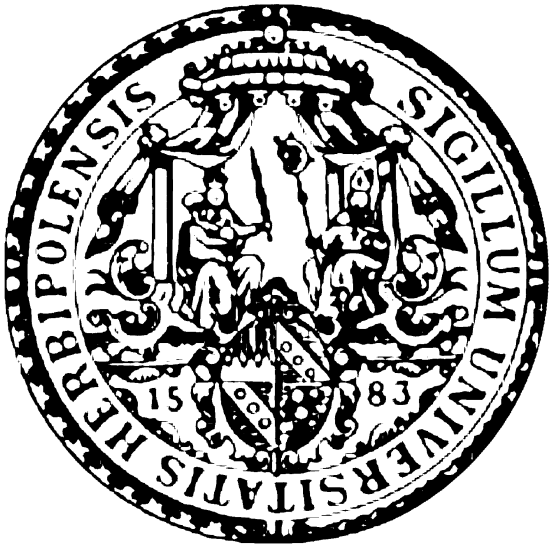

Suppression of Experimental Autoimmune-Encephalomyelitis by Myeloid-Derived Suppressor Cells

Suppression der Experimentellen Autoimmun-Enzephalomyelitis durch Myeloide Suppressorzellen

Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences, Julius-Maximilians-Universität Würzburg, Section of Infection and Immunity

Sarah Sandwick
Manfred B. Lutz Laboratory

June 2012
Würzburg, Deutschland

1.1 Table of Content

1.2 Members of the Thesis Advisory Committee

Primary Supervisor: Dr. Manfred B. Lutz
Supervisor (Second): Dr. Rudolf Martini
Supervisor (Third): Dr. Stephan Kissler
Acting replacement to third chair: Dr. Andreas Beilhack

Submitted on:

Date of Public Defense:

Affidavit
I hereby confirm that my thesis entitled **Suppression of Experimental Autoimmune-Encephalomyelitis by Myeloid-Derived Suppressor Cells** is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

22 June 2012, Würzburg, Germany _____
Sarah Sandwick

1.3 Acknowledgements

I would like to dedicate this research to my grandmother, Gladys K. Sandwick. She was instrumental in inspiring me to push my limits.

I would like to thank Dr. Andreas Beilhack for the gracious hospitality in his laboratory during our animal hardships and the use of his bioluminescence imager and fluorescent microscope. I would like to thank my thesis committee for their helpful annual discussions. I would like to thank my supervisor, Dr. Manfred Lutz for his funding and support, and the assistance of translating of my summary into German. I would like to thank Dr. Thomas Hünig for his support. I would also like to thank the Immunomodulation Graduate school group and Immunology department for opening up many scientific discussions and collaborations. I would like to thank Dr. Jörg Wischhusen, whose knowledge is inspiring, and his lab members for the many collaborations and mini-project. I would like to also thank Dr. Ari Waisman (Mainz, Transregio 52 collaboration) for our EAE support and CNS isolation protocols.

I would like to thank my lab mates, Dr. Eliana Ribechini, Anja Döhler, Katrien Pletinckx, Gudren Schnell, Dr. Regina Laube, Cinthia Silva-Vilches, Theresa Schneider, Katrin Schweizer, for their support and ideas. I would like to especially thank Dr. Eliana Ribechini for all of her guidance and support for that I owe you endlessly. I also would like to give an extra thank you to Anja Döhler for helping with the summary translation, as well as formatting and proof-reading of this document. I would like to thank Janos Groh (Rudolf Martini Lab) and Ana-Laura Jordan-Garrote for their assistance in different areas of expertise, neurology and bioluminescence imaging. I would like to thank Ahmed Adel Seida for his knowledge and helpful hints in establishing our RT-PCRs. Although the data was is not shown in this thesis, I would also like to thank Anoop Chandran for his knowledge and assistance in the microRNA mini-project performed in Dr. Jörg Wischhusens lab.

I would like to thank Dr. Karin Elflein for her hard work and dedication to the embryo transfer for our mouse facility. I would like to thank Dr. Heinz Wiendl for the use of his transgenic mice. I would like to thank Dr. Andre Gessner (Former Erlangen) for providing MyD88/TLR4 KO, Dr. Christine Krempl for IFN-aR KO mice (Würzburg), and Dr. Micheal Lohoff for IRF-1 KO mice (Marburg) for the function assays. I would like to thank our collaborators in Erlangen Dr. Ulricke Schliecher and Dr. Christian Bogden for their gift of IFN-gR1 KO and iNOS- KO bone marrow used in our experiments. I would like to thank Dr. Friedericke Bereberich-Siebelt for the gift of the VAV-CRE and VAV-CRE X C/EBP-b fl/fl mice used for the transcription factor experiments

I would like to thank my family, especially my husband, Jerry Potter, and dogs, Ren and Bernie, for their support and putting up with my really late nights in the lab. I also would like to thank my parents for their support. I would especially like to thank Dr. Roger Sandwick for the many proof-reads of this document.

This research was funded in part by SFB 581.

1.4 Abbreviations:

Arg-1	arginase-1
B7-H1	B7-homologue family member-1
BBB	Blood brain barrier
BLI	biolumenscence imaging
BM	bone marrow
BM-IMC	bone marrow generated immature myeloid cell
BM-MDSC	bone marrow generated myeloid derived suppressor cells
BM-DC	bone marrow generated dendritic cell
BSA	Bovine Serum Albumin
C/EBP-b	CAAT/enhancer binding protein-beta
CD	Cluster of Differentiation
CFA	Complete Freunds Adjuvant
CFSE	carboxyfluorescein diacetate succinimidyl ester
CLR	C-Type Lectin receptor
CNS	central nervous system
CpG	Cytosine phosphate guanine
DC	dendritic cells
DNA	deoxyribosenucleic acid
dsDNA	double stranded DNA
dsRNA	double stranded RNA
EAE	Experimental Autoimmune Encephalomyelitis
ELISA	Enzyme linked immunoabsorbant assay
FACS	flow cytometry
FLT-3L	Fms like tyrosine kinase 3 ligand
GM-CSF	granulocyte macrophage colony stimulating factor
i.c.	intracranially
i.p.	intraperitoneally
i.v.	intravenously
IFN-b	Interferon beta
IFN-g	Interferon gamma
IL	interleukin
IMC	Immature myeloid cell
iNOS	inducible nitric oxide synthase
Iono	Ionomycin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunorecptor tyrosine-based inhibition motif
Jak	Janus kinase
LPS	Lipopolysaccharide
Luc+	Luciferase positive
MBP	myelin basic protein
MDSC	myeloid derived suppressor cells
MHC	Major Histocomptability complex
MLR	mixed lymphocyte reaction
MOG	myelin oligodendricyte protein
MS	Multiple Sclerosis
MyD88	Myeloid differentiation factor 88
NF-kB	nuclear factor kappa light chain enhancer of activated B cells
NO	Nitric oxide

OVA	ovalbumin
PAMP	Pathogen associated molecular pattern
PD-1	Programmed Death Receptor -1
PMA	phorbol 12-myristate 13-acetate
Poly (I:C)	Polyinosinic:polycytidylic acid
PRR	Pathogen recognition receptor
RBC	red blood cell
RNA	ribosenucleic acid
ROS	Reactive oxygen species
s.c.	subcutaneously
SEB	staphylococcus enterotoxin subunit B
STAT	Signal Transducer and Activator of Transcription
TCR	T-cell receptor
Teff	T effector cell
TLR	Toll-like receptor
Th1	T helper cells (Type 1)
Th2	T helper cells (Type 2)
Th17	T helper cells (Type 17)
Tr1	Regulatory T cells (Type I)
Treg	Regulatory T cell
WT	wildtype

1.5 List of Figures/Tables

1.6 Summary

Autoimmune diseases, unwanted overshooting immune responses against self antigens, are due to an imbalance in immunity and tolerance. Although negatively impacting cancer prognosis, myeloid derived suppressor cells (MDSC), with their potent suppressive capabilities, might be applicable in a more beneficial light when applied in to autoimmunity. As previous shown MDSC have protective roles in Experimental Autoimmune Encephalomyelitis (EAE) (Zhu et al., 2007), the established inducible mouse model for the autoimmune disease multiple sclerosis (MS). This decrease in disease severity indicates *in vitro* generated immature myeloid cells (IMC) from bone marrow (BM) as precursors of MDSC are promising candidates for cellular therapy. Important to any cellular therapy by adoptive transfer, the major questions regarding BM-IMC efficacy was addressed within the thesis. This thesis attempts to elucidate how BM-IMC operate in EAE. This thesis defines the factors within the autoimmune microenvironment that lead to the activation of BM-MDSC, where BM-IMC home once delivered *in vivo*, and the protective mechanisms BM-IMC employ.

To emulate BM cells when they first enter circulation through the blood, BM-IMC were injected intravenously (i.v.). BM-IMC are protective with no regard to the various routes delivered (i.v., i.p.). They protect to a lesser extent when pre-activated before injection. BM-IMC suppress by causing a delay and/or by decreasing the severity of the disease via a mechanism yet determined.

To understand the migration pattern of BM-IMC after i.v. injection, *in vivo* kinetics experiments employing bioluminescence imaging were performed. This techinique allows for whole *in vivo* mouse imaging daily, allowing the tracking of cell migration over days within a single mouse. During steady-state, BM-IMC circulate and appear to accumulate in the spleen by day 4 after injection, whereas they alternatively home to inflammatory sites (immunization site), draining lymph nodes, and the spleen within mice with low grade EAE. Visualization of CM-DiI-labelled BM-IMC by fluorescence microscopy could locate BM-IMC injected cells outside the white pulp, as they were co-localizing in the regions stained with CD169 or outside, but not within the follicles of spleens on day 4. Consistant with these findings, the attempt to analyze the phenotype of these cells by flow cytometry was problematic as these cells seem to adhere strongly to collagen also indicating the cells are located in the collagenous area of the marginal zone and the red pulp.

To determine factors influencing MDSC activation, we utilized different stimuli through a high throughput method detecting release of nitric oxide (NO). Extracts from yeast, fungi, and bacteria were observed to activate MDSC to produce nitric oxide. Surprisingly, material mimicking viral DNA (CpG) and RNA (poly I:C), and several self glycolipids, could not activate the MDSC to produce NO. Upon attempts to understand synergistic effects between microbial pathogens and host cytokines, IFN-g was determined to boost the signal of pathogen stimuli, whereas IL-17, another cytokine which causes pathology during EAE, and IFN-b, a drug used in therapy to treat MS, did not cause any additional effects. Activation of BM-MDSC was determined by the microbial pathogens components LPS, curdlan, and zymosan, to induce upregulation of B7-H1 on the cell surface. BM-MDSC did not increase any

co-stimulatory markers, such as CD40, CD80, CD86, CD70, or the co-inhibitory marker, PD-L2. On day 1 after EAE induction, endogenous MDSC populations when stimulated showed an increase in B7-H1 expression and a downregulation of CD80. After further analysis, these cells were concluded to be mostly granulocytic cells (Ly-6G^{+}). As the B7-H1 ligand PD-1 is upregulated in chronic diseases and correlates to an exhausted phenotype, the PD-1 : B7-H1 interaction was a good candidate for the mechanism our cells may employ for their suppressive capacity. To investigate this interaction, fixed BM-IMC deficient in B7-H1 were incubated with restimulated memory T cells. BM-IMC deficient in B7-H1 resulted in a significant loss of T cell suppression, as compared to the wildtype control BM-IMC. To assess this interaction *in vivo*, we injected wildtype (WT) and B7-H1$^{-/-}$ BM-IMC into mice followed by induction of EAE to assess whether B7-H1 mediated this suppression. The lack of B7-H1 did not alter their suppressive capacity under these conditions, contrary to other findings which have described this interaction to be important in their suppressive capacity when administered post EAE induction (Ioannou et al., 2012). Interestingly, EAE mice pre-treated with BM-IMC had similar amounts of cytokine production in the CNS after restimulation. Spleens from BM-IMC injected mice had increased amounts of Arg-1 suggesting suppression is via oxidation or recruitment by soluble mediators may lead to this protection. We speculate this may inhibit T cell reactivation in the CNS.

We conclude that MDSC, upon further testing in human, might be a potential candidate as a suppressive cellular therapy for MS. This thesis provides insight into the ability of MDSC to suppress the severity of EAE. The suppressive capacity of MDSC is not altered by the current therapy, IFN-b, therefore it may be possible to utilize in a combinational therapy. Our findings also demonstrate that if given as a preventive cellular therapy, the cells would home to the spleen and suppress the activated T cells through direct release of NO, but not B7-H1. Further roles of PD-1 in EAE protection may need to be elucidated.

1.7 Zusammenfassung

Autoimmunerkrankungen, unerwünschte, überschießende Immunantworten gegen Selbstantigene, resultieren aus einem Ungleichgewicht von Immunität und Toleranz. Obwohl sie einen negativen Einfluss auf Tumorerkrankungen haben, könnten Myeloide Suppressorzellen (MDSC) durch ihre potenten immunsuppressiven Eigenschaften, in einem besseren Licht bei Anwendung gegen Autoimmunerkrankungen erscheinen. Wie zuvor gezeigt, können MDSC eine protektive Rolle bei der Experimentellen Autoimmunenzephalomyelitis (EAE) entfalten, dem etablierten induzierbaren Mausmodel für die Autoimmunerkrankung Multiple Sklerose (MS). Die Verminderung der Erkrankungssymptome deutet darauf hin, dass *in vitro* aus Knochenmark (BM) generierte unreife myeloide Zellen (IMC) als Vorläufer von MDSC viel versprechende Kandidaten für eine Zelltherapie darstellen. Da für jede Art der Zelltherapie die Effektivität der transferierten Zellen eine entscheidende Rolle spielt, sollte in dieser Arbeit die Funktionalität von BM-IMC untersucht werden. Diese Dissertation erarbeitet wie BM-IMC bei der EAE funktionieren. Die Arbeit versucht die Faktoren innerhalb der Autoimmun-Mikroumgebung zu definieren, welche zur BM-MDSC Aktivierung führen, wohin applizierte BM-IMC *in vivo* wandern und welche protektiven Mechanismen BM-IMC anwenden.

Um nachzubilden, wie BM Zellen bei ihrem Eintritt in das Blut sich in der Zirkulation verhalten, wurden BM-IMC intravenös injiziert. Die injizierten gemischten BM-IMC verhielten sich protektiv, unabhängig von der Art der Injektion (i.v., i.p.). Sie sind jedoch weniger protektiv, wenn sie voraktiviert injiziert wurden. BM-IMC supprimieren auf eine Weise, dass sie eine Verzögerung und/oder Verminderung der Erkrankungssymptome bewirken, wobei die dafür zugrunde liegenden Mechanismen noch nicht definiert sind.

Um die Wanderungsmuster der BM-IMC nach i.v. Injektion zu verstehen, wurden *in vivo* Kinetikexperimente mittels der Biolumineszenz-Darstellung durchgeführt. Diese Technik erlaubt eine tägliche Betrachtung der gesamten lebenden Maus, so dass die Zell-Wanderungsmuster über Tage in derselben Maus aufgezeichnet werden können. Unter homöostatischen Bedingungen zirkulieren BM-IMC bis sie nach 4 Tagen in der Milz akkumulieren, wogegen sie alternativ zu Entzündungsherden wandern (Immunisierungssstelle), in Lymphknoten und Milz in Mäusen mit milden EAE Symptomen. Deren Lokalisierung konnte durch Fluoreszenzmikroskopie von CM-DiI-markierten BM-IMC in der roten Pulpa der Milz an Tag 4 lokalisiert werden. In Übereinstimmung mit diesem Befund, waren durchflusszytometrische Phänotyp-Analysen problematisch, da die Zellen fest an Kollagenfasern gebunden schienen, was als weiterer Hinweis auf ihre Kollagenbindung dienen kann.

Um Faktoren zur Aktivierung zu bestimmen, wurden verschiedene MDSC Stimuli benutzt und deren Freisetzung von Stickstoffmonoxid (NO) mittels einer Hochdurchsatzmethode bestimmt. Es konnte nachgewiesen werden, dass Extrakte aus Hefen, Pilzen und Bakterien MDSC aktivieren und zur NO Produktion führen. Überraschenderweise konnten DNS- (CpG) oder RNS-Bestandteile (Poly I:C) mit viralen Charakteristika oder verschiedenen Selbst-Glykolipide keine NO Freisetzung hervorrufen. Darüber hinaus konnte für das Zytokin IFN-g eine wichtige verstärkende

Rolle gezeigt werden, wobei ein anderes bei der EAE-Pathogenese beteiligte beteiligtes Zytokin, IL-17, und auch IFN-b, eine Substanz zur Therapie der MS, keinerlei Effekte zeigten. Untersuchungen nach MDSC-Aktivierung mit den mikrobiellen Komponenten LPS, Curdlan und Zymosan zeigten eine Hochregulation des B7-H1 Moleküls auf der Zelloberfläche. Andere kostimulatorische Marker, wie CD40, CD80, CD86, CD70 oder der inhibitorische Marker PD-L2 nahmen nicht zu. Einen Tag nach EAE-Induktion exprimierten auch die endogene MDSC Populationen nach Stimulation eine erhöhte B7-H1 und eine erniedrigte CD80 Expression. Nach weiterer Analyse konnten diese Zellen überwiegend als granulozytär (Ly-6G+) eingestuft werden. Da der B7-H1-Ligand PD-1 bei chronischen Erkrankungen hochreguliert wird, und mit einem verbrauchten Phänotyp korreliert, sollte die PD-1:B7-H1 Interaktion als guter Kandidat für den Suppressionsmechanismus untersucht werden. Fixierte B7-H1-defiziente BM-IMC wurden auf ihre Suppressorfunktion auf Gedächtnis-T-Zellen getestet. B7-H1-defiziente IMC zeigten eine signifikant niedrigere Suppression, im Vergleich zu Wildtyp BM-IMC. Um diese Interaktion *in vivo* zu untersuchen, wurden Wildtyp- oder B7-H1-defiziente BM-IMC in Mäuse injiziert und danach EAE induziert um auch hier eine B7-H1-vermittelte Suppression nachzuweisen. Die Abwesenheit von B7-H1 veränderte jedoch die suppressiven Eigenschaften unter diesen Bedingungen nicht, im Gegensatz zu anderen beschriebenen Befunden bei denen eine wichtige suppresive Rolle bei Injektion nach EAE-Induktion beschrieben wurde. Interessanterweise zeigten Mäuse, welche mit BM-IMC vorbehandelt wurden, eine vergleichbare Zytokinfreisetzung im ZNS nach Restimulation. Milzen zeigten nach BM-IMC Injektion auch erhöhte Mengen Arginase-1 könnte dies auf eine Suppression durch oxidative Mediatoren hindeuten. Man kann also annehmen, dass so eine Reaktivierung der T Zellen im ZNS verhindert wird.

Zusammenfassend stellen MDSC, nach weiteren Tests im Menschen, einen möglichen Kandidaten zur suppresiven Zelltherapie bei MS dar. Diese Arbeit erlaubt Einblicke in die Fähigkeit von MDSC die EAE zu unterdrücken. Die suppressive Fähigkeit von MDSC ist durch die gegenwärtige IFN-b Therapie unbeeinflusst, so dass eine kombinatorische Therapie möglich wäre. Die Ergebnisse zeigen auch, dass bei präventiver Gabe eine Migration in die Milz zu beobachten ist, wo Suppression der aktivierten T Zellen durch NO Freisetzung, jedoch nicht B7-H1, stattfindet. Um eine weitere Beteiligung von PD-1 bei der EAE abzuklären bedarf es zusätzlicher Analysen.

Translation performed by Dr. Manfred Lutz and Anja Döhler

2 INTRODUCTION

2.1 Immune Responses

Immunology is the study of the balance between tolerance and activation of immune cells in response to self and foreign antigens. As the human body consists of more than 90% cells of non-human origin (Turnbaugh et al., 2007) mostly consisting of commensal bacteria, understanding this balance is important. Physical barriers, such as the gastrointestinal and respiratory tracts and skin, and soluble mediators, like anti-microbial substances and defensins, provide primary defenses by keeping pathogens out of the body (Abbas AK, 2010). The immune system fights pathogens managing to surpassed the physical barriers (Abbas AK, 2010). The immune system can regulate self tissues in cases of uncontrollable growth (tumors and cancer) and mediates removal of damaged or dying tissues (Abbas AK, 2010). The immune system consists of both innate and adaptive immune cells, which generally play different roles in fighting pathogens, but work in concert through communication (Abbas AK, 2010). The function of the immune system in regulating tolerance and defending from pathogens is a constant process. Although there is much functional overlap within the immune system, dysfunction of immune cells shows major effects. An example of the problems resulting from disregulation of the immune system is the appearance of autoimmune diseases (Abbas AK, 2010). Autoimmune diseases, activated by various triggers both genetic and environmental in nature, are induced by an overshooting response of the immune system against self tissues. This generally leads to dehabilitatation of the individual. Autoimmunity will be discussed in greater detail later in the thesis.

2.1.1 Innate Immunity

The innate immune cells and soluble factors, i.e., complement, are the first responders (Abbas AK, 2010). Innate immunity consists of macrophages, dendritic cells (DC), monocytes, granulocytes and NK cells that recognize a limited number of conserved structures of repeating components of pathogens, called pattern associated molecular patterns (PAMP), by using pattern recognition receptors (PRR) upon their surface. PRR are specifically programmed within the cell's germline DNA (Abbas AK, 2010). PRR consist of many different classes of receptors, such as toll-like receptors (TLR), c-type lectins receptor (CLR), NOD-like receptors, and RIG-I like receptors, which together recognize different types of antigens and lead to various further immune responses (Abbas AK, 2010). Each PRR recognizes a specific PAMP, i.e., bacterial cell walls, leading to different subsequent signalling resulting in activation (Abbas AK, 2010). This signalling activates the innate immunity to upregulate other molecules or the release of proinflammatory cytokines to assist in furthering the immune response through activating and directing the adaptive immunity (Abbas AK, 2010).

Dendritic cell-associated C-type lectin-1 (Dectin-1), a CLR recognizing carbohydrate antigen, is expressed predominately on myeloid cells, but can also be expressed on other immune cells (Brown and Gordon, 2001). Dectin-1 functions by first recognizing beta-glucans (repeating glucose polysaccharides linked by glycosidic bonds) on fungal and yeast pathogens, such as curdlan and zymosan, respectively (Brown and Gordon, 2001), or other undetermined molecular patterns on other

pathogens like mycobacteria (Rothfuchs et al., 2007; Yadav and Schorey, 2006). Once interacting with the beta-glucan, Dectin-1 signals through Syk-CARD9 for Th17 responses (LeibundGut-Landmann et al., 2007). Curdlan signalling with GM-CSF leads to synergic signals in DC, thereby inducing increased costimulatory molecules and more cytokine production, including IL-6 and IL-12p70 (Min et al., 2012). Other glycan receptors, like galactin-1, have been reported to support more tolerogenic signals into DC (Ilarregui et al., 2009).

Possibly the most well known family of PRR are the TLRs (Abbas AK, 2010). These receptors recognize specific PAMPs from a variety of sources, including bacterial, i.e., gram negative cell walls, such as lipopolysachharide (LPS), viral, i.e., viruses encoded by double stranded RNA (dsRNA), also mimicked by poly (I:C), or double straded DNA (dsDNA), mimicked by cysteine and guanine rich DNA regions (CpG), in nature (Abbas AK, 2010). Of these discovered 12 TLR, the majority signal via a common adaptor protein myeloid differentiation factor-88 (MyD88). Exceptions to MyD88 signalling by signalling alternatively through the TRIF dependent pathway include TLR3 and TLR4 (Akira et al., 2001). TLR are typically located where the corresponding type of pathogen they detect are mostly located. Bacterial components, e.g. LPS, signal through the extracellular TLR 2/4, whereas the intracellular pathogens, e.g. viruses, trigger through TLR 3, TLR 7, TLR 8, TLR 9, which are found in endosomes (Abbas AK, 2010). Signalling through TLR can further activate the cell to regulate other receptors, costimulatory/coinhibitory molecules, or cytokines, which then control effector functions of the adaptive immunity (Abbas AK, 2010).

The innate immunty is important in several ways: Phagocytosing, otherwise known as the engulfment of particulate antigens (e.g. microbes), and the clearing dead tissue and debris through "eat-me" receptors and the sampling the soluble environment through uptake of liquid antigens, termed pinocytosis (Abbas AK, 2010). Ingestion of pathogens or damaged cell tissues are digested in the lysosomes into small proteins and subsequently loaded as peptides into major histocompatability complexes (MHC) (Abbas AK, 2010). MHC, classified into class I and II, are based on the length of the peptide which fits into its groove and the location, i.e., extracellular vs intracellular (Abbas AK, 2010) of the pathogen supplying these peptides. The presenting of the peptide through MHC molecules to the scanning T cells is termed antigen presentation (Abbas AK, 2010). The recognition of the peptide-MHC complex occurs by the T cell receptor (TCR) of the T cell (Abbas AK, 2010). The MHC I molecule presents smaller peptides, 9-mers, usually of intracellular origin, which are recognized by CD8 cells, while the MHC II presents 15-mer peptides usually of extracellular origin to CD4 T cells (Abbas AK, 2010). As T cells recognize proteins, this immune response generated is peptide-based, but innate cells are not limited to this source of antigen and can also load glycolipids upon another presenting molecules, called CD1d, for presentation to NKT cells (Abbas AK, 2010). The presentation of antigen delivers signals into the adaptive immune cell activating it (Abbas AK, 2010).

2.1.2 Adaptive Immunity

Adaptive responses work in concert with the innate immune responses, but have a different mode of response. As its name implies, the process is adaptive and slower to mount, but it does support memory responses (Abbas AK, 2010). Innate immunity primes the adaptive immune responses and directs the effector actions of the adaptive immunity. The two major players in adaptive immunity are of the lymphocyte family:

T lymphocytes (T cells) and B lymphocytes (B cells) (Abbas AK, 2010). The T and B cells are considered adaptive because their T cell receptor or B cell receptor, respectively, changes during the course of infection to "adapt" through selection for cells that have better ability to recognize specific pathogens (Abbas AK, 2010).

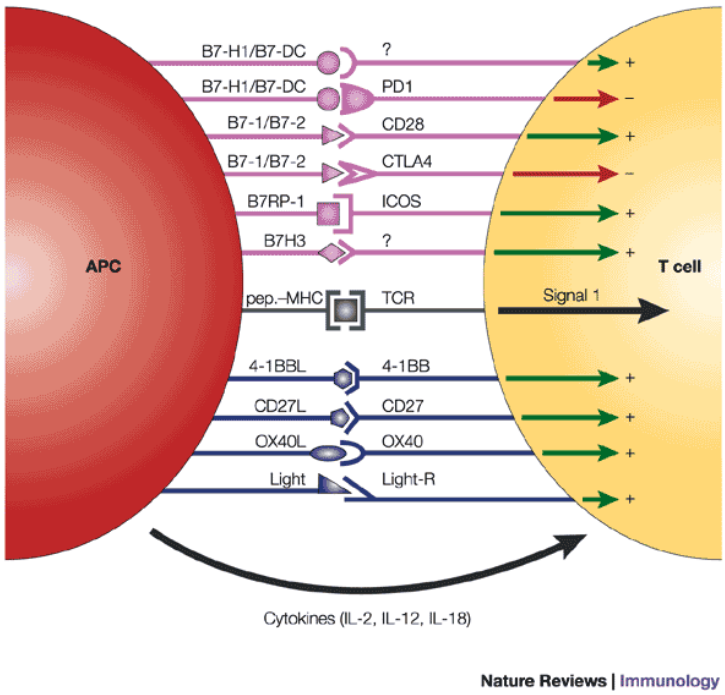

Figure I.1 Depiction of 3-step APC-T cell interaction for T cell activation. Signal 1: the MHC-peptide-TCR (grey, center) interaction gives the first positive signal into the T cell. Signal 2: possible co-stimulation or co-inhibitory signals (pink, top), gives second activation signal into T cell. Signal 3: The release of cytokines by the APC direct T cell polarization. Figure from (Pardoll, 2002).

2.1.3 Immune Activation

Dendritic cells (DC) recognize and process antigen from the tissue through phagocytosis (Abbas AK, 2010). After migrating to the nearest draining lymphoid organ, this antigen is processed and presented. Once the DC reaches the lymphoid organ, it moves into the white pulp, where it interacts with T cells (Abbas AK, 2010). Normally, immune cells are constantly scanning one another within the lymphoid organs. This close contact and constant short cell-cell interaction provides a tonic TCR signal, otherwise known as survival signals (Romer et al., 2011). Some suggest the length the time of this interaction decides which type of response is mounted (Corse et al., 2011).

For activation of a specific subset of T cells, CD4 or T-helper cells, a specific series of events must occur. Naive T cells migrate to secondary lymphoid organs where the association with required interaction partners is performed in a step-wise fashion (Abbas AK, 2010) (described in Figure I.1). The first signal is through MHC presentation of peptide antigens to the TCR of T cells (Abbas AK, 2010; Pardoll, 2002). The second signal is delivered upon the co-stimulatory signal of the B7 molecule (CD80 / CD86) interaction with CD28 (Abbas AK, 2010; Pardoll, 2002). Finally, the third signal given to the T cell is from cytokines released by the DC. These cytokines direct the T cell specific polarization (Abbas AK, 2010; Pardoll, 2002) resulting in different effector phenotypes. CD4 polarized cells have the

following classifications: Th1 cells which release IFN-g during times of inflammation and viral infection (Abbas AK, 2010); Th2 cells, which release IL4, IL-5, IL-13 during parasitic infection and allergy (Abbas AK, 2010; Nurieva and Chung, 2010); Th17 cells which release IL-17, IL-21, IL-23 in times of wound healing and autoimmunity (Abbas AK, 2010; Nurieva and Chung, 2010); T regulatory cells (Tregs) which suppress in numerous ways, e.g. releasing TGF-b and IL-10 while involved in controlling immune responses (Abbas AK, 2010); and lastly, a newer defined population of T follicular cells involved in B cell maturation and autoimmunity (Nurieva and Chung, 2010). Recently, it has been suggested the polarizations into different T-helper subsets may be induced by the strength and time of interaction of between the MHC and the TCR (Corse et al., 2011), caused by the maturation status of the DC (Pletinckx et al., 2011).

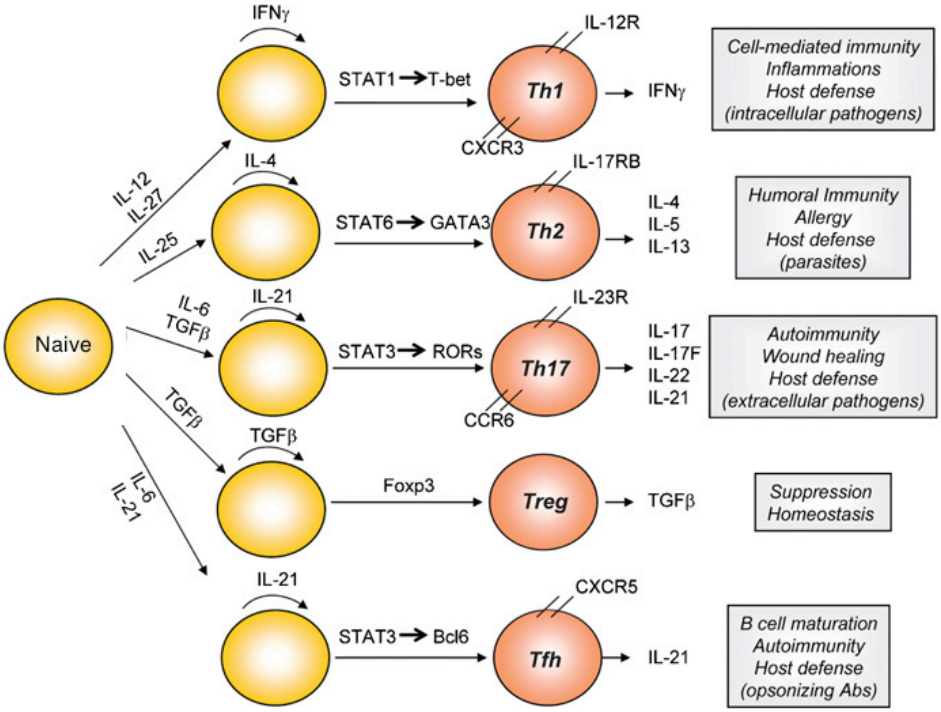

Figure I.2 Polarization in CD4 T cells. Naive T cells when activated are given signals by polarizing cytokines activating different transcription factors, which shape their "polarization" into different effector T helper cells. Figure modified from (Nurieva and Chung, 2010).

Once they encounter a pathogen presented by the innate immune response, specific T cells will selectively proliferate (Abbas AK, 2010). Due to different cytokine signals given to the T cell during priming, the T cell will polarize in a different manner, releasing different effector cytokines which best combat the type of protein antigen recognized (Abbas AK, 2010). These T cells go through an expansion phase where increased amounts of effector T cells are generated to combat the infection. A small portion of these cells will then futher differentiate into memory T cells, which can be recalled upon subsequent exposure to the same pathogen (Abbas AK, 2010).

2.1.4 Spleen: Structure and Function

The spleen functions as the largest filter of blood in the body (Kraal, 1992), acts as reservoir of monocytes (Swirski et al., 2009), and plays a vital role in systemic

immune responses as it functions as a secondary lymphoid organ. The spleen is thought to play a essential role in the immune reaction against blood-borne pathogens. Due to its multi-functionality, organization of this organ is crucial. The spleen is organized into stroma, the structural portion, and parenchyma, the cellular portion. The stroma consists of mostly reticular fibroblast cells which comprise the fibrous capsule of connective tissue surrounding the entire spleen and other splenic connective tissue, such as vessels. The parenchyma is made up of the leukocyte population, consisting of T cells, B cells, macrophages, monocytes and other immune cells (Mebius and Kraal, 2005).

The parenchyma of the spleen consists of two major zones: the red pulp and the white pulp (Mebius and Kraal, 2005). The border between the red and white pulps is considered the marginal zone where macrophages normally reside (Mebius and Kraal, 2005). The blood enters the spleen through the chords of the red pulp, which must pass through the major filter of the spleen, by forcing the cells through slits formed by stress fibers and parallel endothelial cells to enter the sinuses (Mebius and Kraal, 2005). This is the primary site of turnover of dead cells, breakdown of cellular debris, and RBC degradation (Mebius and Kraal, 2005). Tyrosine nitration has been shown to interfere with phosphorylation and targets the protein for degradation (Gow et al., 1996). To enter the white pulp, cells are required to undergo an active migration involving G-protein coupled receptors. This G-protein coupled receptor migration into the white pulp can be inhibited by the administration of pertussis toxin (Cinalli et al., 2005), inducing an accumulation of T and B cells within the red pulp (Cyster and Goodnow, 1995). Upon maturation DC through CCR7 undergo an active migration to enter the white pulp. The white pulp, where the adaptive immune responses occur, is further broken down into the T cell and B cell areas. The interaction between DC and T cells occur in the T cell zone, where T cells become activated and move to the border of the T-B cell zone, where they can further interact with B cells (Abbas AK, 2010). The red pulp consists of mostly of plasmablasts, monocytes, and macrophages and is the main site for RBC degradation and iron recycling (Mebius and Kraal, 2005). It also has been reported that the red pulp is where memory CD8 cells may home (Bajenoff et al., 2010). A reservoir of monocytes, located in the cords of the subcapsular red pulp, is released upon stress signals to injured tissue to initiate wound healing (Swirski et al., 2009). During overexpression of TNF-a or chronic inflammation, the spleen structure changes dramatically, remodeling its organization and expanding to allow more infiltrating cells, and developing the formation of more germinal centers (Abbas AK, 2010).

