RESTRICTING THE DEVELOPMENT OF AUTOIMMUNE DISEASE BY SKEWING THE PATHOGENIC TH17 CELLS TO PROTECTIVE TREGS

Doctoral Thesis

by

Mohammad Adeel Zafar

(Regd. No. 2018BMZ0008)

DEPARTMENT OF BIOMEDICAL ENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY ROPAR

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In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

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Mohammad Adeel Zafar: Restricting the Development of Autoimmune Disease by Skewing the Pathogenic Th17 Cells to Protective Tregs.

DECLARATION OF ORIGINALITY

I, at this moment, declare that the work presented in the thesis entitled "RESTRICTING THE DEVELOPMENT OF AUTOIMMUNE DISEASE BY SKEWING THE PATHOGENIC TH17 CELLS TO PROTECTIVE TREGS" has been solely authored by me. It presents the results of my independent investigation/research conducted from January 7th, 2019 to December 11th, 2023, under the supervision of Prof Javed Naim Agrewala, Professor, Department of Biomedical Engineering, IIT Ropar and co-supervision of Dr Srivatsava Naidu, Assistant Professor, Department of Biomedical Engineering, IIT Ropar. To the best of my knowledge, it is an original work, both in terms of research contents and narrative, and has not been submitted or accepted elsewhere, in part or whole, for the award of any degree, diploma, fellowship, associateship or similar title of any university or institutions. Further due credit has been attributed to the relevant state-of-the-art collaborations with appropriate citations and acknowledgements in line with the established ethical norms and practices. I also declare that any idea/data/facts/figures/source stated in my thesis has not been fabricated/falsified/ misrepresented. All the principles of academic honesty and integrity have been followed. The Institute reserves the right to withdraw the thesis from the archive and revoke the associated degree conferred if the idea is unoriginal, manufactured, or plagiarized. Additionally, the Institute reserves the right to appraise all concerned sections of society for their information and necessary actions. If accepted, I now consent for my thesis to be available online in the institute's open-access repository, inter-library loan, and the title & abstract to be made available to outside organizations.

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DEDICATED SOLEY TO

Almighty Lord and My Beloved Parents

CERTIFICATE

This is to certify that the thesis entitled "Restricting the Development of Autoimmune Disease by Skewing the Pathogenic Th17 Cells to Protective Tregs", submitted by Mr Mohammad Adeel Zafar (2018BMZ0008) for the award of the degree of Doctor of Philosophy of the Indian Institute of Technology Ropar, is a record of *bona fide* research work carried out under my guidance and supervision. To the best of my knowledge and belief, the work presented in this thesis is original. It has not been submitted, either in part or whole, for the award of any other degree, diploma, fellowship, associateship or similar title of any university or institution.

The thesis has reached the standard of fulfilling the requirements of the regulations relating to the degree.

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LAY SUMMARY

Autoimmune diseases represent a collection of disorders wherein the body's immune system, notably T cells and B cells, erroneously direct their attack towards the body's own tissues and organs. This detrimental immune response ultimately results in dysfunction of the affected cells and tissues.Notable examples include type 1 diabetes, rheumatoid arthritis, and multiple sclerosis. While various autoimmune diseases may focus on distinct bodily systems and employ differing mechanisms, they share a common pathogenic root, often driven by autoreactive T cells and B cells. A pertinent instance lies in MOG_{35-55}, a pivotal immunodominant constituent of the myelin sheath, which serves as insulation for neurons. In the context of multiple sclerosis, pathogenic T cells directed against MOG_{35-55} precipitate the destruction of the myelin sheath. This results in irreversible neuronal damage, leading to compromised motor function, muscle control, vision, and cognitive faculties.

Unfortunately, autoimmune diseases presently lack a vaccine or a definitive cure. Recent research endeavours have illuminated potential avenues for managing the symptoms of autoimmune diseases. These studies have indicated that antigen-specific regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSC) hold promise in dampening the pathogenic T cell response. Furthermore, MDSC can promote the conversion of undifferentiated T cells into protective Tregs. Additionally, antigen-specific memory Tregs exhibit the ability to recall antigens and mount a robust response upon future encounters with the same antigen. Consequently, effectively preventing disease progression.

Immunosuppressive drugs like rapamycin have the remarkable ability to generate Tregs. Further, immunization with autoantigen (MOG_{35-55}) generates a myelin specific pathogenic T cells (Th17 cells) biased immune response resulting in pathogenesis and development of mice model of multiple sclerosis i.e., experimental-autoimmune-encephalomyelitis (EAE). Based on the above facts, we capitalized on rapamycin’s unique property to transform autoantigen reactive pathogenic T cells into auto-antigen specific protective Tregs. We demonstrated that animals immunized with the cocktail, showed a substantial increase in the pool of MOG reactive Tregs and MDSC with the suppressive phenotype. This was accompanied by a decline in Th17 cells. Furthermore, there was a significant increase in the population of MOG_{35-55} specific memory Tregs and the vaccinated animals displayed no symptoms of EAE upon exposure to a sensitisingdose of MOG_{35-55}. This study introduces a novel stratagem to

revert pathogenic MOG_{35-55} reactive Th17 cells towards protective MOG_{35-55} specific Tregs by using immunosuppressive molecules.

