 2008), their role in autoimmune diseases remain controversial. For SLE, pDCs play a crucial role in its pathogenesis, through secretion of type I IFNs (Ronnblom and Pascual, 2008), and depletion of pDCs during the onset of EAE and autoimmune arthritis exacerbated the autoimmune pathology (Bailey-Bucktrout et al., 2008), (Jongbloed et al., 2009). On the other hand, depletion of pDCs during the initiation of the autoimmune response, leads to disease amelioration (Isaksson et al., 2009). pDCs have also been implicated in the maintenance as well as re-establishment of tolerance through the induction of Foxp3$^+$ Treg cells suggesting a regulatory role in of this cell subset in autoimmune diseases (Ochando et al., 2006). In this study we aimed at elucidating the precise mechanisms by which pDCs promote or regulate autoimmunity, focusing on the priming of the autoimmune response.

To address this objective we used an antibody-mediated depletion of pDCs, which efficiently depleted this specialized DC subset from the periphery of mice. Depletion of pDCs during the priming of EAE, the experimental model that resembles MS, resulted in an insufficient activation and proliferation of MOG-specific T cell responses in the draining lymph nodes that was associated with a significant decrease of disease pathology and delayed clinical onset. Our results support that depletion of pDCs during the priming of the autoimmune response has a beneficial effect towards autoimmunity; however others demonstrate that depletion during the onset of the disease exacerbated disease pathology. Several hypotheses could be formulated to explain the differential role of pDCs in the priming and effector phase of autoimmunity. Thus, a) pDCs could uptake and present autoantigen in the dLNs activating directly the autoantigen-specific T cells, b) pDCs might be required for the efficient activation and migration of cDCs during the priming of the immune response, c) depletion of pDCs might change the balance of

cytokines/chemokines affecting therefore other cell populations that participate in the initiation and effector phase of autoimmunity.

To delineate the mechanisms by which absence of pDCs during the priming of autoimmunity led to disease amelioration, we asked whether other cell populations are affected upon pDC depletion. We first looked on cDCs that have the unique ability to prime naïve $CD4^+$ T cells in the dLNs. Based on our results, absence of pDCs did not alter the ability of cDCs to uptake antigen and to accumulate in the dLNs. These findings suggest that the insufficient priming of T cell responses in the absence of pDCs could not be attributed to the lack of APCs carrying the antigen in the dLNs. In contrast, we observed that pDC depletion significantly increased the frequency and numbers of $CD11b^+Gr1^+$ MDSCs in the BM and peripheral lymphoid organs. $CD11b^+Gr1^+$ MDSCs consist a myeloid cell population with the potential to perturb immune responses (Nagaraj and Gabrilovich, 2010) (discussed below). Based on this, we hypothesize that accumulation of MDSCs in the lymphoid organs upon pDC-depletion suppress the priming of autoantigen-specific effector cells and thus limiting disease development. How pDCs regulate the expansion and accumulation of MDSCs in BM and peripheral lymphoid compartments is not known. One possibility is that depletion of pDCs increases myelopoiesis in the BM, as a compensatory mechanism, and thus the expansion of MDSCs. In support of this hypothesis, we observed increased frequency of MDSCs in the BM of pDC-depleted mice as compared to the non-depleted control group. This is also in agreement with a recent study demonstrated that ablation of cDCs leads to an increased expansion of myeloid cells in the BM and the induction of a myeloproliferation syndrome in cDC-depleted animals (Birnberg et al., 2008). Another mechanism through which depletion of pDCs could direct the accumulation of MDSCs in the periphery, is through the induction of chemokines and/or cytokines that could attract the egress of MDSCs from the BM. For example, it has been shown that increased MCP-1 during bacterial infections enchances the migration of monocytic cells from the BM to the periphery (Serbina and Pamer, 2006). Whether, MCP-1 (or other chemokines) is increased in pDC-depleted animals compared to control group remains to be defined.

MDSCs comprise a heterogeneous population of myeloid precursors of macrophages, dendritic cells and granulocytes and characterized by the co-expression of Gr-1 and CD11b. They can be further divided into cells with monocytic (M-MDSCs) or granulocytic (G-MDSCs) morphology, defined as $CD11b^{+}Ly6C^{+}Ly6G^{-}$ or $CD11b^{+}Ly6C^{low}Ly6G^{+}$ respectively (Gabrilovich et al., 2007; Ribechini et al., 2010; Youn et al., 2008). Extensive studies have established a prominent role of MDSCs in the regulation of immune responses in mice during cancer (Gabrilovich and Nagaraj, 2009; Nagaraj et al., 2009; Rabinovich et al., 2007), infections (Delano et al., 2007; Nagaraj et al., 2009) and transplantation (Boros et al., 2010; Garcia et al., 2010; Marigo et al., 2010) whereas in humans, MDSC accumulation at tumor site, down-regulate antitumor immunity promoting therefore tumor surveillance and growth (Marigo et al., 2008; Serafini et al., 2006). Although the importance of MDSCs in antitumor immunity is well defined, their role in the regulation of autoimmune pathology is poorly understood. Our data have begin to shed some light on their role in autoimmunity.

Accumulation of MDSCs in the periphery of pDC depleted mice and amelioration of EAE in the previous experiments, led us to the hypothesis that MDSCs might contribute to the resolution of inflammation during autoimmune responses. Therefore we next studied the role of MDSC population, as a component of the immunoregulatory network of immune system, during the course of EAE.

In this study, we provide for the first time compelling evidence for a pivotal role of granulocytic $CD11b^{hi}Ly6G^{+}$ MDSCs (G-MDSCs) in the regulation of CNS autoimmune inflammation. Thus, *in vivo* transferring of highly purified G-MDSCs ameliorated EAE, significantly reduced the expansion of autoreactive T cells in the draining LNs and constrained pathogenic Th1 and Th17 immune responses, in a PD-L1/IFN- γ -dependent fashion. Importantly, our results document, an important role of G-MDSCs in patients with MS, since this subset was significantly increased in the periphery during active disease and potently suppressed autologous T cell proliferation *in vitro*. Together, these data highlight the potential of G-MDSCs to serve as a novel target for pharmacologic intervention in autoimmune inflammatory diseases.

Whether G-MDSCs described here represent a subset of neutrophils or are undifferentiated myeloid suppressor cells is not clear at present. G-MDSCs isolated from MOG/CFA immunized mice, had a high SSC profile and phenotypically displayed features of neutrophils, such as multi-lobed nucleus and expression of the typical marker Ly6G. Mounting evidence suggests that similar to macrophages, polymorphonuclear cells (PMNs) are versatile cells and could acquire diverse functions depending upon the microenvironment. Thus, in the malignancy setting, tumor-associated neutrophils (TANs) were polarized into either an anti-tumorogenic (N1) or a pro-tumorogenic (N2) population depending upon the tumor milieu (Fridlender et al., 2009). Moreover, in a systemic inflammatory model, two operationally different PMN populations were characterized denoted as PMN-I and PMN-II producing high levels of IL-12 and IL-10 respectively (Tsuda et al., 2004). Similarly, in an infectious disease setting, mycobacteria-exposed neutrophils, secrete high levels of IL-10, possessing thus anti-inflammatory properties (Zhang et al., 2009). We found, that in the autoimmune environment generated by the injection of a self-antigen in adjuvant, G-MDSCs secrete elevated levels of IL-10 but not IL-12, a profile consistent with the PMN-II regulatory cells described above. Irrespective of the differentiation status of G-MDSCs in the autoimmune setting, our data undoubtedly establish a regulatory role of granulocytic “neutrophil”-like myeloid cells in the resolution of autoimmune inflammation. Further phenotypic characterization of the regulatory granulocytic cells and development of genetic approaches for specific depletion of the polarized PMN subsets is needed to reassess their potential in regulation of immune responses.

Our work provides strong evidence for a G-MDSC regulation of EAE at the target tissue. Adoptive transfer of G-MDSCs in MOG/CFA immunized mice reduced inflammation and demyelination in the spinal cord that in turn correlated with delayed disease onset and amelioration of clinical symptoms. Importantly, we also observed an increased accumulation of G-MDSCs at the meningeal lesions of spinal cord suggesting that G-MDSCs could exert their function not only at the peripheral lymphoid organs but also at the target tissue. This is in agreement with recent data demonstrating that MDSCs suppress T- cell function, preferentially at the inflammatory site in a mouse model of prostate cancer (Haverkamp et al., 2011). The mechanism involved in the G-MDSC

trafficking, migration and specific localization is unknown. One possibility is that pro-inflammatory cytokines, such as IL-1ß (Bunt et al., 2006; Song et al., 2005; Tu et al., 2008), and chemokines produced during the early infiltration of immune cells into the tissue could direct the extravasation of G-MDSCs. Alternatively, products of Th1 and/or Th17 cells infiltrated the CNS, could mediate such process. For example, IL-17 has been implicated in granulocyte recruitment during inflammatory responses (Li et al., 2010). Finally, T cell Ig and mucin domain (Tim-3) expressed on Th1 cells was recently reported to facilitate G-MDSC recruitment through binding to galectin-9 (Dardalhon et al., 2010) . Further investigation on the molecules and/or growth factors involved in the specific trafficking of G-MDSCs at the inflammatory sites is needed.

While the function of MDSCs in a malignant disease environment has been previously addressed (Peranzoni et al., 2010), the mechanisms underling their suppressive activity in an autoimmune setting have not been explored. In our experiments, G-MDSCs isolated from MOG/CFA immunized mice and exposed to IFN-γ in vitro, fail to express NO, Arg-1 and ROS, which have been closely linked to MDSC-mediated suppression of antitumor immunity. These findings suggest that the autoimmune environment might induce a novel regulatory “signature” on G-MDSCs. Indeed, further characterization revealed a significant IFN-γ-mediated up-regulation of the inhibitory molecule PD-L1. Thus, adoptive transfer of PD-L1-deficient G-MDSCs during the course of EAE failed to suppress disease pathology, and to limit the expansion of encephalitogenic T cells in the draining LNs. Published data have established a pivotal role of the PD-1/PD-L1 pathway in the regulation of an autoimmune response (Francisco et al., 2010). Thus, PD-L1-deficient mice develop greatly exacerbated EAE compared to control littermates that was associated with enhanced autoantigen-specific T cell responses (Keir et al., 2006; Latchman et al., 2004). Our data extent those findings, as they point to a PD-L1-dependent G-MDSC-mediated regulation of EAE. Furthermore, we did not detect expression of the other B7 family inhibitory molecule PD-L2 by G-MDSCs, indicating that this receptor is not involved in the G-MDSC-mediated inhibition of disease. This finding could explain the increased susceptibility of PD-L1$^{-/-}$ but not PD-L2$^{-/-}$ mice in MOG-induced EAE (Carter et al., 2007; Latchman et al., 2004). In line with our G-MDSC phenotypic data, another study demonstrated increased expression of PD-L1 in MDSCs

isolated from tumor-bearing mice; however, in this report MDSC-mediated suppression was PD-L1-independent (Youn et al., 2008). The disparity in these results could be explained by the increased plasticity of the MDSCs population and is consistent with the concept that MDSC phenotype and function would greatly depend on the microenvironmental milieu. It should be noted that, apart from PD-L1, G-MDSCs from MOG/CFA-immunized mice secreted significant amounts of the immunosuppressive cytokine IL-10, indicating that several, non-mutually exclusive mechanisms, might contribute to the G-MDSC-mediated resolution of autoimmunity.