2.1.5 Adaptive Immune Development: Tolerance

The thymus and the BM are considered the primary lymphoid organs and the sites of development of the T cell and B cells, respectively (Abbas AK, 2010). The thymus is where the development of T cells pass through several check points before the cells can exit into the circulation (Abbas AK, 2010). This process, termed central tolerance, is divided into positive and negative selection; both lead to the T cell's ability to act against foreign pathogens and not against self (Abbas AK, 2010). Immature T cells expressing both CD4 and CD8 enter the thymus and undergo positive selection (Abbas AK, 2010). Positive selection occurs by the selection within the thymus which selects for the cells which bind to the self major histocompatibility complex (MHC) molecule with low avidity, therefore preventing harm to the host organism (Abbas AK, 2010). This selection ensures only single positive CD4 or CD8

expression on the T cells, restricting them to interact with only MHC I or MHC II peptide presentation (Abbas AK, 2010). During this step, the cells expressing high avidity will be eliminated (clonal deletion) by apoptosis or they will have their receptor altered (receptor editing) (Abbas AK, 2010). Those which lack the interaction with the TCR die due to lack of signals (neglect), whereas the selected ones with the right avidity to self MHC receive positive survival signals to continue into the medullary chords, where they enter negative selection (Abbas AK, 2010). Located within the medullary chords of the thymus are cells with the particular ability to express self molecules found in every tissue which play a role in the selection process (Abbas AK, 2010). Selection of T cells with the inability to bind these self antigens with high avidity is termed negative selection (Abbas AK, 2010). Unfortunately, this process of selection is not 100%, as some cells can escape and become autoreactive. These self-reactive T cells can be pathogenic, leading to autoimmune diseases. Once the T cells exit the thymus, they still have mechanisms of peripheral tolerance, which keep them in check (Abbas AK, 2010). Peripheral tolerance mostly is based on the APC-T cell interaction. This interaction is a very tightly controlled process which regulates the immune response against foreign antigens and protection of self (Abbas AK, 2010). Peripheral tolerance mechanisms include anergy, deletion, and suppression (Abbas AK, 2010).

2.1.6 Tolerance by Anergy

When the immune interaction between T cells and DC occur, but lack the second signal, the cells become non-functional or anergic (Abbas AK, 2010). Another way to induce anergy is through co-inhibitory signals, such as the B7-homologue-1 (B7-H1) interacting with programmed death receptor-1 (PD-1) or with cytotoxic T-lymphocyte antigen-4 (CTLA-4) on T cells (Abbas AK, 2010). These inhibitory factors deliver signals into the T cell through the immunoreceptor tyrosine-based inhibition motif (ITIM) rather than the immunoreceptor tyrosine-based activation motif (ITAM). Therefore, instead of becoming activated, the cells fall into an anergic state (Abbas AK, 2010).

2.1.7 Programmed Death Receptor-1 and B7-family member homologue-1

Freeman et al. (Freeman et al., 2006) have termed T cells with the loss of their effector function during chronic infections, such as HIV and LCMV, “exhausted.” The phenotype of “exhausted” cells is that they display an increase in programmed death receptor-1 (PD-1) expression, as well as have decreased effector functions, such as cytokine release, cytotoxic lysis, and proliferative capacity (Figure I.3) (Freeman et al., 2006). These cells during the course of infection are deleted as PD-1 makes them more susceptible to apoptosis mediated death (Bhadra et al., 2012). PD-1 has been reported to play a role not only in the peripheral tolerance, but also has a role in positive selection during central tolerance (Nishimura et al., 2000).

PD-1 expression was first discovered on dying cells, but unlike its name, it doesn’t trigger cell death, but rather anergy by triggering inhibitory signals into the cell (Ishida et al., 1992; Tsushima et al., 2007). After triggering PD-1, the cells increase motility (Fife et al., 2009), maybe explaining this anergy, by decreasing the opportunity for immune cell-cell interactions. Interestingly, PD-1 is found on resting Tregs (Raimondi et al., 2006). The interaction of PD-1 with its ligand B7-H1, also known as programmed death receptor ligand-1 (PD-L1), increases Tregs, but the mechanisms to do so are not clear. Immature DC have been described to have higher

expression of PD-1 compared to mature DC (Kuipers et al., 2006). Also, expression of its ligand, B7-H1, on DC is regulated by factors regulating maturation (Liu et al., 2007) through IRF-1, Erk, p38 signalling (Karakhanova et al., 2010; Lee et al., 2006). Further studies have shown PD-1 expression on DC regulate IL-6, MIP-a, IL-12, and TNF-a production (Pena-Cruz et al., 2010; Yao et al., 2009).

Ligands for PD-1 are B7-H1, also referred to as PD-L1, and B7-DC, also known as PD-L2. These ligands are differentially expressed on different cell types and at different times. B7-H1 is constitutively expressed in immune restricted organs, such as the CNS, eyes, and placenta (Hori et al., 2006; Petroff et al., 2002). The expression of B7-H1 is not restricted to these cell types, as B7-H1 can also be expressed on other non-hematopoetic cells and most immune cells, including T cells, B cells, monocytes, and DC (Freeman et al., 2000; Yamazaki et al., 2002). The other ligand, PD-L2, expressed mostly on myeloid cells and a few other hematopoetic cells, is highly expressed by cells within the thymus, whereas B7-H1 expression is mainly localized to the spleen (Liang et al., 2003). In various cell types, B7-H1 has been described to be upregulated upon IFN-g stimulation (Lee et al., 2005; Liu et al., 2007). B7-H1 can also interact with other B7 family members, such as CD80 (Butte et al., 2007). This interaction overlaps the binding of CD80 with PD-1 and CTLA-4, which still results in inhibitory signals into both cells (Butte et al., 2007).

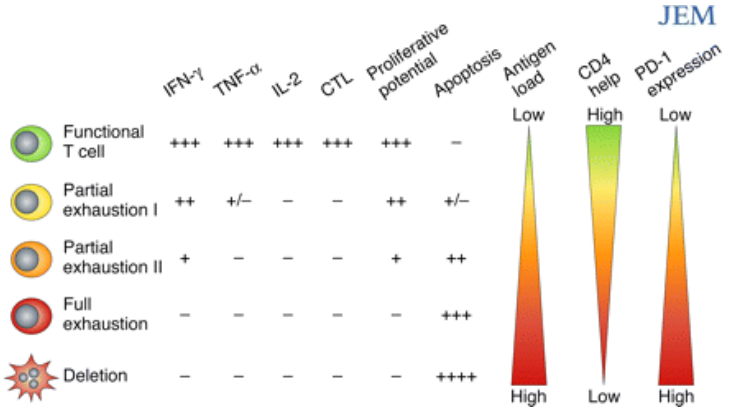

Figure I.3 The exhausted phenotype of T cells includes an increase of PD-1 expression and a decrease of proliferation and proinflammatory cytokines. Figure from (Freeman et al., 2006).

During experimental autoimmune encephalomyelitis (EAE), a mouse model for the study of the autoimmune disease multiple sclerosis (MS) discussed in more detail later in this thesis, B7-H1 and PD-1 expression increases in the CNS increasing with time correlating with disease score, with the peak between weeks 2 and 3 (Salama et al., 2003). Mice lacking B7-H1 and PD-1 genes present with more severe symptoms (Ortler et al., 2008). Experiments performed with blocking B7-H1 and PD-1 show similar importance of these genes in protecting against EAE (Salama et al., 2003) (Zhu et al., 2006). Interestingly, it is claimed that during EAE, B7-H1 can selectively suppress Th1 responses better than Th17 responses, *in vivo* (Schreiner et al., 2008).

2.1.8 Suppression as a Mode of Tolerance

Suppression is another important tolerance mechanism (Abbas AK, 2010). Various mechanisms to inhibit the immune response through the use of cytokines and the

deprevation of required amino acids are employed by different cell types (Abbas AK, 2010). One mechanism of suppression is via the famous Tregs. Although immune suppression is not a new topic, as originally proposed in 1970s (Gershon, 1975), Tregs, once questionable to the scientific community due to the inability to clearly define their exact function, were only recognized recently by the research community as important components of the immune system. Phenotypically, they are characterized by their expression of CD4, interleukin-2 receptor-alpha (CD25), and forkhead-winged-helix transcription factor (Foxp3) (Sakaguchi et al., 2010). There is a long list of various ways in which these cells can regulate the immune system (Sakaguchi et al., 2010), most commonly reported is their ability to release TGF-b. Their important role in regulating immune responses, even during steady-state, is seen in scurfy mice. Scurfy mice, mice containing a mutation in the FoxP3 gene (Bennett et al., 2001), lack regulation by self-reactive T cells and as a consequence have severe autoimmune diseases. In humans, this mutation causes a condition termed IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), leading to, as the name implies, very severe autoimmune diseases (Sakaguchi et al., 2010). Another suppressive cell type is the type 1 T regulatory (Tr1) cells which are most notably the producers of IL-10 (Roncarolo et al., 2006).

Myeloid cell-based suppression has been described in various cases by triggering of various receptors, such as the scavenger receptors, glutocorticoid receptor, and cytokine receptors, as well as storage of low-oxidized lipids (Abbas AK, 2010).

2.2 Myeloid Derived Suppressor Cells

First gaining attention in the fields of tumors and cancer as a medical obstacle to overcome, Myeloid Derived Suppressor cells (MDSC) are now recognized as playing roles in other fields, such as transplant immunology (Highfill et al., 2010) and our current focus, autoimmunity (Yin et al., 2010). MDSC, particularly noted for their potent suppressive activity, have been reported in some cases to even cause complete suppression of responding T cells at only comprising 3% of the cultured cells (Mazzoni et al., 2002). MDSC are part of innate immunity. They are comprised of a heterogenous population with the only determining characteristics being their suppressive function and phenotypic expression of CD11b and Gr-1 (mouse) (Bronte et al., 1998). There are no clearly defined populations, although several general myeloid markers found in human MDSC. In the mouse, the Gr-1-containing cells can be further differentiated by the expression of the glycoproteins lymphocyte antigens-6, Ly-6G and Ly-6C (Greifenberg et al., 2009; Ribechini et al., 2009). The Ly-6 family has been described as phosphatidylinositol anchored cell surface glycoproteins. The triggering of Ly-6 molecules results in various effects, including T cell activation (Ortega, 1986)(Malek, 1986) and migration (Hanninen et al., 2011). Interestingly, the expression of Ly-6C and Ly-6G are not limited to just MDSC. Ly-6C expression was observed upon others cell types including, but is not limited to, memory T cells, whereas Ly-6G is found, among others cell types, also on neutrophils. $CD11b^{+}$/Ly-$6C^{low}$Ly-$6G^{+}$ subset represents the PMN-MDSC, whereas the $CD11b^{+}$/Ly-$6C^{high}$Ly-$6G^{-}$ subset is the mono-MDSC (Movahedi et al., 2008). These two subsets also can be differentiated by the nuclei. PMN-MDSC exhibit a phenotypic ring nucleus while the monocytic fraction has a round nucleus consistant with cells of the monocytic lineage. MDSC residing in the spleen can be identified by their surface markers during steady-state (Ribechini et al., 2010), but only those isolated from mice containing tumors or inflammation are suppressive (Gabrilovich, 2012). This lack of

suppression is thought to be caused by dilution of MDSC by other cells which are not suppressive, such as eosinophils, neutrophils, plasmacytoid DC and others, but expressing the same markers (Greifenberg et al., 2009). Alternatively, it is hypothesized that steady-state MDSC lack stimulating signals required for their activation, therefore the diversion away from steady-state differentation into DC, macrophages, and granulocytes does not occur (Gabrilovich and Nagaraj, 2009).

The generation of myeloid cells occurs in the BM. Defined from hematopoetic stem cells, the myeloid cell lineage differentiates through a common myeloid precursor, where it then via appropriate signals can differentiate into common myeloid progenitor cells (Gabrilovich and Nagaraj, 2009). These common myeloid progenitor cells differentiate into monocytes and/or IMC and can be further programmed into various cell types, such as DC, macrophages, and granulocytes, during steady-state, or under pathological conditions into MDSC (Gabrilovich and Nagaraj, 2009). MDSC have reportedly require growth factors or cytokines i.e., GM-CSF (Dolcetti et al., 2010), G-CSF, M-CSF, stem-cell factor (SCF), VEGF, IL-6, and IL-3, to regulate the differentiation of IMC and MDSC (Gabrilovich et al., 2012).

2.2.1 Transcription factors relating to MDSC

In tumors, various transcription factors are described to play roles in the generation or activation of MDSC (Gabrilovich, 2012). Involved in general myeloid cell lineage development and therefore also involved in MDSC development and activation are several transcription factors, including CAAT/enhancer binding protein-beta (C/EBP-b) (Auffray et al., 2009), PU.1 (Auffray et al., 2009), and signal transducer and activator of transcription (STAT) 1,3,6 (Greifenberg et al., 2009). C/EBP-b has been reported to promote the generation of activated MDSC *in vitro* (Marigo et al., 2010) and mice deficient for C/EBP-b cells cannot differentiate into MDSC (Condamine and Gabrilovich, 2011; Hirai et al., 2006; Zhang et al., 2010). It has not been clearly shown if C/EBP-b is required for differentiation or for activation.

2.2.2 Myeloid derived suppressor cell activation

IMC are known in different conditions to be activated by different factors (Gabrilovich, 2012). Previously described factors which lead to the activation of MDSC are proinflammatory cytokines, IFN-g, and bacterial factors or inflammatory factors, such as the TLRs and IL-1b. A combination of IFN-g and LPS leads to activation of MDSC. This activation leads to nitric oxide production in supernatant and suppressive capacity when tested in a MLR (Greifenberg et al., 2009). Pathogenic factors activate many different Jak-STAT pathways within the MDSC, which in turn activates nuclear factor kappa beta (NF-kB) leading to many effector functions, including NO (Gabrilovich, 2012). Since the definition of this population is based on functionality, the specific factors can activate each subset differently, leading to different overall effector functions.

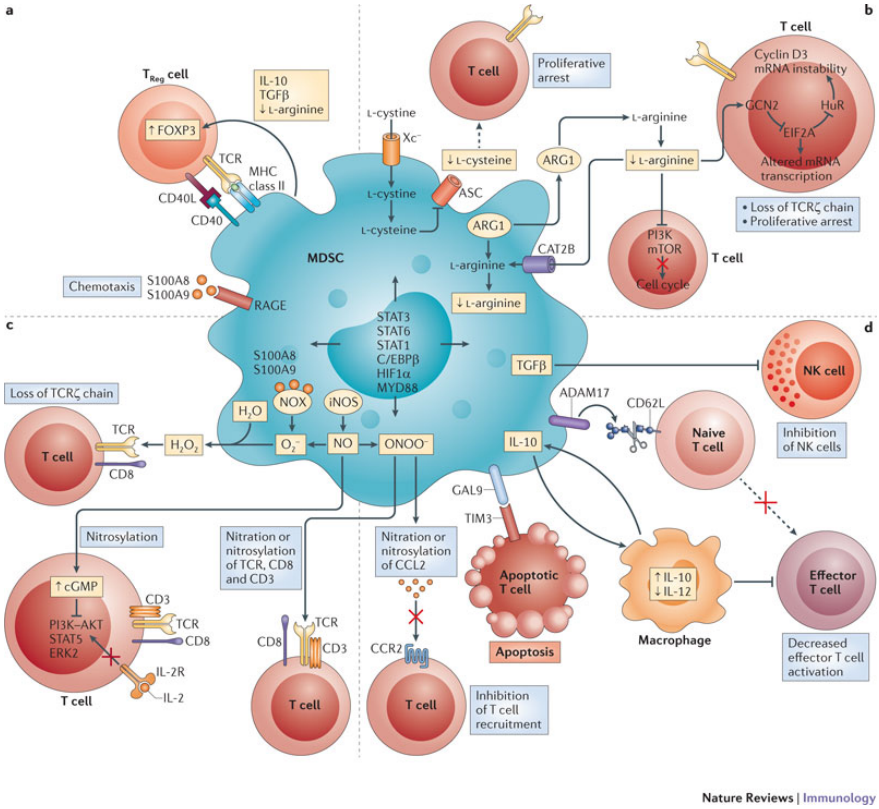

Figure I.4 Suppressive mechanisms of MDSC. A | induction of T regs, **B** | deprevation of amino acids, **C** | blocking T cell activation **D** | affecting migration through nitrosylation or cleavage of chemokines, direct "kill" signals, or release of anti-inflammatory cytokines. Figure from (Gabrilovich et al., 2012)

2.2.3 Suppressive Capacity of MDSC

The suppressive abilities of MDSC can be cell-cell contact based and/or soluble mediated. The mechanisms employed by the MDSC can be the consumption of amino acids, the induction of T regulatory cells, or the release of short-lived soluble mediators, such as urea, NO, and other reactive oxygen species, which can denature the TCR (Gabrilovich and Nagaraj, 2009). Mono-MDSC primarily are the main producers of NO through inducible nitric oxide synthase (iNOS), whereas the PMN-MDSC have been reported to use mostly arginase-1 (Arg-1) (Movahedi et al., 2008). MDSC subsets have been reported to produce the enzymes, Arg-1 and/or iNOS. Both of which are mediators of L-arginine metabolism, therefore both can reduce the local quantity of L-arginine (Rodriguez et al., 2002). In the case of Arg-1, its released from MDSC (Munder et al., 2006) suppresses is not only via decreasing the local L-arginine, but also by increasing the concentrations of the metabolized L-arginine endproduct, urea. Urea inhibits the re-expression of CD3-zeta chain in activated T cells after internalization, thus preventing activation signals (Rodriguez et al., 2002) (Figure I.4). iNOS expression in MDSC leads to the production of NO, a potent molecule controlling many functions. In our *in vitro* based suppression assays, suppression by NO accounts for a considerable amount of the suppression. In the case of T cells, NO nitrosylates tyrosine residues preventing activation signals into the T cell (Gabrilovich et al., 2012). Molon et al. have also shown the ability of MDSC to nitrosylate chemokines (Molon et al., 2011). The combination of NO and Arg-1 causes production of further modes of suppression, such as the creation of reactive oxygen species (ROS) (Gabrilovich et al., 2012). Interestingly, Indoleamine 2,3-

dioxygenase, an enzyme employed by T regs as a suppressive mechanism by depletion of the available tryptaphan, and NO inhibit each other (Hucke et al., 2004).

MDSC suppress DC in many ways. One mode of suppression occurs during the differentiation process by redirecting differentiation of DC-progenitors into MDSC, rather than the steady-state process, which normally would yield DC (Ribechini et al., 2010). Another way MDSC can suppress DC is through the release of NO, which inhibits MHC II expression on the DC, thereby removing their ability to present antigen (Harari and Liao, 2004). NO from MDSC have been observed to inhibit T cell activation by preventing JAK-3 and STAT-5 activation, thereby inducing apoptosis in T cells (Gabrilovich and Nagaraj, 2009). MDSC have other mechanisms by which it can suppress various cell types, but whether this suppression is antigen-specific has remained controversial. Reports have shown that MDSC require antigen-specificity for their suppression (Solito et al., 2011). Others state antigen specificity increases the suppressive capacity in a MHC II-dependent manner (Nagaraj et al., 2012). Contrary to these findings, others have described MDSC during hypoxic conditions can suppress non-specifically (Corzo et al., 2010). Other cell-cell contact mediated suppression has been observed. B7-H1 expression on MDSC can interact with PD-1 on Tregs to induce a Treg-mediated suppression during ovarian cancer (Liu et al., 2008).

2.2.4 Migration of MDSC

Auffray et al. have described two major monocyte populations differentiated by their expression of the cytokine receptor CCR2 and CXCR1 (Auffray et al., 2009). CCR2 expressing monocytes, namely classical monocytes, have been reported for their inflammatory migration, whereas $CX3CR1^{hi}$ monocytes, termed non-classical monocytes, have reported mostly as the precursor cells for resident myeloid cells (Auffray et al., 2009). These two cell types have been observed to have differing expression of Ly-6C/Ly-6G (Gr-1) (Geissmann et al., 2003). The CCR2 population has Gr-1 expression, whereas the CX3CR1 population does not. Due to inflammatory signals during pathological times, monocytes are released from the BM using the CCR2 (Serbina and Pamer, 2006); these monocytes can also use this CCR2 to migrate into spleen and inflamed tissue (Geissmann et al., 2003).

2.3 Autoimmunity

Autoimmunity is the failure of self-tolerance, leading to reactivity against self antigens (Abbas AK, 2010). Inflammation may be a prerequisite of consequence of autoimmunity. The immune system has two ways to regulate itself: increasing pro-inflammatory responses in the case of battling infection and increasing anti-inflammatory responses, in the case of wound healing. These pro- and anti-inflammatory responses are controlled by particular cytokines. Central and peripheral tolerance evolved as a "checks and balances" way to keep the immune systems controlled (Abbas AK, 2010). Cancer and autoimmunity are two ways in which the immune system can go awry. Cancer usually results in immune suppression, whereas autoimmunity results in immune hyperreactivity. Autoinflammation is driven by the innate immunity resulting in inflammation, whereas autoimmunity is caused by the adaptive immune system, primarily in auto-reactive T and B cells. Normally, the immune system can determine the difference between self and non-self antigens; therefore it only attacks those pathogen invaders, like bacteria and viruses, which find their way into the host. During autoimmune diseases there is an attack of normal host

tissue causing massive inflammation. This occurs primarily via adaptive immune responses such as auto-antibodies secreted from B cells or auto-reactive T cells (Abbas AK, 2010).

Autoimmunity is usually accompanied by inflammation. At the site of inflammation, the innate immune system releases reactive oxygen species, which may lead to the alteration of cell proteins. Those altered glycoproteins and lipids on the cell surface then can be interpreted as foreign by the immune system (Moody and Besra, 2001).

2.3.1 Multiple Sclerosis: The Disease

Multiple sclerosis (MS) is a dehabilitating autoimmune disease characterized by the demyelination of nerves and axonal degradation caused by inflammatory conditions within the CNS (Glass et al., 2010). This disease is devastating, largely because of the damage which occurs to the axons influencing motor function. The cause of the disease is unknown, but studies have found that other diseases where viral lesions in the brain cause inflammation and infiltration of immune cells often result in a similar encephalopathy. MS is a worldwide concern affecting at least 1 in 1000 people (Fugger et al., 2009). There are four different classical MS types – relapsing remitting, primary progressive, secondary progressive, and progressive relapsing – but these are just broad categories as each person develops MS quite differently (Loma and Heyman, 2011). The most common form is the relapsing remitting disease, characterized by its intermittent episodes (Loma and Heyman, 2011). Although women are more prone to develop MS (Fugger et al., 2009) due to its possible link with estrogen, both genetic and environmental factors have been suggested in initiating the disease (Ercolini and Miller, 2006). Currently, MS is a tragic disease; while there are many therapies attempting to reduce the severity of the disease, there is no known cure to date.

2.3.2 Animal model for MS: Experimental Autoimmune Encephalomyelitis

Experimental Autoimmune Encephalomyelitis (EAE) is the commonly used mouse model for MS (Raine, 1984). EAE most closely emulates the first episode of the relapsing-remitting model of MS. EAE can be induced by a combination of an emulsion of myelin protein and oil-containing mycobacteria injected subcutaneously, providing a depot for slow release of self antigen, and i.p. injection of pertussis toxin, which among many functions opens the blood brain barrier (Hofstetter et al., 2002; Linthicum et al., 1982), induction of Th17 cytokines in the CNS (Zhao et al., 2008), and stops G-protein mediated migration, mimicks the first wave of the relapsing remitting disease in humans. Although the mechanisms which cause this immune attack on the myelin sheath have been studied in depth, there is still much left to be elucidated. All models of EAE are driven by auto-reactive CD4+ T cells against myelin proteins, most commonly used are myelin basic protein (MBP) and proteolipid (PLP), and myelin oligodendrocyte glycoprotein (MOG). The importance of CD4 T cells within this model has been shown by the ability to induce EAE via the adoptive transfer of activated MOG-specific CD4 T cells, termed “passive” EAE (Stinissen et al., 1998). Although EAE is considered to be a CD4 driven model, the effects of other cells playing a contributing role in EAE induction should not be discounted. Various reports have shown the role of CD8 T cells during EAE (Ford and Evavold, 2005; Sun et al., 2001; Sun et al., 2003), whereas others have shown the importance of myeloid cells and B cells. The emulsion of peptide and complete Freund’s adjuvant (CFA) causes a relatively strong activation of cells in the local area,

activating the innate immune response. CFA is the most commonly used way to induce EAE and other autoimmune diseases, as it serves as a depot, prolonging the lifetime of the injected antigen and the mtb component induces the expansion of Mac+ IMC (Billiau and Matthys, 2001). Other adjuvants besides mtb, the major antigen component of CFA, have been shown to induce EAE; these include, CpG, LPS, Poly(I:C) and zymosan, which are TLR agonists (Mills, 2011). Interestingly, this TLR signalling is not required in the specific autoimmune-targeted organ for the induction of the disease (Hansen et al., 2006). This local activation of DC, macrophages, and resident microglia activate the T cells, but to date, only a few reports of adoptive transfer of DC induced EAE (Weir et al., 2002). One claim has shown the importance of the administration route, as only DC injected s.c., but not i.v. or i.p. could induce disease (Aghdami et al., 2008). It is important to note that induction of other autoimmune disease models by DC has been shown. DC are not only important for the activation of the autoreactive T cells, but help maintain local immune responses, perpetuating the disease (Ludewig et al., 1998)

2.3.3 Multiple Sclerosis: Therapeutic FDA-Approved Drugs

Currently offered for treatment of MS is the IFN-beta-1b therapy, with several drugs on the market including Avonex, Betaseron, Extavia, and Rebif (Loma and Heyman, 2011). IFN-beta, a type I interferon, is a cytokine normally released in response to viral infections to suppress the immune response. IFN-beta has been shown to block IFN-g mediated disintegration of endothelial tight junctions (Minagar et al., 2003) and *in vitro* to enhance BBB integrity (Kraus et al., 2004). When given IFN-b, patients with relapsing-remitting MS reduced severity and the duration of episodes by reducing the size, amount, and duration of lesions as observed by MRI (Gupta et al., 2005). Frequently, MS patients use combination therapy with the few other drugs on the market, with different active ingredients, such as glatiramer acetate (Copaxone), a fingolimod (Gilenya), and mitoxantrone (Novantrone) (Loma and Heyman, 2011). Natalizumab, a monoclonal antibody of vasculature cell adhesion molecule-1 (VCAM-1), was another popular therapeutic drug until serious side effects limited it usage (Loma and Heyman, 2011). This drug has been shown to block entry of cells into the CNS, but it also has been shown to have problematic side effects in patients that have a co-infection of JC virus (Loma and Heyman, 2011). Currently, there are no cellular therapies in practice, although cellular therapy of Tregs has been shown promising for protection in EAE.

2.3.4 Trafficking and Entry into CNS

The blood-brain barrier (BBB) is a structure limiting entry from the blood into the brain, thereby regulating the movement of materials into and out of the neural organ. The BBB was discovered when trypan blue or evans blue dyes injected into the blood stream could not enter the CNS, and when injected into CNS did not exit to the periphery (de Vries et al., 1997). This led to the belief that the CNS is an immune-privileged organ. Fairly recently, healthy individuals with an intact BBB showed, although limited in numbers, immune cells patrolling the CNS. This changed our view of the CNS from an immune restricted organ to an immune-restricted organ (Romo-Gonzalez et al., 2012). The difficult job of the immune cells is to protect the CNS from invading pathogens, while inducing the least possible bystander damage. The BBB normal restriction of entry into the CNS can be disrupted due to various causes. Although unknown for MS, it has been suggested the leaky BBB may be induced by antibiotics, infection, or inflammation (de Vries et al., 1997). Some

suggest BBB permeability is caused by co-infection of viruses, linking it to the release of IFN-g locally, as IFN-g, TNF-a, IL-1b, and IL-6 increase the permeability of tight junctions *in vitro* (de Vries et al., 1996; de Vries et al., 1997). Interestingly, NO is indicated to decrease BBB permeablization (Wong et al., 2004). Whatever the reason, the leaky BBB allows for the entry of re-activated T cells into the brain. There are two cycles for entry of T cells in the brain; the first being the entry of Th1 cells into the spinal chord and subsequently release IFN-g inducing inflammation. During the second round of entry, the Th17 cells enter through the choroid plexus via CCR6 - CCL20 dependent mechanism (Liston et al., 2009) and release IL-17 (Goverman, 2009), inducing further chemokine recruitment of other cells to induce EAE pathology (O'Connor et al., 2008). Rothhammer et al. has shown the entry requirements of Th1 cells are different than that of Th17 cells; Th1 cells require VLA-4, whereas the Th17 require LFA-1 (Rothhammer et al., 2011). Goverman showed the ratio of Th17 cells to Th1 cells is an important factor within the CNS in inducing inflammation (Goverman, 2009). In the CNS, T cells interact with the activated microglia with high expression of MHCII and display myelin protein from surrounding damaged cells. This interaction causes further activation of T cells by other myelin epitopes, in a process termed epitope spreading (Klehmet et al., 2004). This epitope spreading could be due to T cells containing dual TCR expression for both myelin proteins and possible viral proteins, as shown in EAE with CD8 cells (Ji et al., 2010). Epitope spreading has been reportedly reduced by nitric oxide in EAE due to its regulation effect on autoreactive T cells (Fenyk-Melody et al., 1998; Shi et al., 2001). Recognition of self can occur due to molecular mimicry of viruses with self (Stohlman and Hinton, 2001). When confronting the challenges of a virus, the immune system can select T cells with certain epitopes to better adapt, but as the virus sequences are similar to self tissue molecules, host tissue may be attacked. There is sequence homology between myelin basic protein, found in neurons, and human herpes virus 6 (Tejada-Simon et al., 2003) and Epstein Barr virus (Holmoy et al., 2004; Lang et al., 2002; Wucherpfennig and Strominger, 1995).

As microglia are the largest population of immune cells residing within the CNS (Gordon, 1986) and are innate immune cells, they act as first responders (Kreutzberg, 1996) to any insult, such as pathogens, trauma, inflammation, with the brain. In MS patients, the clustering of microglia, resident CNS cells similar to macrophages but normally lacking the expression of MHC II, are found before infiltrates and demyelination occur (Adams and Kubik, 1952; Seitelberger, 1973). Upon activation, microglia upregulate MHC molecules, produce proinflammatory cytokines and NO, as well as can phagocytose pathogens, dead cells, and unfortunately in the case of multiple sclerosis, also myelin, the protective coating which ensheaths nerves (Nakajima and Kohsaka, 1993). Roughly 30% of these CD11b+ cells within the brain are DC.

It is known that during chronic inflammation the spleen is a site of systemic infection priming, whereas acute infections usually have the priming occur in the lymph nodes. EAE has been observed to have splenomegaly, an increased size of the spleen, due to increased influx of myeloid cells (Billiau and Matthys, 2001). Interestingly, GM-CSF, the growth factor we use to generate our BM-MDSC and also has been show to mobilize cells from the BM, is required for disease development of EAE as GM-CSF knockout mice are resistant to EAE, and neutralization of GM-CSF attenuates EAE (Roncarolo et al., 2006). This role of GM-CSF in EAE pathogenesis may be due to

its ability to enhance TNF-a-induced IL-6 (Brissette et al., 1995) and the production of IL-23 (Sonderegger et al., 2008), both mediators of Th17 cells, by DC during the effector phase of EAE. In addition, GM-CSF has been reportedly released from the Th17 subset (McGeachy, 2011). Microglia have been reported to display a DC phenotype upon presence of high concentrations of GM-CSF *in vitro* (Esen and Kielian, 2007). Interestingly, mice lacking GM-CSF are defective in myeloid cell recruitment to the brain (McQualter et al., 2001). Similar findings demonstrated administration of GM-CSF i.c. resulted in increased amount of cells phenotypically resembling DC with an activated phenotype (Mausberg et al., 2009). Trafficking of monocytes which differentiate into antigen presenting dendritic cells and macrophages are important for the induction of disease, as these cells are required for the reactivation or priming of the T cells once they enter the CNS (Ifergan et al., 2008). Entry can also be caused by CCL2 upregulation within the CNS, which allows inflammatory cells to enter the perivascular cuffs, but does not necessarily cause any clinical signs of disease (Fuentes et al., 1995; Toft-Hansen et al., 2006). DC after migrating across the BBB and receiving GM-CSF and TGF-b signals have been shown to secrete Th1 and Th17 polarizing cytokines within the perivasculature cuffs (Ifergan et al., 2008).

Although other cells contribute to the disease, it has been thought that MS is a T cell driven disease. Flügel et al. have reported that MBP-specific T cells migrating to the spleen by day 3-4 after injection must be first primed in the spleen before heading to the brain (Flugel et al., 2001). Activated T cells and other inflammatory cells may enter the CNS via the interaction of very late antigen-4 (VLA-4) and vasculature cell adhesion molecule-1 (VCAM-1), which is upregulated in MS. This interaction is specifically targeted in the case of the drug, Natizumab.

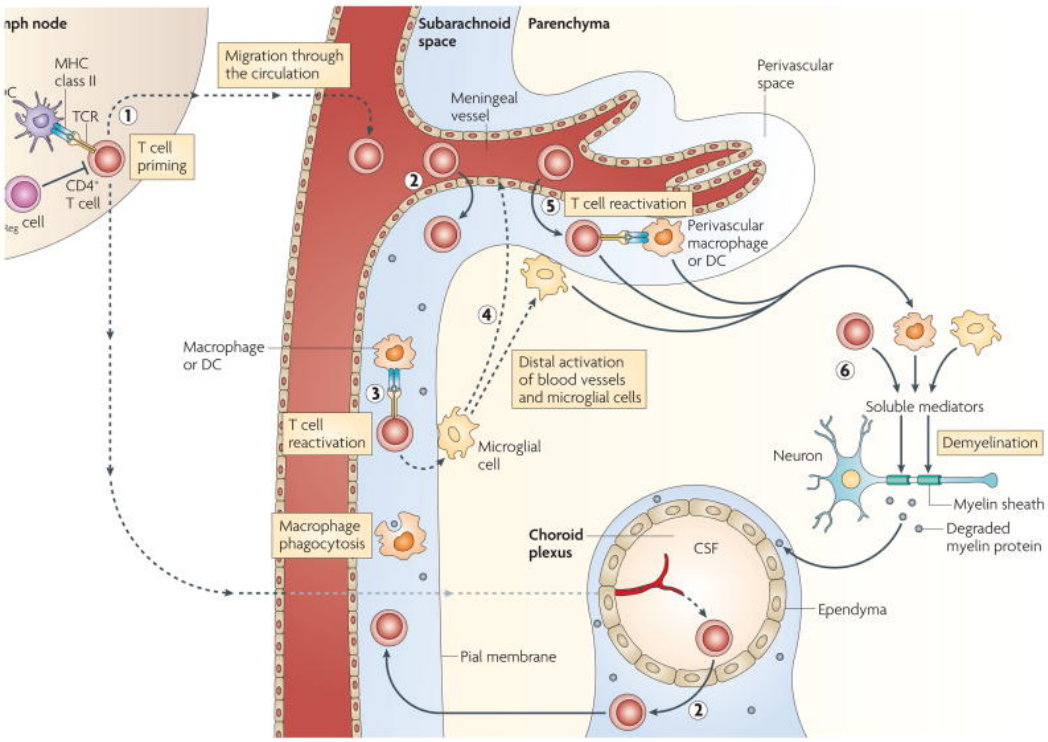

Figure I.5 T cells priming and entry into CNS. T cells get primed in the periphery and have two modes of entry into the CNS. Figure from (Goverman, 2009).