In the future, this study has the potential to open avenues for the development of a vaccine to prevent and treat autoimmune diseases.

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ABBREVIATIONS

AIDs Autoimmune Diseases

Treg Regulatory T cells

TGF Transforming growth factor

Ab Antibody

Ag Antigen

ANOVA Analysis of variance

APC Antigen-presenting cells

BMDC Bone marrow-derived dendritic cell

BSA Bovine serum albumin

CCR Chemokine receptor

CFA Complete Freund's adjuvant

CD Cluster of differentiation

CTL Cytotoxic T lymphocytes

DAMP Damage associated molecular pattern

DC Dendritic cells

ddH_2O Double distilled water

DNA Deoxyribonucleicacid

FBS Fetal bovine serum

Fig Figure

GM-CSF Granulocyte-macrophage colony-stimulating factor

HLA Human Leukocyte Antigen

HSP Heat shock protein

IFA Incomplete Freund's adjuvant

IFN-γ	Interferon-γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
NF-κB	Nuclearfactor kappa B
NO	Nitric oxide
NP	Nanoparticle
OD	Optical density
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBST	1X PBS + 0.1% Tween-20
pH	Negative logarithm of hydrogen ion concentration
PRR	Pattern recognition receptors
PVDF	Polyvinylidene difluoride
r	Recombinant
R&D	Research and Development
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute
s.c.	Subcutaneous
SD	Standard deviation
SN	Supernatants
TCR	T cell receptor

Th	T helper
TLR	Toll-Like Receptor
TNFα	Tumor necrosis factor-alpha
UN	United Nations
Vol.	Volume
WHO	World Health Organization

Symbols

α	Alpha
β	Beta
γ	Gamma
κ	Kappa
±	Plus-minus
µ	Micro
≤	Less than or equal to
≥	More than or similar to

Units of measurement

%	Percentage
×g	Centrifugal force equal to gravitational force
µg	Microgram
µl	Microlitre
°C	Degrees Celsius

d	day
h	Hour(s)
kDa	kilo Dalton
kHz	kilo Hertz
M	Molar
mg	Milligrams
min	Minutes
ml	Milliliter
mM	Millimolar
mV	Millivolts
ng	Nanogram
nM	Nanomolar
nm	Nanometer
OD	Optical density
V	Volts

Techniques

ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence assisted cell sorting
PCR	Polymerase chain reaction
qRT-PCR	Real-time Quantitative Reverse Transcription PCR

IHCImmunohistochemistry

Chemicals

DMSO Dimethylsulphoxide

H_2SO_4 Sulphuric acid

H_2O_2 Hydrogen peroxide

HCl Hydrochloric acid

HRP Horseradish peroxidase

KH_2PO_4 Potassium dihydrogen phosphate

$KHCO_3$ Potassium bicarbonate

MES 2-(N-morpholino)ethane sulfonic acid

$MgCl_2$ Magnesium chloride

Na_2CO_3 Sodium carbonate

Na_2HPO_4 Disodium hydrogen phosphate

NaCl Sodium chloride

$NaHCO_3$ Sodium bicarbonate

NaN_3 Sodium azide

NaOH Sodium hydroxide

OPD Ortho phenylenediamine

PPD Purified Protein Derivative

SDS Sodium dodecyl sulphate

TMB Tetramethylbenzidine

β-ME/2-ME β- or 2- mercaptoethanol

ABSTRACT

During multiple sclerosis, MOG_{35-55} reactive pathogenic Th17 cells destroy the myelin sheath surrounding nerve fibers. There is no vaccine or durable cure for the disease. Rapamycin has the remarkable ability to differentiate stimulated naïve T cells into regulatory T cells (Tregs). In this study, we exploited on rapamycin's unique property to transform MOG_{35-55} reactive pathogenic Th17 cells into MOG_{35-55} specific protective Tregs. We demonstrated that mice, which were vaccinated with a combination of MOG_{35-55} and rapamycin, displayed no symptoms of experimental autoimmune encephalomyelitis (EAE) upon re-exposure to a morbific dose of MOG_{35-55}. Notably, there was a significant increase in the population of MOG_{35-55}-specific memory Tregs that primarily expressed immunosuppressive phenotype ($CD39^{hi}$, $CD73^{hi}$, $PD1^{hi}$, $Tim3^{hi}$, and $IL\text{-}10^{hi}$). This was accompanied by a substantial decline in the population of Th17 cells. Additionally, a substantial increase in the pool of myeloid-derived suppressor cells (MDSC) with the suppressive phenotype ($TGF\text{-}\beta^{hi}$, $IL\text{-}10^{hi}$, $CD103^{hi}$, $LFA\text{-}1^{lo}$, and $FasL^{hi}$) was observed. The underlying mechanism of this phenomenon was deciphered to be through autophagy; as evidenced by the modulation of autophagy markers $mTOR^{lo}$, $FIP200^{hi}$, $Atg13^{hi}$, $ULK1^{hi}$, $beclin^{hi}$, and $LC3^{hi}$ in both Tregs and MDSC. This study introduces a novel stratagem to revert pathogenic MOG_{35-55} reactive Th17 cells towards protective MOG_{35-55} specific Tregs through rapamycin. In the future, this study has the potential to open new avenues for developing a vaccine to protect and cure multiple sclerosis.