Our findings regarding the IFN-γ-dependent up-regulation of PD-L1 are of interest. Although IFN-γ levels are abundant both in the periphery and the target organ during the course of EAE and in MS patients, its role in the clinical outcome of the disease in both human and mice remains controversial (Axtell et al., 2010; Lees et al., 2008; Stromnes et al., 2008). Our results support a regulatory role of IFN-γ in the effector phase of disease since up-regulation of PD-L1 expression by G-MDSCs was greatly dependent on IFN-γ. This is in agreement with studies demonstrating that IFN-γ enhances the MDSC suppressive function (Gallina et al., 2006; Huang et al., 2006; Movahedi et al., 2008) and blocking of IFN-γ totally reversed the inhibitory activity of granulocytic MDSCs in a tumor mouse model (Movahedi et al., 2008). Our data reconcile findings that demonstrate exacerbation of EAE in IFN-$\gamma^{-/-}$ and IFN-γ $R^{-/-}$ mice or during neutralization of this cytokine (Chu et al., 2000; Ferber et al., 1996; Heremans et al., 1996; Lublin et al., 1993; Willenborg et al., 1996). At the human level, a clinical trial using IFN-γ to treat MS patients led to disease exacerbations (Panitch et al., 1987); however, it was later demonstrated that IFN-γ could induce apoptosis in human oligodentrocytes precipitating thus the autoimmune response (Pouly et al., 2000; Vartanian et al., 1995). In this context and based on our findings, it is worth postulating that exposure of G-MDSCs to IFN-γ during the course of the disease leads to up-regulation of PD-L1 expression which subsequently might serve as a regulatory mechanisms in controlling pathology and facilitating disease remission.

Promoting and establishing immune modulation could potentially be a beneficial therapeutic strategy in patients with MS. Our data demonstrate that granulocytic CD33+CD15+MDSCs are significantly enriched in the periphery of MS patients with active

disease and importantly, they inhibit the activation and proliferation of autologous effector T cell in vitro. Whereas, our study involves a relatively limited sample of MS patients nevertheless, the results obtained suggest that MDSCs mobilization in the peripheral blood of MS patients is particularly robust at disease onset, raising the possibility that such mobilization may contribute to the clinical recovery. Of note, disease remission is known to be more often complete after the initial attack of the diseases (MS onset) as compared to subsequent clinical events (relapses). Since available treatments in MS are only partially effective, development of new therapies that specifically target the inflammatory autoimmune response is mandatory (Feldmann and Steinman, 2005; Hohlfeld and Wekerle, 2004).

Overall, our findings establish a critical role of G-MDSCs in the regulation of EAE and MS and provide novel insights into the mechanisms that limit inflammation during autoimmune diseases. Understanding the mechanisms that are involved in disease recovery may provide important insights into aberrant pathways that account for the chronic and progressive form in patients with MS. The tolerogenic and immunosuppressive properties of G-MDSCs demonstrated herein could be exploited for the development of more cell-specific based therapies in patients with autoimmune inflammatory diseases.

8. FUTURE DIRECTIONS

The data presented here have provided evidence for a novel role of MDSCs in the regulation of autoimmune diseases. Furthermore, pDCs have been shown to regulate the recruitment of MDSCs in the peripheral lymphoid compartments, since pDC-depletion markedly increased the frequency of MDSC in the periphery of mice. Based on our data several questions have been raised that could be further explored. The most fundamental of these questions are listed next:

7.1 How autoimmune inflammation affects MDSCs function? Identify inflammatory mediators that control MDSCs (a) differentiation (b) expansion (c) recruitment and (d) activation.

First we will investigate if the autoimmune inflammatory environment affects MDSCs maturation and how this correlates with their suppressive activity. One hypothesis is that inflammatory mediators upregulated during autoimmune responses might increase the differentiation of MDSCs and therefore decrease their immunoregulatory function in EAE mice. To address this hypothesis:

a) MDSCs isolated from BM and spleen of naive or MOG/CFA immunized mice will be analyzed for surface maturation markers (MHCII, CD80, CD86, CD40), but also will be used for suppression assays in order to examine their suppressive activity in vitro. For suppression assay, MDSCs will be cocultured with naive $CD4^+$ T cells in the presence of anti-CD3/CD28 and T cell proliferation will be assessed based on CFSE dilution, CD25 upregulation and cytokine production (IL-2, IFNγ). This will be the first indication about the role of inflammation in MDSCs maturation status and suppressive activity. To confirm the results from the previous in vitro experiments, we could perform adoptive transfer experiments of MDSCs in EAE mice and monitor disease development. If our hypothesis is correct, it could provide additional evidence about the biological significance of MDSCs in the progression or amelioration of EAE.

b) In order to detect soluble mediators or genes affected during autoimmune inflammation and control MDSCs immunoregulatory role, we indent to:

- Examine cytokine/chemokine profile using bead array immunoassay, in the serum of naive, EAE mice and tumor bearing mice at different phases of the disease. Cytokine profile in naive and EAE mice at different phases of the disease, will help us to select soluble mediators up- or down regulated during autoimmune inflammation that may correlate with MDSCs accumulation (Figure 11-Results section) and regulate their recruitment in the periphery of EAE mice. In addition, comparing the cytokine profile of EAE and tumor-bearing mice, will provide evidence about inflammatory mediators that are differentially affected in EAE and cancer mouse models and might play a role in MDSC differentiation. In cancer the goal is to identify and modulate factors that induce the differentiation of MDSCs and therefore their inflammatory function, whereas in autoimmunity these factors could be target in order to keep MDSCs immature and suppressive.

- Determine gene expression profile, by DNA Microarrays in MDSCs isolated from Spleen and BM of naïve, EAE mice and tumor-bearing mice, at different phases of disease. These results will provide important evidence on surface-markers, signaling mediators and transcription factors that may implicated in the expansion, activation and differentiation of MDSCs during autoimmune inflammation. In addition we will show if there is differential gene expression in MDSCs between the different microenvironment of cancer and autoimmune inflammation.

c) Results from the experiments described at (b), may reveal candidate molecules implicated in MDSCs function. The role of these molecules could be further examined in functional experiments:

- Treatment of BM cells isolated from naive mice with different cytokines/growth factors selected from cytokine bead array results (b). After 3 days of culture study the effect in MDSC expansion and differentiation

based on the frequency of MDSCs in total BM cells and the expression of cell-surface maturation markers (MHCII, CD80, CD86, CD40).

- Administration or neutralization selected cytokines/chemokines in EAE mice:
  - ° Recruitment of MDSCs in the periphery of EAE mice in response to cytokine or chemokine administration, will be assessed by the numbers of MDSCs accumulated in spleen and dLNs of untreated or cytokine-treated EAE mice. Recruitment of MDSCs in response to a specific chemokine will be assessed by in vitro migration assay. In this case MDSCs isolated from MOC/CFA immunized mice will be cultured in the presence of the recombinant chemokine in transwell plates. To assess if this chemokine directly attracts MDSCs in vivo: naive mice will be administrated i.p. with the recombinant chemokine and then injected intravenously with CFSE-labeled MDSCs isolated from MOG/CFA immunized mice. After 24h recruitment of CFSE-labeled MDSCs will determine in mesenteric LNs and spleen of naïve or MDSC-treated mice.
  - ° Activation as well as maturation status of MDSCs will assessed by production of suppressive factors (iNOs, ArgI and ROS) and by the expression of surface markers (MHCII, CD80, CD86, CD40, PD-L1, PD-L2) in the MDSCs from spleen of treated or untreated EAE mice. In addition, in vitro suppressive assays with MDSCs as previously described (a) in the presence of these cytokines will directly show how treatment with growth factors affect MDSCs suppressive activity.
  - ° In order to examine how these cytokines affects the expansion of MDSCs, mice could be injected with Brdu and 24h after injection MDSCs from spleen and BM will analysed for Brdu incorporation.
- To assess if suppressive factors such as iNOs, ArgI, ROS, produced by MDSCs are indispensable for their suppressive activity, we will perform in vitro suppression assays (as described in 7.1 (a)) with MDSCs deficient for iNOs, ArgI or with wt MDSCs

and addition of ROS inhibitor (diphenylene iodonium (DPI)) in the culture. Adoptive transfer of wt MDSCs or MDSCs deficient for these factors in the EAE mice will provide direct evidence about their contribution in MDSCS function.

Identification of the factors that are indispensable for MDSCs immunoregulatory fuction in autoimmune inflammation might have not only biological, but also therapeutic significance in the treatment of autoimmune diseases.

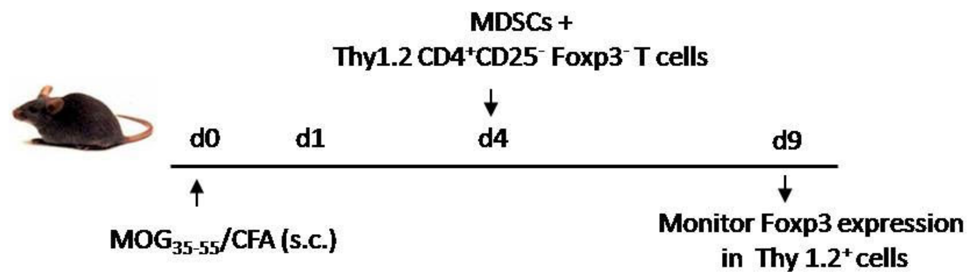
7.2 Study whether MDSCs could facilitate the induction of Treg cells

T regs have been implicated in the suppression of myelin-reactive T cells and thus the inhibition of autoimmune response (Korn et al., 2007). In tumor-bearing mice, MDSCs play an active role in the development of $Foxp3^+$ Treg cells (Huang et al., 2006). In contrast, another study showed that MDSCs were not involved in Treg induction (Movahedi et al., 2008). Therefore, we intent to study if MDSCs could induce the development Tregs in autoimmune inflammation, either through the production of suppressive cytokines (IL-10, TGFβ) or through direct cell-cell interactions as follows:

a) Study the kinetics of Treg cells ($CD4^+Foxp3^+$ T cells) at different phases of EAE in dLNs and Spleen of Control and MDSC- transferred EAE mice. Correlation of Tregs expansion with MDSCs expansion during EAE , might link MDSCs and Tregs in a common immunoregulatory network.

b) Examine the suppressive function of Tregs isolated from dLNs of Control and MDSC- transferred EAE mice. For this purpose, we will perform in vitro suppression assays with titrating amounts of $CD4^+foxp3^+$ T cells cocultured with naive CD4+CD25- T cells activated by anti-CD3/CD28 ab.

c) To address whether MDSCs promote de novo differentiation of naïve T cells into $Foxp3^+$ Tregs: MDSCs isolated from EAE mice and naïve Thy1.2 $CD4^+CD25^-$ T cells from Foxp3-GFP reporter mice, coinjected into Thy1.1 MOG/CFA immunized mice. After 7 days of transfer isolate peripheral lymphoid organs and monitor Foxp3 expression in $Thy1.2^+CD4^+$ cells.



d) Examine if suppressive molecules expressed by MDSCs such as IL-10, TGFβ, NO, Arginase I and PD-L1 promote Treg induction. For this, we will isolate from the appropriate knock-out mice MDSCs deficient for IL-10, TGFβ, iNOs, ARG I and PDL1 as well as wt MDSCs and perform the adoptive transfer experiment as described in c).