2.3.5 Suppressive Cellular therapies in EAE

Cellular therapy, the application of supplementing an animal with cells to replenish the defective immunity, is not a new concept. Cellular therapy has been studied extensively in mice, but now the application in humans is beginning to be accepted and even approved. Interestingly, there are current trials using stem cell treatment to humans with MS, but as these trials are not finished, the results are unclear. Previous studies have shown the adoptive transfer of Tregs in mice to be beneficial, i.e., dramatically reduced disease scores, when given as a treatment for EAE (Kohm et al., 2002). As discussed earlier, a mutation or lack in genes involving Foxp3 lead to very serious autoimmuity, i.e., IPEX mice. Cellular therapies are not limited to T cells. Endogenous and adoptively transferred regulatory B (B10) cells have provided protection against EAE by a mechanism appearing to be independent of the those used by regulatory T cells (Matsushita et al., 2010). Cellular therapies using the innate immunity have been employed. Our lab has shown that BM-DC stimulated with TNF-a, which creates semi-mature DC, which can suppress EAE through immune deviation and activation of IL-10 producing Tr1 cells (Menges et al., 2002; Pletinckx et al., 2011). As these cellular therapies proved effective, we were interested in determining if MDSC can be applied in a similar manner.

2.3.6 Myeloid Derived Suppressor Cells in Autoimmune Diseases/EAE

When we began this project, there had not been much discussion about MDSC in autoimmune diseases. Previous work was performed in analyzing the suppression of various adaptive immune cells, including CD8 and NKT, but most of this work was done in tumor and cancer settings (Ostrand-Rosenberg and Sinha, 2009). Recently, there have been some reports on the ability of MDSC to suppress various disease models such as type I diabetes (Yin et al., 2010), GVHD (Highfill et al., 2010), and EAE (Zhu et al., 2007). MDSC accumulation after CFA injection is described to be a reason why adjuvant therapy one cannot induce an immune response after boosting with CFA or live mycobacteria (O'Connor et al., 2012).

MDSC were first described as a protective monocyte population observed during EAE (King et al., 2009). During the peak of EAE, the CNS has shown 30% of infilitrates were immature monocytes, phenotypically with $CD11b^{+}Ly\text{-}6C^{hi}$ F4/80 and CD93 (Zhu et al., 2007). In this report, the authors analyzed the endogenous population kinetics, which increased during the disease to the peak in BM, blood, spleen and CNS (Zhu et al., 2007). It has been shown using Gr-1 antibodies, which are known to bind the same epitopes as Ly-6C and Ly-6G cause the downregulation of the marker Ly-6C but not depletion (Ribechini et al., 2009) of myeloid cells, causes a delayed or completely protective effect (McColl et al., 1998). As Ly-6C is a memory marker for T cells, the encephalitic T cells deletion could not be discounted from this study.

It has been shown that GM-CSF release of $Ly\text{-}6C^{hi}$ precursors from the BM prevents exhaustion of the myeloid populations, possibly DC and macrophages, residing within the CNS, thus suggesting increased $Ly\text{-}6C^{hi}$ cells would be benefical to be applied to prevent demyelination (King et al., 2009). Indications of suppressive cells within the lymph node have been reported as the transfer of lymph node cells, but not blood cells have been shown to prevent induced EAE (Englert and Hempel, 1981). Another indication of the protective effect of IMC is a report discussing IMC in adjuvant

therapy, which protects from EAE, by the boost of CFA or live bacteria to the original CFA injection. This action caused the expression of CXCR10 and CXCR16 attracted T cells to the draining LN rather than the CNS (O'Connor et al., 2012).

During the course of our study, it was published that B7-H1 is required for the PMN-MDSC fraction to be supressive late during EAE (Ioannou et al., 2012). These researchers showed that the granulocytic subset given after EAE induction was the suppressive fraction. They also demonstrated that PMN-MDSC is no longer suppressive when it lacks the B7-H1 gene.

2.4 Scope of Study

One of the areas of focus of the Manfred B. Lutz laboratory at the University of Würzburg is to understand mechanisms of tolerance. Our laboratory utilizes DC and MDSC to study different ways to manipulate the immune system to suppress unwanted responses in the cases of autoimmunity, transplant, and allergy, and to maintain tolerance during steady-state. MDSC have been studied intensively in other fields, but little has been applied to autoimmunity. As their negative effect of suppressing the immune system during times of tumors and cancer is an unwanted effect, we set out to see whether this effect, if manipulated, could be beneficial in overshooting responses, such as autoimmunity. Our ability to generate larger quantities of MDSC *in vitro* combined with the inducible EAE as a model allowed us the ability to assess these cells to suppress autoimmunity. The aim of this project is broken up into three categories:

1. The analysis of the factors leading to activation of MDSC.

2. The identification of mechanisms MDSC utilizes to suppress T cell responses under both Th1 and Th17 conditions.

3. The localization of IMC after i.v. injection.

3 Materials and Methods

3.1 Materials

3.1.1 Stimuli

Stimulation with different compounds was used to analyze different factors activating BM-IMC into BM-MDSC.

LPS	0.1 µg/ml (E.coli 0127:B8, Sigma-Aldrich), stored at -20°C.
Curdlan	10 mg/mL in PBS (WAKO), particulate resuspension, stored at -20°C.
Zymosan A	10 mg/mL stock in PBS (Sigma-Aldrich), particulate resuspension, use at 40 µg/mL, stored at -20°C.
IFN-g	10 mg/mL in PBS (Peprotech), use at 1 µg/mL, stored at -20°C.
IFN-b-1a	100 U/µL stock in dH20 (Peprotech), use at 500 U/mL, stored at -20°C.
IFN-b-1b	100 U/µL stock in dH20 (Peprotech), use at 500 U/mL, stored at -20°C.
IL-17	2 µg/mL stock in PBS (Peprotech), 10 ng/mL in PBS, stored at -20°C.
Poly (I:C)	1 mg/mL stock in H_2O (HMW, InvivoGen), 2 µg/mL in PBS, stored at -20°C.
CpG	100 pMol/uL stock in PBS (Eurofins MWG/operon, sequence: TCCATGACTTCCTGATG), 600 pMol/mL in PBS, stored at -20°C.
SEB	1 mg/mL stock (Sigma), use at 100 ng/mL or 10 ng/mL, stored at -20°C.

Stimulation with antibodies for proliferation of T cells:

Anti-CD3	3.6 µg/mL, stock at 3.6 mg/mL (gift from Dr. Hünig, Würzburg), store at 4°C
Anti-CD28	0.5 µg/mL, stock at 2.3 mg/mL (clone E18, gift from Dr. Hünig, Würzburg), store at 4°C

3.1.2 Media, buffers, solutions

Culture medias of cells:

cRPMI:

RPMI 1640 supplemented with 10 % of heat inactivated Fetal Calf Serum (Invitrogen, PAA gold), 2 mM L-Glutamine (PAA), 100 U/mL Penicillin and 100 µg/mL Streptomycin (PAA), 50 µM beta-mercaptoethanol (sigma).

GM-CSF:

Grown from hybridoma in cRPMI, yields roughly 400 ng/mL GM-CSF, use at 10% for culturing of BM-cells, supplemented every 3rd day.

HL-1:

HL-1 (Lonza) supplemented with 2 mM L-Glutamine (PAA), 100 U/mL Penicillin and 100 µg/mL Streptomycin (PAA), 50 µM beta-mercaptoethanol (Sigma-Aldrich).

Freezing Media:
For cell (hybridomas) storage in liquid nitrogen. 10% DMSO (Sigma-Aldrich) and 90% heat inactivated-Fetal Calf Serum (Invitrogen, PAA gold). First freeze down in Mr. Frosty, containing iso-propanol at -80°C (for slow freezing process), once completely frozen for long term storage, place into liquid nitrogen.

Buffers:

4% Paraformaldehyde Solution:
4 g Paraformaldehyde (Sigma-Aldrich) resuspended in PBS. Sodium hydroxide was added to allow paraformaldehyde to dissolve. Heated until 100^{o}C for 1h (pH 7.4), stored for use at 4°C. For fixation, use 10-30mins at 4^{o}C.

FACS Buffer (2.4G2 Supernatant):
Hybridoma cell line grown in cRPMI producing an antibody specifically binding tp FcgRII/III, equivalent to Fc Block (CD16/32).

Phosphate buffered saline (PBS) without Magnesium and Calcium:
Calcium and Magnesium have been shown to activate DC, therefore we use the PBS without these components. The solution consists of 4 mM KH_2PO_4, 16 mM Na_2HPO_4 and 115 mM NaCl at pH 7.3.

10X PCR Tissue Homogenization Buffer:
A solution was made consisting of 500 mM KCl and 100 mM Tris in H_2O, the pH was then adjusted to pH 8. To this solution the following were added 0.1 mg/mL gelatine, 0.45 % Nonident P-40, 0.45 % Tween 20, 100 mL dH20. The solution was then sterile filtered before use.

RBC Lysis Buffer:
Gey's Solution: For removal of RBC. For 70 mL, 20 mL Stock A, 5 mL Stock B, 5 mL Stock C and 40 mL sterile dH_2O were combined. The solution was filter through a sterile filter (without autoclaving) and stored at 4 °C or RT. For lysis, 1 mL of Gey's solution was added to splenocytes for 2 min. The cells were washed thoroughly and spun down for use in different assays.

Gey's Stock Solution A (1 L):
NH_4Cl (35 g), KCl (1.85 g), Na_3PO_4-$12H_2O$ (1.5 g), KH_2PO_4 (0.12 g), and glucose (5 g) diluted in H_2O.

Gey's Stock Solution B (0.1 L):
$MgCl_2$-$6H_2O$ (0.42 g), $MgSO_4$-$7H_2O$ (0.14 g) and $CaCl_2$ (0.34 g) diluted in dH_2O.

Gey's Stock Solution C (0.1L):
$NaHCO_3$ (2.25 g) diluted in dH_2O.

3.1.3 Griess Reagent:

For analysis of NO production through nitrite detection. For working reagent, Griess Reagent A was mixed in equal volumes of Griess Reagent B fresh for every usage. Then Griess Reagent A/B was mixed with equal amounts of supernatant. Reaction was allowed for 10 minutes. Colormetric assay was run on the Spectramax at 492 nm.

Griess Reagent A:

0.1 % Naphthylethylenediamine dihydrochloride (Sigma) in dH_2O. Stored at 4^oC; away from light.

Griess Reagent B:

1 % Sulfanilamide (Sigma) in 5 % H_3PO_4/dH_2O. Stored at 4 °C.

Standard:

$NaNO_2$

3.1.4 Anaesthesia:

Sleeping Mouse Kax:

For roughly 10 mice. Injections administered i.p. at 100 µL/mouse, solution of 144 µL Ketavet (Ketamin at 100 mg/mL), 90 µL Rompun (Xylazin 2%; 10 mg/mL), and 760 µL PBS was prepared.

Lethal Anesthesia:

For roughly 30 mice injections ip at 70 µL/mouse a solution of 1440 µL Ketavet (Ketamin at 100 mg/mL), 900 µL Rompun (Xylazin 2%; 10 mg/mL), and 760uL PBS was prepared.

Isofluran:

Isofuran inhalation was given to anesthesize mice during EAE induction. Sleeping is induced through inhalation. The oxygen was controlled, level 2 mixing with water and Level 3 isofluran to anesthesize mice. Mice were then placed into a chamber for the initial anesthesia, followed by placement of only the head into a nozzle during injections.

CO_2 afixiation:

CO2 was given to until mice were completely afixiated, followed by cervical dislocation to assure mice were sacrificed.

3.1.5 *In vivo* tracking

D-Luciferin firefly:

4 g of D-luciferin (Biosynth) was reconstituted in 165 ml aqua-dest H_2O. Aliquots were stored at -20 °C. Mice were injected with 200 µL i.p. with 200 µL of mouse kax (sleeping) i.p. and 10 minutes were allowed for complete circulation and for best signal for imaging.

CFSE:

CFSE labels intracellular proteins within the cell. The cells then are visable in the FITC channel. Stock concentration is 5.6 mM. Aliquots are stored at -20°C. Cells were labeled for *in vitro* proliferation assays at 0.93 µM, whereas cells injected *in vivo* were labeled with 1.87 µM in PBS.

eFluor 670:

eFluor670 (eBioscience) labels cells in a similar way to that of CFSE, with the exception that it labels the cells so they are visable in the APC channel. Stock concentration is 5 mM. Aliquots are stored at -20 °C.

CM-DiI:

CM-DiI (Molecular Probes, Invitrogen) is a membrane labeling lipophilic dye. Stock is stored frozen at -20 °C, freshly diluted with DMSO for the concentration of 2 mg/mL. The dye is used by incubating 10^7 cells in PBS with 10 µM (1 / 200 dilution) of the stock dye for 5 mins at 37 °C followed by a 15 min incubation at 4 °C. Cells are visable in the PE channel.

3.1.6 Radioactive [^{3}H] Thymidine

Radioactive [^{3}H] thymidine: To assess proliferation, a radioactively labeled isotope of thymidine was added to cells. This intercalates into the DNA, as the cells divide there will be a brighter signal. A 50 µCi/mL solution was prepared by dilution of [^{3}H] thymidine (Hartmann Analytic) in serum free media. The concentration used in the assay was 1 µCi/well in a 96 well plate.

3.1.7 Experimental Autoimmune Encephalomyelitis

Oil-water emulsion was created using 1:1 ratio of CFA containing extra heat-killed Mtb to MOG peptide. Emulsion was performed using a three-way stop cock.

$MOG_{35—55}$ Peptide (EVGWYRSPFSRVVHLYRNGK):

A solution was prepared at 2mg/mL in ddH2O by dilution. (Synthesized by HPLC, Charite, Berlin)

Pertussis toxin:

A solution was prepared by dilution at 0.1 µg/uL in PBS. (List Biological Laboratories)

Complete Freund's Adjuvant (CFA):
10 mL vial (Sigma-Aldrich) containinig 1 mg of Mtb and 10 mL oil.

Heat killed Mycobacteria (strain H37RA):
10 mg vial (DIFCO Laboratories)

3.1.8 Enzymes and inhibitors

L-NMMA:
An inhibitor of iNOS. Stock solution diluted first in DMSO, then in RPMI at 50 mM. This solution was then diluted into cell culture at 500 ug/mL.

3.1.9 GM-CSF Supernatant

Supernatant was obtained as previously published by Stockinger et al (Zal et al., 1994). In brief, GM-CSF gene transfected plasmacytoma X63-Ag8 cell line was cultured in cRPMI medium until medium was orange-yellow. The cells were then pelleted, supernatant was taken, and filtered through a 0.2 µm filter. The supernatant was then tested via ELISA to assure the concentration was above 400 ng/mL. Supernatant was frozen at -20 °C until use. Supernatant was used at 10 % in growth of BM-DC and BM-IMC cultures.

3.1.10 Mice

Mice were used at 6 – 16 weeks of age. Mice were bred in the animal facilities of the center for molecular medicine (ZEMM) and of the Institute for Virology and Immunbiology, University of Würzburg, Würzburg, Germany, or at Charles River. Breeding pairs for C57Bl/6 PD-$1^{-/-}$ and C57Bl/6 B7-$H1^{-/-}$ mice were a kind gift of Heinz Wiendl, Neurology Clinic, Würzburg University. Albino.C57Bl/6J and Luciferase+ CD45.1+ C57Bl/6J mice were kindly provided by Andreas Beilhack, ZEMM, Würzburg University.

BALB/c
A mouse used for allo-MLR because of its different MHC genes I-Ab)

C3H.TLR4$^{-/-}$/MyD88$^{-/-}$ (control C3H.TLR4$^{-/-}$) mice
A transgenic mouse which lacks MyD88 adaptor protein and the TLR4 gene. The control mice lack only the TLR4 gene. (Provided as a gift by Andre Gessner, formerly of Uni-Erlangen)

C57BL/6J
Our control mouse. MHCII molecules are all I-Ad

C57Bl/6J.albino mice
A transgenic mouse containing a changed for the hair color allele. This mouse provides less interference of the luciferase bioluminescence compared to black hair counterparts. (Provided as a gift by Andreas Beilhack, ZEMM, Würzburg).

C57Bl/6J.B7-H1$^{-/-}$ mice

A transgenic mouse which lacks the gene B7 homologue 1 (B7-H1). This is one of the ligands which interacts with PD-1, therefore it was used for analysis of the PD-1 : B7-H1 interaction. Confirmation was performed via PCR for the neocassette. (Provided as a gift by Heinz Wiendl, Neurology Clinic, Würzburg). Mouse line made by Lieping Chen.

C57Bl/6.IFN-gR1$^{-/-}$ mice

These mice lack the interferon gamma receptor. (Provided as a gift from Dr. Ulricke Schieber/Dr. Christian Bogden)

C57Bl/6.iNOS$^{-/-}$ mice

These mice lack inducable nitric oxide synthase. (Provided as a gift from Dr. Ulricke Schiecher/Dr. Christian Bogden)

C57Bl/6.IRF-1$^{+/-}$ and C57Bl/6.IRF-1$^{-/-}$ mice

These mice lack the signalling gene for Interferon regulatory factor-1 either in one allele (IRF$^{+/-}$) or both allels (IRF$^{-/-}$). (Provided as a gift from Dr. Micheal Lohoff, Marburg)

C57Bl/6J.Luc^{+}CD45.1 mice

A transgenic mouse contain a luciferase-expressing L2G85 reporter gene construct, which had been inserted through a pronuclear injection into FVB/N mice (Cao et al., 2004) and subsequently backcrossed over 14 generations on to C57BL/6 mice. Luciferase cleaves a substrate called luciferin which allows for *in vivo* analysis. These mice also have the allele for CD45.1 that allows differentiation from C57Bl/6 mice which express the CD45.2 allele. (Provided as a gift by Andreas Beilhack, ZEMM, Würzburg) Confirmation was performed via PCR in the Beilhack lab.

C57Bl/6J.Luc^{+}IFN-aR$^{-/-}$ mice

These mice lack IFN-I receptor genes. A transgenic mouse which produces luciferase. (Provided as a gift by Christine Krempl, Virology, Würzburg)

C57Bl/6J.OTII mice

These mice are transgenicly altered so that the TCR of CD4 T Cells is specific for the recognition of OVA.

C57Bl/6J.PD-1$^{-/-}$

A transgenic mouse which lacks the gene for programmed death receptor-1 (PD-1). Used for analysis of the PD-1-B7-H1 interaction. Confirmation for the loss of PD-1 is performed via PCR for the neo-cassette. (Provided as a gift by Heinz Wiendl, Neurology Clinic, Würzburg). Line made by Honjo.

C57Bl/6J.VAV-CRE mice

These mice have the expression of the CRE-recombinase under the VAV promoter. This promoter is expressed in all cells, therefore all cells within this mouse have the expression of CRE. This mouse is generally used for breeding with mice that have lox-p sites, which allows for the mouse to lack a particular gene as the CRE recombinase can cleaves these sites. These mice were used as control mice for these mice which lack the gene. (Provided as a gift from Dr. Friederike Bereberich-Siebelt, Pathology, Würzburg.)

C57Bl/6J.VAV-CRE X CAAT/enhancer binding protein-beta fl/fl mice

These mice lack the expression of C/EBP-b. When activated VAV-CRE promoter, in all cells, when activated then can cleave the flox sites around the C/EBP-b, which excizes the gene. (Provided as a gift from Dr. Friederike Bereberich-Siebelt, Pathology, Würzburg.)

3.1.10.1 Antibodies

Primary Antibodies:

Antibody	Company	Staining
CD11b	Biolegend	Surface
Ly-6C	BD	Surface
Ly-6G	Biolegend	Surface
CD80	BD	Surface
CD86	BD	Surface
CD40	Miltenyi	Surface
PD-1	eBioscience	Surface
B7-H1	BD	Surface
CD4	Biolegend	Surface
CD8	Biolegend	Surface
IL-17	eBioscience	ICC
IFN-g	BD	ICC
Foxp3	eBioscience	Intranuclear
Ki67	BD	Intranuclear

Secondary antibodies:
Anti-Rat Alexa 647 (Jackson Labs)

3.2 Methods

3.2.1 Harvesting, and culturing Primary murine cells

Bone Marrow Immature Myeloid Cells and Immature Dendritic Cells

Mice were sacrificed for their BM. The skin around the hind legs was trimmed off and the feet were cut at the ankle joint. The leg bones were separated from the pelvic/hip bone. The muscle/tendons were cleaned off via rubbing a paper towel over the bones. Bones were then placed in ethanol for one minute. The ends were then clipped off with scissors. The BM was then flushed out using a 10 mL syringe with a 27 gauge needle (grey) containing PBS. The BM was collected in a 15 mL conical tube, spun down to a pellet, and resuspended in cRPMI. Cells were counted and

plated at $3X10^6$ cells/10 cm sterile petri dish in a total of 10 mL cRPMI supplemented with GM-CSF. Cells were incubated in a 7% CO_2 incubator. BM-IMC can be harvested at day 3. Immature DC need to be fed with 10 mL cRPMI supplemented with GM-CSF on day 3 and day 6 for use on day 8.

Splenocytes

Mice were sacrificed and spleens were collected. A single cell suspension was created by mushing the whole spleen through a plastic cell strainer (0.2 μm) to create a single cell suspension. Cells were then spun down, resuspended in Geys solution for 1-3 min, washed and spun down in PBS. The cells were then resuspended in cRPMI. For samples with injected BM-IMC, the collagenous bundle found in spleen would then be separated. To make a single cell solution, DNAse I (1mg/mL, Roche) and Collagenase III (1mg/mL, Wothington, CA) were added for 20 min at RT.

Lymph nodes

Mice were sacrificed and inguinal lymph nodes are collected. A single cell suspension is created by mushing whole lymph nodes through a plastic cell strainer (0.2 μm) to create a single cell suspension. Cells were then spun down and resuspended in cRPMI for tissue culture or for FACS analysis.

Central Nervous System: Isolation of Lymphocytes

Mice were sacrificed and brain and spinal chord were removed. The organs were then cut into small pieces, incubated with DNase I (1mg/mL, Roche) and Collagenase NB4 (1 mg/mL, Serva) for 45 mins. The cells were then lysed through a needle resuspended in 30 % Percoll and placed over a 70 % Percoll gradient. The cells were then spun down without a break for 30 mins. The lymphocyte fraction of cells, found within the buffy layer, were then washed and spun down. Cells were then resuspended in cRPMI and used for further analysis.

3.2.2 Stimulation

Stimulation of Bone marrow generated immature myeloid cells

BM-IMC were stimulated using concentrations listed in the materials section. Stimulation was performed in particular ways for each assay. BM-IMC were stimulated overnight for analysis of surface markers and intracellular cytokines. BM-IMC were placed into MLRs for three days.

T cells Restimulation

Splenocytes were harvested (see above) and cells were seeded at $4X10^5$ cells/well in a 96 round bottom plate in a total of 200 μL/well. Specific memory responses were analyzed via addition of different concentrations of MOG peptide, or aspecific stimulation could be analyzed by addition of anti-CD3/anti-CD28 antibodies.

3.2.3 Sorting Immature Myeloid cell subsets via MACS

BM-IMC were generated as normal. D3 bulk cultures were then sorted using Ly-6G$^-$ Biotin antibody, incubating in 2.4G2 supernatant for 30mins at 1:100 μL, Samples were washed. Resuspended in PBS and incubated with anti-biotin MACS-microbeads (Miltenyi Biotec) for 30 minutes. Washed with PBS. Cells were placed over a MS column for positive selection of Ly-6G. Cells which flowed through were the Ly-6G

negative fraction. Columns were washed and plunged to receive the Ly-6G positive fraction.

3.2.4 Fixation of subsets for MLR

Cells were sorted using MACS the incubated overnight in cRPMI supplemented with GM-CSF. The previously described protocol for fixation and washing by Munster et al was slightly modified. Briefly, cells were harvested and fixed with 4% PFA for 30 mins, then washed with PBS and then placed in cRPMI overnight to wash out any residual PFA which might affect the interaction with the T cells. Cells were then added to EAE splenocytes restimulated with either anti-CD3/anti-CD28 or with MOG peptide at 10 µg/mL.

3.2.5 Proliferation

CFSE

Cells (bulk splenocytes) were stained in 10.5 mL of PBS for 15 mins at RT. The CFSE is neutralized by FCS and then washed. T cells were then incubated for 6 days in a CO_2 incubator.

[^{3}H] Thymidine Incorporation

Culturing:
Cells were cultured in a 96 well plate containing 200 µL/well for 2 days at 7% CO_2.

Pulse:
Radioactive [^{3}H] -methyl-thymidine is reconsitituted in serum free media at the concentration of 50 µCi/mL. For proliferation assessment, 20 µL/well was added providing each well 1 µCi per a total of 200 µL cell solution.

Harvest:
Harvest was performed using a Tomtec 96-well harvester (PerkinElmer). The 96 well plates were first harvested onto glass-fiber filters (PerkinElmer). The filter was then dried in an incubator for over an hour. Followed by the addition of Scintallation buffer. Each filter was then heat sealed in an individual plastic sample bag (PerkinElmer) and counting, as performed by the 1450 Microbeta counter. Readouts were given in Microsoft Excel format.

3.2.6 Flow cytometry

Flow cytometry: Surface Marker Staining

Staining is performed in 100 µL of 2.4G2 supernatant at 1:100 dilution of antibody. Cells were then incubated at 4°C for 30 mins. Cells were then washed and either aquired by FACS LSRII using FACS-Diva software.or continued using intracellular or intranuclear staining.

Flow cytometry: Intracellular Staining

Staining was performed as normal using cell surface staining protocol (see above). Cells were then fixed with 4 % PFA for 30mins. Cells were then washed with PBS, spun down, and resuspended in permeabilization solution (0.5 % Saponin) for 30

mins. Cells were then spun down and resuspended in 100 µL Perm Buffer containing 1:100 dilution of antibody. Cells were then incubated for 1 h at 4°C. Cells were washed with PBS to remove the Saponin and aquired by FACS LSRII using FACS-Diva software.

Flow cytometry: Intranuclear Staining (Foxp3, eBioscience kit)

Staining was performed as normal using cell surface staining protocol (see above). Cells were then fixed with 100 µL Fix/Perm buffer (eBioscience, freshly diluted 1 part fixation/permeabilization concentrate with 3 parts Fix/Perm buffer) for 30 mins. Cells were then spun down, resuspended in 100 µL Perm solution (eBioscience, freshly diluted 1 part permeabilization buffer diluted in 9 parts dH2O) for 30mins. Cells were then spun down and resuspended in 100 µL Perm Buffer containing 1:100 µL dilution of antibody. Cells were then incubated for 1 h at 4°C. Cells were washed with PBS to remove the perm buffer and aquired by FACS LSRII using FACS-Diva software.

Flow Cytometry: Flow Jo Analysis

Events aquired on FACS-Diva software was then analyzed using Flow jo software (Treestar). Statistics of percentages were calculated using Microsoft Excel.

3.2.7 Histology by Immunofluorescence staining

Staining for surface antigens (CD169)

Fresh frozen tissue was fixed for 30 min with 4% PFA. Tissue was then blocked using Blocking buffer (5% BSA in PBS with 0.3% TritonX) for 30 min. Tissue was then stained overnight with antibodies (1:100) in staining buffer (1% BSA in PBS with 0.3% TritonX). Tissue was washed three times for 5 min. Tissue was then incubated for 1 h with secondary antibody (1:300) in staining buffer (1% BSA in PBS with 0.3% TritonX). Tissue was washed three times for 5 min. Tissue was coversliped with Fluoromount G and analyzed via Fluorescent microscopy.

3.2.8 Cell tracking

Biolumenescence in vivo Imaging

Mice were injected with 200 µL anaesthesia and 200 µL luciferin. Ten minutes were given to allow to luciferin to circulate the entire mouse. The mouse was then placed under a camera. The same settings were used every time with 5 min exposure. The photons emitted from the cleavage of the luciferin by the cells are captured into pictures. These pictures were then overlayed onto a normal photograph of the mouse/organ to display where the emission was located. Analysis was performed by gating with ROI generates the Photons/second within the area of interest. These values can be graphed with Microsoft Excel.

3.2.9 Soluble factors

Nitric Oxide

Released NO is measured via a colormetric assay. To 50 µL supernatant, 50 µL of freshly mixed Griess Reagent (see above) was added and incubated for 10 min. The $NaNO_2$ concentration of samples and standards was measured at 492 nm. Standard curves were created based on the NaNO2 optical density (OD) readings. From this standard curve, samples concentrations were calculated.

Enzyme linked immunoabsorbant Assay

Enzyme linked immunoabsorbant Assay (ELISA) is a very common technique involving antibodies followed by an enzyme-substrate color forming reaction to quantify how much of an antigen is in sera or supernatant. In this case, GM-CSF ELISA were performed using the instructions suggested by the manufacturer (BD Optia kits).

3.2.10 PCR

PCR for B7-H1$^{-/-}$ mice

Ear notches were taken from mice and diluted in a 1x Tissue homogenization buffer containing a 200 µL of buffer and 5 µL of proteinase K/ sample. Samples were then digested overnight at 56 °C. Inactivation of proteinase K was performed at 96 °C for 10 mins. PCR was performed useing reagents from Fermentas. The master mix included 10x ammonium sulfate buffer, 25 mM MgCl2, 10 mM dNTP, 10 µM primers, 5 U/µL Taq polymerase, water and 1 µL digested tissue DNA for a total of 25 µL.

Primer sequences:
Primer sequences were synthesized by Sigma-Aldrich.

B7-H1	**FOR:**	5‘AGAACGGGAGCTGGACCTGCTT~GTTAG3‘
	REV:	5 ‘ATTGACTTTCAGCGTGATTCGCTTGTAG 3‘
	NEO:	5 ‘TTCTATCGCCTTCTTGACGAGTTCTTCTG 3‘

PCR Program:
Thermocycling was performed using an initial 2 min hot start at 94 °C, followed by 40 cycles of 30 s at 94 °C (Denaturing), 30 s at 60 °C (Annealing), 8 s at 72 °C (Elongation), and completed with another 30 s at 72 °C (final Elongation) before holding at 4 °C.

PCR Product lengths:
PCR products were run on a 2 % agarose gel (0.5% TBE buffer and corresponding amount of agarose, boiled, followed by addition of ethidium bromide) at 100V. The WT PCR product is 250 bp in length while the B7-H1$^{-/-}$ band detected by the neo cassette is 450 bp in length.

PCR for PD-1$^{-/-}$ mice

Ear notches were taken from mice and diluted in a 1x Tissue homogenization buffer containing a 200 µL of buffer and 5 µL of proteinase K/ sample. Samples were then digested overnight at 56 °C. Inactivation of proteinase K was performed at 96 °C for 10 mins. PCR was performed useing reagents from Fermentas. The master mix included 10x ammonium sulfate buffer, 25 mM MgCl2, 10 mM dNTP, 10 µM primers, 5 U/µL water and 1 µL digested tissue DNA for a total of 25 µL.

Primers sequence:
Primer sequences were synthesized by Sigma-Aldrich.

PD-1	**FOR:**	5' CCG CCT TCT GTA ATG GTT TG 3'
	REV:	5' TGT TGA GCA GAA GAC AGC TAG 3'
NEO	**FOR:**	5' GCC CGG TTC TTT TTG TCA AGA CCG A 3'
	REV:	5' ATC CTC GCC GTC GGG CAT GCG CGC C 3'

PCR Program:
Thermocycling was performed using an initial 2 min hot start at 94 °C, followed by 35 cycles of 30 s at 94 °C (Denaturing), 30 s at 54 °C (Annealing), 45 s at 72 °C (Elongation), and completed with another 30 s at 72 °C (final Elongation) before holding at 4 °C.

PCR Product lengths:
PCR products were run on a 2 % agarose gel (0.5% TBE buffer and corresponding amount of agarose, boiled, followed by addition of ethidium bromide) at 100V. The WT PCR product is 690 bp in length while the PD-1$^{-/-}$ band detected by the neo cassette is 400 bp in length.

qRT-PCR for Arginase-1 mice

Splenocytes we placed into 1mL of Trizol (Ambion) at 2X10^6/mL.

RNA Isolation:
To thawed trizol samples, 200 uL of Chlorophorm was added. Samples were shaken hard by hand for roughly 20 s. Samples were then incubated on ice for 5mins. Before spinning down, samples were then shaken hard by hand for another roughly 20 s. The aqueous phase (clear portion) was removed and placed into new eppendorf tube. Isopropanol was added in equal volumes. Samples were shaken hard by hand for roughly 20 s, followed by an incubation on ice for another 5 mins. Samples were spun down and supernatant was removed, recovering only the pellet. The pellet was washed 2 times with 70 % Ethanol. Samples were allowed to air-dry. Concentration of the isolated RNA was observed using the Nano-drop technology.

DNAse Reaction:
DNA was removed from the samples using DNase I (Roche). Extracted RNA was incubated in a solution containing 10x reaction buffer, DNase I (1U/µL) and H2O at 37°C for 15 mins, 50 mM EDTA was added followed by another incubation at 65°C for 10 mins. Samples were then read for reverse transcription into cDNA.

cDNA:
Reverse transcription was performed to obtain cDNA. To do so, RNA was added into a master mix containing Oligo(dt)18 primer with DEPC H_2O. This solution was then mixed and incubated at 70 °C for 5 mins. The mix was then added to a secondary master mix containing 10 mM dNTP, 5X reaction buffer, BioScript Reverse Transcriptase enzyme (Bioline), and DEPC H_2O. This solution was then incubated at 45 °C for 1 h, followed by subsequent incubation of the samples at 70 °C for 10 mins. Samples were then stored at 4 °C

qRT-PCR*:*
Samples were prepared for analysis by qRT-PCR. 2 µL of cDNA was added to SyBr green master mix containing 2x SYBR green and light cycler H_2O and forward and reverse primers (5 µM, Sigma-Aldrich.). Arg-1 primer sequences were selected from a paper discussing Arg-1 in murine macrophages (Pourcet et al., 2011). 18S, due to its stability, and that myeloid cells upon activation increase beta-actin, was selected as the control (Bas et al., 2004; Schmittgen and Zakrajsek, 2000). Sequence for the 18S primers was selected from (Schmittgen and Zakrajsek, 2000).

Arg-1	**FOR:** 5’	GGAATCTGCATGGGCAACCTGTGT 3’
	REV: 5’	AGGGTCTACGTCTCGCAAGCCA 3’
18S	**FOR:** 5’	GTAACCCGTTGAACCCCATT 3’
	REV: 5’	CCAT CCAATCGGTAG TAGCG 3’

Program:
Thermocycling was performed using the iCycler (BioRad) an initial 15 min hot start at 94 °C, followed by 40 cycles of 30 s at 94 °C (Denaturing), 30 s at 60 °C (Annealing), 30 s at 72 °C (Elongation), and completed with another 1 min at 95 °C and 1 min at 60°C (final Elongation). Temperature was then placed at 55 °C and incrementally increased every 15 s after the initial 30 s.

Analysis:
Values obtained qRT-PCR were further analyzed using Microsoft Excel. Arg-1 values were subtracted from the control (18S) values obtaining a value considered delta CT. The delta CT values of the experimental group (EAE + BM-IMC) were subtracted from the average delta CT values of the control group (EAE), this value is considered deltadelta CT. To assess the logarithmic phase of the PCR values were analyzed using the formula $[2^{(-\text{deltadeltaCT})}]$.

3.2.11 Induction of Experimental Autoimmune Encephalomyelitis

BM-IMC were injected i.v. 3 to 4 days before induction. BM-IMC injected mice were immunized with 150 µg of the myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide emulsified in complete Freund's adjuvant followed by administration of pertussis toxin on day 0 and day 2.