Keywords: Multiple Sclerosis, Vaccine, Rapamycin, MOG_{35-55}

CHAPTER 1
INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease characterized by demyelination and incapacitating effects on the central nervous system (CNS). Inflammatory lesions within the CNS are associated with the presence of Th17 cells, which play a crucial role in the pathogenesis of the disease. Autoimmune memory persists in the context of MS, making it challenging to eliminate autoreactive memory T cells and B cells that pose a constant threat of disease recurrence (*Devarajan P et al., 2013*). Thus, the primary perpetrators of autoimmune disorders are autoreactive Th17 cells, whereas regulatory T cells (Tregs) are responsible for protecting them from autoimmunity. Tregs are particularly effective in inhibiting Th17 cells (*Rojas JI et al., 2010*). Although, rapamycin, cyclosporine A and FK506 are currently used to treat AIDs but can only partially subsidize the symptoms.

Studies in the experimental autoimmune encephalomyelitis (EAE), amurine model of MS have demonstratedthe pathogenic role of Th17 cells in the onset and progression of the disease (*Matsui M et al., 2008*). EAE can be induced in susceptible mouse strains by injecting MOG_{35-55} peptide emulsified incomplete Freund's adjuvant (CFA), resulting in the expansion of MOG_{35-55}-reactive autoimmune Th17 cells ($Th17^{MOG}$), the orchestrator of the CNS inflammation (*Rostami A and Ciric B, 2013*). Interestingly, rapamycin has shown promising results in suppressing the generation of Th17 cells and induction and enhancement of stable and functional Tregs (*Kopf H et al., 2015; Battaglia M et al., 2005*). Consequently, we hypothesised that skewing MOG-reactive pathogenic Th17 cells to MOG-specific protective Tregs could control the severity of EAE and havethe potential to ameliorate MS in humans. Therefore, we investigated the role of rapamycin in shifting the generation of MOG_{35-55}-specific naive CD4 T cells into protective MOG_{35-55}-specific Tregs ($Tregs^{MOG}$). Furthermore, we assessed whether $Tregs^{MOG}$ differentiates into memory Tregs to achieve long-term protection against EAE.

Our findings revealed that vaccination of animals with MOG peptide in combination with rapamycin successfully induced the generation of MOG-specific memory $Tregs^{MOG}$ which drasticallydiminished the development of EAE. This novel vaccination strategy holds promise to generate protective autoantigen-reactive Tregs against other autoimmune diseases.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Overview of Immune System. The immune system is a complex and highly orchestrated network of cells, tissues, and molecules designed to safeguard the body against infections, diseases, and other foreign invaders. It comprises two primary branches. the innate immune system and the adaptive immune system. The innate immune system serves as the initial line of defense against pathogens and remains in a constant state of readiness, offering immediate but non-specific responses to threats. Key components of the innate immune system encompass macrophages and dendritic cells. These cells not only recognize and engulf pathogens but also kickstart an inflammatory response and present antigens to activate the adaptive immune system. Natural Killer (NK) cells, meanwhile, possess the remarkable ability to detect and eliminate infected or aberrant cells, including cancer cells, without the prerequisite of prior exposure. Neutrophils, classified as phagocytic cells, play a pivotal role in combating bacterial infections. The Complement System, comprising proteins, plays a crucial role in identifying and obliterating pathogens through a process known as complement activation. in contrast, the adaptive immune system confers specific and long-lasting immunity. While it takes time to develop, it boasts a memory function that enables more effective responses upon subsequent encounters with the same pathogen. Key constituents of the adaptive immune system encompass T cells and B cells. T cells comprise helper T cells and cytotoxic T cells, with the former orchestrating immune responses and the latter directly assaulting infected or aberrant cells. B cells, on the other hand, generate antibodies, proteins capable of recognizing and neutralizing specific pathogens. Recent advancements have culminated in the development of monoclonal antibody therapies for various diseases. Antigen-presenting cells (APC), including dendritic cells, serve as essential intermediaries by presenting antigens to T cells, thereby initiating the adaptive immune response. Recent breakthroughs in the field of immunology have yielded highly targeted immunotherapies such as CAR-T cell therapy for cancer treatment, along with a deepened understanding of immune memory and its manipulation for innovative vaccination strategies. Moreover, advancements in genomics and immunogenetics are facilitating personalized approaches to immunotherapy and vaccine development. Research into the influence of the gut microbiome on immune function is garnering significant attention, potentially paving the way for therapeutic interventions. Additionally, the burgeoning field of how metabolism influences immune responses holds promise for addressing various diseases. These trends underscore the enduring significance of immunology in both comprehending and addressing a broad

spectrum of health conditions. Researchers are continually exploring novel avenues to harness the potency of the immune system to enhance health outcomes.

2.2 Cells of Immune System. Most of the immune cells arise from hematopoietic stem cells (HSC). HSC are unique cells present in the bone marrow and differentiate into various cell types of the immune system. HSC have lifelong self-renewing ability and give rise to cells with restricted self-renewable capacity.