7.3 How pDC depletion affects MDSCs accumulation in the lymphoid compartments during an inflammatory response?

a) Investigate if pDC depletion affects soluble mediators that are secreted during inflammation:

- Serum, dLNs and spleen of control and pDC-depleted mice, immunized or not with MOG/CFA, will be collected and analyzed using the cytometric bead –array immunoassay for the presence of multiple chemokines/cytokines/growth factors: CCL2, KC, IL-1β, IL-6, IL-10, TGF-β, GM-CSF, G-CSF, MCSF. These factors are upregulated during inflammation.

b) Determine genes that are differentially expressed by MDSCs in control or pDC-depleted MOG/CFA immunized mice:

- Using DNA microarray we will determine differentially expressed genes on MDSCs isolated from spleen and BM of control and pDC-depleted MOG/CFA immunized mice.

c) Identification of candidate molecules from the previous experiments, will allow us to perform functional experiments i.e in vivo neutralization or blocking assay and directly provide evidence for MDSCs recruitment in the peripheral lymphoid organs.

7.4 Examine whether depletion of pDCs affects myelopoiesis, leading to increased generation of MDSCs.

During 'emergency' situations such as infections, host defense sense the pathogens in the periphery and induce rapid expansion of myeloid progenitors in bone marrow in order to combat infection (‘emergency myelopoiesis (Basu et al., 2000). Similarly, in the condition of stress that we induce by the pDC –depletion , the host may sense the absence of pDCs and trigger a feedback loop mechanism resulting in myeloid expansion in BM in order to compensate the pDCs absence.

a) Determine the levels of growth factors, that are established inducers of myelopoiesis such as GMCSF, MCSF, Flt3 and G-CSF in the serum of control and pDC-depleted mice (immunized or not with MOG/CFA). Elevated levels of these growth factors after pDC depletion, will provide an evidence that these factors may trigger the accumulation MDSCs in the BM and spleen of pDC-depleted mice.

b) Measure cellularity of spleen and BM in the control and pDC-depleted MOG/CFA immunized group. In addition we will perform Colony Forming Unit (CFU) assay in order to assess hematopoesis. In this assay we will compare hematopoietic precursor frequencies by measure CFU in BM and spleen isolated from the two groups.

c) Assess MDSCs proliferation by in vivo Brdu incorporation assay. For this purpose we perform Brdu injection in the cnt and pDC-depleted MOG/CFA immunized group, at day4 after immunization, and analyse after 24h by FACs for the percentage of proliferating MDSCs in spleen and BM

7.5 To investigate whether MDSC-mediated amelioration of autoimmunity is specifically induced upon pDC depletion or can be achieved through systemic ablation of other myeloid cell populations.

a) Deplete cDCs ($CD11c^+$ fraction) in MOG /CFA immunized mice and examine myelopoiesis and MDSCs accumulation in BM. For cDCs depletion, Cd11cDTR (B6) mice will be injected with Diphtheria toxin one day before MOG/CFA (D0) and analyzed four days after immunization for:

- The frequency of MDSCs in LNs, Spleen and BM by FACs in cDC-depleted MOG/CFA immunized CD11c:DTR mice and controls (MOG/CFA) as well as in naïve and non-immunized cDC -depleted mice.
- The levels of cytokines or growh factors that have been identified to affect MDSCs accumulation in pDC-depleted mice (see 7.3), in order to assess whether pDC depletion have a similar effect. In addition we will perform CFU assay and study the cellularity in spleen and BM of: controls (MOG/CFA), cDC-depleted MOG/CFA immunized CD11c:DTR mice, naïve and non-immunized cDC -depleted mice.

7.6 Delineate the role of MDSCs in patients with Multiple Sclerosis:

In our study we show that MDSCs are accumulated in the periphery of subjects with active multiple sclerosis (MS) whereas they are significantly decreased in the peripheral blood of patients in remission. In addition isolation of MDSCs of active MS patient suppressed the activation and proliferation of autologous $CD4^+$ T cells *ex vivo.* Additional objectives that were generated from these experiments are discussed below.

7.6.1 Could failure of disease resolution in progressive MS patients attributed to a MDSC dysfunction?

Multiple sclerosis RR patients are characterized by periods of relapses followed by periods of remissions. Although MS patients with the progressive form of disease characterized by a gradual but steady progression of disability, without obvious remissions. The reasons underlying these different disease courses in patients remains

undefined. In our study we show that MDSCs are associated with disease activity in RR patients, suppress T cell responses in vitro and might play a role in the induction of the remission phase of disease. Therefore we formulate the hypothesis that progressive MS patients that cannot achieve remission, might have either dysfunctional MDSCs or inadequately accumulation of MDSCs in the periphery. To address this hypothesis we will perform the following experiments:

a) Analyze MDSCs in relapsing/remitting MS patients versus progressive MS patients:

- Isolate PBMCs from Peripheral blood of progressive or RR patients in active phase of the disease and analyze by FACs the Frequency of MDSCs.

- Isolate RNA from MDSCs from progressive vs RR patients in active or remitting phase of the disease and perform DNA microarray analysis in order to compare the gene expression profile.

- Perform suppression assays with MDSCs Isolated from progressive vs RR patients (in active or remitting phase of the disease). To do so we will coculture MDSCs with autologous naïve $CD4^+$ T cells and measure T cell proliferation, activation and cytokine production as described in (a). This experiment will show us if MDSCs from progressive MS patients have defects in their suppressive activity and therefore they are not able to limit inflammation in these patients.

- Collect serum from progressive vs RR patients in active or remitting phase of the disease and analyse cytokine profile using bead array immunoassay. Identification of cytokines/chemokines that are differentially produced between these patients will provide evidence about candidate molecules that may regulate MDSCs function or recruitment in the periphery of MS patients. The role of these molecules will be further assessed by functional in vitro experiments or in vivo experiments in mice.

7.6.2 How autoimmune inflammation affects the functionality of MDSCs during active disease.

a) Examine whether MDSC suppressive activity is affected by the disease status. Compare healthy, active MS patients and patients in remission.

- Co-culture MDSCs isolated from and three group of patients with naïve CFSE labeled $CD4^+$ T cells in the presence of anti-CD3/CD28 ab. Then monitor T cells proliferation and activation based on CFSE dilution and CD25 upregulation and cytokines levels (IL-2, IFNγ) in the cultured supernatants.
- RNA isolation from MDSCs from the three groups and DNA microarrays in order to detect differential gene expression
- Serum collection from the three subject groups in order to analyze the cytokine profile by using cytometric bead array immunoassay.

7.6.3 What are the mechanisms involved in the MDSC-mediated suppression of autoimmune T cell responses in MS patients?

Perform suppression assay as described in a) in the presence of anti-PD-L1 blocking ab, or inhibitors for iNOS, Arg I, ROS in order to assess which of these suppressor molecules mediate the suppressive function of MDSCs in humans. In addition, the role of other suppressive molecules detected from DNA microarrays will be investigated. Identification of molecules that regulates the suppresive activity of MDSCs will offer new therapeutic targets for disease amelioration through.

7.6.4 Investigate if treatment of MS patients with rIFNb affects the expansion/activation of MDSCs. Experiments in MS patients and EAE mice

IFN-β is one of the most widely used treatments for multiple sclerosis. However, a major limitation with IFN-β is that a 30%–50% of patients with multiple sclerosis do not respond to IFN-β therapy. Therefore we intent to investigated if treatment with IFNβ affects activation , expansion or suppressive activity of MDSCs.

(a) Experiments in humans

- MDSCs from active MS patients (no IFNb treatment) will be cultured in vitro with LPS, in the presence or absence of rhIFNβ and then analyzed for:
    - surface markers of activation/differentiaton such as HLADR, CD14, CD15, CD80, CD86, PDL1, PDL2, CTLA4
    - cytokines (IL-10, TGFβ, , IL-12, IL-23, TNF, IL-1β) secreted in supernatants, and NOS2 or Arginase I in cell lysates by western blot.
    - Suppressive function using suppression assays as described in 7.6.2 (a).
- MDSCs from MS patients before and after treatment with IFNβ will be also analyze for surface marker expression, and suppressive activity as mentioned above.

(b) Experiments in mice

- Culture BM cells from naïve B6 mice with GMCSF, in the presence or not of rmIFNβ. After 3 days examine the frequency (expansion) of MDSCs by FACS.
- Administrate or neutralize IFNb (rmIFNb or anti-IFNb ab) in MOG/CFA immunized mice and examine expansion of MDSCs in dLNs, spleen and BM of Control or treated mice. Examine the levels of cytokines/chemokines implicated in MDSCs expansion and activation such as CCL2, KC, IL-6, IL-10, GM-CSF, G-CSF, MCSF, in serum collected from the three group of mice.

7.7 In vitro generation of MDSCs for therapeutic purposes

The efficacy of exogenously applied MDSC in inhibiting autoimmune disease in certain murine models suggests that they could be harnessed as a cellular therapy for autoimmune disease. The use of *in vitro* expanded MDSCs offers a promising therapeutic strategy for the treatment of human autoimmune diseases. However more extensive studies in animal models are needed before MDSC as cellular therapy could be entertained for a phase I trial. Therefore we intent to expand in vitro murine MDSCs and at the same time retain their immunoregulatory function.

a) BM cells from MOG/CFA immunized mice will cultured with rmGM-CSF for 3 days. In addition based on the results from previous experiments (see 7.1), different cytokine cocktails will be added in the BM culture in order to find the most efficient combination for MDSCs expansion.

- First we will, study the frequency of MDSCs in BM cultures, in the different conditions, in order to assess which cytokine cocktail induce better expansion of MDSCs.

- Determine the phenotype of MDSCs collected from BM at day 0 (before the expansion with cytokine cocktail) and at day 3 (after treatment), in order to study if there are any differences in the expression of activation/maturation markers (MHCII, CD40, CD80,CD86, PD-L1) or in the production of suppressive molecules such as iNOs, Arginase I, ROS, IL-10, TGF-β. To do so, MDSCs from D0 or D3 will treated or not with LPS and then measure expression of surface markers and ROS production using FACs, secreted cytokines in the cultured supernatants by ELISA, ArgI and iNOs expression in cell lysates by western blot.

- Examine the suppressive activity of MDSCs from BM at day 0 and day 3, in order to study if there any differences in their suppressive function after expansion in the culture. To do so we will perform suppression assays (as described in 7.1.(a)).

- Finally we will perform adoptive transfer of BM-generated MDSCs in EAE mice, to confirm their suppressive activity in vivo.

Overall, our data as well as the proposed experiments will provide novel insights into the regulatory pathways of MS as well as the mechanisms that limit inflammation during autoimmune diseases. Characterization of the immunosuppressive properties of MDSCs in an autoimmune setting may open new avenues for the development of more specific cell-based therapies in patients with autoimmune inflammatory diseases.

9. REFERENCES

Akkaraju, S., W.Y. Ho, D. Leong, K. Canaan, M.M. Davis, and C.C. Goodnow. 1997. A range of CD4 T cell tolerance: partial inactivation to organ-specific antigen allows nondestructive thyroiditis or insulitis. *Immunity* 7:255-271.

Albert, L.J., and R.D. Inman. 1999. Molecular mimicry and autoimmunity. *N Engl J Med* 341:2068-2074.