4 RESULTS

4.1 Confirming bone marrow generation of myeloid derived suppressor cells and their corresponding splenic myeloid derived suppressor cell subsets in untreated wildtype C57Bl/6 mice

MDSC, described by their CD11b, Gr-1 expression and suppressive function, are normally found in low quantities within the normal untreated WT mouse (2-4%) (Gabrilovich and Nagaraj, 2009; Kusmartsev and Gabrilovich, 2006). As Gr-1 can be broken down into two further markers, utilizing these surface markers identifies two subsets, PMN-MDSC (Ly-6C^{low}, Ly-6G^{+}) and Mono-MDSC (Ly-6C^{high}, Ly-6G^{-}) (Bunt et al., 2006). To confirm our ability to phenotype MDSC ex vivo, we performed surface stainings of spleens and could confirm this population makes up roughly 2% of our steady-state spleens (Figure R1). Unfortunately, with the yield being only 2% of the spleen and the fact that these populations are not suppressive during steady-state conditions (Ribechini et al., 2010), made it hard to utilize it as a cellular therapy. Therefore, we switched to BM-MDSC generation by culturing BM in the presence of GM-CSF for 3 days as previous established by Roßner et al. This BM-MDSC protocol allows the generation of a considerable amount of cells, of which can suppress proliferating T cells (Rossner et al., 2005). To confirm that we could generate the same cells, we flushed BM from WT C57Bl/6 mice and cultured them in the presence of GM-CSF for 3 days creating BM-IMC. The cells expressed CD11b, Ly-6C, and Ly-6G, consistant with previous findings in the lab (Figure R2). To assess functionality, cells were stimulated overnight with LPS and IFN-g to push the cells into BM-MDSC, which have been described to produce NO and suppress T cells. We could confirm BM-MDSC can produce NO compared to the untreated BM-IMC and they can supress when added to stimulated splenocytes (Figure R2).

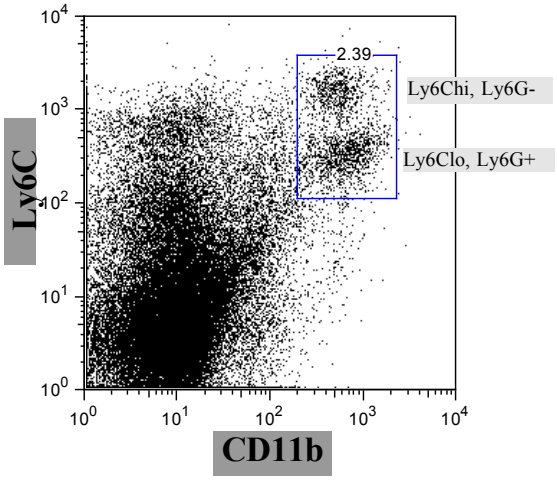

Figure R1. Phenotype of splenic cells from C57Bl/6 mouse.
A single cell solution was made from spleens from untreated WT C57Bl/6 mice. Briefly, spleen cells were filtered through a T-cup strainer, and RBC lysed for flow cytometric analysis. Flow cytometry staining for MDSC markers CD11b, Ly-6C, Ly-6G markers. Plot show is representative of many experiments.

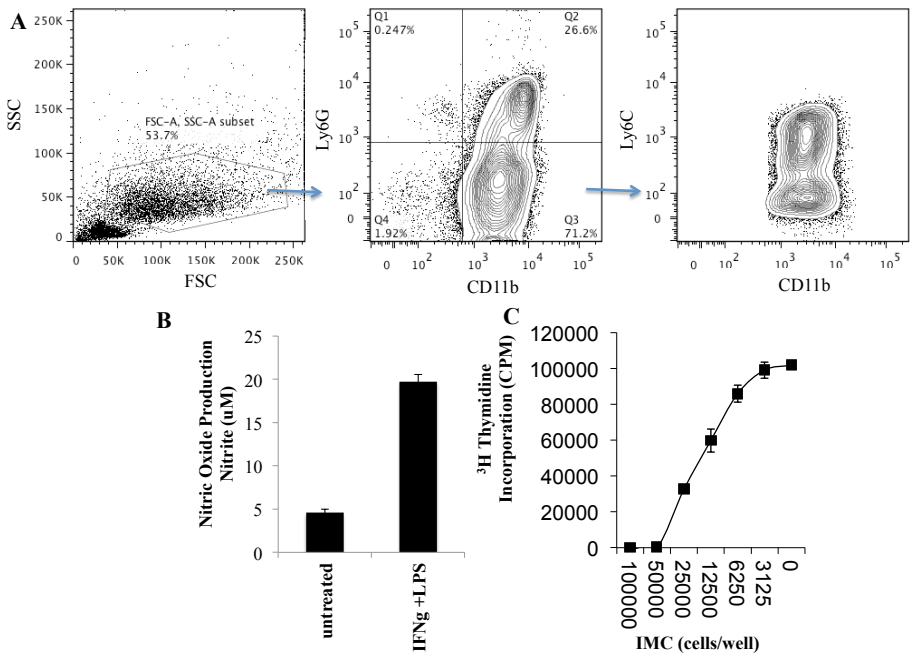

Figure R2. Phenotype and functionality of BM-MDSC from C57Bl/6 mouse. A | BM-BM-IMC Phenotype. D3 BM-IMC grown in the presence of GM-CSF from a WT C57Bl/6 mouse. Flow cytometry staining for MDSC markers CD11b, Ly-6C, Ly-6G. Plots shown are representative of many experiments. **B |** D3 BM-IMC were stimulated with LPS/IFN-g overnight or left unstimulated. Supernatants were analyzed for NO production using the Griess Reagent. Plot show is representative of many experiments. **C |** D3 BM-IMC were added to anti-CD3/anti-CD28 stimulated C57Bl/6 splenocytes. Cells were incubated for 2 days then pulsed with radioactively labeled [^{3}H] thymidine to assess proliferation. Plot show is representative of many experiments.

4.2 CAAT/enhancer binding protein-beta may influence development and activation of mono-BM-MDSC.

Bronte et al. has described C/EBP-b as a critical factor for MDSC suppression (Marigo et al., 2010). As C/EBP-b has been described as controlling emergency granulopoiesis (Hirai et al., 2006), we thought we would test whether this transcription factor was important for development of BM-IMC or if these cells require it for activation into BM-MDSC. To answer this question we utilized C/EBP-b fl/fl X VAV-Cre mice, which lack C/EBP-b in all cells, and assessed the result compared to their control mice, VAV-Cre, which have fully functional C/EBP-b, just expressing CRE under the VAV promoter. The active VAV promoter, expressed in all cells, transcribes CRE recombinase, which then is able to cleave at the loxp sites surrounding the C/EBP-b gene. In the case of the control mouse, the CRE recombinase has no loxp sites to cleave, therefore there is no removal of any gene. BM-IMC generated from these mice were used for assessment of the different MDSC cell subsets. D3 BM-IMC had a decreased percentage of Ly-6C^{high}Ly-6G^{-} cells (mono-MDSC) by 35%, although not significant (p=0.081). Interestingly, this change had very little influence, 10% reduction (p=0.1), on the percentage of Ly-6C^{lo}Ly-6G^{+} cells (PMN-MDSC) yielded in the D3 culture of BM-IMC (Figure R3). We investigated the role of C/EBP-b on activation, by culture of bulk BM-IMC in the presence of LPS/IFN-g overnight to assess the supernatants for the production of NO.

There was an observed significant decrease in the NO production (Figure R3). Unfortunately, we cannot discount the lack of 35% of the mono-BM-MDSC subset contributing to the decrease. Therefore, one must sort the populations and then test for NO to accurately analyze this activation. To characterize the splenic populations *in vivo*, we assessed whole splenocytes for MDSC markers. Interestingly, the organs seemed comparable to the control mice (Figure R3C). We conclude that BM-IMC, which arise during inflammation, require C/EBP-b for generation of mostly the generation of mono-MDSC, whereas the PMN-MDSC is slightly altered or unaffected. This lack of mono-MDSC generation most likely accounts for the decreased NO production, but as the reduction in NO was more than 35%, there seemingly may be further impairment of their activation. This possible intrinsic defect needs to be investigated in further detail. This effect in subsets is BM-IMC generation specific as the steady-state splenic MDSC subsets remain at comparable levels to their control counterparts. We hypothesize this difference may be attributed to our growth factor, GM-CSF, occurring more in inflammatory conditions rather than during steady-state. Further changes in the ability of these *in vivo* populations after inflammation or infection would be interesting to analyze.

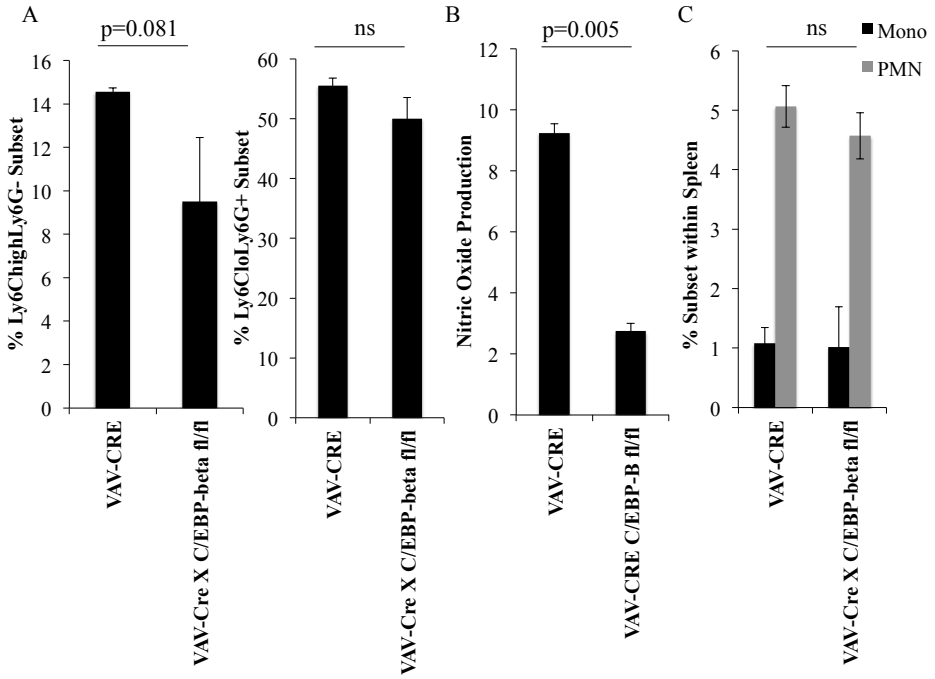

Figure R3. BM-IMC require CAAT/enhancer binding protein-beta for differentiation and activation. A | D3 BM-IMC from mice lacking C/EBP-b (VAV-CRE X C/EBP-b fl/fl) and their controls (VAV-CRE) were generated in the presence of GM-CSF. Percentage of subsets were compared by flow cytometry for MDSC markers CD11b, Ly-6C, and Ly-6G. Results are from 3 mice/group. Statistics was performed using a student T-test, p values did not reach significance. **B |** D3 BM-IMC from mice lacking C/EBP-b (VAV-CRE X C/EBP-b fl/fl) and their controls (VAV-CRE) were stimulated with LPS/IFN-g overnight. Supernatants were analyzed via Griess Reagent for NO production. Results are from 3 mice/group. Statistics was performed using a student T-test. P values are significant (p=0.005). **C |** Splenocytes from mice lacking C/EBP-b were analyzed by flow cytometry for MDSC makers CD11b, Ly-6C, and Ly-6G. Results are from 3 mice/group. Statistics was

performed using a student T-test. P values did not reach significance.

4.3 Induction of nitric oxide by bone marrow generated myeloid derived suppressor cells with various pathogens and interferon gamma requires MyD88, IFN-gR1, and IRF-1 signalling, but not activation by nucleic acids and self-glycolipids.

To understand if BM-MDSC activate in the presence of the autoimmune diseases microenviroment to become suppressive, we investigated activation of BM-MDSC by various compounds relating to EAE, including cytokines, pathogens, and glycolipids. The stimuli were selected based on their effects on DC to polarize T cells in different manners, i.e., Th1 or Th17. A combination of LPS/IFN-g, polarizing in a Th1/CD8 manner (Abbas AK, 2010; Re and Strominger, 2001), was previously elucidated to be a strong stimulator of BM-MDSC production of NO (Greifenberg et al., 2009), therefore we used this as a positive control. To assess Th17-polarizing pathogens on BM-MDSC NO production, curdlan (Higashi et al., 2010) (a 1,3 beta-glycan purified from fungi), zymosan A (Veldhoen et al., 2006) (a yeast-derived 1,3 beta-glycan similar to curdlan but a more crude extract possibly contaminated with endotoxin), and an obvious choice as a component in CFA, heat-killed mycobacteria (Mtb) (Veldhoen et al., 2006; Zenaro et al., 2009), were selected. A link between NO production and EAE severity has been previously observed as iNOS deficient mice have more severe EAE. We found that the three extracts from yeast, fungi, and bacteria can activate the MDSC to produce NO (Figure R4), therefore conclude that both Th1 and Th17-like polarizing stimuli can activate MDSC functionality.

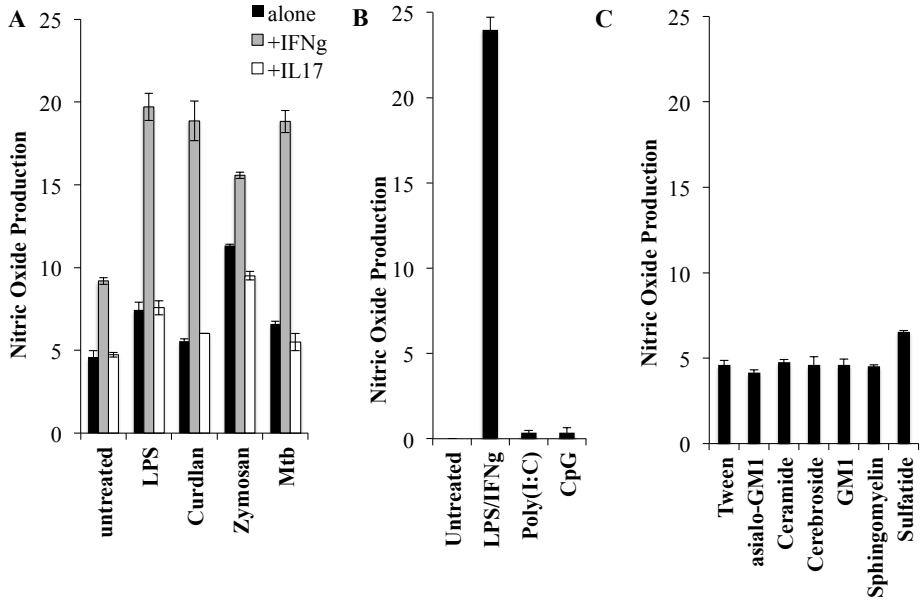

Figure R4. Different bacterial, fungal, and yeast stimuli can induce bone marrow generated myeloid derived suppressor cell activation, whereas nucleic acids and self glycolipids cannot. A | D3 C57Bl/6.BM- IMC were stimulated with different bacterial or fungal stimuli in the presence of T effector cell cytokines, IFN-g or IL-17, overnight or left unstimulated. Supernatants were analyzed for NO production (Nitrites, μM). Graph is representative of 3 independent experiments. **B |** D3 C57Bl/6.BM-IMC were stimulated with nucleic acids, CpG and poly (I:C) or left unstimulated. Supernatants were analyzed for NO production. **C |** D3 C57Bl/6.BM- IMC cultured in the presence of

GM-CSF were stimulated with self glycolipids or left unstimulated. Supernatants were analyzed for NO production. No significance was reached by student T-test.

As LPS and IFN-g signals are known to activate BM-IMC in a synergistic manner, we wanted to investigate if similar effector cytokine-pathogen combination can stimulate BM-IMC under Th17 conditions. Therefore, BM-IMC were stimulated using a combination of IL-17 with the pathogenic stimuli. We did not see any increased NO production (Figure R4), whereas the NO production by all pathogens tested could be boosted by IFN-g (Figure R4). We conclude the combination of pathogen stimuli, such as LPS, curdlan, zymosan A, and mycobacteria, with IFN-g work in a synergistic effect to boost the suppressive capacity of BM-IMC into BM-MDSC through production of NO, whereas Th17 cytokines do not.

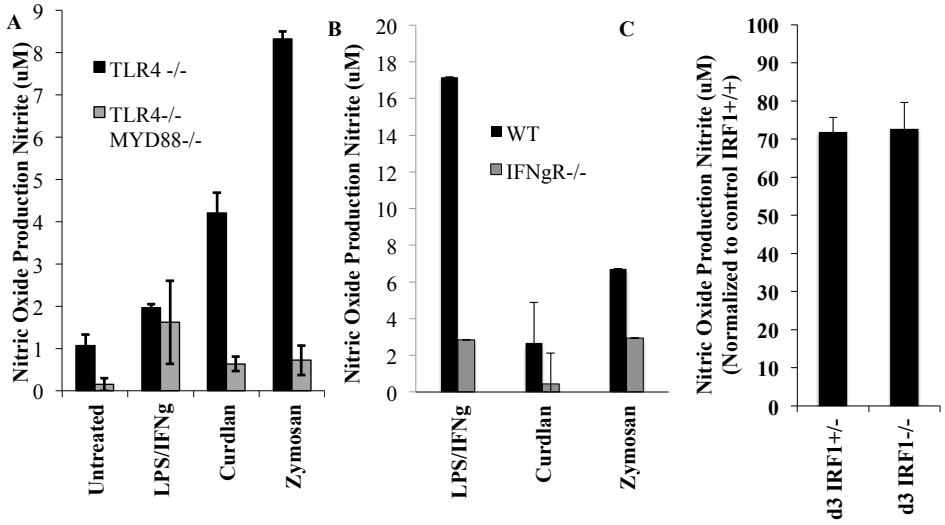

Figure R5. Different stimuli can induce nitric oxide production by BM-MDSC through MyD88, IFN-gR1, and partially through IRF-1 signalling. A | D3 BM-IMC from mice lacking TLR4 and MyD88 signalling molecules were stimulated with different pathogenic overnight or left unstimulated. Supernatants were analyzed for NO production. **B |** D3 BM- IMC lacking IFN-gR1 were stimulated with different pathogenic overnight or left unstimulated. Supernatants were analyzed for NO production. **C |** D3 BM-IMC deficient in one (IRF-$1^{+/-}$) or both alleles (IRF-$1^{-/-}$) of IRF-1 were stimulated with LPS/IFN-g overnight or left unstimulated. Supernatants were analyzed for NO production. Results of two independent experiments were normalized to stimulated control $IRF^{+/+}$ BM-IMC. Decrease reaches significance by student T-test.

As some links have been associated between viral pathogens and inflammation to MS, therefore the question arises if viral pathogens or dead tissue can activate MDSC to become suppressive. To analyze if viral nucleic acids or if loose nucleic acids which may become available during infection and inflammation by release of intracellular contents by dead or necrotic cells, may activate the MDSC (Barker et al., 1999; Gallucci et al., 1999; Sauter et al., 2000), stimulation with dsDNA, such as CpG, and dsRNA, such as poly (I:C) was performed. In accompaniment with damaged myelin sheath, self glycolipids were observed within the CNS during active lesions in MS. To understand if this may activate MDSC, BM-IMC were stimulated with a small panel of self glycolipids. Suprisingly, neither material from nucleic acids, nor self

glycolipids (Figure R4) could not activate the MDSC to produce NO. We conclude that pathogens, but not cellular material alone could activate MDSC.

As this boosting effect was only seen upon IFN-g administration, we wanted to identify the signalling mechanisms in MDSC to induce NO production. Myeloid differentiation primary response gene (MyD88) has been shown to be required for activation of MDSC *in vivo* under septic conditions (Delano et al., 2007). To investigate if Th17 stimuli activate BM-IMC iNOS through TLR-MyD88 signalling pathway or if they can alternatively signal through dectin-1, we performed experiments employing BM-IMC generated from $TLR4^{-/-}$ mice and $TLR4^{-/-}$ $MyD88^{-/-}$ mice. Overnight treatment with of $TLR4^{-/-}$ mice and $TLR4^{-/-}$ $MyD88^{-/-}$ BM-IMC with pathogenic stimuli was performed and NO production was detected by Griess reagent. BM-IMC NO production in by all stimuli tested is induced through the MyD88-TLR pathway (Figure R5). The model has been proposed that MDSC require two signals, MyD88 and IFN-g signaling, that are required for activation of iNOS for subsequent suppressive function. Therefore, we wanted to analyze the requirement of IFN-g signaling on the activation of BM-IMC. To do this, we performed an experiment generating BM-IMC from IFN-$gR1^{-/-}$ mice. BM-IMC were incubated overnight in the presence of bacterial stimuli and analyzed the NO production. Without IFN-gR1, BM-IMC cannot produce NO (Figure R5). Therefore, both the proposed multi-signal requirement for activation of iNOS by BM-IMC is validated. To investigate downstream signaling leading to the activation of iNOS, experiments were performed with BM-IMC from one or both alleles of IRF-1 knockout were analyzed for NO production. Upon activation with LPS / IFN-g, both IRF-1 knockout mice had equalivently reduced levels of NO production, roughly 30% (Figure R5). Indicating IRF-1 has a limited role in induction of iNOS, but their must be other players involved in the downstream signalling.

4.4 Interferon-beta, used in multiple sclerosis drug therapy, does not influence myeloid derived suppressor cell activation or suppressive capacity.

Type I interferons play an important role in regulating disease severity. MS patients receiving IFN-b reduces the severity of clinical symptoms. Furthermore, Prinz et al. (Prinz et al., 2008) has demonstrated that IFN-aR deficient mice have stronger EAE disease severity correlating with a CD11b population. Interestingly, IFN-a has been shown to block MDSC suppressive function *in vivo* (Zoglmeier et al., 2011). Therefore, we wanted to investigate the effects of poly (I:C), which have been determined to induce IFN-b production in other cell types, such as DC (Okahira et al., 2005) and IFN-b itself directly on MDSC. Therefore, to elucidate if IFN-b might activate or prevent activation of MDSC, BM-IMC were stimulated with IFN-b and poly (I:C) overnight in the presense or absence of the activating stimuli LPS/IFN-g. NO production was analyzed by Griess Reagent. NO production was unaltered compared to the LPS/IFN-g treated BM-IMC, and IFN-b treatment alone was comparable to untreated BM-IMC (Figure R6). We conclude IFN-b does not alter

BM-IMC activation to produce NO. To discover if IFN-b may play a role in stimulating or inhibiting MDSC function through other mechanisms besides NO, the suppressive capacity in the presense of IFN-b was assessed. No change in suppressive capacity was observed (Figure R6). It is unknown if BM-MDSC themselves may produce sufficient amounts of IFN-a/b, which may cause this unaltered suppressive capacity upon the presence of exogenous IFN-b, as it possibly could already be at saturating levels. BM-IMC activation by IFN-a or IFN-b through their receptor IFN-aR was tested using knockout mice. Suppressive capacity by NO production and a functional suppression assay both confirmed BM-MDSC functionality was unaltered (Figure R6C). Therefore, we can conclude that IFN-b therapy may not directly influence the MDSC alone during treatment of MS, as BM-IMC activation and suppressive capacity is unaltered in its presence.

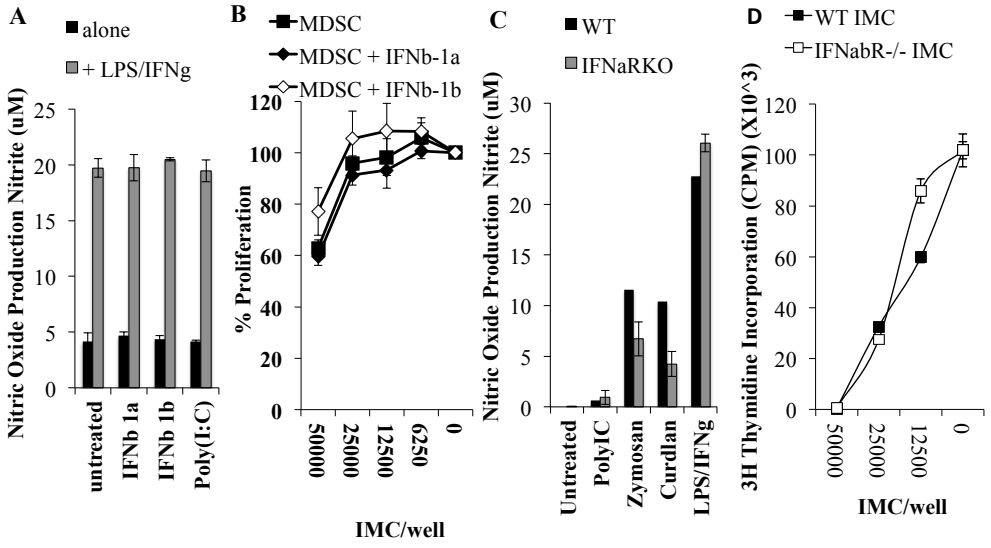

Figure R6. Bone marrow generated myeloid derived suppressor cell are not influenced by interferon-beta. **A |** D3 C57Bl/6 BM-IMC were stimulated with IFN-b-1a, IFN-b-1b, or poly (I:C) in the presence or absence of LPS/IFN-g overnight or left unstimulated. Supernatants were analyzed for NO production by Griess Reagent. Student T-test was performed no statistical difference was found. Results based on 2 separate experiments, one representative experiment shown. **B |** D3 BM-IMC from C57Bl/6 mouse were added to stimulated splenocytes in the presence or absense of IFN-b-1a and IFN-b-1b. Radioactive [^{3}H] thymidine was used to assess proliferation. No significant differences as performed by student T-test. Results of two separate experiments. **C |** D3 BM-IMC from IFN-aR$^{-/-}$ mice were stimulated with IFN-b overnight or left unstimulated. Supernatants were analyzed for NO production by Griess Reagent. Student T-test was performed, no statistical difference was observed. Results based on 3 mice. **D |** D3 BM-IMC from C57Bl/6.IFN-aR$^{-/-}$ or C57Bl/6.WT mice were added to C57Bl/6 splenocytes stimulated with anti-CD3/anti-CD28. Radioactive [^{3}H] thymidine was used to assess proliferation. No statistical difference was observed by student T-test. Results of 3 mice.

4.5 Both *in vitro* generated and endogenous myeloid derived suppressor cells specifically upregulate B7-H1, but no change occurs in co-stimulatory markers on both subsets 24h after pathogen stimulation.

To investigate potential cell-to-cell contact mechanisms BM-MDSC employ, analysis

of BM-MDSC surface expression was performed. One would hypothesize, as BM-IMC are suppressive, co-inhibitory markers rather than co-stimulatory markers would be expressed upon activation. Surface expression of MDSC after their activation by LPS, curdlan, zymosan was investigated by flow cytometry. Gating was performed individually on each subset, Mono-MDSC ($CD11b^+/Ly\text{-}6C^+$) and PMN-MDSC ($CD11b^+Ly\text{-}6C^+Ly\text{-}6G^+$). Both populations upregulated the expression of B7-H1, but did not increase any co-stimulatory markers such as CD40, CD80, CD86, CD70, or tolerance markers B7-H2 (Figure R7). As BM-IMC upregulate B7-H1 upon stimulation, we checked the expression of this marker on endogenous splenic MDSC, to determine if similar things happen *in vivo*. EAE induction was performed on day 0. After 24 h, spleens from both EAE and untreated mice were re-stimulated for 4 h with PMA/Iono. MDSC populations were assessed by FACS for expression of B7-H1. During steady-state conditions, there is no B7-H1 expression on the splenic fraction of MDSC, but one day post EAE induction, increased B7-H1 expression and a downregulation of CD86 (Figure R8) was observed upon restimulation. To understand if BM-IMC changes the balance by increasing the amount of B7-H1 in the system, spleens were examined according to the same protocol, but administering a BM-IMC injection three days prior to EAE induction. In this case, splenocytes receiving BM-IMC had no differences (Figure R8) compared to their untreated controls in their expression of B7-H1 intensity on MDSC.

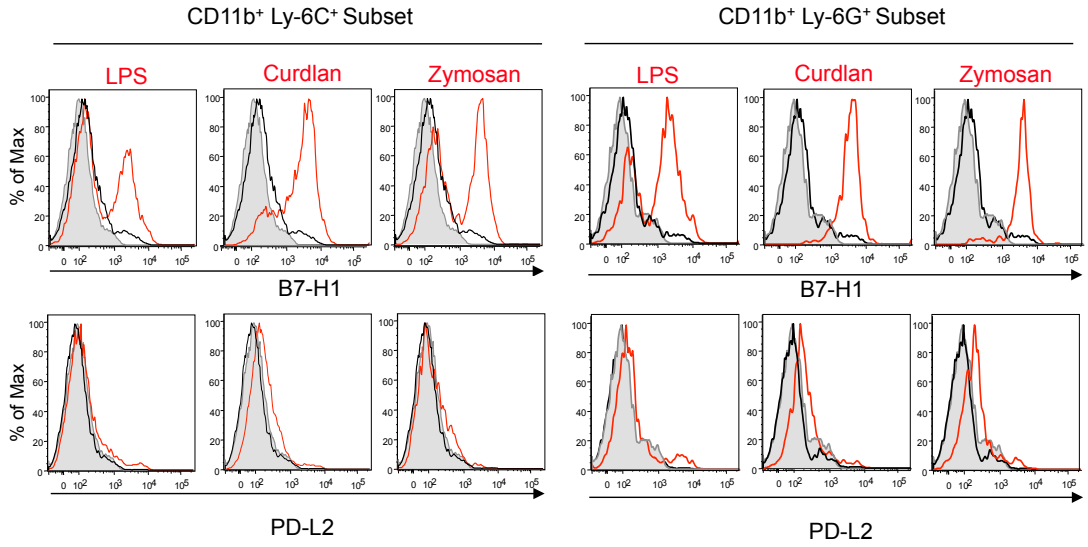

Figure R7. Phenotype of BM-MDSC from C57Bl/6 mouse. D3 C57Bl/6.BM-IMC stimulated with LPS/IFN-g, curdlan, or zymosan overnight or left unstimulated (black). Flow cytometry staining for activation markers gated upon MDSC subsets: CD11b+ Ly-6C+ Ly-6G+ or CD11b+ Ly-6C+ Ly-6G-, B7-H1, and PD-L2. Isotype control is depicted in grey. Result of 3 independent experiments. Results of one experiment shown.

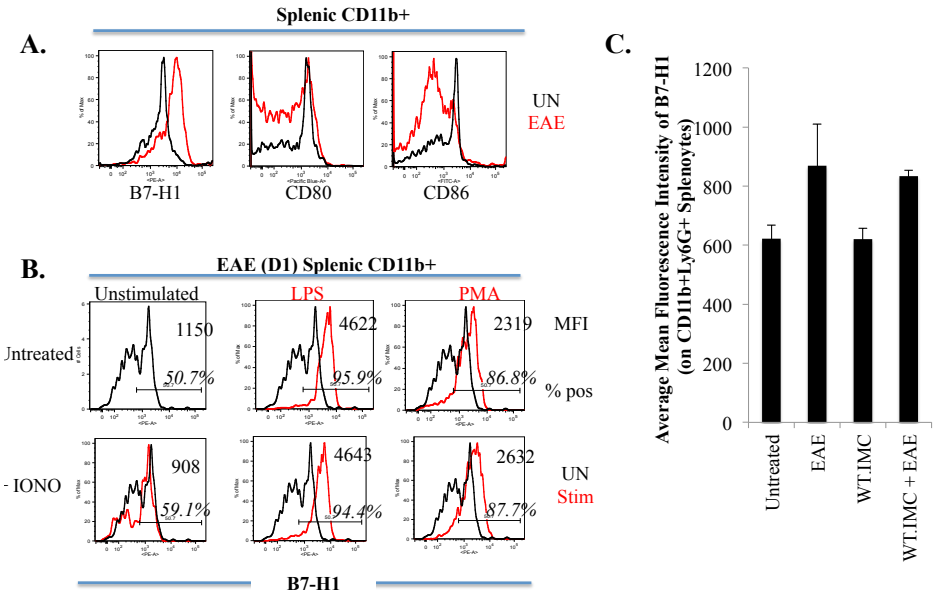

Figure R8. Phenotype of Endogenous MDSC from C57Bl/6 mouse after activation. A | C57Bl/6 mice were either induced with EAE (D0) or left untreated. Splenocytes were restimulated on D1 *ex vivo* using PMA/Iono for 4 h. Surface markers of MDSC were assessed as shown pre-gated on $CD11b^+$ $Ly\text{-}6C^+$ $Ly\text{-}6G^+$ or $CD11b^+$ $Ly\text{-}6C^+$ $Ly\text{-}6G^-$. Results of 2 independent experiments. One experiment shown. **B |** C57Bl/6 mice were either induced with EAE (D0). Spleens were restimulated on D1 for 4 h with PMA or LPS in the presence or absence of Iono, expression of B7-H1 on MDSC was assessed. Results of 2 independent experiments. One experiment shown. **C |** Mice were injected with BM-IMC three days prior to induction of EAE (D0). Spleens were restimulated on D1 for 4 h with PMA/Iono, B7-H1 expression of MDSC was assessed. Results of 3 mice/group.

4.6 Bone marrow generated immature myeloid cells injected into mice cause suppression of experimental autoimmune encephalomyeolitis induced by myelin oligodendrocyte glycoprotein$_{35\text{-}55}$.

To establish a possible role for MDSC as a cellular therapy in autoimmune diseases BM-IMC were injected D-3 before EAE injection to assess the ability to suppress the disease severity *in vivo*. Compared to WT controls, the clinical score of MDSC injected mice were lower (Figure R9). On the peak day, day 14, mice were sacrificed and underwent further evaluation, including analysis of infiltrates in the CNS, including brain and spinal chord sections, and in the spleen. Immune infiltrates were analyzed within the CNS and spleen (Figure R9) through intracellular cytokine analysis, and also the organs were placed embedded into OCT, a compound used for freezing your tissue, for immunofluorescence stainings. Further analysis revealed there were slightly increased infiltrates within the CNS from untreated as compared to MDSC injected mice WT-BM-IMC, while analysis of the spleens showed an increased overall cellularity from MDSC injected mice (Figure R9). These infiltrates showed no change in the pathogenic cytokines, IFN-g and IL-17, in MDSC injected mice in neither the IL-17, nor the IFN-g after restimulation with PMA/Iono (Figure R10). To evaluate the ability of BM-IMC to suppress EAE, various changes were performed to the protocol. The evaluation of BM-IMC injected via various route was performed. The protection between the two delivery methods (i.p. vs i.v.) showed comparable protection as compared to the EAE control group. Next, we attempted to

assess whether pre-activated BM-MDSC for 4 h with LPS / IFN-g instead of untreated BM-IMC would protect more as BM-MDSC produce nitric oxide. Pre-activated BM-MDSC seemed to protect via a delay, but not in severity, we believe this intermediate interaction may due either to changes in integrins after activation causing a differential homing or due to the activated BM-MDSC secretes the NO before it reaches the location where BM-IMC suppress, where BM-IMC become activated and suppress.