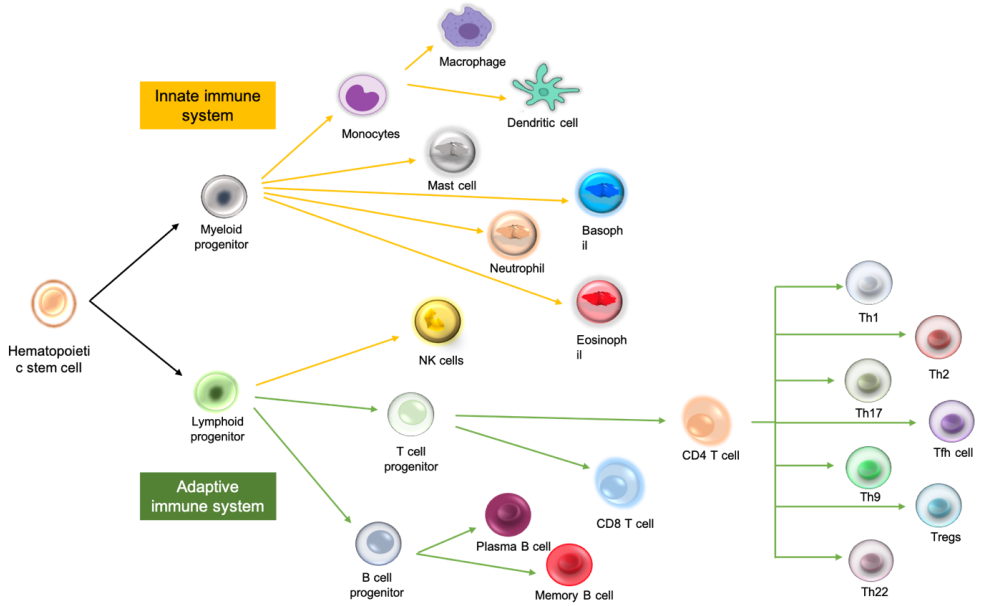


*Fig.1. Development of different cells of the Immune system (Figure adapted from Peprotech Inc, 2021).*The figure explains the origin of different cells of the immune system from hematopoietic stem cells. Hematopoietic stem cells differentiate into progenitors of myeloid or lymphoid cell lineages. Myeloid progenitors give rise to cells of innate immune system and lymphoid progenitors gives rise to cells of adaptive immune system.

Usually, the differentiation of HSC into different hematopoietic lineages is believed to occur in a hierarchical mode. HSC first develop into a short-term self-renewing HSCwhich further become progenitor with erythroid and myeloid (PEM) potential. Further, PEM develops into myeloid and lymphoid lineage (PLM), which are direct precursorsto common myeloid progenitors and common lymphoid progenitors. Generally, cells of the adaptive immune system arise from common lymphoid progenitor while cells of the innate immune system are

generated from common myeloid progenitor except for NK cells which originatefrom lymphoid progenitor (*Figure 1*).

2.2.1 Macrophages. The word macrophage is derived from the Greek words 'macro = large' and 'phage = to eat' which means eating of macromolecules. These cells are real housekeepersas they not only engulf pathogens and cancer cells but also clear off the cellular debris and help in wound healing after the infection is controlled. These cells have a specialized system of recognition receptors known as pattern recognition receptors (PRR) to detect the molecular patterns present inthe pathogens (PAMP). PRR help the macrophage recognise and endocytose pathogens and initiate immune response. Since these cells patrol almost every tissue and perform different functions, they have been divided into different types depending on the tissue they are present in e.g., Kupffer cell in the liver, microglia in the central nervous system, alveolar macrophages in respiratory tracts, splenic macrophages in spleen and others.

Macrophages employ another mechanism to carry out their effector function by secreting either pro-inflammatory or anti-inflammatory cytokines. Depending on the specific cytokines they release, macrophages are categorized into two subtypes: M1 and M2 macrophages. Another distinguishing characteristic between M1 and M2 macrophages is their metabolism of arginine. M1 macrophages metabolize arginine into the inflammatory molecule nitric oxide, whereas M2 macrophages convert arginine into the reparative molecule ornithine (*Murphy K and Weaver C, 2006*). M1 macrophages play a crucial role in the immune response by secreting pro-inflammatory cytokines such as IL-6, TNF-α, IL-23, Type 1 interferons, and IL-1β. These cytokines facilitate an inflammatory response against infections and cancer. Conversely, M2 macrophages secrete anti-inflammatory cytokines like TGF-β and IL-10, which serve to regulate inflammation and promote tissue repair.

2.2.2 Dendritic cells.Dendritic cell is a very important cell of the innate immune system since these are the only ones that can activate naive T cells. Dendritic cells work as a bridge between innate and adaptive immunity. Functionally they are very similar to macrophages in a way that both of them patrol the tissue and have the ability of phagocytosis. Macrophages after phagocytosis remain at the site of tissue damage to facilitate tissue repair while dendritic cells migrate to the nearest organ-lymph node and present the antigen to naïve T cells. The

ability of dendritic cells to migrate and present the antigens to naïve T cells makes them a unique professional antigen-presenting cell.