Almand, B., J.I. Clark, E. Nikitina, J. van Beynen, N.R. English, S.C. Knight, D.P. Carbone, and D.I. Gabrilovich. 2001. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol* 166:678-689.

Ang, D.K., C.V. Oates, R. Schuelein, M. Kelly, F.M. Sansom, D. Bourges, L. Boon, P.J. Hertzog, E.L. Hartland, and I.R. van Driel. 2010. Cutting edge: pulmonary Legionella pneumophila is controlled by plasmacytoid dendritic cells but not type I IFN. *J Immunol* 184:5429-5433.

Asselin-Paturel, C., A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Briere, and G. Trinchieri. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol* 2:1144-1150.

Asselin-Paturel, C., G. Brizard, J.J. Pin, F. Briere, and G. Trinchieri. 2003. Mouse strain differences in plasmacytoid dendritic cell frequency and function revealed by a novel monoclonal antibody. *J Immunol* 171:6466-6477.

Axtell, R.C., B.A. de Jong, K. Boniface, L.F. van der Voort, R. Bhat, P. De Sarno, R. Naves, M. Han, F. Zhong, J.G. Castellanos, R. Mair, A. Christakos, I. Kolkowitz, L. Katz, J. Killestein, C.H. Polman, R. de Waal Malefyt, L. Steinman, and C. Raman. 2010. T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis. *Nat Med* 16:406-412.

Bailey-Bucktrout, S.L., S.C. Caulkins, G. Goings, J.A. Fischer, A. Dzionek, and S.D. Miller. 2008. Cutting edge: central nervous system plasmacytoid dendritic cells regulate the severity of relapsing experimental autoimmune encephalomyelitis. *J Immunol* 180:6457-6461.

Barker, C.F., and R.E. Billingham. 1977. Immunologically privileged sites. *Adv Immunol* 25:1-54.

Baron, J.L., J.A. Madri, N.H. Ruddle, G. Hashim, and C.A. Janeway, Jr. 1993. Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma. *J Exp Med* 177:57-68.

Barrat, F.J., T. Meeker, J. Gregorio, J.H. Chan, S. Uematsu, S. Akira, B. Chang, O. Duramad, and R.L. Coffman. 2005. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J Exp Med* 202:1131-1139.

Basu, S., G. Hodgson, H.H. Zhang, M. Katz, C. Quilici, and A.R. Dunn. 2000. "Emergency" granulopoiesis in G-CSF-deficient mice in response to Candida albicans infection. *Blood* 95:3725-3733.

Bave, U., M. Magnusson, M.L. Eloranta, A. Perers, G.V. Alm, and L. Ronnblom. 2003. Fc gamma RIIa is expressed on natural IFN-alpha-producing cells (plasmacytoid dendritic cells) and is required for the IFN-alpha production induced by apoptotic cells combined with lupus IgG. *J Immunol* 171:3296-3302.

Bave, U., G. Nordmark, T. Lovgren, J. Ronnelid, S. Cajander, M.L. Eloranta, G.V. Alm, and L. Ronnblom. 2005. Activation of the type I interferon system in primary Sjogren's syndrome: a possible etiopathogenic mechanism. *Arthritis Rheum* 52:1185-1195.

Bettelli, E., M.P. Das, E.D. Howard, H.L. Weiner, R.A. Sobel, and V.K. Kuchroo. 1998. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J Immunol* 161:3299-3306.

Bingisser, R.M., P.A. Tilbrook, P.G. Holt, and U.R. Kees. 1998. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. *J Immunol* 160:5729-5734.

Birnberg, T., L. Bar-On, A. Sapoznikov, M.L. Caton, L. Cervantes-Barragan, D. Makia, R. Krauthgamer, O. Brenner, B. Ludewig, D. Brockschnieder, D. Riethmacher, B. Reizis, and S. Jung. 2008. Lack of conventional dendritic cells is compatible with normal development and T cell homeostasis, but causes myeloid proliferative syndrome. *Immunity* 29:986-997.

Boonstra, A., C. Asselin-Paturel, M. Gilliet, C. Crain, G. Trinchieri, Y.J. Liu, and A. O'Garra. 2003. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. *J Exp Med* 197:101-109.

Boros, P., J.C. Ochando, S.H. Chen, and J.S. Bromberg. 2010. Myeloid-derived suppressor cells: natural regulators for transplant tolerance. *Hum Immunol*

Bronte, V., P. Serafini, C. De Santo, I. Marigo, V. Tosello, A. Mazzoni, D.M. Segal, C. Staib, M. Lowel, G. Sutter, M.P. Colombo, and P. Zanovello. 2003. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. *J Immunol* 170:270-278.

Bronte, V., M. Wang, W.W. Overwijk, D.R. Surman, F. Pericle, S.A. Rosenberg, and N.P. Restifo. 1998. Apoptotic death of CD8+ T lymphocytes after immunization: induction of a suppressive population of Mac-1+/Gr-1+ cells. *J Immunol* 161:5313-5320.

Bronte, V., and P. Zanovello. 2005. Regulation of immune responses by L-arginine metabolism. *Nat Rev Immunol* 5:641-654.

Brown, A., D.E. McFarlin, and C.S. Raine. 1982. Chronologic neuropathology of relapsing experimental allergic encephalomyelitis in the mouse. *Laboratory investigation; a journal of technical methods and pathology* 46:171-185.

Brys, L., A. Beschin, G. Raes, G.H. Ghassabeh, W. Noel, J. Brandt, F. Brombacher, and P. De Baetselier. 2005. Reactive oxygen species and 12/15-lipoxygenase contribute to the antiproliferative capacity of alternatively activated myeloid cells elicited during helminth infection. *J Immunol* 174:6095-6104.

Buer, J., A. Lanoue, A. Franzke, C. Garcia, H. von Boehmer, and A. Sarukhan. 1998. Interleukin 10 secretion and impaired effector function of major histocompatibility complex class II-restricted T cells anergized in vivo. *J Exp Med* 187:177-183.

Bunt, S.K., V.K. Clements, E.M. Hanson, P. Sinha, and S. Ostrand-Rosenberg. 2009. Inflammation enhances myeloid-derived suppressor cell cross-talk by signaling through Toll-like receptor 4. *J Leukoc Biol* 85:996-1004.

Bunt, S.K., P. Sinha, V.K. Clements, J. Leips, and S. Ostrand-Rosenberg. 2006. Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. *J Immunol* 176:284-290.

Cao, W., and Y.J. Liu. 2007. Innate immune functions of plasmacytoid dendritic cells. *Curr Opin Immunol* 19:24-30.

Carter, L.L., M.W. Leach, M.L. Azoitei, J. Cui, J.W. Pelker, J. Jussif, S. Benoit, G. Ireland, D. Luxenberg, G.R. Askew, K.L. Milarski, C. Groves, T. Brown, B.A. Carito, K. Percival, B.M. Carreno, M. Collins, and S. Marusic. 2007. PD-1/PD-L1, but not PD-1/PD-L2, interactions regulate the severity of experimental autoimmune encephalomyelitis. *J Neuroimmunol* 182:124-134.

Cauley, L.S., E.E. Miller, M. Yen, and S.L. Swain. 2000. Superantigen-induced CD4 T cell tolerance mediated by myeloid cells and IFN-gamma. *J Immunol* 165:6056-6066.

Cella, M., F. Facchetti, A. Lanzavecchia, and M. Colonna. 2000. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. *Nat Immunol* 1:305-310.

Cella, M., M. Salio, Y. Sakakibara, H. Langen, I. Julkunen, and A. Lanzavecchia. 1999. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp Med* 189:821-829.

Chang, J.T., E.M. Shevach, and B.M. Segal. 1999. Regulation of interleukin (IL)-12 receptor beta2 subunit expression by endogenous IL-12: a critical step in the differentiation of pathogenic autoreactive T cells. *J Exp Med* 189:969-978.

Chu, C.Q., S. Wittmer, and D.K. Dalton. 2000. Failure to suppress the expansion of the activated CD4 T cell population in interferon gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. *J Exp Med* 192:123-128.

Cisse, B., M.L. Caton, M. Lehner, T. Maeda, S. Scheu, R. Locksley, D. Holmberg, C. Zweier, N.S. den Hollander, S.G. Kant, W. Holter, A. Rauch, Y. Zhuang, and B. Reizis. 2008. Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. *Cell* 135:37-48.

Coccia, E.M., M. Severa, E. Giacomini, D. Monneron, M.E. Remoli, I. Julkunen, M. Cella, R. Lande, and G. Uze. 2004. Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. *Eur J Immunol* 34:796-805.

Colonna, M., G. Trinchieri, and Y.J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat Immunol* 5:1219-1226.

Critchfield, J.M., M.K. Racke, J.C. Zuniga-Pflucker, B. Cannella, C.S. Raine, J. Goverman, and M.J. Lenardo. 1994. T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. *Science* 263:1139-1143.

Cua, D.J., B. Hutchins, D.M. LaFace, S.A. Stohlman, and R.L. Coffman. 2001. Central nervous system expression of IL-10 inhibits autoimmune encephalomyelitis. *J Immunol* 166:602-608.

Cua, D.J., J. Sherlock, Y. Chen, C.A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S.A. Lira, D. Gorman, R.A. Kastelein, and J.D. Sedgwick. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 421:744-748.

Curotto de Lafaille, M.A., and J.J. Lafaille. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 30:626-635.

Dai, J., N.J. Megjugorac, S.B. Amrute, and P. Fitzgerald-Bocarsly. 2004. Regulation of IFN regulatory factor-7 and IFN-alpha production by enveloped virus and lipopolysaccharide in human plasmacytoid dendritic cells. *J Immunol* 173:1535-1548.

Dardalhon, V., A.C. Anderson, J. Karman, L. Apetoh, R. Chandwaskar, D.H. Lee, M. Cornejo, N. Nishi, A. Yamauchi, F.J. Quintana, R.A. Sobel, M. Hirashima, and V.K. Kuchroo. 2010. Tim-3/galectin-9 pathway: regulation of Th1 immunity through promotion of CD11b+Ly-6G+ myeloid cells. *J Immunol* 185:1383-1392.

Davidson, A., and B. Diamond. 2001. Autoimmune diseases. *N Engl J Med* 345:340-350.

Delano, M.J., P.O. Scumpia, J.S. Weinstein, D. Coco, S. Nagaraj, K.M. Kelly-Scumpia, K.A. O'Malley, J.L. Wynn, S. Antonenko, S.Z. Al-Quran, R. Swan, C.S. Chung, M.A. Atkinson, R. Ramphal, D.I. Gabrilovich, W.H. Reeves, A. Ayala, J. Phillips, D. Laface, P.G. Heyworth, M. Clare-Salzler, and L.L. Moldawer. 2007. MyD88-dependent expansion of an immature GR-1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis. *J Exp Med* 204:1463-1474.

Delves, P.J., and I.M. Roitt. 2000. The immune system. First of two parts. *N Engl J Med* 343:37-49.

Di Pucchio, T., B. Chatterjee, A. Smed-Sorensen, S. Clayton, A. Palazzo, M. Montes, Y. Xue, I. Mellman, J. Banchereau, and J.E. Connolly. 2008. Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. *Nat Immunol* 9:551-557.

Diaz-Montero, C.M., M.L. Salem, M.I. Nishimura, E. Garrett-Mayer, D.J. Cole, and A.J. Montero. 2009. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol Immunother* 58:49-59.