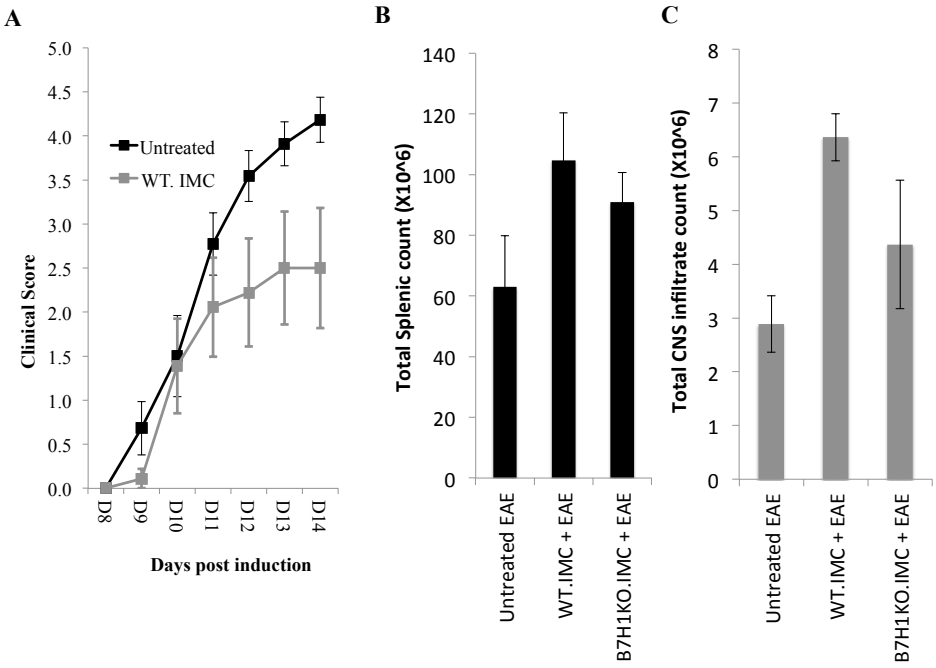

Figure R9. Brain section of EAE mice show increased infiltrates

A | Mice were allowed to develop clinical symptoms until the peak of the disease (day 14), then mice were sacrificed, the **B |** spleen and **C |** CNS were removed, lymphocytes were isolated by collagenase NB4 and DNase I digestion and overlayed on a percoll gradient and cell numbers were assessed. Mice were allowed to develop clinical symptoms until the peak of the disease (day 14), then mice were sacrificed, CNS were removed and counted. Significant differences, as performed by student T test, between control untreated EAE and BM-IMC treated EAE mice were obsesrved at later time points Days 12 – 14, $p<0.05$.

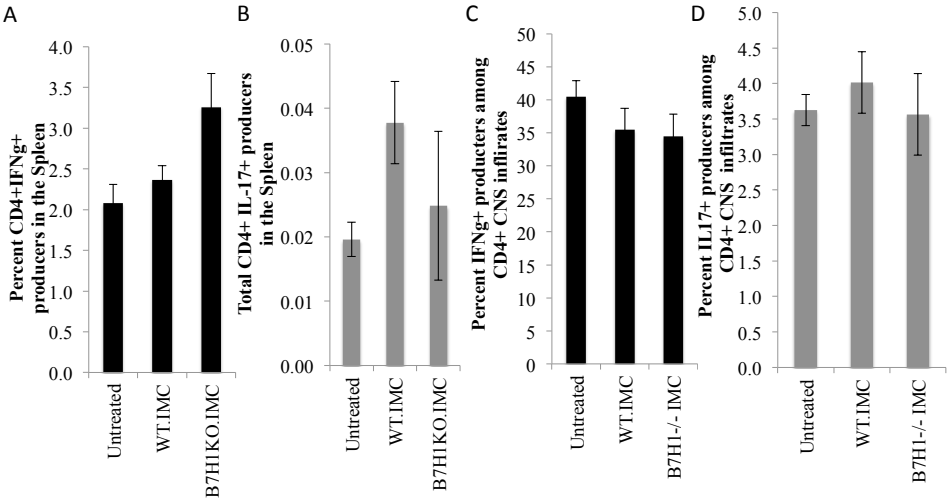

Figure R10. Bone marrow generated immature myeloid cells may slightly protect EAE mice.
A / B | Mice were induced with EAE. At the peak of the disease (day 14) mice were sacrificed and spleens were restimulated with PMA/Iono in the presence of brefeldin A for 4h. ICC was performed to assess the amount of IFN-g (A) and IL-17 (B) production in CD4 cells. Significant was assessed by student T test. This is one representative experiment of 2. **C / D |** Mice were induced with EAE. At the peak of the disease (day 14) mice were sacrificed CNS isolated. Digestion was performed with collagenase NB4 and DNase I digestion and overlayed on a percoll gradient. Cells were restimulated for 4 h with PMA/Iono and brefeldin A. IFN-g (C) and IL-17 (D) production was examined by intracellular cytokine staining.

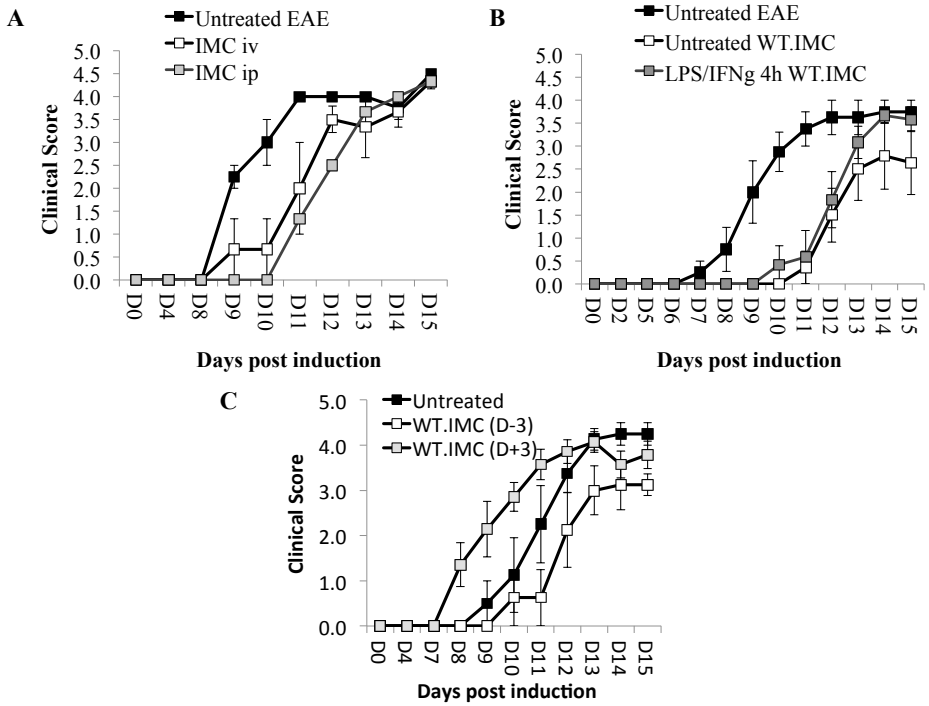

Figure R11. Bone marrow generated immature myeloid cells slightly protect and/or delay onset of EAE in mice without regard to route of administration, but may be affected by activation status and timing.
A | C57Bl/6 mice were injected with BM-IMC/PBS (i.v. or i.p.; Day -3) then MOG- EAE was induced (Day 0, 2). Clinical Score was assessed (3mice/group). A significant delay was observed, significance analyzed by day using student T-test. **B |** C57Bl/6 mice were injected with BM-IMC or BM-IMC stimulated 4h with LPS/IFN-g (i.v.; Day -3) then MOG-EAE was induced (Day 0, 2). Clinical Score was assessed (3 mice/group). A significant delay was observed, significance analyzed by day using

student T-test. **C |** C57Bl/6 mice were injected with BM-IMC (iv; Day -3, Day +3) then MOG-EAE was induced (Day 0, 2). Clinical Score was assessed. A significant delay and decreased severity was observed with our Day -3 cells (3 mice), but earlier onset was observed upon IMC treatment on Day+3 (7 mice) Significance analyzed by day using student T-test.

4.7 Increased suppression of specific memory cells by bone marrow generated immature myeloid cells is dependent upon the expression of PD-1 on splenocytes *in vitro*.

To test the ability of MDSC suppression by the B7-H1 : PD-1 interaction, analysis of naïve T cells was performed by a suppression assay utilizing B7-H1 deficient BM-IMC (Figure R12) with OTII specific T cells. No difference in suppressive capacity was observed. Similar results were also observed upon stimulation of allogeneic (Balb/c) splenocytes stimulated with antibody (data not shown). As PD-1 functionality has been described in the phenotype of cells during chronic diseases and infections, a suppression assay utilizing memory cells was performed. $PD1^{-/-}$ and wildtype splenocytes were taken at the peak of EAE and restimulated specifically or aspecifically with antibodies, suppression by BM-IMC was assessed. There were no significant differences seen between the groups (Figure R12). C57Bl/6 mice induced with MOG-EAE were sacrificed on day 14, spleens were re-stimulated with MOG peptide. BM-IMC stained with Ly-6G-biotin, followed by the addition of streptavidin microbeads. MACS separation was performed to obtain PMN-MDSC and Mono-MDSC subsets. These subsets were fixed for 30 minutes to allow assessment of only surface cell-cell contact mediated interactions. To fully assure our fixative did not alter the proliferating T cells, cells were incubated in media overnight. These fixed-MACS-sorted D3-BM-IMC subsets from C57Bl/6.WT or $C57Bl/6.B7\text{-}H1^{-/-}$ mice were added to the restimulated splenocytes. Proliferation was assessed by radioactive [^{3}H] thymidine. Proliferation showed lack of B7-H1 releases BM-IMC regulation on antigen specific T cells (Figure R13).

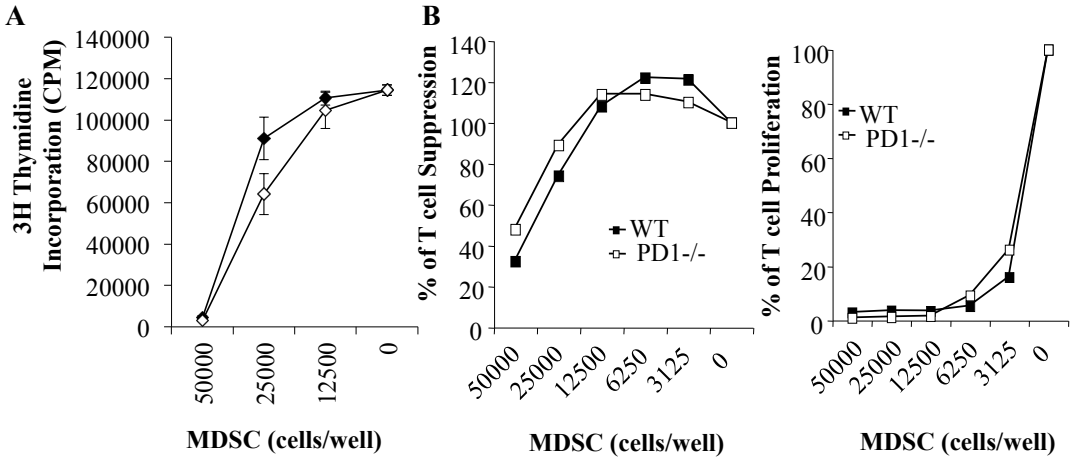

Figure R12. Bone marrow generated immature myeloid cells suppress T cells with no influence of PD-1

A | C57Bl/6 (filled) and B7-H1 knockout (open) BM-IMC were titred into OVAII peptide (1uM) stimulated OTII splenocytes. Cells were pulsed with radioactive [^{3}H] thymidine to assess proliferation.

Results of 2 experiments are shown. Statistics was performed using a student T-test. P values were not of significance. **B |** C57Bl/6 and PD-1 knockout mice were induced with EAE (Day 0, 2). At the peak (day 14), splenocytes restimulated specifically with MOG peptide (left) and aspecifically using aCD3/aCD28 (right), in the presence of BM-IMC. Cells were pulsed with radioactive [^{3}H] thymidine to assess proliferation. Results of 3 mice are shown. Statistics was performed using a student T-test. P values were not of significance.

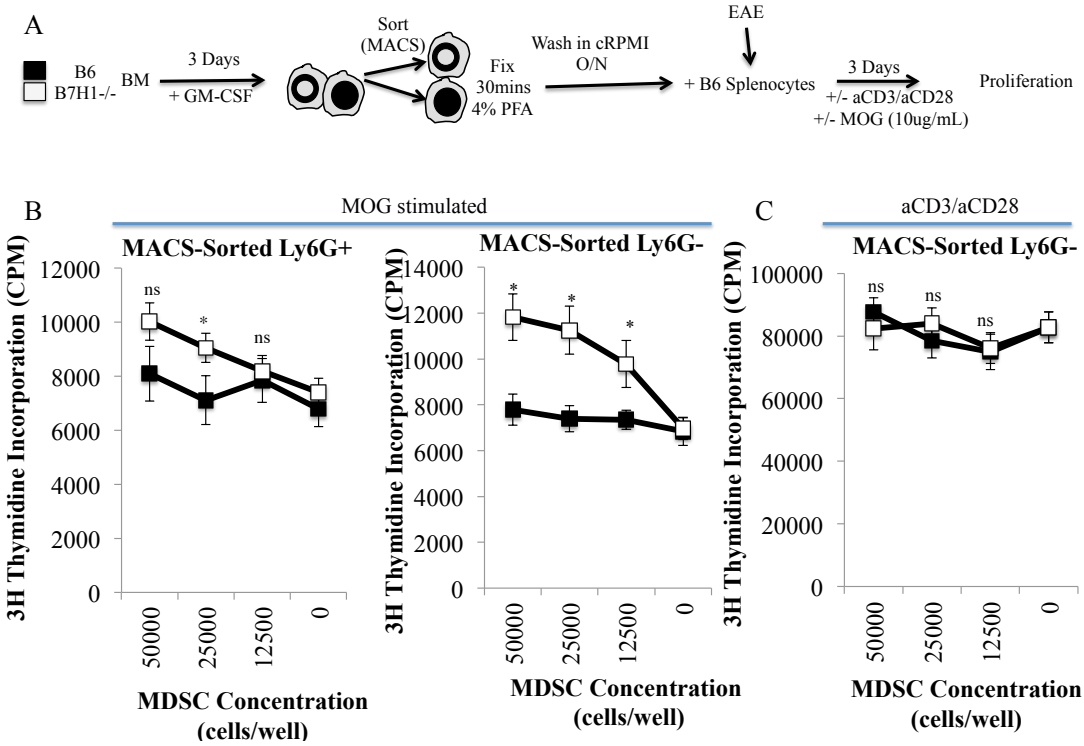

Figure R13. Bone marrow generated immature myeloid cells suppress memory cells more than effector cells through interaction between B7-H1 and PD-1

A | C57Bl/6 mice were induced with EAE (Day 0, 2). At the peak (day 14), splenocytes restimulated specifically using MOG peptide, in the presence of fixed-MACS-Sorted Ly-6G+ and Ly-6G- subsets from BM-IMC of either B7-H1 deficient or compentent mice. Cells were pulsed with radioactive [^{3}H] thymidine to assess proliferation. Results of 3 mice are shown. Statistics was performed using a student T-test. Significant differences were found between the B7-H1 knockout and wildtype BM-IMC ($p<0.05$). A trend was observed for the Ly-6G+ subset, but p values were not all of significance. **B |** C57Bl/6 mice were induced with EAE (Day 0, 2) and allowed to develop disease until the peak (day 14). Splenocytes restimulated aspecifically using anti-CD3/anti-CD28 peptide, in the presence of fixed-MACS-sorted Ly-6G- subsets from BM-IMC of either B7-H1 deficient or compentent mice. Results of 3 mice are shown. Cells were pulsed with radioactive [^{3}H] thymidine to assess proliferation. Statistics was performed using a student T-test. P values were not of significance.

4.8 Bone marrow generated immature myeloid cells expression of B7-H1 does not change the ability to suppress experimental autoimmune encephalomyelitis severity, but PD-1 expression in recipient mice does.

As MDSC increase expression of B7-H1 upon activation was already demonstrated both *in vitro* and *in vivo* and B7-H1 expression was required for regulation of memory cell proliferation *in vitro*, assessment of the B7-H1 : PD-1 interaction *in vivo* still needed to be elucidated. To confirm this interaction C57Bl/6 and C57Bl/6.B7-H1$^{-/-}$

BM-IMC were injected into mice (iv; Day -3), followed by EAE induction (Day 0, 2). Clinical Score was assessed. This allowed for protection by both groups (Figure R14). Although B7-H1 on BM.IMC seemingly does not play a role in the suppression of EAE, confirmation was performed utilizing splenocytes from PD-$1^{-/-}$ mice. Injection of BM-IMC into C57Bl/6 or C57Bl/6.PD-$1^{-/-}$ mice (iv; Day -3), EAE (Day 0, 2) induced, and Clinical Score was assessed. Demonstrating protection only in C57Bl/6 mice, indicates a role of MDSC suppression of EAE via interaction with PD-1 (Figure R14).

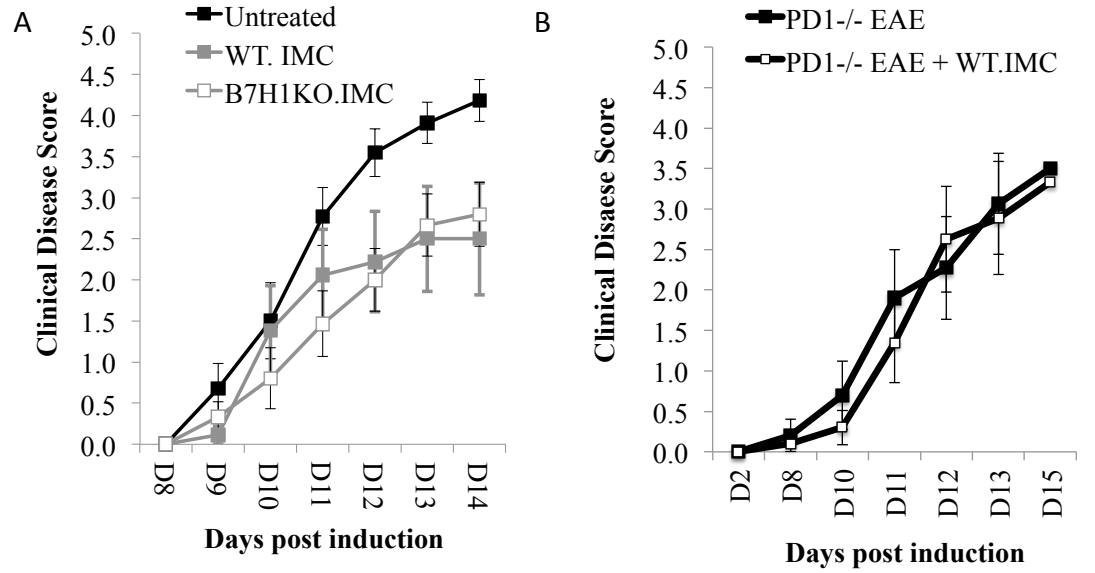

Figure R14. Direct or indirect interaction with PD-1 may play a role in BM-IMC suppression *in vivo*, whereas B7-H1 expression itself upon the BM-IMC does not influence protective capacity in EAE.

A | C57Bl/6 mice were injected with B7-H1-/- or WT BM-IMC/PBS (iv; Day -3) then MOG- EAE was induced (Day 0, 2). Clinical Score was assessed. Significant decrease in severity was observed in both groups. Statistical analysis was performed by student T-test. **B |** C57Bl/6 or C57Bl/6.PD-$1^{-/-}$ mice were injected with C57Bl/6 BM-IMC/PBS (iv; Day -3) then MOG-EAE was induced (Day 0, 2). Clinical Score was assessed. No difference between the groups was observed by student T-test.

4.9 Kinetics of bone marrow generated immature myeloid cells accumulate in the spleen, homing to the red pulp and marginal zones.

To identify BM-IMC migration and localization *in vivo* after intravenous injection, bioluminescence imaging (BLI) techniques were employed. To utilize this technique, BM-IMC can be generated from mice containing a vector encoding for the enzyme luciferase. In the presence of luciferin, luciferase will cleave the luciferin into products emitting photons (luminescence). The detection of photon emission can be detected using a special camera system, allowing daily assessment of migration and localization of the injected BM-IMC within the same mouse. BLI, a safer method as compared to radioactive labeling, has the advantage of one detecting only live cells, however the method itself lacks some sensitivity. If cells are in low quantities and/or diluted over the whole body, detection is not possible. As BM-IMC may not proliferate after injection, increased amounts of BM-IMC were injected to allow for proper detection. As black hair may absorb some signal, C57Bl/6.albino mice were

used as recipients. To understand BM-IMC localization of BM-IMC in steady-state mice, mice were injected with Luc+BM-IMC. After 24h, BM-IMC were anaesthesized, luciferin was injected observed in the lungs and spleen *ex vivo* (Figure R15). To understand their homing during steady-state or inflammatory, kinetics of Luc+BM-IMC migration in untreated and EAE mice were assessed daily subsequent to the injection of BM-IMC. It was observed that BM-IMC accumulate in the spleen during steady-state conditions, whereas during inflammation, the cells are not only recruited to the spleen but also recruited to the site of inflammation (the subcutaneous injection of CFA) and to the site draining the inflammed immune priviledged organ, the cervical lymphnode (Figure R16). To understand if BM-IMC can actively home from tissue to the spleen, BM-IMC subsets were inject sc in the footpad. Migration was analyzed, determining BM-IMC travel to the draining lymph node (Figure 17). We speculate BM-IMC cannot exit or possibly may differentiate into resident lymph node cells.

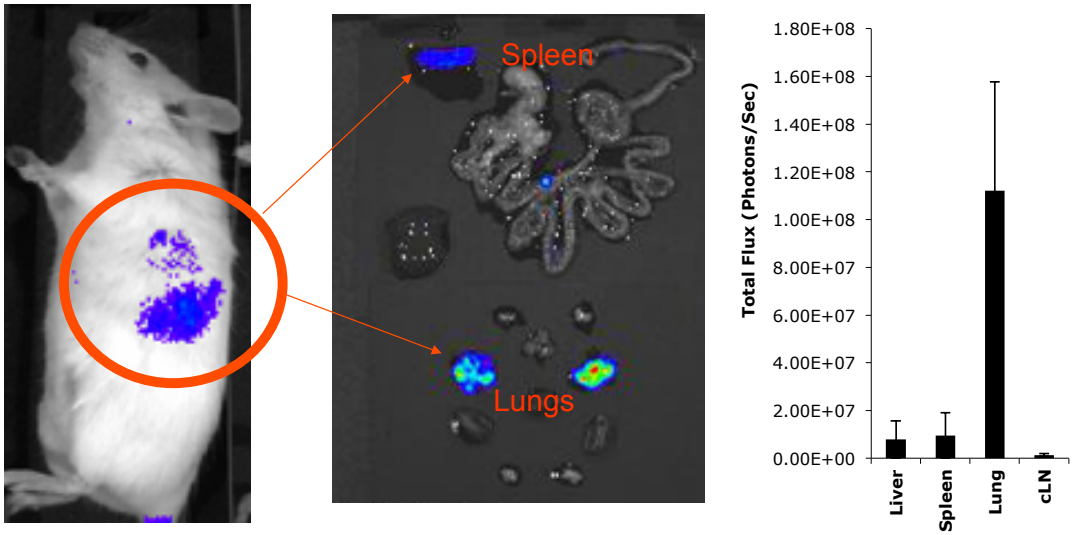

Figure R15. Imaging shows bone marrow generated immature myeloid cells accumulate mostly in lungs and spleen after 24h

C57Bl/6.albino mice were injected with Luc+BM-IMC 24h after i.v. administration *in vivo* and *ex vivo* imaging was performed upon injection of luciferin. Data representative of 3 separate mice, one mouse *in vivo* and *ex vivo* image depicted. Analysis performed on 3 separate mice *ex vivo* organs was performed. Lumenescence quantified per organ as photons detected/second.

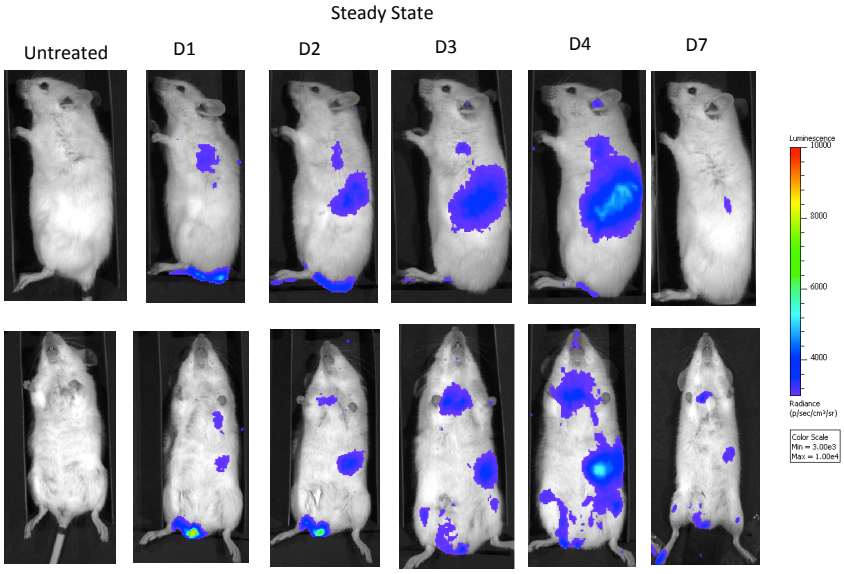

Figure R16. Imaging depicting kinetics of BM-IMC migration *in vivo*, suggests bone marrow generated immature myeloid cells accumulate in spleen after 4 days.
C57Bl/6 albino mice were injected with Luc+ BM-IMC. BLI imaging was performed daily upon injection of luciferin. Mice with low grade EAE (score 2; tail paralysis) were also injected and imaged. Data displayed of one mouse, but representative distributation of several mice.

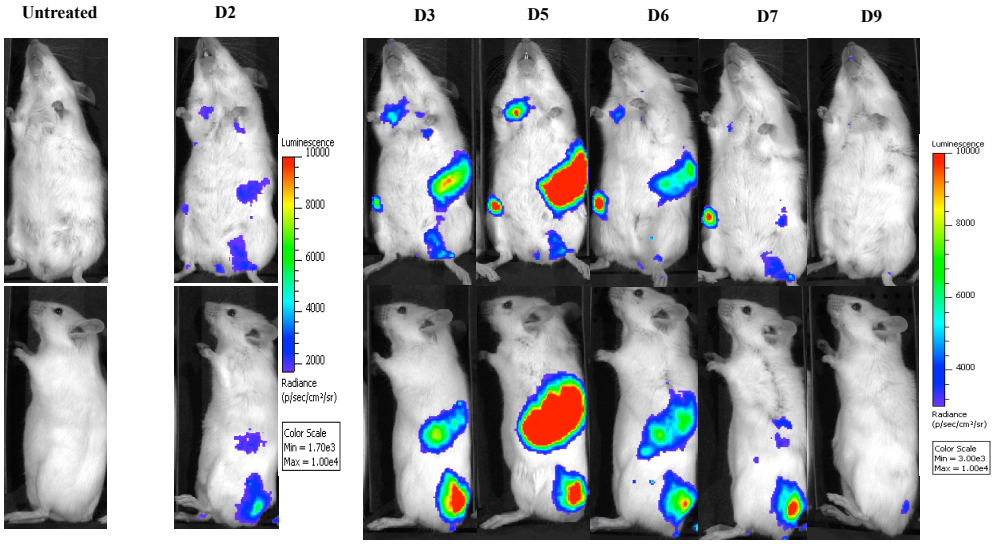

Figure R17. Imaging illustrates in addition to spleen, bone marrow generated immature myeloid cells seemingly are recruited to CFA/MOG injection site and draining lymph nodes.
A | C57Bl/6 albino mice were injected s.c. in one foot pad with FACS-Sorted Luc+ mono-BM-IMC and PMN-BM-IMC or bulk. BLI imaging was performed 24h after injection. Data representative of two experiments, one mouse depicted.

To attempt to quantify the BM-IMC and characterize BM-IMC, *ex vivo* analysis of the organ was performed. BM-IMC labeled with CM-DiI, a dye which labels membranes, were injected i.v. into C57Bl/6 mice. After 4 days, a single cell spleens suspension was lysed of RBC. A white collagenous "blob" arises within the cell suspension. This "blob" was placed on a microscope slide for analysis. As seemingly all CM-DiI BM-IMC were found within this "blob," no cells were discovered via flow cytometry

for PE (Figure R18). To understand BM-IMC localizing within the spleen, fluorescence microscopy was employed. CM-DiI BM-IMC injected i.v. into C57Bl/6 mice were allowed to migrate in vivo for 4 days, thereafter spleens were fresh frozen for cryosection analysis. CM-DiI BM-IMC were seeming located within the marginal zone and red pulp areas of the spleen, but not in the white pulp (Figure 18).

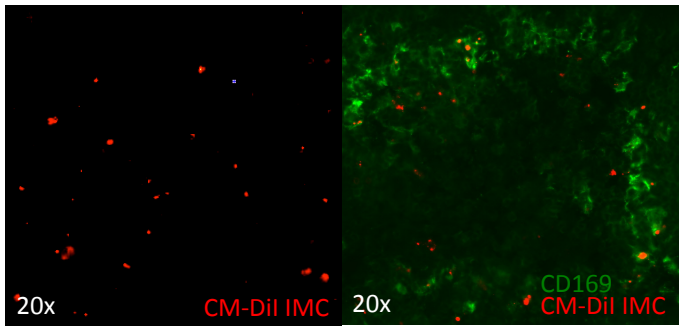

Figure R18. Imaging shows bone marrow generated immature myeloid cells accumulate in spleen after 4 days, localizing to outside the white pulp.
A | C57Bl/6 mice were injected with CM-DiI labelled BM-IMC. On day 4, spleen was digested and lysed. A white "blob" structure was observed within the single cell suspension was placed onto a microscope slide and analysed for PE fluorescence. **B** | C57Bl/6 mice were injected with CFSE labelled BM-IMC. After 4 days, spleens were removed and fresh frozen in OCT. Staining was performed using CD169. Sections were analyzed for CM-DiI BM-IMC (Swirski et al.) and CD169 (Green), localization seems restricted to outside the white pulp.

4.10 Bone marrow generated immature myeloid cells induce increased spleen cellularity by induction of proliferation of splenic cells.

BM-IMC localize in the spleen, but their mechanism of action to suppress was still unclear. Therefore, to investigate this mechanism BM-IMC were injected i.v. into C57Bl/6 mice. After 4 days, mice were induced with EAE. On day 7 after induction, spleens were removed and counted. We found an increase of cell numbers within the spleen (Figure R19). As $PD1^{-/-}$ mice were not protected, we analyzed the spleen counts. There were no differences observed between BM-IMC injected and untreated EAE spleens in PD-1 deficient recipient mice (Figure 19), indicating that the increase observe may cause protection. Further assessment of the B7-H1 interaction with PD-1 as a possible role in the increases in spleen cellularity was performed as BM-IMC from mice lacking B7-H1 or WT were injected into EAE mice on Day 5 after induction and assessed the spleens at day 9. No differences between the WT-BM-IMC and the $B7\text{-}H1^{-/-}$ BM-IMC in comparison to spleen size (Figure 19) were observed. As BM-IMC delivered after EAE were not protective, it might suggest protection is correlated to timing or the locality of particular cells in which BM-IMC interact.

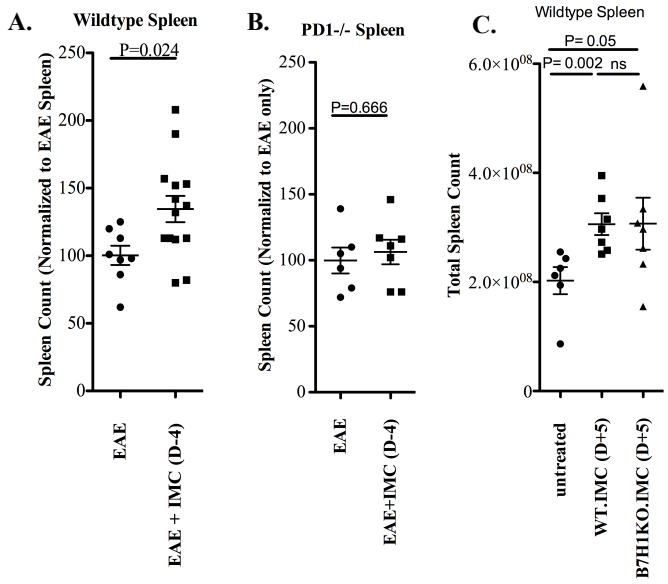

Figure R19. Increased spleen cellularity in wildtype, but not program death receptor 1 knockout mice injected with bone marrow generated immature myeloid cells.
A | C57Bl/6 mice were injected with BM-IMC (D-4), followed by induction of EAE (D0, D2), spleens were removed and counted. Cell numbers were normalized to the control EAE spleen. Statistics was performed using a student T-test. **B |** C57Bl/6 and C57Bl/6.PD-1$^{-/-}$ mice were injected with BM-IMC (D-4), followed by induction of EAE (D0, D2). Day 7 after EAE induction, spleens were removed and counted. Cell numbers were normalized to the control EAE spleen. Statistics was performed using a student T-test. **C |** C57Bl/6 mice were induced with EAE (D0, D2). Mice were subsequently injected with BM-IMC from C57Bl/6.B7-H1$^{-/-}$ or C57Bl/6 mice (D+5), Day 9 after EAE induction, spleens were removed and counted. Statistics was performed using a student T-test.

In vitro, we noticed the effect of MDSC to induce proliferation (Figure R20) upon interaction of MDSC with resting T cells in the presence of a superantigen, staphylococcus enterotoxin B (SEB). This superantigen would force the interaction of the MHCII molecule of the MDSC to interact with the TCR of the T cell without antigen. Steady-state spleens should not have many activated T cells to suppress, and steady-state spleens are known not to have suppressive MDSC functionality, suggesting the effects of protection are more that of delivery of a tonic signal. To determine if the spleens are bigger because BM-IMC induces proliferation of cells within the spleen, BM-IMC were injected i.v. into mice. After 4 days, spleens were harvested, labelled with CFSE and placed in culture for 6 days unstimulated. Proliferation was assessed via flow cytometry. We found an increase of cell proliferation in CD11b, B220, CD4, and CD8 cells (Figure R21). Upon further analysis of the proliferating subsets of CD4, we found an increase in proliferation of CD4 T regulatory cells, as compared to CD4 effector cells (Figure R22).

To confirm this "tonic" signal by BM-IMC after injection instead of some activated function caused by soluble mediators, such as Arg-1 and NO, we compared EAE spleens to that of BM-IMC injected EAE spleens for the expression levels of Arg-1 (Figure 23). We found significant increases in the relative expression level. To analyze BM-IMC directly or indirectly effected the recruitment or proliferation, BM-

IMC lacking one of the signals controlling activation and subsequent effector function *in vitro,* IFN-gR1, or the effector function itself, iNOS, were knocked out. No size changes occurred between the size of the spleens in these groups and they displayed similar proliferation results to that of WT BM-IMC (Figure R24).

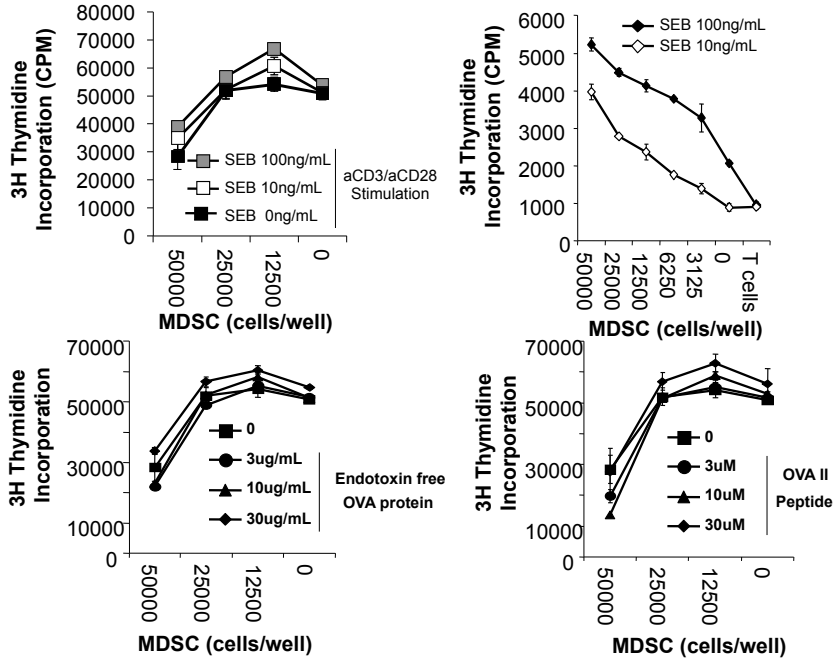

Figure R20. Bone marrow generated immature myeloid cells induce proliferation in unstimulated synergistic T cells upon addition of superantigen.