Dendritic cells like any other immune cell express major histocompatibility complex I and II (MHC I and MHC II) on their surface. These MHC molecules help the dendritic cells to present the antigens to T cells (CD4, CD8) in the form of antigen-MHC complex (Ag-MHC). Ag-MHC I complex interacts with TCR of CD8 T cells while Ag-MHC II complex interacts with TCR of CD4 T cells. As a result of Ag-MHC-TCR interaction naïve T cell converts into activated T cells. Activated T cells then proliferate and start producing their respective cytokines.

Dendritic cells are divided into two types, classical dendritic cells (cDC1, cDC2) and plasmacytoid dendritic cells (pDC). Classical DCs mainly help in the detection of extracellular pathogens and immunity against intracellular pathogens and cancerous cellsby activating $CD8^+$ cytotoxic T cells. pDC help in anti-viral immunity by producing interferons that are anti-viral in nature. The presence of other non-classical subsets of DC is also reported that originate from monocyte lineage i.e., monocyte-derived dendritic cells (MO-DC) and Langerhans cells (*Mildner A and Jung S, 2014*). Based on their cytokine profile and microenvironment DC can perform both pro-inflammatory and anti-inflammatory activity.

2.2.3 Natural Killer Cells. Natural Killer (NK) cells that perform cytotoxic action are part of the innate immune system. Albeit, being part of the innate immune system, these cells originate from lymphoid progenitors. Due to their dual characteristic, these cells are also termed group 1 innate lymphoid cells(ILC). These cells are best known for their ability to protect against viral infection and cancer. NK cells like other innate immune cells patrol into tissue and interact with every nucleated cell. MHC I present on nucleated cells interacts with inhibitory receptors present on NK cells to inhibit the function of NK cells. Cancerous and virally infected cells have downregulated/abnormal expression of MHCI that results in improper inhibition of NK cells. Additionally, cancerous cells secrete chemicals that can activate NK cells. After activation, NK cells release perforin and granzyme for their cytotoxic activity or secrete cytokines to attract other inflammatory cells for an organized immune response.

NK cells are functionally similar to CD8 T cells but they differ in a way that NK cells do not require any prior exposure/training against the harmful substances. They just naturally kill

every cell that does not show normal characteristics hence the name natural killer cell is given to them.

2.2.4 Lymphocytes. Lymphocytes are the key players of the adaptive immune system and originate from the lymphoid progenitor. These cells are generated in bone borrow but some of them migrate to the thymus before becoming mature cells. Cells that are formed and matured into bone marrow are termed B cells while those which are generated in bone marrow but mature into thymus are known as T cells (CD4, CD8). T cells can be differentiated from other cells based on the presence of a T cell receptor (TCR) that recognizes antigens in the context of an MHC molecule. Cells are very specific in their function as they are trained against a particular antigen. These cells protect by producing an array of proteins namely antibodies, cytokines etc. The average life span of cells ranges from a week to a month but some of them live very long for years after exposure to an antigen. Cells that live long for years are known as memory cells which can protect future upon encounter with the same antigen.

2.2.4.1 B cells. B cells are generally known for their ability to produce antibodies. These antibodies destroy bacteria, viruses or any harmful substances through neutralization, agglutination and opsonization. Another function of B cells is to help CD4 T cell activation in response to a low amount of antigen or autoantigens (*Bouaziz JD et al., 2007*). B cells upon encounter with an antigen undergo clonal expansion and becomeplasma B cellsto produce antibodies specific to an encountered antigen.

2.2.4.2 CD8 T cells. T cell that expresses CD8 on their surface are known as cytotoxic T cells. The major function of cytotoxic T cells is to provide cellular immunity against intracellular pathogens (e.g., viruses) or cancer. Cancerous or foreign antigen presented by the affected cell through MHCI is recognized by CD8 T cells. Upon activation, the CD8 T cell secretes perforin and granzyme around the point of contact to induce apoptosis in the affected cell.

2.2.4.3 CD4 T cells. Another subset of T cells is CD4 T cells also known as helper T cells. The major function of this cell is to help in the activation/function of innate immune cells, B cells or cytotoxic T cells. Helper T cells are further divided into different subsets depending on their cytokine profile. Activated CD4 T cell takes up one of the pre-defined subsets of helper T cell depending on the cytokine signal produced by the dendritic cell during antigen

presentation e.g., Th1 cells, Th2 cells, Th17 cells, Tfh cells, Treg, etc. (*Figure 2*). Once differentiated these cells proliferate rapidly and secrete their signature cytokines to perform their designated function.