Diveu, C., M.J. McGeachy, and D.J. Cua. 2008. Cytokines that regulate autoimmunity. *Curr Opin Immunol* 20:663-668.

El-behi, M., A. Rostami, and B. Ciric. 2010. Current views on the roles of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* 5:189-197.

Ezernitchi, A.V., I. Vaknin, L. Cohen-Daniel, O. Levy, E. Manaster, A. Halabi, E. Pikarsky, L. Shapira, and M. Baniyash. 2006. TCR zeta down-regulation under chronic inflammation is mediated by myeloid suppressor cells differentially distributed between various lymphatic organs. *J Immunol* 177:4763-4772.

Farkas, L., K. Beiske, F. Lund-Johansen, P. Brandtzaeg, and F.L. Jahnsen. 2001. Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions. *Am J Pathol* 159:237-243.

Feldmann, M., and L. Steinman. 2005. Design of effective immunotherapy for human autoimmunity. *Nature* 435:612-619.

Ferber, I., G. Schonrich, J. Schenkel, A.L. Mellor, G.J. Hammerling, and B. Arnold. 1994. Levels of peripheral T cell tolerance induced by different doses of tolerogen. *Science* 263:674-676.

Ferber, I.A., S. Brocke, C. Taylor-Edwards, W. Ridgway, C. Dinisco, L. Steinman, D. Dalton, and C.G. Fathman. 1996. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol* 156:5-7.

Filipazzi, P., R. Valenti, V. Huber, L. Pilla, P. Canese, M. Iero, C. Castelli, L. Mariani, G. Parmiani, and L. Rivoltini. 2007. Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. *J Clin Oncol* 25:2546-2553.

Fisher, G.H., F.J. Rosenberg, S.E. Straus, J.K. Dale, L.A. Middleton, A.Y. Lin, W. Strober, M.J. Lenardo, and J.M. Puck. 1995. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell* 81:935-946.

Foell, D., H. Wittkowski, T. Vogl, and J. Roth. 2007. S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. *J Leukoc Biol* 81:28-37.

Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.

Forabosco, P., E. Bouzigon, M.Y. Ng, J. Hermanowski, S.A. Fisher, L.A. Criswell, and C.M. Lewis. 2009. Meta-analysis of genome-wide linkage studies across autoimmune diseases. *European journal of human genetics : EJHG* 17:236-243.

Francisco, L.M., P.T. Sage, and A.H. Sharpe. 2010. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* 236:219-242.

Fridlender, Z.G., J. Sun, S. Kim, V. Kapoor, G. Cheng, L. Ling, G.S. Worthen, and S.M. Albelda. 2009. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 16:183-194.

Fujinami, R.S., and M.B. Oldstone. 1985. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science* 230:1043-1045.

Fujinami, R.S., M.B. Oldstone, Z. Wroblewska, M.E. Frankel, and H. Koprowski. 1983. Molecular mimicry in virus infection: crossreaction of measles virus phosphoprotein or of herpes simplex virus protein with human intermediate filaments. *Proc Natl Acad Sci U S A* 80:2346-2350.

Furlan, R., C. Cuomo, and G. Martino. 2009. Animal models of multiple sclerosis. *Methods Mol Biol* 549:157-173.

Gabrilovich, D., T. Ishida, T. Oyama, S. Ran, V. Kravtsov, S. Nadaf, and D.P. Carbone. 1998. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. *Blood* 92:4150-4166.

Gabrilovich, D.I., V. Bronte, S.H. Chen, M.P. Colombo, A. Ochoa, S. Ostrand-Rosenberg, and H. Schreiber. 2007. The terminology issue for myeloid-derived suppressor cells. *Cancer Res* 67:425; author reply 426.

Gabrilovich, D.I., and S. Nagaraj. 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 9:162-174.

Gallina, G., L. Dolcetti, P. Serafini, C. De Santo, I. Marigo, M.P. Colombo, G. Basso, F. Brombacher, I. Borrello, P. Zanovello, S. Bicciato, and V. Bronte. 2006. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *J Clin Invest* 116:2777-2790.

Ganguly, D., G. Chamilos, R. Lande, J. Gregorio, S. Meller, V. Facchinetti, B. Homey, F.J. Barrat, T. Zal, and M. Gilliet. 2009. Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. *J Exp Med* 206:1983-1994.

Garcia, M.R., L. Ledgerwood, Y. Yang, J. Xu, G. Lal, B. Burrell, G. Ma, D. Hashimoto, Y. Li, P. Boros, M. Grisotto, N. van Rooijen, R. Matesanz, F. Tacke, F. Ginhoux, Y. Ding, S.H. Chen, G. Randolph, M. Merad, J.S. Bromberg, and J.C. Ochando. 2010. Monocytic suppressive cells mediate cardiovascular transplantation tolerance in mice. *J Clin Invest* 120:2486-2496.

Gilliet, M., W. Cao, and Y.J. Liu. 2008. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol* 8:594-606.

Giordanengo, L., N. Guinazu, C. Stempin, R. Fretes, F. Cerban, and S. Gea. 2002. Cruzipain, a major Trypanosoma cruzi antigen, conditions the host immune response in favor of parasite. *Eur J Immunol* 32:1003-1011.

Gold, R., C. Linington, and H. Lassmann. 2006. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* 129:1953-1971.

Gomez-Garcia, L., L.M. Lopez-Marin, R. Saavedra, J.L. Reyes, M. Rodriguez-Sosa, and L.I. Terrazas. 2005. Intact glycans from cestode antigens are involved in innate activation of myeloid suppressor cells. *Parasite Immunol* 27:395-405.

Goni, O., P. Alcaide, and M. Fresno. 2002. Immunosuppression during acute Trypanosoma cruzi infection: involvement of Ly6G (Gr1(+))CD11b(+)immature myeloid suppressor cells. *Int Immunol* 14:1125-1134.

Goubier, A., B. Dubois, H. Gheit, G. Joubert, F. Villard-Truc, C. Asselin-Paturel, G. Trinchieri, and D. Kaiserlian. 2008. Plasmacytoid dendritic cells mediate oral tolerance. *Immunity* 29:464-475.

Greifenberg, V., E. Ribechini, S. Rossner, and M.B. Lutz. 2009. Myeloid-derived suppressor cell activation by combined LPS and IFN-gamma treatment impairs DC development. *Eur J Immunol* 39:2865-2876.

Grouard, G., M.C. Rissoan, L. Filgueira, I. Durand, J. Banchereau, and Y.J. Liu. 1997. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J Exp Med* 185:1101-1111.

Gutcher, I., and B. Becher. 2007. APC-derived cytokines and T cell polarization in autoimmune inflammation. *J Clin Invest* 117:1119-1127.

Hadeiba, H., T. Sato, A. Habtezion, C. Oderup, J. Pan, and E.C. Butcher. 2008. CCR9 expression defines tolerogenic plasmacytoid dendritic cells able to suppress acute graft-versus-host disease. *Nat Immunol* 9:1253-1260.

Hafler, D.A., A. Compston, S. Sawcer, E.S. Lander, M.J. Daly, P.L. De Jager, P.I. de Bakker, S.B. Gabriel, D.B. Mirel, A.J. Ivinson, M.A. Pericak-Vance, S.G. Gregory, J.D. Rioux, J.L. McCauley, J.L. Haines, L.F. Barcellos, B. Cree, J.R. Oksenberg, and S.L. Hauser. 2007. Risk

alleles for multiple sclerosis identified by a genomewide study. *N Engl J Med* 357:851-862.

Haile, L.A., R. von Wasielewski, J. Gamrekelashvili, C. Kruger, O. Bachmann, A.M. Westendorf, J. Buer, R. Liblau, M.P. Manns, F. Korangy, and T.F. Greten. 2008. Myeloid-derived suppressor cells in inflammatory bowel disease: a new immunoregulatory pathway. *Gastroenterology* 135:871-881, 881 e871-875.

Haverkamp, J.M., S.A. Crist, B.D. Elzey, C. Cimen, and T.L. Ratliff. 2011. In vivo suppressive function of myeloid-derived suppressor cells is limited to the inflammatory site. *Eur J Immunol* 41:749-759.

Heremans, H., C. Dillen, M. Groenen, E. Martens, and A. Billiau. 1996. Chronic relapsing experimental autoimmune encephalomyelitis (CREAE) in mice: enhancement by monoclonal antibodies against interferon-gamma. *Eur J Immunol* 26:2393-2398.

Hestdal, K., F.W. Ruscetti, J.N. Ihle, S.E. Jacobsen, C.M. Dubois, W.C. Kopp, D.L. Longo, and J.R. Keller. 1991. Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. *J Immunol* 147:22-28.

Heward, J., and S.C. Gough. 1997. Genetic susceptibility to the development of autoimmune disease. *Clin Sci (Lond)* 93:479-491.

Hoeffel, G., A.C. Ripoche, D. Matheoud, M. Nascimbeni, N. Escriou, P. Lebon, F. Heshmati, J.G. Guillet, M. Gannage, S. Caillat-Zucman, N. Casartelli, O. Schwartz, H. De la Salle, D. Hanau, A. Hosmalin, and C. Maranon. 2007. Antigen crosspresentation by human plasmacytoid dendritic cells. *Immunity* 27:481-492.

Hofstetter, H.H., S.M. Ibrahim, D. Koczan, N. Kruse, A. Weishaupt, K.V. Toyka, and R. Gold. 2005. Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. *Cell Immunol* 237:123-130.

Hohlfeld, R., and H. Wekerle. 2004. Autoimmune concepts of multiple sclerosis as a basis for selective immunotherapy: from pipe dreams to (therapeutic) pipelines. *Proc Natl Acad Sci U S A* 101 Suppl 2:14599-14606.

Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.

Horwitz, M.S., L.M. Bradley, J. Harbertson, T. Krahl, J. Lee, and N. Sarvetnick. 1998. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat Med* 4:781-785.

Huang, B., P.Y. Pan, Q. Li, A.I. Sato, D.E. Levy, J. Bromberg, C.M. Divino, and S.H. Chen. 2006. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res* 66:1123-1131.

Infante-Duarte, C., H.F. Horton, M.C. Byrne, and T. Kamradt. 2000. Microbial lipopeptides induce the production of IL-17 in Th cells. *J Immunol* 165:6107-6115.

Isaksson, M., B. Ardesjo, L. Ronnblom, O. Kampe, H. Lassmann, M.L. Eloranta, and A. Lobell. 2009. Plasmacytoid DC promote priming of autoimmune Th17 cells and EAE. *Eur J Immunol* 39:2925-2935.

Ito, T., M. Yang, Y.H. Wang, R. Lande, J. Gregorio, O.A. Perng, X.F. Qin, Y.J. Liu, and M. Gilliet. 2007. Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J Exp Med* 204:105-115.

Izaguirre, A., B.J. Barnes, S. Amrute, W.S. Yeow, N. Megjugorac, J. Dai, D. Feng, E. Chung, P.M. Pitha, and P. Fitzgerald-Bocarsly. 2003. Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells. *J Leukoc Biol* 74:1125-1138.

Jahnke, U., E.H. Fischer, and E.C. Alvord, Jr. 1985. Sequence homology between certain viral proteins and proteins related to encephalomyelitis and neuritis. *Science* 229:282-284.

Jego, G., A.K. Palucka, J.P. Blanck, C. Chalouni, V. Pascual, and J. Banchereau. 2003. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. *Immunity* 19:225-234.