A | Spleens of untreated C57Bl/6 mice were stimulated with anti-CD3/anti-CD28 in the presence of SEB. BM-IMC were titred into the wells to assess suppressive capactiy. After 2 days, cells were pulsed with radioactive [^{3}H] thymidine overnight, proliferation was assessed. Results of two independent experiments, one shown. Statistical analysis by student T-test show no significant differences between the groups. **B |** Spleens of untreated C57Bl/6 mice were stimulated with SEB in the presence of BM-IMC. Wells were also seeded with only splenocytes without SEB as controls (labeled T cells in graph). After 2 days, cells were pulsed with radioactive [^{3}H] thymidine overnight, proliferation was assessed. Significant increases from control T cells were observed with increasing concentrations of MDSC. **C |** Splenocytes from OTII transgenic animals were activated by various concentrations of OVA protein to assess the ability of MDSC to process and present antigen to suppress specifically. No significant differences could be detected by student T test. **D |** Splenocytes from OTII transgenic animals were activated by various concentrations of OVAII peptide to assess the ability of MDSC to suppress specifically. No significant differences could be detected by student T test.

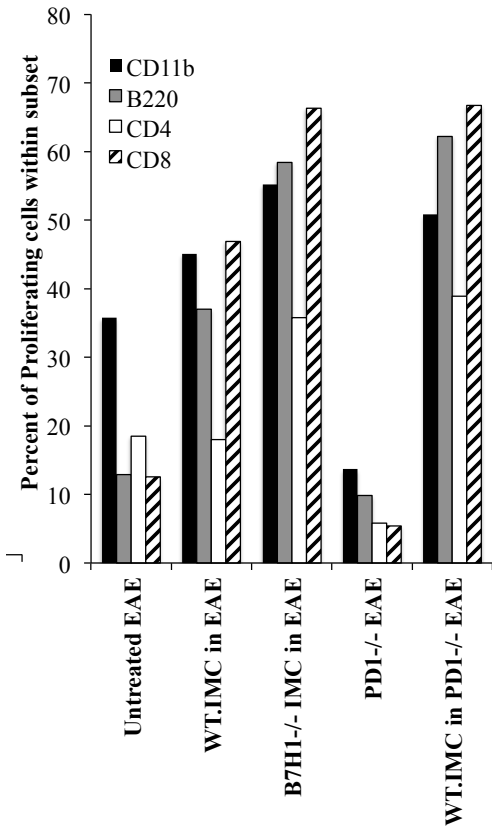

Figure R21. Proliferation of splenic cell subsets induced by injection of immature myeloid cells.
A | C57Bl/6 mice were injected with BM-IMC (D-4), followed by induction of EAE (D0, D2), spleens were removed and counted. **B |** C57Bl/6 and C57Bl/6.PD-1$^{-/-}$ mice were injected with BM-IMC (D-4), followed by induction of EAE (D0, D2). Day 7 after EAE induction, spleens were removed and counted. **C |** C57Bl/6 mice were induced with EAE (D0, D2). Mice were subsequently injected with BM-IMC from C57Bl/6.B7-H1$^{-/-}$ or C57Bl/6 mice (D+5), Day 9 after EAE induction, spleens were removed and counted. **D |** C57Bl/6 were injected with C57Bl/6.IFN-gR1$^{-/-}$, C57Bl/6.iNOS$^{-/-}$ or C57Bl/6 BM-IMC (D-4), were injected i.v. into C57Bl/6 mice. After 4 days, spleens were harvested, RBC were lysed with Gey's solution, labelled with CFSE and placed in culture for 6 days unstimulated. Proliferation was assessed via flow cytometry for CD11b, B220, CD4, and CD8 cells.

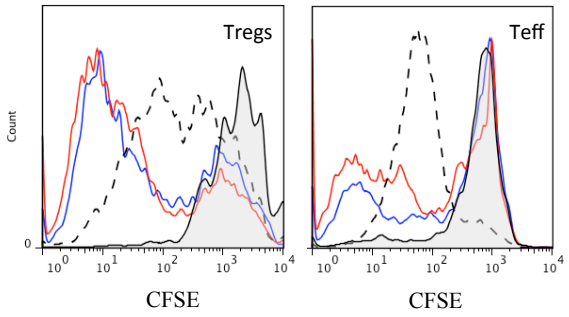

Figure R22. Bone marrow generated immature myeloid cells induce increased Foxp3 proliferation relative to T effector cells *ex vivo*.
A | BM-IMC were injected i.v. into C57Bl/6 mice. After 4 days, spleens were harvested, RBC were lysed with Gey's solution, labelled with CFSE and placed in culture for 6 days unstimulated. BM-IMC injected EAE mice (red, blue), un-injected EAE mice (grey), and *in vitro* stimulated with aCD3/anti-CD28 stimulated (dashed) proliferation of effector and regulatory T cells was assessed via flow cytometry. Data are representative of several experiments. One representative plot is depicted.

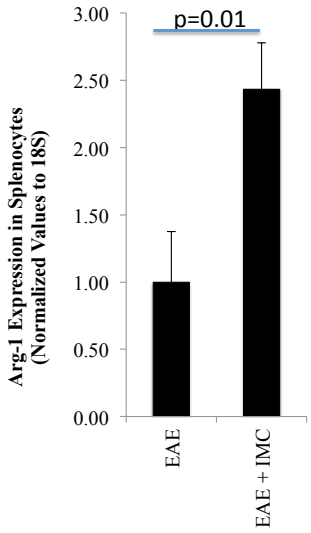

Figure R23. Relative arginase-1 expression is significantly increased in the spleens of EAE pre injected with bone marrow generated immature myeloid cells as compared to control EAE spleens.

BM-IMC were injected i.v. into C57Bl/6 mice. After 4 days, EAE was induced. 7 days after EAE inductions spleens were taken, RBC were lysed with Gey's solution, qRT-PCR was performed. Data is representative of 4 mice (EAE) and 7 mice (EAE+IMC). Statistical significance obtained by student T-test.

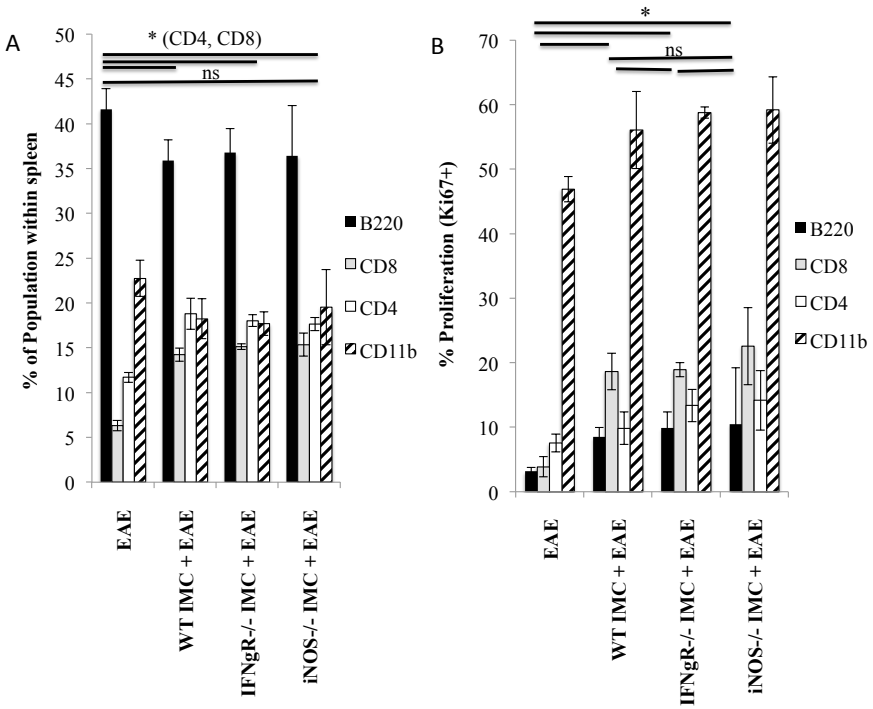

Figure R24. Bone marrow generated immature myeloid cells proliferative function is unaltered by iNOS or IFN-gR1 indicating their activation of NO is not required for their effects.

A | C57Bl/6 were injected with C57Bl/6.IFN-gR1$^{-/-}$, C57Bl/6.iNOS$^{-/-}$ or C57Bl/6 BM-IMC (D-4), were injected i.v. into C57Bl/6 mice. After 4 days, spleens were harvested, RBC were lysed with Gey's solution, labelled with CFSE and placed in culture for 6 days unstimulated. Cells were gated on CD11b, B220, CD4, and CD8 for the different major cell types. Percentage of the cell types was calculated. **B |** C57Bl/6 were injected with C57Bl/6.IFN-gR1$^{-/-}$, C57Bl/6.iNOS$^{-/-}$ or C57Bl/6 BM-IMC (D-4), were injected i.v. into C57Bl/6 mice. After 4 days, spleens were harvested, RBC were lysed with Gey's

solution, labelled with CFSE and placed in culture for 6 days unstimulated. Proliferation was assessed via flow cytometry for Ki67 postive cells with the different cell populations (CD11b, B220, CD4, and CD8).

5 Discussion

As autoimmune diseases are very dehabilitating and the opportunity of cellular therapies is a viable option, we asked the question: *Can MDSC cure EAE?* Within this thesis we display evidence that BM-IMC can be administered as a preventive therapy for EAE, which hopefully may be translatable to other autoimmune disease therapies as well, in the future. The data displayed in the thesis suggests this partial protection are mediated by a non-specific stimulation of seemingly all cells within the spleen through soluble mediators amd possibly signaling through PD-1.

5.1 Establishing the model of Cellular therapy by MDSC in EAE

Utilizing *in vitro* generated BM-IMC we attempted to clarify the potential of MDSC as a cellular therapy for the cure of EAE. EAE was selected based on its ability to be induced, rather than a spontaneous model, because it allows us to assess our cells at specific time points within the disease.

As a proof of concept, generation of BM-IMC *in vitro* reported by Rossner et al. (Rossner et al., 2005) was established in order to yield enough MDSC for cellular therapy. As expected this protocol yielded the same phenotype of BM-MDSC with suppressive function and ability to produce NO (Figure R2) as previously described. To establish EAE, the dose of EAE (CFA/MOG and pertussis toxin) from Brandl et al. (Brandl et al., 2010) was adjusted to better suit our current mouse facility, as commensal bacteria play a role in generating the EAE severity (Lee, 2011)(Ochoa-Reparaz et al., 2009) (Savidge, 2007). Once the EAE was optimal and BM-IMC generated, BM-IMC as a cellular therapy in EAE was pursued. To confirm BM-IMC could protect from EAE disease severity, an administration similar to that of the previously established protective semi-mature DC injection (Menges et al., 2002) was selected. To begin BM-IMC was administered only once, already providing partial protection. Mice receiving BM-IMC had a reduced clinical severity. Interestingly, our protection ranged from a delay, a reduction or both. Most commonly, we would find the reduction. During the course of the thesis the average disease score during each individual experiment ranged in severity for the control mice without changes in the dose, therefore we hypothesize this unknown factor, which may contribute in controling the EAE severity alone may also influence BM-IMC suppressive capacity as well. As EAE is driven by the Th1 and Th17 effector subsets, we investigated if suppression is mediated by suppression of Th1, which had been reported previously in tumor models, or if suppression of Th17 occurs in addition. To answer the questions about their protective effect, a detailed analysis of factors which influence BM-IMC to become suppressive was performed.

5.2 Transcriptional factors in MDSC development and activation

C/EBP-b has been described as a transcription factor for the generation of activated MDSC. To clarify if C/EBP-b plays a role in the differentation or the activation, we assessed cells from mice lacking the gene for C/EBP-b, in attempt to analyze the effects on BM-IMC generation and BM-MDSC activation separately. Although neither decrease is of significant (p=0.1), a 35% and 10% decrease in the generation of mono-MDSC and PMN-MDSC was observed respectively. To truly validate this observed trend, one must repeat with more mice. As C/EBP-b reportedly influences IL-6 signalling (Screpanti et al., 1995), and IL-6 may play a role in generation of MDSC (Bunt et al., 2007), together they might elucidate why a slight trend in decreased mono-MDSC generation is observed. Most likely, as this decrease is not significantly significant, the development in our system from BM precursors to BM-IMC (a steady-state condition) suggests generation may be more regulated by C/EBP-a, as C/EBP-b has been described as required only for "emergency" granulopoiesis (Hirai et al., 2006). "Emergency" granulopoesis is the accelerated generation of the neutrophils occurring in times of infection and inflammation, usually leading to neutorophilia (Panopoulos and Watowich, 2008). It has been noted that C/EBP-b deficient mice retain all normal hematopoietic cells and normal granulopoeisis (Hirai et al., 2006; Screpanti et al., 1995). This steady-state development is only blocked upon addition of activating signals, i.e., LPS/IFN-g. This theory was contradicted by Koeffler et al., which noted neutrophils from C/EBP-b deficient mice do not respond normally to GM-CSF or G-CSF growth factors, enhancing their apoptosis (Akagi et al., 2008). Also, it has been shown that C/EBP-b can compensate for lack of C/EBP-a, so it is possible that the inverse compensation in our system can occur equally. Interestingly, others have shown that C/EBP-b is not required for the generation of precursors, but rather the re-pooling of the granulocytes after the neutropenia (Cain et al., 2011).

The other question, *does the activation of BM-IMC require C/EBP-b*, was investigated by a functional assay. The activation of BM-IMC with LPS/IFN-g was subsequent analyzed for NO production. There was a significant decrease in the production of NO, but one cannot exclude decreased populations of mono-IMC, the major producers of NO within the culture, did not influence this production. To gain better understanding into if these cells require C/EBP-b for activation, one must sort these cells first before assessing the NO production. It has been shown mesenchymal stem cells utilize C/EBP-b for induction of iNOS (Xu et al., 2009). Interestingly, it has been shown that IL-1b, but not IFN-g, nor IFN-g downstream signalling molecules, STAT-1 and IRF-1, is required for C/EBP-b activation of iNOS (Teng et al., 2002). To understand if the PMN-MDSC function was affected, we should also further investigate our BM-IMC for Arg-1 upon activation, although it already has been reported that in macrophages IL-4 signalling through C/EBP-b mediates Arg-1 (Gray et al., 2005). The functionality of MDSC seemingly requires C/EBP-b as both effector function enzymes, iNOS (Eberhardt et al., 1998) and Arg-1 (El Kasmi et al., 2008), are under its regulation. Therefore we conclude in our system that C/EBP-b is

partially involved in generation of mono-MDSC, and to a lesser extent the generataion of PMN-MDSC. The activation of the cells may also be influenced, but further testing is required.

5.3 Activation requirements of BM-IMC by Th1 and Th17 microenviroment stimuli, *in vitro*

To investigate the effect of stimuli and signals MDSC may encounter during EAE, incubation of various stimuli with BM-IMC and analysis of activation was performed. To study the effects of various factors on BM-IMC, NO detection was established as a high-throughput fashion to assess whether different stimuli can activate mono-BM-IMC. Griess reaction has been previously described to detect NO through a colormetric assay though detection of $NaNO_2$ (end product) (Griess, 1879). This test can be utilized as a characterization tool to assess whether the stimuli can or cannot activate MDSC with LPS/IFN-g, a known activator (Greifenberg et al., 2009), employed as the positive control. Pathogenic stimuli from bacteria, fungi, and yeast were able to activate BM-IMC. This activation was enhanced by IFN-g, but not IL-17. This activation of iNOS is dependent on IFN-gR1 and MyD88 signalling and occurs partially through IRF-1 signalling. This mechanism has been described in suppressive monocytes in a transplantation model, identifying signaling through a IFN-gR-, IRF-1-, STAT-1-dependent release of NO via iNOS (Garcia et al., 2010). Alternatively other potential stimuli found during these inflammatory conditions, such as nucleic acid and self glycolipids do not activate BM-IMC, at least not functionally by inducing iNOS.

Screening of various pathogenic stimuli with known capabilities to produce different effector cytokines in T cells upon stimulation by DC. All of the pathogens assessed, from both bacterial, LPS and Mtb, and fungal/yeast origin, zymosan and curdlan, activated MDSC to produce NO. We could show that zymosan, curdlan, and heat-killed mycobacteria (H37RA), with their known signalling through either dectin and/or TLR2, can induce NO to the same capacity as LPS/IFN-g require MyD88 signalling (in this case, through TLR2) only. It has been found that MyD88 co-signalling with Syk pathways in MDSC has been shown to lead to release of other suppressive or regulatory factors, such as IL-10 (Zhang et al., 2009). Cell wall components such as LPS, curdlan, and zymosan have been reported to activate other myeloid cells, such as DC and macrophages (Kataoka et al., 2002). Although it has been reported live Mtb inhibit iNOS (Miller et al., 2004), this is an active process, therefore heat-killed Mtb is unable to actively prevent the co-localization with the phagosomes subsequently allowing the observed NO production. Therefore, Th17-like inducing stimuli may also employ NO in MDSC to suppress T cell responses in a similar manner to that of Th-1 stimuli.

Interestingly, NO has been described to regulate Th17 cells by decreasing IL-23R expression and decreasing their subsequent proliferation, but interestingly not affecting their induction (Niedbala et al., 2011), suggesting NO might work as a

negative feedback mechanism. These reports, together with reports that zymosan-induced EAE disease severity is transient, linked to reduced cytokines levels related to both induction (TGF-b, IL-6) and sustainance (IL-23) of Th17 as compared to Mtb-induced EAE (Veldhoen et al., 2006), suggests a link between the increased Th17 induction NO further downregulates Th17 cytokines. This idea is supported by the observed increase of NO production by MDSC when stimulated with zymosan compared to Mtb.

MS has been characterized by increased numbers of T cells with reactivity to glycolipids (Shamshiev et al., 1999). Self glycolipids can be a source of autoimmune diseases through molecular mimicry of self tissues by bacterial pathogens, e.g. *Borrelia* burgdorferi (Martin et al., 2001), or viral pathogens, e.g. human herpes virus 6 (Tejada-Simon et al., 2003) and Epstein Barr virus (Holmoy et al., 2004; Lang et al., 2002; Wucherpfennig and Strominger, 1995), or induced by reactive oxygen species alteration of self, which is then recognized as foreign to the host. Interestingly, biophysical studies have revealed affinity for the sequence of some microbial peptides is lower than that of the same sequence in auto-antigen peptides (Harkiolaki et al., 2009). In our studies, self glycolipids (ceramide, cerebroside, asialoGM-1, GM-1, ganglioside, sphingomyelin, and sulfatides) themselves were not stimulatory, as shown by equilvalent NO production to untreated controls. Self glycolipids also did not have an effect on the suppressive capacity. This may be explained as sphingomyelin has been shown not to be able to activate NF-kB (Sun et al., 2010), but as other glycolipids, such as sulfatides, can induce activation of NF-kB (Jeon et al., 2008). This activation through NF-kB might not have a huge impact as other signals might be required in addition. Further studies will have to be performed to assess the recognition of self glycolipids by MDSC after potential oxidation by NO and other reactive oxygen species, or in combination of other stimuli, mimicking conditions found in inflammation.

To examine if genetic material lost from dying cells or from viruses may activate MDSC, we tested the effect of CpG, dsDNA, and poly (I:C), dsRNA on MDSC's production of NO through Griess reaction, but neither of these activated MDSC. This finding is contradictory of a previous report's claims of poly (I:C) inducing activation of BM-MDSC (Liu et al., 2011). This may be due to the signalling mechanism. It has been described that stimulation with pathogen stimuli, from bacteria or fungi, signal through MyD88/TLR4, whereas it has been shown that dsRNA signals through TLR3 can act via a TRIF-dependent, MyD88-independent mechanism (Abbas AK, 2010). We investigated whether signals from our bacterial and fungal pathogens require MyD88 for activation, as some Th17-like stimuli can also signal through dectin-1 to induce Th17 polarization by DC. MDSC lacking the MyD88 adaptor protein cannot produce NO from any of the stimuli, indicating the role of MyD88 signalling is essential for the activation of NO. The role of MyD88 has been observed in MDSC from mice with sepsis (Delano et al., 2007). Interestingly, CpG has been

reported to suppress the generation of MDSC by causing their further differentiation (Zoglmeier et al., 2011).

It has also been noticed that MDSC demonstrate a synergistic effect upon a combined stimulation with LPS/IFN-g (Greifenberg et al., 2009). As we were interested in understanding MDSC during Th1 and Th17 conditions, we investigated the role of IL-17 in MDSC activation. It has been reported that MDSC have the ability to produce TGF-b (Li et al., 2009; Terabe et al., 2003), which may present an interesting role of MDSC in regulating Th17. Griess reagent was employed to analyze if MDSC can be activated specifically by IL-17 and if this activation could act in concert with other stimuli to synergistically boost the NO production in the same manner as IFN-g. Similarly to the inability of IL-17 to influence macrophage NO production (Jovanovic et al., 1998), BM-IMC did not secrete any NO after stimulation with IL-17, nor did IL-17 boost the amount of NO produced by other stimuli. Interestingly, this boost seems to be restricted to IFN-g. It has been reviewed in macrophages that the synergy occurs between TLR signalling (Schroder et al., 2006), resulting in IFN-g signalling through IFN-gR resulting in STAT-1 translocation and NF-kB activation. The overall result of this synergy results in increased macrophage NO and other effector functions. IFN-g and TLR seem to influence the expression of each other's receptors, as IFN-gR signalling changes the expression of TLR2 and TLR4 (Schroder et al., 2006). To analyze this signalling we used IFN-gR1 deficient mice, in the presence of bacteria and fungi components. We observed a reduction in NO production in this system. Colleagues have assessed the role of IFN-gR1 in MDSC ability to suppress T cell responses and have found that MDSC lacking IFN-gR1 do not suppress as well as those with IFN-gR expression (Ribechini, unpublished). Interferon regulatory factor-1 (IRF-1) plays a major role in regulating IFN-gR signalling further downstream, such as induction of iNOS (Kamijo et al., 1994), IL-1b (Tamura et al., 1995), and type I IFN (Miyamoto et al., 1988).

IRF-1, a downstream element of IFN-g signaling, has been notably associated with susceptibility to EAE (Buch et al., 2003; Tada et al., 1997) and progression of MS (Fortunato et al., 2008). With the use of IRF-1 knockout MDSC, we further showed that IRF-1 signalling only plays a partial role in production of NO within MDSC. This partial decrease was seen by knockdown in only one allele and was not further decreased by the full knockout. This differs from the common result seen in literature based on other cell types. The ability of IRF-1 knockout mice upon LPS/IFN-g stimulation to produce iNOS has been reported by various groups (Kamijo et al., 1994; Martin et al., 1994; Salkowski et al., 1996). Corresponding most likely to this small reduction in iNOS, IRF-1 only slightly reduced the ability of MSDC to suppress T cells in a suppressor assay (Ribechini, unpublished). IRF-1 has been shown to play a role in IFN-g mediated regulation and constitutive expression of B7-H1 (Lee et al., 2006). Indications suggests that other IRF molecules, such as interferon consensus sequence binging protein / interferon regulatory factor-8, are involved in signalling through CSF, IFN-g and TLRs, and modulating an important transcription factor in

myeloid cells (Nerlov and Graf, 1998), PU.1, IRF-8 has been shown to have importance in MDSC activation (Stewart et al., 2009), as IRF-8 and PU.1 have been reported to be induce Arg-1 activity. Therefore, the role of interferons and their regulation is crucial to the development and effector functions of MDSC.

5.4 IFN-b treatment does not directly influence BM-IMC *in vitro*.

As IFN-g and its regulation plays a major role in MDSC, we wanted to assess another interferon family member, but from the Type I interferons, IFN-beta. Type I interferons play a major role in regulation of the immune system, but unlike Type II interferons, namely IFN-g, the type I IFN, IFN-a and IFN-b, are contributed by innate immune cells, predominantly plasmacytoid DC (Abbas AK, 2010). It has been shown that IFN-b and IFN-g play opposing roles in immune activation and their signalling has been discovered to negatively regulate each other (Rayamajhi et al., 2010). Alternatively, it has been suggested low levels of type I IFN are required to sustain IFN-g signaling (Nguyen et al., 2000; Takaoka et al., 2000). As IFN-g boosts NO production in MDSC, assessment of IFN-b, a cytokine given for treatment of MS patients, on MDSC activation and function was performed. IFN-b was found to neither activate nor suppress the activation of MDSC NO production. Nor does it influence the ability to suppress by the addition of exogenous IFN-b-1a and IFN-b-1b, and other factors which normally lead to increased IFN-b production (such as poly (I:C)), into the suppression assay, or influence suppression in BM-IMC which lack IFN-aR. This result differs from reports stating the presence of autocrine IFN-b release peritoneal macrophages from their suppressive phenotype *in vitro* (Hamilton et al., 2010). This effect was interestingly noted to correspond with activation by TRIF, but not MyD88 signalling (Hamilton et al., 2010). It has been also reported through the use of IFN-aR blocking antibodies that IFN-aR is required for Ly-6C^{hi}, but not Ly-6C$^{int.}$ differentiation from BM (Seo et al., 2011), but as their culture conditions did not include GM-CSF this indicates GM-CSF may negate IFN-a/b required signalling *in vitro*. Therefore, we conclude that the IFN-b therapy does not directly influence BM-MDSC *in vitro*. Recently, MDSC indirect suppression of type I and II interferons was demonstrated. MDSC reduce the production of IFN-g and IFN-b by other immune cells at the activation level, i.e., though nitrosylation of STAT-1 in cancer (Mundy-Bosse et al., 2011) leading to less activation and therefore less effector cytokine function. The treatment of MS by a potential combination therapy of IFN-b and MDSC cellular therapy may be attractive as the cytokine IFN-b itself has not been discussed as being altered, although this has never been addressed. The induction of type I IFNs, lack direct influence on MDSC, may influence other members of the immune system, such as enhancing function and quantity of Tregs (de Andres et al., 2007) and/or negatively regulating Th17 (Guo et al., 2008). Therefore administration of a combination therapy including IFN-b and MDSC may provide better outcomes in overcoming autoimmunity.

5.5 Migration of BM-MDSC

Knowledge of localization of cells given as cellular therapy is required to assess mechanisms *in vivo*. To understand the cell trafficking, BLI was employed to observe *in vivo* kinetics of the intraveneously injected BM-IMC. Upon i.v. injection, BM-IMC seem to spread over the mouse, but slowly accumulated in the spleen, where peak accumulation was on day 4. The cell numbers in the spleen subsequently decreased, we speculate by possibly either death or recirculation. This homing process reflects current theory described by Swirski et al., as they describe the spleen as a reserviour for monocytes (Swirski et al., 2009). To understand where in the spleen the cells were located, we then analyzed the spleen for our labeled BM-IMC. Interestingly, we located them back in the outside the white pulp. iNOS+ monocytes have been reported during Listeria infection to interact with memory CD8+ T cells in the red pulp (Bajenoff et al., 2010).

To answer where IMC migrate/home to mediate their suppression, BM-IMC were administered during low grade EAE. BM-IMC migrated similarly to the spleen when administered into a mouse displaying EAE symptoms and steady-state, but also to the site of inflammation (CFA/MOG injection site) and the draining lymphnodes during times of inflammation. It had been described that in migration of endogenous cells during tumors high amounts of MDSC accumulate in the liver, spleen, blood, BM, and tumor site (Ilkovitch and Lopez, 2009). As our test was not sensitive enough to see the cells within the blood, this data corresponds nicely with our results displaying the MDSC in the spleen, and inflammation site. Using a model of using sponges as an artificial site of inflammation, it was reported endogenous MDSC migrate to the site of inflammation (Ribechini et al unpublished), where it was shown NO was produced (Witte and Barbul, 2002). Interestingly, we had seen additional signal of our cells within the lymph nodes, which had been seen previously in our lab within the endogenous population, but this increase was so small, it had previously been discounted (Ribechini, unpublished). O'Connor et al. have described similar localization of IMC during adjuvant immunotherapy, which induces further recruitment of T cells to the lymph nodes rather than the brain whereby inducing protection. Interestingly, Zhu et al. has published the observation of IMC in the brain by using the CD45.1 marker suggesting the cells exert their ability to suppress in the brain itself (Zhu et al., 2011). The recruitment to the spleen may be similar to that of the endogenous CD11b population, which also migrates to the spleen during EAE (Zhu et al., 2007). This recruitment and accumulation of endogenous CD11b cells to the spleen has been observed by Mtb (Billiau and Matthys, 2001), thereby explaining the observed splenomegaly (Wang et al., 2010). This increased CD11b population has been observed *ex vivo* to be suppressive, providing an indication that the CD11b population may play a role in suppression of the disease (Zhu et al., 2007).

From these observations, we attempted to use fluorescent labeling to look at the BM-IMC injected cells *ex vivo* from the spleen to characterize their expression. Unfortunately, much difficulty was encountered during the process of recovering the

BM-IMC from the spleen. Many conventional methods were attempted to recover them for analysis by flow cytometry, but these proved unsuccessful. Finally, after another trial, we noticed a "blob" of collagenous-like material that would always appear in the cell suspension. We plan to use collagenase staining on paraffin sections to see if our cells colocalize with these collagen fibers within the spleen. Interestingly, it has been described that Arg-1 end product, proline, regulates proliferation and collagen deposition during asthma, cancer, parasite infection as part of the wound healing response (Das et al., 2010). Also, NO has been described in positively regulating collagen formation, as well as having roles in cell proliferation, and wound contraction (Witte and Barbul, 2002). This mass turned out to contain our cells, as they could be observed by microscopy. After several attempts to digest the "blob" using collagenase III and DNase I, we still could not isolate our cells from this mass. More experiments must be performed to determine the optimal method to retrieve these cells, possibly using other methods of RBC lysis in combination with digestion of other matrix proteins. It has been observed in humans that CD14+ monocytes can differentiate into endothelial cells through the presence of adiponectin (Yang et al., 2006), a molecule involved in the promotion of angiogenesis. MDSC surrounding the blood vessel release NO into the blood stream during tumors (Yang, 2010).

5.6 Protection by BM-IMC

Once we established that BM-IMC protect against EAE, we then altered the protocol to establish the method providing the best protection. MDSC have generally a differentially described mode of suppression as compared to the reportedly protective semi-mature DC (Pletinckx et al., 2011). The semi-mature DC induce immune tolerance through immune deviation by shifting the response from a Th1 response to either Th2 or IL-10 Tr1 responses (Menges et al., 2002). This induces a less vigorous immune response therefore causing less damage within the CNS (Pletinckx et al., 2011). In other models, MDSC on the other hand have not been observed to cause immune deviation to date, but rather induce suppressive mechanisms which cause the T cells to be dysfunctional (Gabrilovich et al., 2012). Interestingly, MDSC have been shown to induce Tregs (Gabrilovich et al., 2012). We injected BM-IMC at a D-3, and found these mice were still able to suppress EAE. Ioannou et al. published that BM-IMC when injected post EAE induction utilitzed the B7-H1 and PD-1 interaction to mediated protection by the PMN-MDSC (Ioannou et al., 2012) subset. Attempts performed by us and previous members in our lab could not find a protective effect with BM-IMC post-EAE induction. This discrepancy observed may be attributed to possibly different sources of BM-IMC as they are a heterogenous population. We have concluded injection of BM-IMC could only be given preventively to provide protection. To determine whether the cells must be activated *in vivo* or could be pre-activated to provide better protection we injected BM-MDSC matured with LPS/IFN-g instead of BM-IMC. We have shown that injection of activated BM-MDSC, rather than BM-IMC, partially blocks the ability to suppress. Curdlan-activated BM-IMC injection into mice could not be attempted, as curdlan particles are too large. We

postulate the activation of BM-IMC into BM-MDSC causes the release of NO possibly before they reach the necessary location, or maybe even before injection, but this must be further investigated. Also, it has not been investigated whether these cells home to the similar place or interact with the same cells; this is however beyond the scope of this thesis.

To determine if the MDSC can suppress the pathologic causing cytokines IFN-g and IL-17 *in vivo,* we assessed the infiltrates within the CNS of EAE mice receiving pre-treatment of BM-IMC. Overall, there was no statistically significant change in the cytokines. In the spleen, very low amounts of IFN-g and IL-17 were detected even in control EAE mice, this increased in the mice treated with BM-IMC although this change was not significant. No significant change in cytokine production between the groups was observed in the CNS upon restimulation either. We were confused by this finding, because given a reduction in the clinical score, one would not expect to find similar amounts of cytokines which are thought to be involved in the disease progression. As we hypothesized IFN-g, as had been previously shown to be inhibited in several *in vivo* models, but also IL-17, as it was reported to be specifically inhibited by NO (Nath et al., 2010), would have been suppressed in BM-IMC protected EAE mice. When re-evaluating our restimulation for cytokine analysis, we found PMA/Iono, which stimulate the cells through calcium influx (ionomycin) and protein kinase C activation (PMA), without the use of the TCR (Weiss et al., 1986), may not be the best choice for this assay. The TCR has been discussed as a possible place of nitrosylation by NO released from MDSC (Gabrilovich et al., 2012). Therefore, future experiments should assess the cytokine production using other stimuli, for example a specific restimulation using MOG peptide. Interestingly, others have found a reduction in CNS pathological cytokines upon application of BM-IMC after specific restimulation with MOG using CD4+ T cell transgenic mice, 2D2 (Zhu et al., 2011).

It has been shown in brain tissue that upregulation of B7-H1 decreases the CD4 T cell production of IL-17, IFN-g, and IL-10 (Duncan and Miller, 2011), therefore increased B7-H1 can suppress clinical severity in Theiler's virus model of MS. To address the aim of investigating the role of different mechanisms of suppression we first went to a mechanism of tolerance described by Wherry et al. (Freeman et al., 2006), termed "exhaustion." As MS is a chronic disease, and MDSC are also found in chronic inflammatory conditions, this mechanism of B7-H1 and PD-1 interaction may be utilitized by MDSC to be suppressive. The role of B7-H1 interaction with PD-1 on memory T cells was assessed *in vitro*. Our findings displayed that B7-H1 reduced the ability of MDSC to suppress memory T cells. Expression of B7-H1 on endogenous and *in vitro* MDSC was noted upon restimulation, suggesting although the endogenous MDSC have the ability to suppress, it might be a numbers game. It has been shown that MDSC appear in the peripherial blood peaking around day 6 - 9 and in spleen peaking on day 21 in CFA treated mice (Wang et al., 2010), suggesting the BM-IMC delivered at the earlier timepoints gives the mouse the protection. *In vivo*,

we found different results. The expression of B7-H1 upon the injected IMC was not required for suppression of the disease. PD-1 on recipient mice, however did seem to play a role in suppression of EAE by MDSC. There are several factors contributing to these differential results. We believe that this interaction between B7-H1 and PD-1 occurs during chronic situations (Freeman et al., 2006). The timing of our MDSC injection is before the induction and onset of the disease, and therefore it is possible that the cells may die off before they can suppress at these late timepoints as rapid turn over of MDSC has been reported. This NO production may influence other cells inducing suppression, or may activate others to suppress through PD-1. Another explanation could be the cells use another ligand besides B7-H1 to interact with PD-1, but as B7-H2 is not upregulated on the BM-MDSC after stimulation, and other ligands have not been described, we believe this is unlikely. Proof supporting the timing possibility has been reported showing the suppression of EAE by B7-H1 expressed on PMN-MDSC upon MDSC administeration after EAE induction (Ioannou et al., 2012). We however do not see this protection, as BM-IMC injected post-EAE induction does not cause protection in our hands. Therefore, these experiments need to be repeated to confirm this finding, or to see if other factors such as their source of MDSC, may contribute to the differences seen. Interestingly, there are some reports supporting the role of PD-1 within the CNS, as not being related to the exhausted phenotype, but a method employed to mediate tissue damage (Sadagopal et al., 2010).