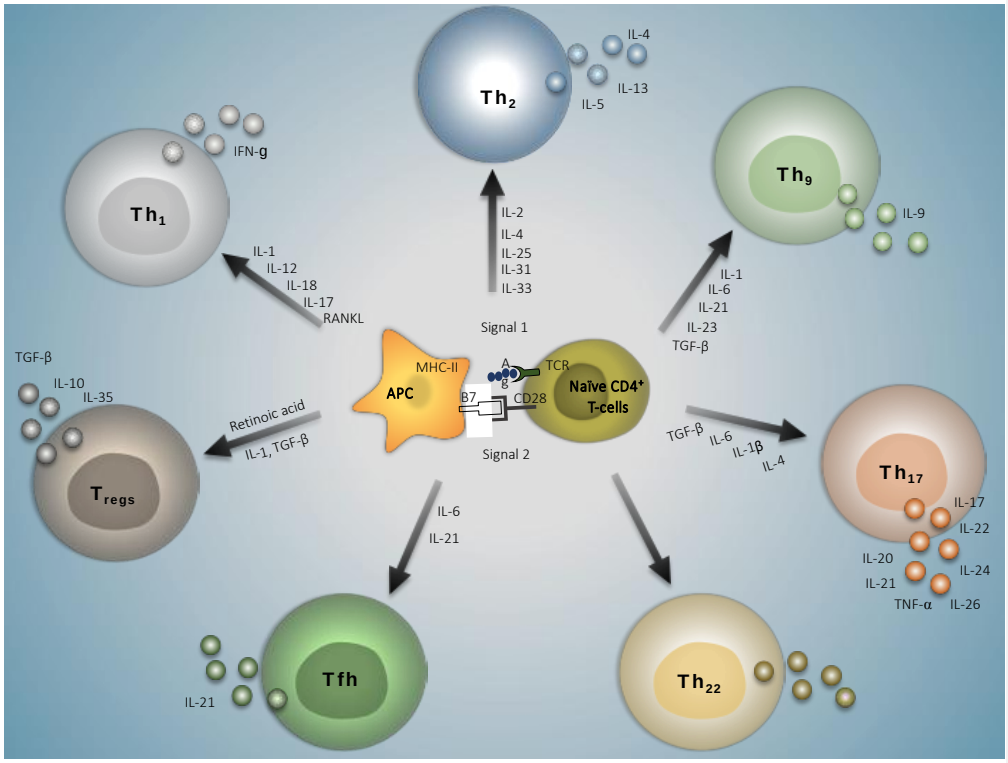


*Figure 2. Differentiation of naïve CD4 T cells into their subsets upon encounter with dendritic cells.*The optimum activation of naïve CD4 T cells requires 3 different signals from antigen-presenting cells (APC) typically in the form of Ag-MHC complex that binds to TCR, co-stimulatory molecules interaction with their corresponding ligands present on T cell and finally cytokines signalling. Based on the type of cytokines secreted by APC, naïve CD4 T cells transform into different subsets of CD4 T cells like Th1, Th2, Th9, Tregs, Th17, Th22 and Tfh.

2.2.4.4 T helper 1 (Th1)cell. Th1 cells are differentiated in response to IL-12 and secrete pro-inflammatory cytokine IFN-γ. The major function performed by these cells is to provide immunity against intracellular pathogens like viruses and microbes. IFN-γ secreted by Th1 cells helps in the activation of macrophages, cytotoxic T cells and B cells.

2.2.4.5 T helper 2 (Th2)cell. Th2 cells differentiate in response to IL-4 and offer protection against multicellular organisms, such as parasites. These specialized cells secrete a range of cytokines, including IL-4, IL-5, and IL-13. IL-4 plays a key role in activating B cells, prompting them to produce antibodies. IL-5 serves as a stimulant for eosinophils, while IL-13 activates epithelial cells, leading to the production of mucus. When Th2 cells are activated in response to allergens, they trigger B cells to produce IgE antibodies. This excess IgE production can lead to allergic reactions.

2.2.4.6 T helper 17 (Th17)cell. Th17 cells are polarized from naïve CD4 T cells in response to IL-6 and TGF-β secreted by dendritic cells. Th17 cells majorly protect against extracellular pathogens and fungal infection. The major cytokine secreted by these cells is IL-17 which helps in the recruitment of neutrophils and macrophages at the site of infection for phagocytosis of foreign substances. IL-23 secreted by dendritic cells during differentiation promotes the pathogenicity of Th17 cells.

2.2.4.7 Regulatory T cells. Regulatory T cells (Tregs) are specialized immune cells responsible for regulating the inflammatory immune response and maintaining homeostasis. Their primary role is to uphold immune tolerance. These cells are generated in response to signals from TGF-β and IL-2 and exert suppressive functions. Tregs are identified by their expression of the transcription factor FoxP3, which serves as a master regulator of their suppressive capabilities (*Kim CH, 2019*). Tregs secrete molecules such as TGF-β and IL-10 to dampen the activity of various immune cell types, including Th1 cells and Th17 cells, CD8 T cells, dendritic cells, and macrophages. Additionally, they can engage in contact-dependent suppression by expressing inhibitory surface molecules like Tim3, PD1, CTLA4, and GITR to modulate the function of other immune cells (*Schmidt A et al., 2012*).

2.2.4.8 T helper cell 9 (Th9). Th9 cells are recently identified helper T cell subset that is differentiate in response to TGF-β and IL-21 and provides immunity to extracellular parasites. IL-9 secreted by Th9 cells promotes the survival and function of Treg cells. It also stimulates mucus production and recruitment of mast cells and eosinophils (*Chen J et al., 2019*).

2.2.4.9 T follicular helper (Tfh)cell. Tfh cells are a specialized subset of T helper cells that are characterized by the expression of CXCR5 and Bcl6 as their master regulators. These

cells provide help to B cells for the generation of germinal centers and long-term humoral response with the help of molecules such as CD40L, IL21 and IL4 (*Crotty S, 2014*).