Jongbloed, S.L., R.A. Benson, M.B. Nickdel, P. Garside, I.B. McInnes, and J.M. Brewer. 2009. Plasmacytoid dendritic cells regulate breach of self-tolerance in autoimmune arthritis. *J Immunol* 182:963-968.

Kamradt, T., P.D. Soloway, D.L. Perkins, and M.L. Gefter. 1991. Pertussis toxin prevents the induction of peripheral T cell anergy and enhances the T cell response to an encephalitogenic peptide of myelin basic protein. *J Immunol* 147:3296-3302.

Karpus, W.J., N.W. Lukacs, B.L. McRae, R.M. Strieter, S.L. Kunkel, and S.D. Miller. 1995. An important role for the chemokine macrophage inflammatory protein-1 alpha in the pathogenesis of the T cell-mediated autoimmune disease, experimental autoimmune encephalomyelitis. *J Immunol* 155:5003-5010.

Kavousanaki, M., A. Makrigiannakis, D. Boumpas, and P. Verginis. 2010. Novel role of plasmacytoid dendritic cells in humans: induction of interleukin-10-producing Treg cells by plasmacytoid dendritic cells in patients with rheumatoid arthritis responding to therapy. *Arthritis Rheum* 62:53-63.

Keir, M.E., M.J. Butte, G.J. Freeman, and A.H. Sharpe. 2008. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* 26:677-704.

Keir, M.E., S.C. Liang, I. Guleria, Y.E. Latchman, A. Qipo, L.A. Albacker, M. Koulmanda, G.J. Freeman, M.H. Sayegh, and A.H. Sharpe. 2006. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med* 203:883-895.

Kerr, E.C., B.J. Raveney, D.A. Copland, A.D. Dick, and L.B. Nicholson. 2008. Analysis of retinal cellular infiltrate in experimental autoimmune uveoretinitis reveals multiple regulatory cell populations. *J Autoimmun* 31:354-361.

Khattri, R., T. Cox, S.A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 4:337-342.

King, I.L., T.L. Dickendesher, and B.M. Segal. 2009. Circulating Ly-6C+ myeloid precursors migrate to the CNS and play a pathogenic role during autoimmune demyelinating disease. *Blood* 113:3190-3197.

Klinman, D.M., A.K. Yi, S.L. Beaucage, J. Conover, and A.M. Krieg. 1996. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc Natl Acad Sci U S A* 93:2879-2883.

Komiyama, Y., S. Nakae, T. Matsuki, A. Nambu, H. Ishigame, S. Kakuta, K. Sudo, and Y. Iwakura. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 177:566-573.

Kool, M., M. van Nimwegen, M.A. Willart, F. Muskens, L. Boon, J.J. Smit, A. Coyle, B.E. Clausen, H.C. Hoogsteden, B.N. Lambrecht, and H. Hammad. 2009. An anti-inflammatory role for plasmacytoid dendritic cells in allergic airway inflammation. *J Immunol* 183:1074-1082.

Korn, T., E. Bettelli, M. Oukka, and V.K. Kuchroo. 2009. IL-17 and Th17 Cells. *Annu Rev Immunol* 27:485-517.

Korn, T., J. Reddy, W. Gao, E. Bettelli, A. Awasthi, T.R. Petersen, B.T. Backstrom, R.A. Sobel, K.W. Wucherpfennig, T.B. Strom, M. Oukka, and V.K. Kuchroo. 2007. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med* 13:423-431.

Kortylewski, M., M. Kujawski, T. Wang, S. Wei, S. Zhang, S. Pilon-Thomas, G. Niu, H. Kay, J. Mule, W.G. Kerr, R. Jove, D. Pardoll, and H. Yu. 2005. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat Med* 11:1314-1321.

Krug, A., A.R. French, W. Barchet, J.A. Fischer, A. Dzionek, J.T. Pingel, M.M. Orihuela, S. Akira, W.M. Yokoyama, and M. Colonna. 2004. TLR9-dependent recognition of MCMV by IPC

and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* 21:107-119.

Kumagai, Y., O. Takeuchi, H. Kato, H. Kumar, K. Matsui, E. Morii, K. Aozasa, T. Kawai, and S. Akira. 2007. Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses. *Immunity* 27:240-252.

Kurtzke, J.F. 1993. Epidemiologic evidence for multiple sclerosis as an infection. *Clinical microbiology reviews* 6:382-427.

Lande, R., J. Gregorio, V. Facchinetti, B. Chatterjee, Y.H. Wang, B. Homey, W. Cao, B. Su, F.O. Nestle, T. Zal, I. Mellman, J.M. Schroder, Y.J. Liu, and M. Gilliet. 2007. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* 449:564-569.

Langrish, C.L., Y. Chen, W.M. Blumenschein, J. Mattson, B. Basham, J.D. Sedgwick, T. McClanahan, R.A. Kastelein, and D.J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201:233-240.

Latchman, Y.E., S.C. Liang, Y. Wu, T. Chernova, R.A. Sobel, M. Klemm, V.K. Kuchroo, G.J. Freeman, and A.H. Sharpe. 2004. PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. *Proc Natl Acad Sci U S A* 101:10691-10696.

Lees, J.R., P.T. Golumbek, J. Sim, D. Dorsey, and J.H. Russell. 2008. Regional CNS responses to IFN-gamma determine lesion localization patterns during EAE pathogenesis. *J Exp Med* 205:2633-2642.

Li, L., L. Huang, A.L. Vergis, H. Ye, A. Bajwa, V. Narayan, R.M. Strieter, D.L. Rosin, and M.D. Okusa. 2010. IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury. *J Clin Invest* 120:331-342.

Liu, C., Y. Lou, G. Lizee, H. Qin, S. Liu, B. Rabinovich, G.J. Kim, Y.H. Wang, Y. Ye, A.G. Sikora, W.W. Overwijk, Y.J. Liu, G. Wang, and P. Hwu. 2008. Plasmacytoid dendritic cells induce NK cell-dependent, tumor antigen-specific T cell cross-priming and tumor regression in mice. *J Clin Invest* 118:1165-1175.

Liu, C.Y., Y.M. Wang, C.L. Wang, P.H. Feng, H.W. Ko, Y.H. Liu, Y.C. Wu, Y. Chu, F.T. Chung, C.H. Kuo, K.Y. Lee, S.M. Lin, H.C. Lin, C.H. Wang, C.T. Yu, and H.P. Kuo. 2010. Population alterations of L-arginase- and inducible nitric oxide synthase-expressed CD11b+/CD14/CD15+/CD33+ myeloid-derived suppressor cells and CD8+ T lymphocytes in patients with advanced-stage non-small cell lung cancer. *Journal of cancer research and clinical oncology* 136:35-45.

Liu, Y.J. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 23:275-306.

Liu, Y.J., H. Kanzler, V. Soumelis, and M. Gilliet. 2001. Dendritic cell lineage, plasticity and cross-regulation. *Nat Immunol* 2:585-589.

Lock, C., G. Hermans, R. Pedotti, A. Brendolan, E. Schadt, H. Garren, A. Langer-Gould, S. Strober, B. Cannella, J. Allard, P. Klonowski, A. Austin, N. Lad, N. Kaminski, S.J. Galli, J.R. Oksenberg, C.S. Raine, R. Heller, and L. Steinman. 2002. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med* 8:500-508.

Lublin, F.D., R.L. Knobler, B. Kalman, M. Goldhaber, J. Marini, M. Perrault, C. D'Imperio, J. Joseph, S.S. Alkan, and R. Korngold. 1993. Monoclonal anti-gamma interferon antibodies enhance experimental allergic encephalomyelitis. *Autoimmunity* 16:267-274.

Lucchinetti, C., W. Bruck, J. Parisi, B. Scheithauer, M. Rodriguez, and H. Lassmann. 2000. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* 47:707-717.

Makarenkova, V.P., V. Bansal, B.M. Matta, L.A. Perez, and J.B. Ochoa. 2006. CD11b+/Gr-1+ myeloid suppressor cells cause T cell dysfunction after traumatic stress. *J Immunol* 176:2085-2094.

Mannick, J.B., A. Hausladen, L. Liu, D.T. Hess, M. Zeng, Q.X. Miao, L.S. Kane, A.J. Gow, and J.S. Stamler. 1999. Fas-induced caspase denitrosylation. *Science* 284:651-654.

Marhaba, R., M. Vitacolonna, D. Hildebrand, M. Baniyash, P. Freyschmidt-Paul, and M. Zoller. 2007. The importance of myeloid-derived suppressor cells in the regulation of autoimmune effector cells by a chronic contact eczema. *J Immunol* 179:5071-5081.

Marigo, I., E. Bosio, S. Solito, C. Mesa, A. Fernandez, L. Dolcetti, S. Ugel, N. Sonda, S. Bicciato, E. Falisi, F. Calabrese, G. Basso, P. Zanovello, E. Cozzi, S. Mandruzzato, and V. Bronte. 2010. Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. *Immunity* 32:790-802.

Marigo, I., L. Dolcetti, P. Serafini, P. Zanovello, and V. Bronte. 2008. Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. *Immunol Rev* 222:162-179.

Marrack, P., J. Kappler, and B.L. Kotzin. 2001. Autoimmune disease: why and where it occurs. *Nat Med* 7:899-905.

Martin-Gayo, E., E. Sierra-Filardi, A.L. Corbi, and M.L. Toribio. 2010. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood* 115:5366-5375.

McFarland, H.F., and R. Martin. 2007. Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol* 8:913-919.

McGeachy, M.J., Y. Chen, C.M. Tato, A. Laurence, B. Joyce-Shaikh, W.M. Blumenschein, T.K. McClanahan, J.J. O'Shea, and D.J. Cua. 2009. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol* 10:314-324.

McGeachy, M.J., L.A. Stephens, and S.M. Anderton. 2005. Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. *J Immunol* 175:3025-3032.

McRae, B.L., M.K. Kennedy, L.J. Tan, M.C. Dal Canto, K.S. Picha, and S.D. Miller. 1992. Induction of active and adoptive relapsing experimental autoimmune encephalomyelitis (EAE) using an encephalitogenic epitope of proteolipid protein. *Journal of neuroimmunology* 38:229-240.

Mencacci, A., C. Montagnoli, A. Bacci, E. Cenci, L. Pitzurra, A. Spreca, M. Kopf, A.H. Sharpe, and L. Romani. 2002. CD80+Gr-1+ myeloid cells inhibit development of antifungal Th1 immunity in mice with candidiasis. *J Immunol* 169:3180-3190.

Mildner, A., M. Mack, H. Schmidt, W. Bruck, M. Djukic, M.D. Zabel, A. Hille, J. Priller, and M. Prinz. 2009. CCR2+Ly-6Chi monocytes are crucial for the effector phase of autoimmunity in the central nervous system. *Brain* 132:2487-2500.

Miller, S.D., C.L. Vanderlugt, W.S. Begolka, W. Pao, R.L. Yauch, K.L. Neville, Y. Katz-Levy, A. Carrizosa, and B.S. Kim. 1997. Persistent infection with Theiler's virus leads to CNS autoimmunity via epitope spreading. *Nat Med* 3:1133-1136.

Mirza, N., M. Fishman, I. Fricke, M. Dunn, A.M. Neuger, T.J. Frost, R.M. Lush, S. Antonia, and D.I. Gabrilovich. 2006. All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res* 66:9299-9307.