5.7 BM-IMC may induce proliferative effects rather than suppressive effects *in vivo*

Upon examining the spleens of receiving BM-IMC, compared to untreated mice, we saw an increase of spleen size. Interestingly, upon one of our attempts to understand if the MDSC suppress specifically by forcing the interaction of MDSC MHC with the TCR of T cells using SEB, we found that untreated "resting" T cells when forced to interact with MDSC proliferated slightly. Therefore, we thought this could be a similar situation, and that during steady-state, the injection of these cells may cause slight proliferation of surrounding cells. To examine this, we injected cells, waited 4 days, and then assessed the induction of proliferatation *ex vivo* by labeling the splenocytes with CFSE and analyzing the dilution. Splenocytes from mice pre-injected with BM-IMC had an increased proliferation in many cell types, including T cells, B cells, and myeloid cells. Previous reports support endogenous Gr1+ cells in the spleen priming of B cells, as their interaction within the red pulp has been seen in Alum injected mice (Jordan et al., 2004). This proliferation in CD4 cells, when examined in closer detail showed increased proliferation of Tregs, as compared to Teff cells. The induction of Tregs by MDSC has been shown, but these studies have been in models of tumor and inflammation (Huang et al., 2006) (Serafini et al., 2008). Interestingly, others have discussed mast cells can induce MDSC to secrete IL-17 under certain conditions leading to the recruitment and increased suppressive capacity of Tregs (Yang et al., 2010). Further studies will have to be performed to analyze if

there is an increased recruitment in addition to proliferative response, although current data suggestions this may not be the case (Yang et al., 2010).

To assess whether this proliferation is regulated by PD-1 interaction with B7-H1, we analyzed spleens from PD-$1^{-/-}$ mice receiving the preinjection of MDSC. Interestingly, spleens from PD-$1^{-/-}$ mice receiving BM-MDSC have no increase of spleen cellularity. Therefore, this proliferation may be induced by interaction of MDSC with PD-1 directly, or indirectly. It has been reported to induce proliferation rather than suppression under certain situations, by utilizing antibodies for PD-1, but not upon interaction with B7-H1 (del Rio et al., 2005). To confirm this data we also tested spleens from mice receiving BM-IMC from B7-$H1^{-/-}$ and WT mice injected post EAE induction. The spleens lacking the expression of B7-H1 did show an increase in splenic cellularity similar to that of WT as compared to untreated EAE controls, implying the spleen cellularity does not relate to the cellular interaction of splenic cells with B7-H1 on MDSC, nor is this increase spleen cellularity the complete reason for this suppressive function as mice given BM-IMC post EAE injection are not protected. These results suggest several possibilities in the mechanisms which injected BM-IMC employ; BM-IMC may prime the spleen before EAE induction, BM-IMC may interact with different cell types during steady-state as compared to inflammatory conditions, BM-IMC may locate in different areas of the spleen, or need activation signals *in vivo* which are induced by the EAE to employ their suppressive capacity.

Our lab has already previously described the importance of two signals for the activation of MDSC when using LPS/IFN-g. These two signals are IFN-g signalling through IFN-gR and LPS signalling through TLR4 (Greifenberg et al., 2009). To understand if BM-IMC must be activated to induce this proliferation, we plan to inject BM-IMC from IFN-g$R1^{-/-}$, $iNOS^{-/-}$, and WT mice, induce EAE and assess the spleens for increased cellularity and proliferation capacity. We found no differences between the BM-IMC groups, displaying the possibility that these cells do not differentiate into MDSC with suppressive function upon injection, therefore not utilizing the effector machinery for suppression. As the mice given post EAE induction injection has similar spleen size increases, this assessment of the functionality requirement needs to be further elucidated. We are currently collaborating to examine this role of iNOS functionality in the injected BM-IMC on the protection associated with EAE mice.

This thesis provides insight into the ability of MDSC as a preventive cellular therapy to suppress the severity of EAE. It also demonstrates where these cells would home, i.e., the spleen, and gives several possibilities for the mechanisms which the cells might utilize in order to suppress, i.e., NO and Arg-1 and possibly modulating effects through PD-1, but not B7-H1.

6 References

Abbas AK, L.A., Pillav S. 2010. Cellular and Molecular and Immunology Updated 6/E. Sauder, an imprint of Elsevier, Philadelphia, PA. 566 pp.

Adams, R.D., and C.S. Kubik. 1952. The morbid anatomy of the demyelinative disease. *The American journal of medicine* 12:510-546.

Aghdami, N., F. Gharibdoost, and S.M. Moazzeni. 2008. Experimental autoimmune encephalomyelitis (EAE) induced by antigen pulsed dendritic cells in the C57BL/6 mouse: influence of injection route. *Experimental animals / Japanese Association for Laboratory Animal Science* 57:45-55.

Akagi, T., T. Saitoh, J. O'Kelly, S. Akira, A.F. Gombart, and H.P. Koeffler. 2008. Impaired response to GM-CSF and G-CSF, and enhanced apoptosis in C/EBPbeta-deficient hematopoietic cells. *Blood* 111:2999-3004.

Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature immunology* 2:675-680.

Auffray, C., M.H. Sieweke, and F. Geissmann. 2009. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annual review of immunology* 27:669-692.

Bajenoff, M., E. Narni-Mancinelli, F. Brau, and G. Lauvau. 2010. Visualizing early splenic memory CD8+ T cells reactivation against intracellular bacteria in the mouse. *PloS one* 5:e11524.

Barker, R.N., L. Erwig, W.P. Pearce, A. Devine, and A.J. Rees. 1999. Differential effects of necrotic or apoptotic cell uptake on antigen presentation by macrophages. *Pathobiology : journal of immunopathology, molecular and cellular biology* 67:302-305.

Bas, A., G. Forsberg, S. Hammarstrom, and M.L. Hammarstrom. 2004. Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scandinavian journal of immunology* 59:566-573.

Bennett, C.L., J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, and H.D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature genetics* 27:20-21.

Bhadra, R., J.P. Gigley, and I.A. Khan. 2012. PD-1-Mediated Attrition of Polyfunctional Memory CD8+ T Cells in Chronic Toxoplasma Infection. *The Journal of infectious diseases* 206:125-134.

Billiau, A., and P. Matthys. 2001. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. *Journal of leukocyte biology* 70:849-860.

Brandl, C., S. Ortler, T. Herrmann, S. Cardell, M.B. Lutz, and H. Wiendl. 2010. B7-H1-deficiency enhances the potential of tolerogenic dendritic cells by activating CD1d-restricted type II NKT cells. *PloS one* 5:e10800.

Brissette, W.H., D.A. Baker, E.J. Stam, J.P. Umland, and R.J. Griffiths. 1995. GM-CSF rapidly primes mice for enhanced cytokine production in response to LPS and TNF. *Cytokine* 7:291-295.

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.

Brown, G.D., and S. Gordon. 2001. Immune recognition. A new receptor for beta-glucans. *Nature* 413:36-37.

Buch, T., C. Uthoff-Hachenberg, and A. Waisman. 2003. Protection from autoimmune brain inflammation in mice lacking IFN-regulatory factor-1 is associated with Th2-type cytokines. *International immunology* 15:855-859.

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.

Bunt, S.K., L. Yang, P. Sinha, V.K. Clements, J. Leips, and S. Ostrand-Rosenberg. 2007. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. *Cancer research* 67:10019-10026.

Butte, M.J., M.E. Keir, T.B. Phamduy, A.H. Sharpe, and G.J. Freeman. 2007. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* 27:111-122.

Cain, D.W., P.B. Snowden, G.D. Sempowski, and G. Kelsoe. 2011. Inflammation triggers emergency granulopoiesis through a density-dependent feedback mechanism. *PloS one* 6:e19957.

Cao, Y.A., A.J. Wagers, A. Beilhack, J. Dusich, M.H. Bachmann, R.S. Negrin, I.L. Weissman, and C.H. Contag. 2004. Shifting foci of hematopoiesis during reconstitution from single stem cells. *Proceedings of the National Academy of Sciences of the United States of America* 101:221-226.

Cinalli, R.M., C.E. Herman, B.O. Lew, H.L. Wieman, C.B. Thompson, and J.C. Rathmell. 2005. T cell homeostasis requires G protein-coupled receptor-mediated access to trophic signals that promote growth and inhibit chemotaxis. *European journal of immunology* 35:786-795.

Condamine, T., and D.I. Gabrilovich. 2011. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. *Trends in immunology* 32:19-25.

Corse, E., R.A. Gottschalk, and J.P. Allison. 2011. Strength of TCR-peptide/MHC interactions and in vivo T cell responses. *J Immunol* 186:5039-5045.

Corzo, C.A., T. Condamine, L. Lu, M.J. Cotter, J.I. Youn, P. Cheng, H.I. Cho, E. Celis, D.G. Quiceno, T. Padhya, T.V. McCaffrey, J.C. McCaffrey, and D.I. Gabrilovich. 2010. HIF-1alpha regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *The Journal of experimental medicine* 207:2439-2453.

Cyster, J.G., and C.C. Goodnow. 1995. Pertussis toxin inhibits migration of B and T lymphocytes into splenic white pulp cords. *The Journal of experimental medicine* 182:581-586.

Das, P., A. Lahiri, A. Lahiri, and D. Chakravortty. 2010. Modulation of the arginase pathway in the context of microbial pathogenesis: a metabolic enzyme moonlighting as an immune modulator. *PLoS pathogens* 6:e1000899.

de Andres, C., C. Aristimuno, V. de Las Heras, M.L. Martinez-Gines, M. Bartolome, R. Arroyo, J. Navarro, S. Gimenez-Roldan, E. Fernandez-Cruz, and S. Sanchez-Ramon. 2007. Interferon beta-1a therapy enhances CD4+ regulatory T-cell function: an ex vivo and in vitro longitudinal study in relapsing-remitting multiple sclerosis. *Journal of neuroimmunology* 182:204-211.

de Vries, H.E., M.C. Blom-Roosemalen, M. van Oosten, A.G. de Boer, T.J. van Berkel, D.D. Breimer, and J. Kuiper. 1996. The influence of cytokines on the integrity of the blood-brain barrier in vitro. *Journal of neuroimmunology* 64:37-43.

de Vries, H.E., J. Kuiper, A.G. de Boer, T.J. Van Berkel, and D.D. Breimer. 1997. The blood-brain barrier in neuroinflammatory diseases. *Pharmacological reviews* 49:143-155.

del Rio, M.L., G. Penuelas-Rivas, R. Dominguez-Perles, P. Ramirez, P. Parrilla, and J.I. Rodriguez-Barbosa. 2005. Antibody-mediated signaling through PD-1 costimulates T cells and enhances CD28-dependent proliferation. *European journal of immunology* 35:3545-3560.

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. *The Journal of experimental medicine* 204:1463-1474.

Dolcetti, L., E. Peranzoni, S. Ugel, I. Marigo, A. Fernandez Gomez, C. Mesa, M. Geilich, G. Winkels, E. Traggiai, A. Casati, F. Grassi, and V. Bronte. 2010. Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF. *European journal of immunology* 40:22-35.

Duncan, D.S., and S.D. Miller. 2011. CNS expression of B7-H1 regulates pro-inflammatory cytokine production and alters severity of Theiler's virus-induced demyelinating disease. *PloS one* 6:e18548.

Eberhardt, W., C. Pluss, R. Hummel, and J. Pfeilschifter. 1998. Molecular mechanisms of inducible nitric oxide synthase gene expression by IL-1beta and cAMP in rat mesangial cells. *J Immunol* 160:4961-4969.

El Kasmi, K.C., J.E. Qualls, J.T. Pesce, A.M. Smith, R.W. Thompson, M. Henao-Tamayo, R.J. Basaraba, T. Konig, U. Schleicher, M.S. Koo, G. Kaplan, K.A. Fitzgerald, E.I. Tuomanen, I.M. Orme, T.D. Kanneganti, C. Bogdan, T.A. Wynn, and P.J. Murray. 2008. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nature immunology* 9:1399-1406.

Englert, D., and K. Hempel. 1981. Suppression of experimental allergic encephalomyelitis by transfer of lymph node cells from Lewis rats pretreated with complete Freund's adjuvant. *International archives of allergy and applied immunology* 66:326-331.

Ercolini, A.M., and S.D. Miller. 2006. Mechanisms of immunopathology in murine models of central nervous system demyelinating disease. *J Immunol* 176:3293-3298.

Esen, N., and T. Kielian. 2007. Effects of low dose GM-CSF on microglial inflammatory profiles to diverse pathogen-associated molecular patterns (PAMPs). *Journal of neuroinflammation* 4:10.

Fenyk-Melody, J.E., A.E. Garrison, S.R. Brunnert, J.R. Weidner, F. Shen, B.A. Shelton, and J.S. Mudgett. 1998. Experimental autoimmune encephalomyelitis is exacerbated in mice lacking the NOS2 gene. *J Immunol* 160:2940-2946.

Fife, B.T., K.E. Pauken, T.N. Eagar, T. Obu, J. Wu, Q. Tang, M. Azuma, M.F. Krummel, and J.A. Bluestone. 2009. Interactions between PD-1 and PD-L1

promote tolerance by blocking the TCR-induced stop signal. *Nature immunology* 10:1185-1192.

Flugel, A., T. Berkowicz, T. Ritter, M. Labeur, D.E. Jenne, Z. Li, J.W. Ellwart, M. Willem, H. Lassmann, and H. Wekerle. 2001. Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. *Immunity* 14:547-560.

Ford, M.L., and B.D. Evavold. 2005. Specificity, magnitude, and kinetics of MOG-specific CD8+ T cell responses during experimental autoimmune encephalomyelitis. *European journal of immunology* 35:76-85.

Fortunato, G., G. Calcagno, V. Bresciamorra, E. Salvatore, A. Filla, S. Capone, R. Liguori, S. Borelli, I. Gentile, F. Borrelli, G. Borgia, and L. Sacchetti. 2008. Multiple sclerosis and hepatitis C virus infection are associated with single nucleotide polymorphisms in interferon pathway genes. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 28:141-152.

Freeman, G.J., A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L.J. Fitz, N. Malenkovich, T. Okazaki, M.C. Byrne, H.F. Horton, L. Fouser, L. Carter, V. Ling, M.R. Bowman, B.M. Carreno, M. Collins, C.R. Wood, and T. Honjo. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *The Journal of experimental medicine* 192:1027-1034.

Freeman, G.J., E.J. Wherry, R. Ahmed, and A.H. Sharpe. 2006. Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *The Journal of experimental medicine* 203:2223-2227.

Fuentes, M.E., S.K. Durham, M.R. Swerdel, A.C. Lewin, D.S. Barton, J.R. Megill, R. Bravo, and S.A. Lira. 1995. Controlled recruitment of monocytes and macrophages to specific organs through transgenic expression of monocyte chemoattractant protein-1. *J Immunol* 155:5769-5776.

Fugger, L., M.A. Friese, and J.I. Bell. 2009. From genes to function: the next challenge to understanding multiple sclerosis. *Nature reviews. Immunology* 9:408-417.

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

Gabrilovich, D.I., S. Ostrand-Rosenberg, and V. Bronte. 2012. Coordinated regulation of myeloid cells by tumours. *Nature reviews. Immunology* 12:253-268.

Gallucci, S., M. Lolkema, and P. Matzinger. 1999. Natural adjuvants: endogenous activators of dendritic cells. *Nature medicine* 5:1249-1255.

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. *The Journal of clinical investigation* 120:2486-2496.

Geissmann, F., S. Jung, and D.R. Littman. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19:71-82.

Gershon, R.K. 1975. A disquisition on suppressor T cells. *Transplantation reviews* 26:170-185.

Glass, C.K., K. Saijo, B. Winner, M.C. Marchetto, and F.H. Gage. 2010. Mechanisms underlying inflammation in neurodegeneration. *Cell* 140:918-934.

Gordon, S. 1986. Biology of the macrophage. *Journal of cell science. Supplement* 4:267-286.

Goverman, J. 2009. Autoimmune T cell responses in the central nervous system. *Nature reviews. Immunology* 9:393-407.

Gow, A.J., D. Duran, S. Malcolm, and H. Ischiropoulos. 1996. Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. *FEBS letters* 385:63-66.

Gray, M.J., M. Poljakovic, D. Kepka-Lenhart, and S.M. Morris, Jr. 2005. Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPbeta. *Gene* 353:98-106.

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. *European journal of immunology* 39:2865-2876.

Griess, P. 1879. *Chemical Ber* 12:

Guo, B., E.Y. Chang, and G. Cheng. 2008. The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice. *The Journal of clinical investigation* 118:1680-1690.

Gupta, S., J.M. Solomon, T.A. Tasciyan, M.M. Cao, R.D. Stone, J.L. Ostuni, J.M. Ohayon, P.A. Muraro, J.A. Frank, N.D. Richert, H.F. McFarland, and F. Bagnato. 2005. Interferon-beta-1b effects on re-enhancing lesions in patients with multiple sclerosis. *Mult Scler* 11:658-668.

Hamilton, M.J., F. Antignano, A. von Rossum, J.L. Boucher, K.L. Bennewith, and G. Krystal. 2010. TLR agonists that induce IFN-beta abrogate resident macrophage suppression of T cells. *J Immunol* 185:4545-4553.

Hanninen, A., M. Maksimow, C. Alam, D.J. Morgan, and S. Jalkanen. 2011. Ly6C supports preferential homing of central memory CD8+ T cells into lymph nodes. *European journal of immunology* 41:634-644.

Hansen, B.S., R.Z. Hussain, A.E. Lovett-Racke, J.A. Thomas, and M.K. Racke. 2006. Multiple toll-like receptor agonists act as potent adjuvants in the induction of autoimmunity. *Journal of neuroimmunology* 172:94-103.

Harari, O., and J.K. Liao. 2004. Inhibition of MHC II gene transcription by nitric oxide and antioxidants. *Current pharmaceutical design* 10:893-898.

Harkiolaki, M., S.L. Holmes, P. Svendsen, J.W. Gregersen, L.T. Jensen, R. McMahon, M.A. Friese, G. van Boxel, R. Etzensperger, J.S. Tzartos, K. Kranc, S. Sainsbury, K. Harlos, E.D. Mellins, J. Palace, M.M. Esiri, P.A. van der Merwe, E.Y. Jones, and L. Fugger. 2009. T cell-mediated autoimmune disease due to low-affinity crossreactivity to common microbial peptides. *Immunity* 30:348-357.

Higashi, T., K. Hashimoto, R. Takagi, Y. Mizuno, Y. Okazaki, Y. Tanaka, and S. Matsushita. 2010. Curdlan induces DC-mediated Th17 polarization via Jagged1 activation in human dendritic cells. *Allergol Int* 59:161-166.

Highfill, S.L., P.C. Rodriguez, Q. Zhou, C.A. Goetz, B.H. Koehn, R. Veenstra, P.A. Taylor, A. Panoskaltsis-Mortari, J.S. Serody, D.H. Munn, J. Tolar, A.C. Ochoa, and B.R. Blazar. 2010. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. *Blood* 116:5738-5747.

Hirai, H., P. Zhang, T. Dayaram, C.J. Hetherington, S. Mizuno, J. Imanishi, K. Akashi, and D.G. Tenen. 2006. C/EBPbeta is required for 'emergency' granulopoiesis. *Nature immunology* 7:732-739.

Hofstetter, H.H., C.L. Shive, and T.G. Forsthuber. 2002. Pertussis toxin modulates the immune response to neuroantigens injected in incomplete Freund's adjuvant: induction of Th1 cells and experimental autoimmune encephalomyelitis in the presence of high frequencies of Th2 cells. *J Immunol* 169:117-125.

Holmoy, T., E.O. Kvale, and F. Vartdal. 2004. Cerebrospinal fluid CD4+ T cells from a multiple sclerosis patient cross-recognize Epstein-Barr virus and myelin basic protein. *Journal of neurovirology* 10:278-283.

Hori, J., M. Wang, M. Miyashita, K. Tanemoto, H. Takahashi, T. Takemori, K. Okumura, H. Yagita, and M. Azuma. 2006. B7-H1-induced apoptosis as a mechanism of immune privilege of corneal allografts. *J Immunol* 177:5928-5935.

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 research* 66:1123-1131.

Hucke, C., C.R. MacKenzie, K.D. Adjogble, O. Takikawa, and W. Daubener. 2004. Nitric oxide-mediated regulation of gamma interferon-induced bacteriostasis: inhibition and degradation of human indoleamine 2,3-dioxygenase. *Infection and immunity* 72:2723-2730.

Ifergan, I., H. Kebir, M. Bernard, K. Wosik, A. Dodelet-Devillers, R. Cayrol, N. Arbour, and A. Prat. 2008. The blood-brain barrier induces differentiation of migrating monocytes into Th17-polarizing dendritic cells. *Brain : a journal of neurology* 131:785-799.

Ilarregui, J.M., D.O. Croci, G.A. Bianco, M.A. Toscano, M. Salatino, M.E. Vermeulen, J.R. Geffner, and G.A. Rabinovich. 2009. Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10. *Nature immunology* 10:981-991.

Ilkovitch, D., and D.M. Lopez. 2009. The liver is a site for tumor-induced myeloid-derived suppressor cell accumulation and immunosuppression. *Cancer research* 69:5514-5521.

Ioannou, M., T. Alissafi, I. Lazaridis, G. Deraos, J. Matsoukas, A. Gravanis, V. Mastorodemos, A. Plaitakis, A. Sharpe, D. Boumpas, and P. Verginis. 2012. Crucial role of granulocytic myeloid-derived suppressor cells in the regulation of central nervous system autoimmune disease. *J Immunol* 188:1136-1146.

Ishida, Y., Y. Agata, K. Shibahara, and T. Honjo. 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *The EMBO journal* 11:3887-3895.

Jeon, S.B., H.J. Yoon, S.H. Park, I.H. Kim, and E.J. Park. 2008. Sulfatide, a major lipid component of myelin sheath, activates inflammatory responses as an endogenous stimulator in brain-resident immune cells. *J Immunol* 181:8077-8087.

Ji, Q., A. Perchellet, and J.M. Goverman. 2010. Viral infection triggers central nervous system autoimmunity via activation of CD8+ T cells expressing dual TCRs. *Nature immunology* 11:628-634.

Jordan, M.B., D.M. Mills, J. Kappler, P. Marrack, and J.C. Cambier. 2004. Promotion of B cell immune responses via an alum-induced myeloid cell population. *Science* 304:1808-1810.

Jovanovic, D.V., J.A. Di Battista, J. Martel-Pelletier, F.C. Jolicoeur, Y. He, M. Zhang, F. Mineau, and J.P. Pelletier. 1998. IL-17 stimulates the production and

expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* 160:3513-3521.

Kamijo, R., H. Harada, T. Matsuyama, M. Bosland, J. Gerecitano, D. Shapiro, J. Le, S.I. Koh, T. Kimura, S.J. Green, and et al. 1994. Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* 263:1612-1615.

Karakhanova, S., S. Meisel, S. Ring, K. Mahnke, and A.H. Enk. 2010. ERK/p38 MAP-kinases and PI3K are involved in the differential regulation of B7-H1 expression in DC subsets. *European journal of immunology* 40:254-266.

Kataoka, K., T. Muta, S. Yamazaki, and K. Takeshige. 2002. Activation of macrophages by linear (1right-arrow3)-beta-D-glucans. Impliations for the recognition of fungi by innate immunity. *The Journal of biological chemistry* 277:36825-36831.

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.

Klehmet, J., C. Shive, R. Guardia-Wolff, I. Petersen, E.G. Spack, B.O. Boehm, R. Weissert, and T.G. Forsthuber. 2004. T cell epitope spreading to myelin oligodendrocyte glycoprotein in HLA-DR4 transgenic mice during experimental autoimmune encephalomyelitis. *Clin Immunol* 111:53-60.

Kohm, A.P., P.A. Carpentier, H.A. Anger, and S.D. Miller. 2002. Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J Immunol* 169:4712-4716.

Kraal, G. 1992. Cells in the marginal zone of the spleen. *International review of cytology* 132:31-74.

Kraus, J., A.K. Ling, S. Hamm, K. Voigt, P. Oschmann, and B. Engelhardt. 2004. Interferon-beta stabilizes barrier characteristics of brain endothelial cells in vitro. *Annals of neurology* 56:192-205.

Kreutzberg, G.W. 1996. Microglia: a sensor for pathological events in the CNS. *Trends in neurosciences* 19:312-318.

Kuipers, H., F. Muskens, M. Willart, D. Hijdra, F.B. van Assema, A.J. Coyle, H.C. Hoogsteden, and B.N. Lambrecht. 2006. Contribution of the PD-1 ligands/PD-1 signaling pathway to dendritic cell-mediated CD4+ T cell activation. *European journal of immunology* 36:2472-2482.

Kusmartsev, S., and D.I. Gabrilovich. 2006. Role of immature myeloid cells in mechanisms of immune evasion in cancer. *Cancer immunology, immunotherapy : CII* 55:237-245.

Lang, H.L., H. Jacobsen, S. Ikemizu, C. Andersson, K. Harlos, L. Madsen, P. Hjorth, L. Sondergaard, A. Svejgaard, K. Wucherpfennig, D.I. Stuart, J.I. Bell, E.Y. Jones, and L. Fugger. 2002. A functional and structural basis for TCR cross-reactivity in multiple sclerosis. *Nature immunology* 3:940-943.

Lee, S.J., B.C. Jang, S.W. Lee, Y.I. Yang, S.I. Suh, Y.M. Park, S. Oh, J.G. Shin, S. Yao, L. Chen, and I.H. Choi. 2006. Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-gamma-induced upregulation of B7-H1 (CD274). *FEBS letters* 580:755-762.

Lee, S.K., S.H. Seo, B.S. Kim, C.D. Kim, J.H. Lee, J.S. Kang, P.J. Maeng, and J.S. Lim. 2005. IFN-gamma regulates the expression of B7-H1 in dermal fibroblast cells. *Journal of dermatological science* 40:95-103.

LeibundGut-Landmann, S., O. Gross, M.J. Robinson, F. Osorio, E.C. Slack, S.V. Tsoni, E. Schweighoffer, V. Tybulewicz, G.D. Brown, J. Ruland, and C. Reis e Sousa. 2007. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nature immunology* 8:630-638.

Li, H., Y. Han, Q. Guo, M. Zhang, and X. Cao. 2009. Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. *J Immunol* 182:240-249.

Liang, S.C., Y.E. Latchman, J.E. Buhlmann, M.F. Tomczak, B.H. Horwitz, G.J. Freeman, and A.H. Sharpe. 2003. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *European journal of immunology* 33:2706-2716.

Linthicum, D.S., J.J. Munoz, and A. Blaskett. 1982. Acute experimental autoimmune encephalomyelitis in mice. I. Adjuvant action of Bordetella pertussis is due to vasoactive amine sensitization and increased vascular permeability of the central nervous system. *Cellular immunology* 73:299-310.

Liston, A., R.E. Kohler, S. Townley, S. Haylock-Jacobs, I. Comerford, A.C. Caon, J. Webster, J.M. Harrison, J. Swann, I. Clark-Lewis, H. Korner, and S.R. McColl. 2009. Inhibition of CCR6 function reduces the severity of experimental autoimmune encephalomyelitis via effects on the priming phase of the immune response. *J Immunol* 182:3121-3130.

Liu, C., C. Zhang, H. Lu, J. Cai, Z. Wang, J. Chen, F. Liu, Z. Wu, X. Liu, and W. Sun. 2011. Poly(I:C) induce bone marrow precursor cells into myeloid-derived suppressor cells. *Molecular and cellular biochemistry* 358:317-323.

Liu, J., A. Hamrouni, D. Wolowiec, V. Coiteux, K. Kuliczkowski, D. Hetuin, A. Saudemont, and B. Quesnel. 2007. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-{gamma} and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. *Blood* 110:296-304.

Liu, Y., B. Zeng, Z. Zhang, Y. Zhang, and R. Yang. 2008. B7-H1 on myeloid-derived suppressor cells in immune suppression by a mouse model of ovarian cancer. *Clin Immunol* 129:471-481.

Loma, I., and R. Heyman. 2011. Multiple sclerosis: pathogenesis and treatment. *Current neuropharmacology* 9:409-416.

Ludewig, B., B. Odermatt, S. Landmann, H. Hengartner, and R.M. Zinkernagel. 1998. Dendritic cells induce autoimmune diabetes and maintain disease via de novo formation of local lymphoid tissue. *The Journal of experimental medicine* 188:1493-1501.

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.

Martin, E., C. Nathan, and Q.W. Xie. 1994. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *The Journal of experimental medicine* 180:977-984.

Martin, R., B. Gran, Y. Zhao, S. Markovic-Plese, B. Bielekova, A. Marques, M.H. Sung, B. Hemmer, R. Simon, H.F. McFarland, and C. Pinilla. 2001. Molecular mimicry and antigen-specific T cell responses in multiple sclerosis and chronic CNS Lyme disease. *Journal of autoimmunity* 16:187-192.

Matsushita, T., M. Horikawa, Y. Iwata, and T.F. Tedder. 2010. Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling experimental autoimmune encephalomyelitis initiation and late-phase immunopathogenesis. *J Immunol* 185:2240-2252.

Mausberg, A.K., S. Jander, and G. Reichmann. 2009. Intracerebral granulocyte-macrophage colony-stimulating factor induces functionally competent dendritic cells in the mouse brain. *Glia* 57:1341-1350.

Mazzoni, A., V. Bronte, A. Visintin, J.H. Spitzer, E. Apolloni, P. Serafini, P. Zanovello, and D.M. Segal. 2002. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J Immunol* 168:689-695.

McColl, S.R., M.A. Staykova, A. Wozniak, S. Fordham, J. Bruce, and D.O. Willenborg. 1998. Treatment with anti-granulocyte antibodies inhibits the effector phase of experimental autoimmune encephalomyelitis. *J Immunol* 161:6421-6426.

McGeachy, M.J. 2011. GM-CSF: the secret weapon in the T(H)17 arsenal. *Nature immunology* 12:521-522.

McQualter, J.L., R. Darwiche, C. Ewing, M. Onuki, T.W. Kay, J.A. Hamilton, H.H. Reid, and C.C. Bernard. 2001. Granulocyte macrophage colony-stimulating factor: a new putative therapeutic target in multiple sclerosis. *The Journal of experimental medicine* 194:873-882.

Mebius, R.E., and G. Kraal. 2005. Structure and function of the spleen. *Nature reviews. Immunology* 5:606-616.

Menges, M., S. Rossner, C. Voigtlander, H. Schindler, N.A. Kukutsch, C. Bogdan, K. Erb, G. Schuler, and M.B. Lutz. 2002. Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. *The Journal of experimental medicine* 195:15-21.

Miller, B.H., R.A. Fratti, J.F. Poschet, G.S. Timmins, S.S. Master, M. Burgos, M.A. Marletta, and V. Deretic. 2004. Mycobacteria inhibit nitric oxide synthase recruitment to phagosomes during macrophage infection. *Infection and immunity* 72:2872-2878.

Mills, K.H. 2011. TLR-dependent T cell activation in autoimmunity. *Nature reviews. Immunology* 11:807-822.

Min, L., S.A. Isa, W.N. Fam, S.K. Sze, O. Beretta, A. Mortellaro, and C. Ruedl. 2012. Synergism between curdlan and GM-CSF confers a strong inflammatory signature to dendritic cells. *J Immunol* 188:1789-1798.

Minagar, A., A. Long, T. Ma, T.H. Jackson, R.E. Kelley, D.V. Ostanin, M. Sasaki, A.C. Warren, A. Jawahar, B. Cappell, and J.S. Alexander. 2003. Interferon (IFN)-beta 1a and IFN-beta 1b block IFN-gamma-induced disintegration of endothelial junction integrity and barrier. *Endothelium : journal of endothelial cell research* 10:299-307.

Miyamoto, M., T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, T. Miyata, and T. Taniguchi. 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. *Cell* 54:903-913.

Molon, B., S. Ugel, F. Del Pozzo, C. Soldani, S. Zilio, D. Avella, A. De Palma, P. Mauri, A. Monegal, M. Rescigno, B. Savino, P. Colombo, N. Jonjic, S. Pecanic, L. Lazzarato, R. Fruttero, A. Gasco, V. Bronte, and A. Viola. 2011. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. *The Journal of experimental medicine* 208:1949-1962.

Moody, D.B., and G.S. Besra. 2001. Glycolipid targets of CD1-mediated T-cell responses. *Immunology* 104:243-251.

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.

Munder, M., H. Schneider, C. Luckner, T. Giese, C.D. Langhans, J.M. Fuentes, P. Kropf, I. Mueller, A. Kolb, M. Modolell, and A.D. Ho. 2006. Suppression of T-cell functions by human granulocyte arginase. *Blood* 108:1627-1634.

Mundy-Bosse, B.L., G.B. Lesinski, A.C. Jaime-Ramirez, K. Benninger, M. Khan, P. Kuppusamy, K. Guenterberg, S.V. Kondadasula, A.R. Chaudhury, K.M. La Perle, M. Kreiner, G. Young, D.C. Guttridge, and W.E. Carson, 3rd. 2011. Myeloid-derived suppressor cell inhibition of the IFN response in tumor-bearing mice. *Cancer research* 71:5101-5110.

Nagaraj, S., A. Nelson, J.I. Youn, P. Cheng, D. Quiceno, and D.I. Gabrilovich. 2012. Antigen-specific CD4(+) T cells regulate function of myeloid-derived suppressor cells in cancer via retrograde MHC class II signaling. *Cancer research* 72:928-938.

Nakajima, K., and S. Kohsaka. 1993. Functional roles of microglia in the brain. *Neuroscience research* 17:187-203.

Nath, N., O. Morinaga, and I. Singh. 2010. S-nitrosoglutathione a physiologic nitric oxide carrier attenuates experimental autoimmune encephalomyelitis. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* 5:240-251.

Nerlov, C., and T. Graf. 1998. PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes & development* 12:2403-2412.

Nguyen, K.B., L.P. Cousens, L.A. Doughty, G.C. Pien, J.E. Durbin, and C.A. Biron. 2000. Interferon alpha/beta-mediated inhibition and promotion of interferon gamma: STAT1 resolves a paradox. *Nature immunology* 1:70-76.

Niedbala, W., J.C. Alves-Filho, S.Y. Fukada, S.M. Vieira, A. Mitani, F. Sonego, A. Mirchandani, D.C. Nascimento, F.Q. Cunha, and F.Y. Liew. 2011. Regulation of type 17 helper T-cell function by nitric oxide during inflammation. *Proceedings of the National Academy of Sciences of the United States of America* 108:9220-9225.

Nishimura, H., T. Honjo, and N. Minato. 2000. Facilitation of beta selection and modification of positive selection in the thymus of PD-1-deficient mice. *The Journal of experimental medicine* 191:891-898.

Nurieva, R.I., and Y. Chung. 2010. Understanding the development and function of T follicular helper cells. *Cellular & molecular immunology* 7:190-197.