2.2.5 Myeloid-derived suppressor cells (MDSC). MDSC are a type of myeloid cell with significant suppressive potential. They constitute a diverse group of immature myeloid cells, contributing to the variety of myeloid cells observed in pathological conditions (*Gabrilovich DI and Nagaraj S, 2009*). MDSC can be classified into two main categories based on their origin: G-MDSC (Granulocytic Myeloid-Derived Suppressor Cells) and M-MDSC (Monocytic Myeloid-Derived Suppressor Cells). These cells tend to accumulate significantly during conditions such as cancer, chronic inflammation, infections, and autoimmunity, primarily to carry out immunosuppressive functions. Their primary targets for immunosuppression are T cells, making them particularly well-suited for dampening T cellmediated autoimmune responses (*Srivastava MK et al., 2010*).

MDSCs perform immunosuppression through different mechanisms namely production of immunosuppressive metabolites (arginase1, IDO, adenosine, PGE2), generation of reactive oxygen (H_2O_2) and reactive nitrogen species (NO), expression of inhibitory and apoptotic molecules on the surface (Tim3/galectin9, PD-L1, Fas-L) and production of inhibitory cytokines (TGF-β, IL-10) (*Figure 3*). The effect of MDSCs on T cells through the above mechanism leads to downregulation of T cell receptor (TCR), induction of apoptosis and inhibition in the proliferation of effector T cells and expansion in the number of Tregs (*Groth C et al., 2019*).

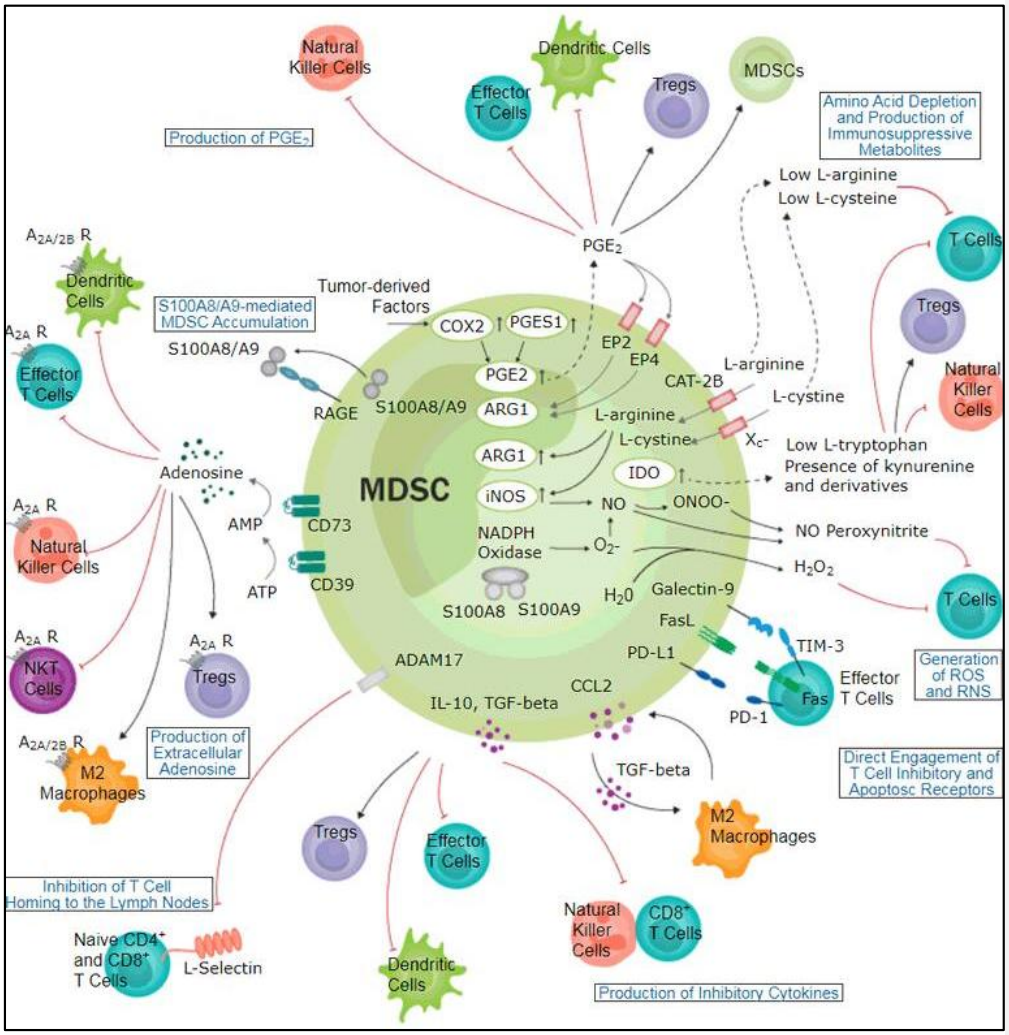


Figure 3. Mechanism of immunosuppression by myeloid-derived suppressor cells (Figure adapted from Bio-Techne, 2019). MDSCs perform their suppressive activities through various mechanisms namely by secreting various cytokines and metabolites, by expressing suppressive and apoptotic molecules and inducing regulatory T cells.