Moseman, E.A., X. Liang, A.J. Dawson, A. Panoskaltsis-Mortari, A.M. Krieg, Y.J. Liu, B.R. Blazar, and W. Chen. 2004. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J Immunol* 173:4433-4442.

Mouries, J., G. Moron, G. Schlecht, N. Escriou, G. Dadaglio, and C. Leclerc. 2008. Plasmacytoid dendritic cells efficiently cross-prime naive T cells in vivo after TLR activation. *Blood* 112:3713-3722.

Movahedi, K., M. Guilliams, J. Van den Bossche, R. Van den Bergh, C. Gysemans, A. Beschin, P. De Baetselier, and J.A. Van Ginderachter. 2008. Identification of discrete tumor-induced

myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood* 111:4233-4244.

Nagaraj, S., M. Collazo, C.A. Corzo, J.I. Youn, M. Ortiz, D. Quiceno, and D.I. Gabrilovich. 2009. Regulatory myeloid suppressor cells in health and disease. *Cancer Res* 69:7503-7506.

Nagaraj, S., and D.I. Gabrilovich. 2010. Myeloid-derived suppressor cells in human cancer. *Cancer J* 16:348-353.

Nagaraj, S., K. Gupta, V. Pisarev, L. Kinarsky, S. Sherman, L. Kang, D.L. Herber, J. Schneck, and D.I. Gabrilovich. 2007. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med* 13:828-835.

Nagaraj, S., A.G. Schrum, H.I. Cho, E. Celis, and D.I. Gabrilovich. 2010. Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. *J Immunol* 184:3106-3116.

Nefedova, Y., S. Nagaraj, A. Rosenbauer, C. Muro-Cacho, S.M. Sebti, and D.I. Gabrilovich. 2005. Regulation of dendritic cell differentiation and antitumor immune response in cancer by pharmacologic-selective inhibition of the janus-activated kinase 2/signal transducers and activators of transcription 3 pathway. *Cancer Res* 65:9525-9535.

Nestle, F.O., C. Conrad, A. Tun-Kyi, B. Homey, M. Gombert, O. Boyman, G. Burg, Y.J. Liu, and M. Gilliet. 2005. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *J Exp Med* 202:135-143.

Ochando, J.C., C. Homma, Y. Yang, A. Hidalgo, A. Garin, F. Tacke, V. Angeli, Y. Li, P. Boros, Y. Ding, R. Jessberger, G. Trinchieri, S.A. Lira, G.J. Randolph, and J.S. Bromberg. 2006. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* 7:652-662.

Ochoa, A.C., A.H. Zea, C. Hernandez, and P.C. Rodriguez. 2007. Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma. *Clin Cancer Res* 13:721s-726s.

Osawa, Y., S. Iho, R. Takauji, H. Takatsuka, S. Yamamoto, T. Takahashi, S. Horiguchi, Y. Urasaki, T. Matsuki, and S. Fujieda. 2006. Collaborative action of NF-kappaB and p38 MAPK is involved in CpG DNA-induced IFN-alpha and chemokine production in human plasmacytoid dendritic cells. *J Immunol* 177:4841-4852.

Pan, P.Y., G.X. Wang, B. Yin, J. Ozao, T. Ku, C.M. Divino, and S.H. Chen. 2008. Reversion of immune tolerance in advanced malignancy: modulation of myeloid-derived suppressor cell development by blockade of stem-cell factor function. *Blood* 111:219-228.

Panitch, H.S., R.L. Hirsch, A.S. Haley, and K.P. Johnson. 1987. Exacerbations of multiple sclerosis in patients treated with gamma interferon. *Lancet* 1:893-895.

Park, H., Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.

Pepper, M., F. Dzierszinski, E. Wilson, E. Tait, Q. Fang, F. Yarovinsky, T.M. Laufer, D. Roos, and C.A. Hunter. 2008. Plasmacytoid dendritic cells are activated by Toxoplasma gondii to present antigen and produce cytokines. *J Immunol* 180:6229-6236.

Peranzoni, E., S. Zilio, I. Marigo, L. Dolcetti, P. Zanovello, S. Mandruzzato, and V. Bronte. 2010. Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr Opin Immunol* 22:238-244.

Perron, H., J.A. Garson, F. Bedin, F. Beseme, G. Paranhos-Baccala, F. Komurian-Pradel, F. Mallet, P.W. Tuke, C. Voisset, J.L. Blond, B. Lalande, J.M. Seigneurin, and B. Mandrand. 1997. Molecular identification of a novel retrovirus repeatedly isolated from patients with multiple sclerosis. The Collaborative Research Group on Multiple Sclerosis. *Proc Natl Acad Sci U S A* 94:7583-7588.

Pettinelli, C.B., and D.E. McFarlin. 1981. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes. *J Immunol* 127:1420-1423.

Poschke, I., D. Mougiakakos, J. Hansson, G.V. Masucci, and R. Kiessling. 2010. Immature immunosuppressive CD14+HLA-DR-/low cells in melanoma patients are Stat3hi and overexpress CD80, CD83, and DC-sign. *Cancer Res* 70:4335-4345.

Pouly, S., B. Becher, M. Blain, and J.P. Antel. 2000. Interferon-gamma modulates human oligodendrocyte susceptibility to Fas-mediated apoptosis. *Journal of neuropathology and experimental neurology* 59:280-286.

Powell, M.B., D. Mitchell, J. Lederman, J. Buckmeier, S.S. Zamvil, M. Graham, N.H. Ruddle, and L. Steinman. 1990. Lymphotoxin and tumor necrosis factor-alpha production by myelin basic protein-specific T cell clones correlates with encephalitogenicity. *Int Immunol* 2:539-544.

Rabinovich, G.A., D. Gabrilovich, and E.M. Sotomayor. 2007. Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol* 25:267-296.

Randolph, G.J., J. Ochando, and S. Partida-Sanchez. 2008. Migration of dendritic cell subsets and their precursors. *Annu Rev Immunol* 26:293-316.

Reizis, B., M. Colonna, G. Trinchieri, F. Barrat, and M. Gilliet. 2011. Plasmacytoid dendritic cells: one-trick ponies or workhorses of the immune system? *Nat Rev Immunol* 11:558-565.

Ribechini, E., V. Greifenberg, S. Sandwick, and M.B. Lutz. 2010. Subsets, expansion and activation of myeloid-derived suppressor cells. *Medical microbiology and immunology* 199:273-281.

Rieux-Laucat, F., F. Le Deist, C. Hivroz, I.A. Roberts, K.M. Debatin, A. Fischer, and J.P. de Villartay. 1995. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* 268:1347-1349.

Rodriguez, P.C., M.S. Ernstoff, C. Hernandez, M. Atkins, J. Zabaleta, R. Sierra, and A.C. Ochoa. 2009. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer Res* 69:1553-1560.

Rodriguez, P.C., and A.C. Ochoa. 2008. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. *Immunol Rev* 222:180-191.

Rodriguez, P.C., D.G. Quiceno, and A.C. Ochoa. 2007. L-arginine availability regulates T-lymphocyte cell-cycle progression. *Blood* 109:1568-1573.

Rodriguez, P.C., D.G. Quiceno, J. Zabaleta, B. Ortiz, A.H. Zea, M.B. Piazuelo, A. Delgado, P. Correa, J. Brayer, E.M. Sotomayor, S. Antonia, J.B. Ochoa, and A.C. Ochoa. 2004. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res* 64:5839-5849.

Ronnblom, L., and G.V. Alm. 2001. A pivotal role for the natural interferon alpha-producing cells (plasmacytoid dendritic cells) in the pathogenesis of lupus. *J Exp Med* 194:F59-63.

Ronnblom, L., G.V. Alm, and M.L. Eloranta. 2009. Type I interferon and lupus. *Curr Opin Rheumatol* 21:471-477.

Ronnblom, L., and V. Pascual. 2008. The innate immune system in SLE: type I interferons and dendritic cells. *Lupus* 17:394-399.

Salio, M., M.J. Palmowski, A. Atzberger, I.F. Hermans, and V. Cerundolo. 2004. CpG-matured murine plasmacytoid dendritic cells are capable of in vivo priming of functional CD8 T cell responses to endogenous but not exogenous antigens. *J Exp Med* 199:567-579.

Salomon, B., and J.A. Bluestone. 2001. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu Rev Immunol* 19:225-252.

Santini, S.M., C. Lapenta, M. Logozzi, S. Parlato, M. Spada, T. Di Pucchio, and F. Belardelli. 2000. Type I interferon as a powerful adjuvant for monocyte-derived dendritic cell development and activity in vitro and in Hu-PBL-SCID mice. *J Exp Med* 191:1777-1788.

Sapoznikov, A., J.A. Fischer, T. Zaft, R. Krauthgamer, A. Dzionek, and S. Jung. 2007. Organ-dependent in vivo priming of naive CD4+, but not CD8+, T cells by plasmacytoid dendritic cells. *J Exp Med* 204:1923-1933.

Saxena, V., J.K. Ondr, A.F. Magnusen, D.H. Munn, and J.D. Katz. 2007. The countervailing actions of myeloid and plasmacytoid dendritic cells control autoimmune diabetes in the nonobese diabetic mouse. *J Immunol* 179:5041-5053.

Schmielau, J., and O.J. Finn. 2001. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res* 61:4756-4760.

Scott, B., R. Liblau, S. Degermann, L.A. Marconi, L. Ogata, A.J. Caton, H.O. McDevitt, and D. Lo. 1994. A role for non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. *Immunity* 1:73-83.

Serafini, P., I. Borrello, and V. Bronte. 2006. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol* 16:53-65.

Serafini, P., R. Carbley, K.A. Noonan, G. Tan, V. Bronte, and I. Borrello. 2004. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. *Cancer Res* 64:6337-6343.

Serafini, P., S. Mgebroff, K. Noonan, and I. Borrello. 2008. Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. *Cancer Res* 68:5439-5449.

Serbina, N.V., and E.G. Pamer. 2006. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol* 7:311-317.

Sharma, M.D., B. Baban, P. Chandler, D.Y. Hou, N. Singh, H. Yagita, M. Azuma, B.R. Blazar, A.L. Mellor, and D.H. Munn. 2007. Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. *J Clin Invest* 117:2570-2582.

Shortman, K., and Y.J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat Rev Immunol* 2:151-161.

Shortman, K., and S.H. Naik. 2007. Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 7:19-30.

Sinha, P., V.K. Clements, A.M. Fulton, and S. Ostrand-Rosenberg. 2007. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer Res* 67:4507-4513.

Sinha, P., V.K. Clements, and S. Ostrand-Rosenberg. 2005a. Interleukin-13-regulated M2 macrophages in combination with myeloid suppressor cells block immune surveillance against metastasis. *Cancer Res* 65:11743-11751.

Sinha, P., V.K. Clements, and S. Ostrand-Rosenberg. 2005b. Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. *J Immunol* 174:636-645.

Song, X., Y. Krelin, T. Dvorkin, O. Bjorkdahl, S. Segal, C.A. Dinarello, E. Voronov, and R.N. Apte. 2005. CD11b+/Gr-1+ immature myeloid cells mediate suppression of T cells in mice bearing tumors of IL-1beta-secreting cells. *J Immunol* 175:8200-8208.

Sospedra, M., and R. Martin. 2005. Immunology of multiple sclerosis. *Annu Rev Immunol* 23:683-747.