O'Connor, R.A., X. Li, S. Blumerman, S.M. Anderton, R.J. Noelle, and D.K. Dalton. 2012. Adjuvant immunotherapy of experimental autoimmune encephalomyelitis: immature myeloid cells expressing CXCL10 and CXCL16 attract CXCR3+CXCR6+ and myelin-specific T cells to the draining lymph nodes rather than the central nervous system. *J Immunol* 188:2093-2101.

O'Connor, R.A., C.T. Prendergast, C.A. Sabatos, C.W. Lau, M.D. Leech, D.C. Wraith, and S.M. Anderton. 2008. Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol* 181:3750-3754.

Ochoa-Reparaz, J., D.W. Mielcarz, L.E. Ditrio, A.R. Burroughs, D.M. Foureau, S. Haque-Begum, and L.H. Kasper. 2009. Role of gut commensal microflora in

the development of experimental autoimmune encephalomyelitis. *J Immunol* 183:6041-6050.

Okahira, S., F. Nishikawa, S. Nishikawa, T. Akazawa, T. Seya, and M. Matsumoto. 2005. Interferon-beta induction through toll-like receptor 3 depends on double-stranded RNA structure. *DNA and cell biology* 24:614-623.

Ortler, S., C. Leder, M. Mittelbronn, A.L. Zozulya, P.A. Knolle, L. Chen, A. Kroner, and H. Wiendl. 2008. B7-H1 restricts neuroantigen-specific T cell responses and confines inflammatory CNS damage: implications for the lesion pathogenesis of multiple sclerosis. *European journal of immunology* 38:1734-1744.

Ostrand-Rosenberg, S., and P. Sinha. 2009. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* 182:4499-4506.

Panopoulos, A.D., and S.S. Watowich. 2008. Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and 'emergency' hematopoiesis. *Cytokine* 42:277-288.

Pardoll, D.M. 2002. Spinning molecular immunology into successful immunotherapy. *Nature reviews. Immunology* 2:227-238.

Pena-Cruz, V., S.M. McDonough, F. Diaz-Griffero, C.P. Crum, R.D. Carrasco, and G.J. Freeman. 2010. PD-1 on immature and PD-1 ligands on migratory human Langerhans cells regulate antigen-presenting cell activity. *The Journal of investigative dermatology* 130:2222-2230.

Petroff, M.G., L. Chen, T.A. Phillips, and J.S. Hunt. 2002. B7 family molecules: novel immunomodulators at the maternal-fetal interface. *Placenta* 23 Suppl A:S95-101.

Pletinckx, K., B. Stijlemans, V. Pavlovic, R. Laube, C. Brandl, S. Kneitz, A. Beschin, P. De Baetselier, and M.B. Lutz. 2011. Similar inflammatory DC maturation signatures induced by TNF or Trypanosoma brucei antigens instruct default Th2-cell responses. *European journal of immunology* 41:3479-3494.

Pourcet, B., J.E. Feig, Y. Vengrenyuk, A.J. Hobbs, D. Kepka-Lenhart, M.J. Garabedian, S.M. Morris, Jr., E.A. Fisher, and I. Pineda-Torra. 2011. LXRalpha regulates macrophage arginase 1 through PU.1 and interferon regulatory factor 8. *Circulation research* 109:492-501.

Prinz, M., H. Schmidt, A. Mildner, K.P. Knobeloch, U.K. Hanisch, J. Raasch, D. Merkler, C. Detje, I. Gutcher, J. Mages, R. Lang, R. Martin, R. Gold, B. Becher, W. Bruck, and U. Kalinke. 2008. Distinct and nonredundant in vivo functions of IFNAR on myeloid cells limit autoimmunity in the central nervous system. *Immunity* 28:675-686.

Raimondi, G., W.J. Shufesky, D. Tokita, A.E. Morelli, and A.W. Thomson. 2006. Regulated compartmentalization of programmed cell death-1 discriminates CD4+CD25+ resting regulatory T cells from activated T cells. *J Immunol* 176:2808-2816.

Raine, C.S. 1984. Biology of disease. Analysis of autoimmune demyelination: its impact upon multiple sclerosis. *Laboratory investigation; a journal of technical methods and pathology* 50:608-635.

Rayamajhi, M., J. Humann, S. Kearney, K.K. Hill, and L.L. Lenz. 2010. Antagonistic crosstalk between type I and II interferons and increased host susceptibility to bacterial infections. *Virulence* 1:418-422.

Re, F., and J.L. Strominger. 2001. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. *The Journal of biological chemistry* 276:37692-37699.

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.

Ribechini, E., P.J. Leenen, and M.B. Lutz. 2009. Gr-1 antibody induces STAT signaling, macrophage marker expression and abrogation of myeloid-derived suppressor cell activity in BM cells. *European journal of immunology* 39:3538-3551.

Rodriguez, P.C., A.H. Zea, K.S. Culotta, J. Zabaleta, J.B. Ochoa, and A.C. Ochoa. 2002. Regulation of T cell receptor CD3zeta chain expression by L-arginine. *The Journal of biological chemistry* 277:21123-21129.

Romer, P.S., S. Berr, E. Avota, S.Y. Na, M. Battaglia, I. ten Berge, H. Einsele, and T. Hunig. 2011. Preculture of PBMCs at high cell density increases sensitivity of T-cell responses, revealing cytokine release by CD28 superagonist TGN1412. *Blood* 118:6772-6782.

Romo-Gonzalez, T., A. Chavarria, and H.J. Perez. 2012. Central nervous system: A modified immune surveillance circuit? *Brain, behavior, and immunity*

Roncarolo, M.G., S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M.K. Levings. 2006. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological reviews* 212:28-50.

Rossner, S., C. Voigtlander, C. Wiethe, J. Hanig, C. Seifarth, and M.B. Lutz. 2005. Myeloid dendritic cell precursors generated from bone marrow suppress T cell responses via cell contact and nitric oxide production in vitro. *European journal of immunology* 35:3533-3544.

Rothfuchs, A.G., A. Bafica, C.G. Feng, J.G. Egen, D.L. Williams, G.D. Brown, and A. Sher. 2007. Dectin-1 interaction with Mycobacterium tuberculosis leads to enhanced IL-12p40 production by splenic dendritic cells. *J Immunol* 179:3463-3471.

Rothhammer, V., S. Heink, F. Petermann, R. Srivastava, M.C. Claussen, B. Hemmer, and T. Korn. 2011. Th17 lymphocytes traffic to the central nervous system independently of alpha4 integrin expression during EAE. *The Journal of experimental medicine* 208:2465-2476.

Sadagopal, S., S.L. Lorey, L. Barnett, D. Sutherland, R. Basham, H. Erdem, S.A. Kalams, and D.W. Haas. 2010. Enhanced PD-1 expression by T cells in cerebrospinal fluid does not reflect functional exhaustion during chronic human immunodeficiency virus type 1 infection. *Journal of virology* 84:131-140.

Sakaguchi, S., M. Miyara, C.M. Costantino, and D.A. Hafler. 2010. FOXP3+ regulatory T cells in the human immune system. *Nature reviews. Immunology* 10:490-500.

Salama, A.D., T. Chitnis, J. Imitola, M.J. Ansari, H. Akiba, F. Tushima, M. Azuma, H. Yagita, M.H. Sayegh, and S.J. Khoury. 2003. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *The Journal of experimental medicine* 198:71-78.

Salkowski, C.A., S.A. Barber, G.R. Detore, and S.N. Vogel. 1996. Differential dysregulation of nitric oxide production in macrophages with targeted disruptions in IFN regulatory factor-1 and -2 genes. *J Immunol* 156:3107-3110.

Sauter, B., M.L. Albert, L. Francisco, M. Larsson, S. Somersan, and N. Bhardwaj. 2000. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of

immunostimulatory dendritic cells. *The Journal of experimental medicine* 191:423-434.

Schmittgen, T.D., and B.A. Zakrajsek. 2000. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *Journal of biochemical and biophysical methods* 46:69-81.

Schreiner, B., S.L. Bailey, T. Shin, L. Chen, and S.D. Miller. 2008. PD-1 ligands expressed on myeloid-derived APC in the CNS regulate T-cell responses in EAE. *European journal of immunology* 38:2706-2717.

Schroder, K., M.J. Sweet, and D.A. Hume. 2006. Signal integration between IFNgamma and TLR signalling pathways in macrophages. *Immunobiology* 211:511-524.

Screpanti, I., L. Romani, P. Musiani, A. Modesti, E. Fattori, D. Lazzaro, C. Sellitto, S. Scarpa, D. Bellavia, G. Lattanzio, and et al. 1995. Lymphoproliferative disorder and imbalanced T-helper response in C/EBP beta-deficient mice. *The EMBO journal* 14:1932-1941.

Seitelberger, F. 1973. Pathology of multiple sclerosis. *Annals of clinical research* 5:337-344.

Seo, S.U., H.J. Kwon, H.J. Ko, Y.H. Byun, B.L. Seong, S. Uematsu, S. Akira, and M.N. Kweon. 2011. Type I interferon signaling regulates Ly6C(hi) monocytes and neutrophils during acute viral pneumonia in mice. *PLoS pathogens* 7:e1001304.

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 research* 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. *Nature immunology* 7:311-317.

Shamshiev, A., A. Donda, I. Carena, L. Mori, L. Kappos, and G. De Libero. 1999. Self glycolipids as T-cell autoantigens. *European journal of immunology* 29:1667-1675.

Shi, F.D., M. Flodstrom, S.H. Kim, S. Pakala, M. Cleary, H.G. Ljunggren, and N. Sarvetnick. 2001. Control of the autoimmune response by type 2 nitric oxide synthase. *J Immunol* 167:3000-3006.

Solito, S., V. Bronte, and S. Mandruzzato. 2011. Antigen specificity of immune suppression by myeloid-derived suppressor cells. *Journal of leukocyte biology* 90:31-36.

Sonderegger, I., G. Iezzi, R. Maier, N. Schmitz, M. Kurrer, and M. Kopf. 2008. GM-CSF mediates autoimmunity by enhancing IL-6-dependent Th17 cell development and survival. *The Journal of experimental medicine* 205:2281-2294.

Stewart, T.J., K.M. Greeneltch, J.E. Reid, D.J. Liewehr, S.M. Steinberg, K. Liu, and S.I. Abrams. 2009. Interferon Regulatory Factor-8 Modulates the Development of Tumor-Induced CD11bGr-1 Myeloid Cells. *Journal of cellular and molecular medicine*

Stinissen, P., R. Medaer, and J. Raus. 1998. Myelin reactive T cells in the autoimmune pathogenesis of multiple sclerosis. *Mult Scler* 4:203-211.

Stohlman, S.A., and D.R. Hinton. 2001. Viral induced demyelination. *Brain Pathol* 11:92-106.

Sun, D., J.N. Whitaker, Z. Huang, D. Liu, C. Coleclough, H. Wekerle, and C.S. Raine. 2001. Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. *J Immunol* 166:7579-7587.

Sun, D., Y. Zhang, B. Wei, S.C. Peiper, H. Shao, and H.J. Kaplan. 2003. Encephalitogenic activity of truncated myelin oligodendrocyte glycoprotein (MOG) peptides and their recognition by CD8+ MOG-specific T cells on oligomeric MHC class I molecules. *International immunology* 15:261-268.

Sun, X., X. Wang, T. Chen, T. Li, K. Cao, A. Lu, Y. Chen, D. Sun, J. Luo, J. Fan, W. Young, and Y. Ren. 2010. Myelin activates FAK/Akt/NF-kappaB pathways and provokes CR3-dependent inflammatory response in murine system. *PloS one* 5:e9380.

Swirski, F.K., M. Nahrendorf, M. Etzrodt, M. Wildgruber, V. Cortez-Retamozo, P. Panizzi, J.L. Figueiredo, R.H. Kohler, A. Chudnovskiy, P. Waterman, E. Aikawa, T.R. Mempel, P. Libby, R. Weissleder, and M.J. Pittet. 2009. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 325:612-616.

Tada, Y., A. Ho, T. Matsuyama, and T.W. Mak. 1997. Reduced incidence and severity of antigen-induced autoimmune diseases in mice lacking interferon regulatory factor-1. *The Journal of experimental medicine* 185:231-238.

Takaoka, A., Y. Mitani, H. Suemori, M. Sato, T. Yokochi, S. Noguchi, N. Tanaka, and T. Taniguchi. 2000. Cross talk between interferon-gamma and -alpha/beta signaling components in caveolar membrane domains. *Science* 288:2357-2360.

Tamura, T., M. Ishihara, M.S. Lamphier, N. Tanaka, I. Oishi, S. Aizawa, T. Matsuyama, T.W. Mak, S. Taki, and T. Taniguchi. 1995. An IRF-1-dependent pathway of DNA damage-induced apoptosis in mitogen-activated T lymphocytes. *Nature* 376:596-599.

Tejada-Simon, M.V., Y.C. Zang, J. Hong, V.M. Rivera, and J.Z. Zhang. 2003. Cross-reactivity with myelin basic protein and human herpesvirus-6 in multiple sclerosis. *Annals of neurology* 53:189-197.

Teng, X., H. Zhang, C. Snead, and J.D. Catravas. 2002. Molecular mechanisms of iNOS induction by IL-1 beta and IFN-gamma in rat aortic smooth muscle cells. *American journal of physiology. Cell physiology* 282:C144-152.

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. *The Journal of experimental medicine* 198:1741-1752.

Toft-Hansen, H., R. Buist, X.J. Sun, A. Schellenberg, J. Peeling, and T. Owens. 2006. Metalloproteinases control brain inflammation induced by pertussis toxin in mice overexpressing the chemokine CCL2 in the central nervous system. *J Immunol* 177:7242-7249.

Tsushima, F., S. Yao, T. Shin, A. Flies, S. Flies, H. Xu, K. Tamada, D.M. Pardoll, and L. Chen. 2007. Interaction between B7-H1 and PD-1 determines initiation and reversal of T-cell anergy. *Blood* 110:180-185.

Turnbaugh, P.J., R.E. Ley, M. Hamady, C.M. Fraser-Liggett, R. Knight, and J.I. Gordon. 2007. The human microbiome project. *Nature* 449:804-810.

Veldhoen, M., R.J. Hocking, R.A. Flavell, and B. Stockinger. 2006. Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis,

but chronic inflammation is needed to sustain disease. *Nature immunology* 7:1151-1156.

Wang, Z., J. Jiang, Z. Li, J. Zhang, H. Wang, and Z. Qin. 2010. A myeloid cell population induced by Freund adjuvant suppresses T-cell-mediated antitumor immunity. *J Immunother* 33:167-177.

Weir, C.R., K. Nicolson, and B.T. Backstrom. 2002. Experimental autoimmune encephalomyelitis induction in naive mice by dendritic cells presenting a self-peptide. *Immunology and cell biology* 80:14-20.

Weiss, A., J. Imboden, K. Hardy, B. Manger, C. Terhorst, and J. Stobo. 1986. The role of the T3/antigen receptor complex in T-cell activation. *Annual review of immunology* 4:593-619.

Witte, M.B., and A. Barbul. 2002. Role of nitric oxide in wound repair. *American journal of surgery* 183:406-412.

Wong, D., K. Dorovini-Zis, and S.R. Vincent. 2004. Cytokines, nitric oxide, and cGMP modulate the permeability of an in vitro model of the human blood-brain barrier. *Experimental neurology* 190:446-455.

Wucherpfennig, K.W., and J.L. Strominger. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 80:695-705.

Xu, G., Y. Zhang, L. Zhang, A.I. Roberts, and Y. Shi. 2009. C/EBPbeta mediates synergistic upregulation of gene expression by interferon-gamma and tumor necrosis factor-alpha in bone marrow-derived mesenchymal stem cells. *Stem Cells* 27:942-948.

Yadav, M., and J.S. Schorey. 2006. The beta-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. *Blood* 108:3168-3175.

Yamazaki, T., H. Akiba, H. Iwai, H. Matsuda, M. Aoki, Y. Tanno, T. Shin, H. Tsuchiya, D.M. Pardoll, K. Okumura, M. Azuma, and H. Yagita. 2002. Expression of programmed death 1 ligands by murine T cells and APC. *J Immunol* 169:5538-5545.

Yang, H., R. Zhang, H. Mu, M. Li, Q. Yao, and C. Chen. 2006. Adiponectin promotes endothelial cell differentiation from human peripheral CD14+ monocytes in vitro. *Journal of cellular and molecular medicine* 10:459-469.

Yang, Z., B. Zhang, D. Li, M. Lv, C. Huang, G.X. Shen, and B. Huang. 2010. Mast cells mobilize myeloid-derived suppressor cells and Treg cells in tumor microenvironment via IL-17 pathway in murine hepatocarcinoma model. *PloS one* 5:e8922.

Yao, S., S. Wang, Y. Zhu, L. Luo, G. Zhu, S. Flies, H. Xu, W. Ruff, M. Broadwater, I.H. Choi, K. Tamada, and L. Chen. 2009. PD-1 on dendritic cells impedes innate immunity against bacterial infection. *Blood* 113:5811-5818.

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.

Zal, T., A. Volkmann, and B. Stockinger. 1994. Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen. *The Journal of experimental medicine* 180:2089-2099.

Zenaro, E., M. Donini, and S. Dusi. 2009. Induction of Th1/Th17 immune response by Mycobacterium tuberculosis: role of dectin-1, Mannose Receptor, and DC-SIGN. *Journal of leukocyte biology* 86:1393-1401.

Zhang, H., H. Nguyen-Jackson, A.D. Panopoulos, H.S. Li, P.J. Murray, and S.S. Watowich. 2010. STAT3 controls myeloid progenitor growth during emergency granulopoiesis. *Blood* 116:2462-2471.

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.

Zhao, C.B., S.W. Coons, M. Cui, F.D. Shi, T.L. Vollmer, C.Y. Ma, S.M. Kuniyoshi, and J. Shi. 2008. A new EAE model of brain demyelination induced by intracerebroventricular pertussis toxin. *Biochemical and biophysical research communications* 370:16-21.

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.

Zhu, B., I. Guleria, A. Khosroshahi, T. Chitnis, J. Imitola, M. Azuma, H. Yagita, M.H. Sayegh, and S.J. Khoury. 2006. Differential role of programmed death-ligand 1 [corrected] and programmed death-ligand 2 [corrected] in regulating the susceptibility and chronic progression of experimental autoimmune encephalomyelitis. *J Immunol* 176:3480-3489.

Zhu, B., J.K. Kennedy, Y. Wang, C. Sandoval-Garcia, L. Cao, S. Xiao, C. Wu, W. Elyaman, and S.J. Khoury. 2011. Plasticity of Ly-6C(hi) myeloid cells in T cell regulation. *J Immunol* 187:2418-2432.

Zoglmeier, C., H. Bauer, D. Norenberg, G. Wedekind, P. Bittner, N. Sandholzer, M. Rapp, D. Anz, S. Endres, and C. Bourquin. 2011. CpG blocks immunosuppression by myeloid-derived suppressor cells in tumor-bearing mice. *Clinical cancer research : an official journal of the American Association for Cancer Research* 17:1765-1775.

7 GSLS Thesis Guidelines

Mandatory Elements

LAB MEETING = GROUP SEMINAR

Required: usually 1h/ week, organized by the research group or the graduate training group:

- Institute of Immunology Seminars (Every Tuesday)
- Lab Meeting (Every Friday)

LITERATURE SEMINAR = JOURNAL CLUB

Required: usually 1h/ week, organized by the research group or the graduate training group

- Institute of Immunology Literature Seminars (Every Wednesday)

RESEARCH TRAINING GROUP SEMINAR (Jour Fixe)

Required: usually once or twice per month

- Immunomodulation (Every Thursday)

OTHER SEMINARS/MEETINGS

- SFB581 Seminars (Some Thursdays)
- Immunology and Virology Seminars (Monday nights)

MEETINGS / SYMPOSIA

Required: 3 International events with scientific participation within 3 years

- Lugano, Switzerland, 2010 (DC and Vaccines Meeting)
- Brussels, Belgium, 2011 (Dendritic Cell and Macrophage Society Meeting)
- Wuerzburg, Germany, 2011 (GSLS Graduate School Meeting)

Non-International events attended

- 2009 Marburg Meeting
- 2011 Transregio 52 Meeting

SCIENTIFIC PUBLICATIONS

Ribechini E, Greifenberg V, Sandwick S, Lutz MB. (2010) Subsets, expansion and activation of myeloid-derived suppressor cells Med Microbiol Immunol. 199(84):273-281. (Review)

RETREAT / SUMMER SCHOOL *(one per year) e.g. organized by the research group, the research training group or the doctoral students* ***Retreat:*** *"period of group withdrawal under a supervisor" (3-4 days)* ***Summer School:*** *"academic session*

during the summer, for remedial or supplementary study" (1-2 weeks)

- Immunomodulation Retreat 2010 Kloster Schontal
- Immunomodulation Retreat 2011, Bad Nereheim

SCIENTIFIC COURSE / SEMINAR / LECTURES *(one per year) organized by the graduate training group, GSLS, doctoral students or offered externally (e.g. method course, statistics, clinical medicine, "Hottest Life Science in Town")*

- 2011 Abbas Book Seminar (Hosted by Immunomodulation)
- 2009 Animal Training Course
- 2009, 2010 German Language Courses

Electives (at least one of the following per year)

TRANSFERABLE SKILLS COURSE / SEMINAR / WORKSHOP

e.g. poster presentation, scientific writing

- 2010 Statistics Course (3 parts)
- 2011 Writing for Publication
- 2011 Grant Writing

CONTRIBUTION TO ACTIVITIES OF THE GSLS

e.g. peer mentor, DSC, teaching activities, organisation of events

- 2011 Assisted in Manfred Lutz section of Student Practical Course

WORKING STAY

e.g. scientific fieldwork, laboratory training, usually outside University of Würzburg

- Transregio 52 collaboration with Dr. Ari Waisman, Mainz, learned EAE CNS isolation techniques.
- Mini-project with Anoop Chandran, Dr. Jörg Wischhusen, Frauenklinik, Würzburg, learned miRNA isolation, poly-adenylation, RT-PCR.

8 Curriculum Vitae

Sarah Jane Sandwick

ACADEMIC PREPARATION:
PhD, Manfred B. Lutz Lab, Universität Würzburg, May 2009 – July 2012
Major: Immunology

MS, Johns Hopkins University with a Thesis, 2009
Major: Biotechnology

BS, University of Southern Maine, 2006 (GPA: 3.17)
Double Major: Biology (emphasis in Biotechnology) and Political Science
Minor: Biochemistry

RESEARCH SKILLS:
General Laboratory Skills: Sample handling, database management, pipetting, and preparation of various solutions, preparations of dilution series, use of pH meter, balance, centrifuge, spectramax, flow cytometers, in vivo/ex vivo bioluminescence imaging, western blotting, ELISA, plaque assay, cell culture including primary cell cultures such as immature myeloid cells, dendritic cells, macrophages, and T cells, surface and intracellular cell staining, CBA, proliferation assays, histology (immuno-fluorescence), isolation of RNA, micro-RNA screenings, BSL2/BSL3/BSL4 tissue culture, and analysis of data.

Animal work:
Mouse work-
General: Blood collection, splenectomies, euthanasia, perfusions
Injections: IV, IP, IM, SC, oral gavage
Dissections for various organs: including liver, pancreas, spleen, bone marrow, spinal chord, brain, lungs, and lymph nodes

Chicken work-
Dissections: spinal motor neuron and forebrain dissections

Vaccines received:
Smallpox, Tularemia, Anthrax, Venezualan Equine Encephalitis, Rift Valley Fever Virus, Yellow Fever

PROFESSIONAL EXPERIENCE:
Universität Würzburg *, May 2009 - Present
Sponsor: SFB-581
Supervisor: Dr. Manfred Lutz
Committee Members: Dr. Stephan Kissler, Dr. Rudolf Martini, Dr. Andreas Beilhack

Projects: To understand the role of myeloid derived suppressor cells (MDSC) in autoimmune diseases and infection. To analyze factors involved in MDSC activation and suppressive mechanisms. Understanding the migration patterns of all subsets under different conditions.

In vivo bioluminescence imaging, Experimental autoimmune encephalomyelitis, asthma models, flow cytometry, immunohistochemistry, cytospins, proliferation assays, ELISAs, intracellular cytokine stainings, literature and progress report seminars, participation in Immunomodulation gradutate program, assistant in student practical course (2011).

United States Army Medical Research Institute for Infectious Disease (USAMRIID) **,
December 2006 – May 2009
Sponsor: Oak Ridge Institute for Science and Education (ORISE)
PI: Dr. Sina Bavari

Projects: (divided into individual mentor's projects)
Dr. Steven Bradfute- Thesis work for MS. Work in BSL4 with T cell epitope mapping of Ebola virus proteins, including mostly ICC and Flow cytometry.
Dr. Mansour Mohamadzadeh- *Lactobacillus acidophilus* studies with dendritic cells, includes Flow cytometry, mouse studies, RNA isolation, ELISA, MTT neutralization assay, T cell proliferation assays, immunohistology.
Dr. Sheli Radoshitzky- Cowpox and Rift Valley Fever Virus studies with transfected cells
Dr. Gordon Ruthel- Bontulinum studies with chicken primary cultures
Dr. Travis Warren- Small molecule therapeutics against Rift Valley Fever Virus, Cowpox (BSL3 agents), mostly propagation of virus stocks, plaque assays.

- Flow cytometry, mouse work. Rotating lab manager (November-December 2008), organized laboratory team building day 2008, mentorship of fellow personnel in BSL3.

IDEXX Laboratories, February 2006 – December 2006
Mycobacterium paratuberculosis (Mpt) ELISA group
Head Supervisor: Dr. Lisa Estey
Lab Supervisors: Jill Kerrick, Dr. Jay Jayarama
Project: Development of ELISA assays to improve a current kit that is on the market which detects *Mycobacterium paratuberculosis* in cow herds.

-ELISA optimization, databasing of samples.

National Aeronautics and Space Administration (NASA) Lyndon B. Johnson Space Center,
June 1, 2005 – August 15, 2005
Sponsor: Maine Space Consortium Grant
PI: Dr. Steve R. Gonda,
Mentors: Racheal Casey and Kamal Emami
Project: Big Blue Mutagenesis Assay

- 3-dimensional cell culture, comparison of agars, Big Blue mutagenesis assay, which includes DNA extraction, transpacking, and cII, presentations, attending seminar presentations. I have done several presentations stemming from this research: An exit presentation, high school presentations, oral presentation at the MSCG annual meeting.

Wise Laboratory of Environmental and Genetic Toxicology***, September 2003 – May 2006
Sponsor: unsponsored and NASA grant
PI/Mentor: Dr. John Pierce Wise Sr.,
Mentor: Amie Holmes
Project: Comparative toxicology studies on human lung cells and bowhead lung cells

- Cytotoxicity, Chromosome Damage, Clonal Expansion, Cell Culture, Media Preparation, Chemical/Metal Preparation, journal presentations, internal presentations, and external

presentations. Research funding awarded from SURF Fellowship (through USM) –Summer 2004 and NASA –Fall/Spring Semester 2004. I won second place at the Northeastern Society of Toxicology Symposium on a poster presentation on this research.

* This research is currently supported by the SFB-581.
* This research was supported in part by an appointment to the Postgraduate Research Participation Program at the US Army Medical Research and Materiel Command administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and the USAMRMC.

** This research was supported by the NASA Grant (Fall 2004 - Feb 2005), while research done during the Summer of 2004 was supported by the Serf Fellowship (USM Grant).

GRANTS RECEIVED:

Fall 2004 - Feb 2005 NASA Grant
Summer 2004 Serf Fellowship (USM Grant)
December 2006-December 2008 USAMRIID Sponsor: This research was supported in part by an appointment to the Postgraduate Research Participation Program at the US Army Medical Research and Materiel Command administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the US Department of Energy and the USAMRMC.

PUBLICATIONS:

Sandwick SJ, Bradfute S, Bavari S. (2011) J. Virol. (in preparation).

Ruthel G, Burnett JC, Nuss JE, Wanner LM, Tressler LE, Torres-Melendez E, Sarah J. Sandwick, Retterer CJ and Bavari S. (2011) Post-Intoxication Inhibition of Botulinum Neurotoxin Serotype A within Neurons by Small-Molecule, Non-Peptidic Inhibitors. Toxins. 3:207-217.

Radoshitzky SR, Dong L, Chi X, Clester JC, Retterer C, Spurgers K, Kuhn JH, Sandwick S, Ruthel G, Kota K, Boltz D, Warren T, Kranzusch PJ, Whelan SP, Bavari S. (2010) Infectious Lassa virus, but not filoviruses, is restricted by BST-2/tetherin. J Virol. 84(20):10569-80.

Ribechini E, Greifenberg V, Sandwick S, Lutz MB. (2010) Subsets, expansion and activation of myeloid-derived suppressor cells Med Microbiol Immunol. 199:273-281. (Review)

Mohamadzadeh M, Duong T, Sandwick SJ, Hoover T, Klaenhammer TR. (2009) Dendritic cell targeting of Bacillus anthracis protective antigen expressed by Lactobacillus acidophilus protects mice from lethal challenge Proc Natl Acad Sci U S A. 106(11):4331-4336.

Holmes, A.L., Wise, S. S., Sandwick, S.J., Lingle, W.L., Negron, V.C., Thompson W.D., and Wise, Sr., J.P. (2006) Chronic Exposure to Lead Chromate Causes Centrosome Abnormalities and Aneuploidy in Human Lung Cells. Cancer Research 66(8):4041-4048.

Holmes, A.L., Wise, S. S., Sandwick, S.J., and Wise, Sr., J.P. (2006) The Clastogenic Effects of Chronic Exposure to Particulate and Soluble Cr(VI) in Human Lung Cells. Mutation Research, 610(1-2): 8-13.

Wise, S.S., Holmes, A.L., Moreland, J.A., Xie, H., Sandwick, S.J., Stackpole, M.M., Fomchenko, E., Teufack, S., May, Jr., A.J., Katsifis, S.P., and Wise, Sr., J.P. (2005) Human Lung Cell Growth Is Not Stimulated by Lead Ions after Lead Chromate-Induced Genotoxicity. Molecular and Cellular Biochemistry 279 (1-2):75-84.

PAPERS PRESENTED AT CONFERENCES:

Moreland, J.A., Teufack, S., Sandwick, S., Dufour, J-F., Wise, S.S., Holmes, A.L., Ketterer, M.E., Hartsock, W.J., Fomchenko, E., Katsifis, S.P., and Wise, Sr., J.P. Particulate Hexavalent Chromium-Induced Cell Cycle Arrest Is Mediated by Cr Ions. Proceedings of the Northeast Society of Toxicology (NESOT) Annual Meeting, November 2003.

Moreland, J.A., Teufack, S., Sandwick, S., Dufour, J-F., Wise, S.S., Holmes, A.L., Ketterer, M.E., Hartsock, W.J., Fomchenko, E., Katsifis, S.P. and Wise, Sr., J.P. Lead Chromate (LC) Induces Cell Cycle Arrest in WTHBF-6 Cells, However Lead Alone Is Mitogenic. Toxicological Sciences 67(S): 270, 2004.

Wise, S.S., Holmes, A.L., Xie, H., Gordon, N., Moreland, J.A., Sandwick, S.J., Stackpole, M.M., Fomchenko, E., Teufack, S., May, Jr., A.J., Katsifis, S.P., Thompson, W.D., and Wise, Sr., J.P. Mechanisms of Particulate Chromate-Induced Genotoxicity. Proceedings of the 3rd Conference on Molecular Mechanisms of Metal Toxicity and Carcinogenesis. September 2004.

Sandwick, S.J., Holmes, A.L., Wise, S.S., Gordon, N., and Wise, Sr., J.P. Lead Ions Disrupt Normal Mitotic Progression in Human Lung Cells after More Chronic Exposures to Lead Chromate. Proceedings of the Annual Meeting of the Northeast Chapter of the Society of Toxicology, October 2004.

Holmes, A.L., Sandwick, S.J., Wise, S.S., Gordon, N., Thompson W.D., and Wise, Sr., J.P. Chronic Exposure to Lead Ions from Lead Chromate Interferes with Normal Mitotic Progression. Toxicological Sciences 84(S-1): 1145, 2005.

Wise, S.S., Holmes, A.L., Sandwick, S.J., Gordon, N., Thompson W.D., and Wise, Sr., J.P. Lead Ions Interfere with Repair of Particulate Chromate-induced Chromosome Aberrations Resulting in Prolonged Chromosome Instability in Human Lung Cells. Proceedings of the American Association for Cancer Research 46:3043, 2005.

Wise, S.S., Holmes, A.L., Sandwick, S.J., and Wise, Sr., J.P. Chronic Exposure to Lead Chromate Induces Premature Anaphase and Inhibits Repair of Chromosome Damage in Human Lung Cells. Proceeding of the American Thoracic Society, 2: A744, 2005.

Holmes, A.L., Wise, S.S., Sandwick, S.J., Lingle, W.L., Negron, V.C., and Wise, Sr., J.P. Chronic Exposure to Lead Chromate Causes Centrosome Abnormalities and Aneuploidy in Human Lung Cells. Proceedings of the Biennial Meeting of the Chromium and Human Health Workshop, August 2005.

Wise, S.S., Holmes, A.L., Sandwick, S.J., and Wise, Sr., J.P. Lead Chromate-Induced Centromeric Abnormalities in Human Lung Cells. Proceedings of the Biennial Meeting of the Chromium and Human Health Workshop, August 2005.

Holmes, A.L., Wise, S.S., Sandwick, S.J., Lingle, W.L., Negron, V.C., and Wise, Sr., J.P. Chronic Exposure to Lead Chromate Causes Centrosome Amplification and

Aneuploidy in Human Lung Cells. Toxicological Sciences 90: 519, 2006.

Holmes, A.L., Wise, S.S., Sandwick, S.J., Lingle, W.L., Negron, V.C., and Wise, Sr., J.P. Lead Chromate-Induced Chromosome Instability is Caused by Centrosome Amplification in Human Lung Cells. Proceedings of the American Association for Cancer Research 47: 1088, 2006.

PAPERS PRESENTED AT LOCAL CONFERENCES:

Sandwick, S., Wise, S.S., Holmes, A.L., and Wise, Sr., J.P. The Effect of Exposure Time on Lead Chromate-Induced Cytotoxicity in Human Lung Cells. Proceedings of University of Southern Maine's Student Research Day 20, 2004.

Moreland, J.A., Teufack, S., Sandwick, S., Dufour, J-F., Wise, S.S., Holmes, A.L., Ketterer, M.E., Hartsock, W.J., Fomchenko, E., Katsifis, S.P. and Wise, Sr., J.P. Lead is Mitogenic to WTHBF-6 cells, but Lead Chromate (LC) Induces Cell Cycle Arrest. Proceedings of University of Southern Maine's Student Research Day 45, 2004.

Sandwick, S., Holmes, A.L., Wise, S.S., and Wise, Sr., J.P. Comparison of Human and Bowhead Lung Cells through Cytotoxicity, Metal Ion Uptake, and Clastogenicity. Proceedings of University of Southern Maine's Student Research Day 11, 2005.

Holmes, A.L., Wise, S.S., Sandwick, S.J., Lingle, W.L., Negron, V.C., and Wise, Sr., J.P. Lead Chromate-Induced Chromosome Instability Is Caused by Centrosome Amplification in Human Lung Cells. Proceedings of University of Southern Maine's Student Research Day 65, 2006.

Sandwick, S.J., Karaczyn, A.A., and Wise, Sr., J.P. *In Vitro* Toxicity and Neoplastic Transformation of Depleted Uranium: Effects on Human Bronchial Epithelial Cells. Proceedings of University of Southern Maine's Student Research Day 58, 2006.