Sozzani, S., W. Vermi, A. Del Prete, and F. Facchetti. 2010. Trafficking properties of plasmacytoid dendritic cells in health and disease. *Trends Immunol* 31:270-277.

Srivastava, M.K., J.J. Bosch, J.A. Thompson, B.R. Ksander, M.J. Edelman, and S. Ostrand-Rosenberg. 2008. Lung cancer patients' CD4(+) T cells are activated in vitro by MHC II cell-based vaccines despite the presence of myeloid-derived suppressor cells. *Cancer Immunol Immunother* 57:1493-1504.

Steinman, L. 1996. Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell* 85:299-302.

Steinman, L. 2007. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med* 13:139-145.

Stromnes, I.M., L.M. Cerretti, D. Liggitt, R.A. Harris, and J.M. Goverman. 2008. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med* 14:337-342.

Stromnes, I.M., and J.M. Goverman. 2006. Passive induction of experimental allergic encephalomyelitis. *Nature protocols* 1:1952-1960.

Suda, T., T. Takahashi, P. Golstein, and S. Nagata. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 75:1169-1178.

Sunderkotter, C., T. Nikolic, M.J. Dillon, N. Van Rooijen, M. Stehling, D.A. Drevets, and P.J. Leenen. 2004. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol* 172:4410-4417.

Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka. 2001. IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol* 19:623-655.

Terabe, M., S. Matsui, J.M. Park, M. Mamura, N. Noben-Trauth, D.D. Donaldson, W. Chen, S.M. Wahl, S. Ledbetter, B. Pratt, J.J. Letterio, W.E. Paul, and J.A. Berzofsky. 2003. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med* 198:1741-1752.

Terrazas, L.I., K.L. Walsh, D. Piskorska, E. McGuire, and D.A. Harn, Jr. 2001. The schistosome oligosaccharide lacto-N-neotetraose expands Gr1(+) cells that secrete anti-inflammatory cytokines and inhibit proliferation of naive CD4(+) cells: a potential mechanism for immune polarization in helminth infections. *J Immunol* 167:5294-5303.

Tisch, R., and B. Wang. 2009. Role of plasmacytoid dendritic cells in type 1 diabetes: friend or foe? *Diabetes* 58:12-13.

Tivol, E.A., F. Borriello, A.N. Schweitzer, W.P. Lynch, J.A. Bluestone, and A.H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541-547.

Tough, D.F., S. Sun, and J. Sprent. 1997. T cell stimulation in vivo by lipopolysaccharide (LPS). *J Exp Med* 185:2089-2094.

Tsuda, Y., H. Takahashi, M. Kobayashi, T. Hanafusa, D.N. Herndon, and F. Suzuki. 2004. Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant Staphylococcus aureus. *Immunity* 21:215-226.

Tu, S., G. Bhagat, G. Cui, S. Takaishi, E.A. Kurt-Jones, B. Rickman, K.S. Betz, M. Penz-Oesterreicher, O. Bjorkdahl, J.G. Fox, and T.C. Wang. 2008. Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell* 14:408-419.

Tuohy, V.K., M. Yu, L. Yin, J.A. Kawczak, J.M. Johnson, P.M. Mathisen, B. Weinstock-Guttman, and R.P. Kinkel. 1998. The epitope spreading cascade during progression of experimental autoimmune encephalomyelitis and multiple sclerosis. *Immunol Rev* 164:93-100.

Ueda, H., J.M. Howson, L. Esposito, J. Heward, H. Snook, G. Chamberlain, D.B. Rainbow, K.M. Hunter, A.N. Smith, G. Di Genova, M.H. Herr, I. Dahlman, F. Payne, D. Smyth, C. Lowe, R.C. Twells, S. Howlett, B. Healy, S. Nutland, H.E. Rance, V. Everett, L.J. Smink, A.C. Lam, H.J. Cordell, N.M. Walker, C. Bordin, J. Hulme, C. Motzo, F. Cucca, J.F. Hess, M.L. Metzker, J. Rogers, S. Gregory, A. Allahabadia, R. Nithiyananthan, E. Tuomilehto-Wolf, J. Tuomilehto, P. Bingley, K.M. Gillespie, D.E. Undlien, K.S. Ronningen, C. Guja, C. Ionescu-Tirgoviste, D.A. Savage, A.P. Maxwell, D.J. Carson, C.C. Patterson, J.A. Franklyn, D.G. Clayton, L.B. Peterson, L.S. Wicker, J.A. Todd, and S.C. Gough. 2003. Association of the T-

cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 423:506-511.

van Eden, W., J. Holoshitz, Z. Nevo, A. Frenkel, A. Klajman, and I.R. Cohen. 1985. Arthritis induced by a T-lymphocyte clone that responds to Mycobacterium tuberculosis and to cartilage proteoglycans. *Proc Natl Acad Sci U S A* 82:5117-5120.

Vartanian, T., Y. Li, M. Zhao, and K. Stefansson. 1995. Interferon-gamma-induced oligodendrocyte cell death: implications for the pathogenesis of multiple sclerosis. *Mol Med* 1:732-743.

Villadangos, J.A., and L. Young. 2008. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity* 29:352-361.

Voisin, M.B., D. Buzoni-Gatel, D. Bout, and F. Velge-Roussel. 2004. Both expansion of regulatory GR1+ CD11b+ myeloid cells and anergy of T lymphocytes participate in hyporesponsiveness of the lung-associated immune system during acute toxoplasmosis. *Infect Immun* 72:5487-5492.

Weber, M.S., T. Prod'homme, S. Youssef, S.E. Dunn, C.D. Rundle, L. Lee, J.C. Patarroyo, O. Stuve, R.A. Sobel, L. Steinman, and S.S. Zamvil. 2007. Type II monocytes modulate T cell-mediated central nervous system autoimmune disease. *Nat Med* 13:935-943.

Willenborg, D.O., S. Fordham, C.C. Bernard, W.B. Cowden, and I.A. Ramshaw. 1996. IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *J Immunol* 157:3223-3227.

Yang, R., Z. Cai, Y. Zhang, W.H.t. Yutzy, K.F. Roby, and R.B. Roden. 2006. CD80 in immune suppression by mouse ovarian carcinoma-associated Gr-1+CD11b+ myeloid cells. *Cancer Res* 66:6807-6815.

Yin, B., G. Ma, C.Y. Yen, Z. Zhou, G.X. Wang, C.M. Divino, S. Casares, S.H. Chen, W.C. Yang, and P.Y. Pan. 2010. Myeloid-derived suppressor cells prevent type 1 diabetes in murine models. *J Immunol* 185:5828-5834.

Youn, J.I., S. Nagaraj, M. Collazo, and D.I. Gabrilovich. 2008. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J Immunol* 181:5791-5802.

Zehntner, S.P., C. Brickman, L. Bourbonniere, L. Remington, M. Caruso, and T. Owens. 2005. Neutrophils that infiltrate the central nervous system regulate T cell responses. *J Immunol* 174:5124-5131.

Zhang, X., L. Majlessi, E. Deriaud, C. Leclerc, and R. Lo-Man. 2009. Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils. *Immunity* 31:761-771.

Zhu, B., Y. Bando, S. Xiao, K. Yang, A.C. Anderson, V.K. Kuchroo, and S.J. Khoury. 2007. CD11b+Ly-6C(hi) suppressive monocytes in experimental autoimmune encephalomyelitis. *J Immunol* 179:5228-5237.

APPENDIX I

Crucial role of granulocytic myeloid-derived suppressor cells in the regulation of central nervous system autoimmune disease

Marianna Ioannou[*†], Themis Alissafi[*†], Iakovos Lazaridis[‡], George Deraos[§], John Matsoukas[§], Achille Gravanis[‡], Vasileios Mastorodemos[¶], Andreas Plaitakis[¶], Arlene Sharpe[ǀ], Dimitrios Boumpas[*†] and Panayotis Verginis[1*†]

[*]Laboratory of Autoimmunity and Inflammation, University of Crete, Medical School, Heraklion, Greece, [†]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, 71300, Heraklion, Greece. [‡]Department of Pharmacology, University of Crete, Medical School, Heraklion, Greece. [§]Department of Chemistry, University of Patras, 26504, Patras, Greece. [¶]Department of Neurology, University of Crete, Medical School, 71003 Heraklion, Greece. [ǀ]Department of Pathology, Harvard Medical School, Brigham & Women's Hospital, Boston, MA 02115, USA.

Abstract

There is a currently unmet need in autoimmune diseases to uncover the mechanisms involved in the natural resolution of inflammation. Herein, we demonstrate that granulocytic myeloid-derived suppressor cells (G-MDSCs) abundantly accumulate within the peripheral lymphoid compartments and target organ of mice with experimental autoimmune encephalomyelitis (EAE) prior to disease remission. *In vivo* transfer of G-MDSCs ameliorated EAE, significantly decreased demyelination and delayed disease onset through inhibition of encephalitogenic Th1 and Th17 immune responses. Exposure of G-MDSCs to the autoimmune milieu led to up-regulation of the programmed death 1 ligand (PD-L1) that was required for the G-MDSC-mediated suppressive function both *in vitro* and *in vivo*. Importantly, MDSCs were enriched in the periphery of subjects with active multiple sclerosis (MS) and suppressed the activation and proliferation of autologous $CD4^+$ T cells *ex vivo*. Collectively, this study reveals a pivotal role of MDSCs in the regulation of multiple sclerosis, which could be exploited for therapeutic purposes.

Under revision at The Journal of Immunology

APPENDIX II

The negative costimulatory molecule PD-1 modulates the balance between immunity and tolerance via miR-21

Dimitrios Iliopoulos[1], Melina Kavousanaki[2,3], **Marianna Ioannou**[2,3],Dimitrios Boumpas[2,3] and Panayotis Verginis[2,3]

---

1 Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA
2 University of Crete, Medical School, Foundation for Research and Technology, Heraklion, Crete, Greece
3 Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Heraklion, Crete, Greece

Abstract

Disruption of the programmed death-1 (PD-1) pathway leads to breakdown of peripheral tolerance and initiation of autoimmunity. The molecular pathways that mediate this effect remain largely unknown. We report here that PD-1 knockout (PD-1$^{-/-}$) mice develop more severe and sustained Ag-induced arthritis (AIA) than WT animals, which is associated with increased T-cell proliferation and elevated levels of IFN-γ and IL-17 secretion. MicroRNA analysis of Ag-specific CD41 T cells revealed a significant upregulation of microRNA 21 (miR-21) in PD-1$^{-/-}$ T cells compared with WT controls. In addition, PD-1 inhibition, via siRNA, upregulated miR-21 expression and enhanced STAT5 binding in the miR-21 promoter area. Computational analysis confirmed that miR-21 targets directly the expression of programmed cell death 4 (PDCD4) and overexpression of miR-21 in cells harboring the 3'UTR of PDCD4 resulted in reduced transcription and PDCD4 protein expression. Importantly, in vitro delivery of antisense-miR-21 suppressed the Ag-specific proliferation and cytokine secretion by PD-1$^{-/-}$ T cells, whereas adoptive transfer of Ag-specific T cells, overexpressing miR-21, induced severe AIA. Collectively, our data demonstrate that breakdown of tolerance in PD-1$^{-/-}$ mice activates a signaling cascade mediated by STAT5, miR-21, and PDCD4 and establish their role in maintaining the balance between immune activation and tolerance.

Published at Eur. J. Immunol. 2011. 41: 1754–1763