2.3 Autoimmunity.

Autoimmune diseases comprise a group of inflammatory conditions notorious for inflicting severe pain and sometimes resulting in permanent damage to the body. Autoimmunity is a term formed from two words: 'auto,' meaning self, and 'immunity,' indicating the immune

system's response. Essentially, it signifies an immune reaction directed against the body's own components, encompassing proteins, cells, tissues, and organs. This immune response can involve inflammation mediated by T cells, B cells, or both. The root cause of this type of immune response against one's own components lies in the breakdown of immunological tolerance. Immunological tolerance refers to the unique ability of the immune system to refrain from recognizing self-components as threats. The body achieves this remarkable feat either by eliminating self-reactive immune cells during development or by concealing self-antigens from the immune system. Such hidden antigens are referred to as cryptic antigens. However, in instances of physical injury or molecular mimicry, cryptic antigens may leak into the bloodstream, leading to the activation of immune cells specific to these self-antigens. These activated cells subsequently launch an inflammatory response targeting the body region where the particular self-antigen is present. This, in turn, results in the destruction of the self-component and its corresponding physiological functions (*Romagnani S, 2006*).

Autoimmune diseases are typically categorized into systemic and organ-specific, based on the location of the affected organ(s). Systemic autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus, result in damage across multiple tissues, while organ-specific autoimmune diseases, like multiple sclerosis and type 1 diabetes, target a particular organ. The underlying causes of autoimmune diseases remain unclear, but several factors can trigger their development, including genetic and environmental influences. A combination of these genetic and environmental factors contributes to the breakdown of immunological tolerance (*Molina and Shoenfeld Y, 2005*). Genetic polymorphism, molecular mimicry, and the release of cryptic antigens are among the leading causes of the breakdown of immunological tolerance (*Figure 4*). In some instances, autoreactive T cells with a high affinity for autoantigens may evade the mechanisms responsible for maturation, further contributing to the breakdown of tolerance.

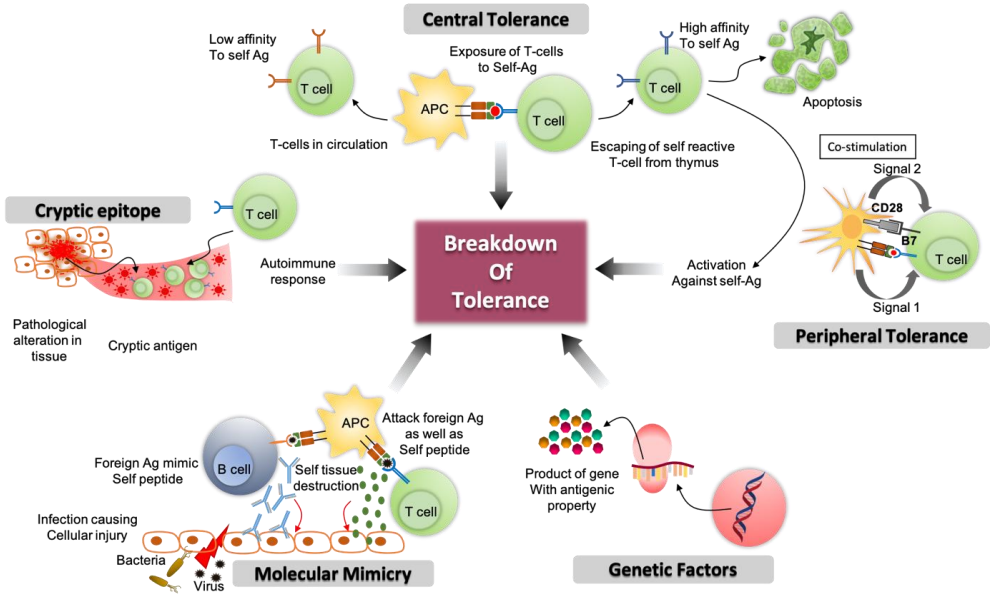


*Figure 4. Breakdown of immunological tolerance.*The breakdown of central immunological tolerance may stem from genetic factors, molecular mimicry, and cryptic antigens. Conversely, the breakdown of peripheral tolerance is linked to the induction of anergy and regulatory T cells.

2.3.1 Rheumatoid Arthritis (RA). RA is a chronic inflammatory disorder affecting multiple joints majorly the upper and lower parts of limbs. The immune system attacks joint lining and synovium responsible for producing synovial fluid resulting in swelling and hampered joint movements. Over time joints become thicker, rigid and tender limiting movement due to extreme pain. At the advanced stage of the disease, bone erosion and joint deformity have also been reported (*Schett G and Gravallese E, 2012*). Although the underlying mechanism behind the pathogenesis of RA is unclear, T cells and B cells orchestrate the inflammatory response responsible for the pathogenesis of RA. CD4 T cells (Th1/Th17) mediated attack against autoantigens of joint proteins is one of the major contributors to RA. B cells also play an important role by generating antibodies against self-antigens and complement-mediated damage of joint tissue e.g., anti-citrullinated peptide antibodies (ACPA) (*Scherer HU et al., 2020*). Inflammatory cytokines secreted by T cells and antibodies secreted by B cells further activate other inflammatory cells like macrophages, dendritic cells and synovial fibroblasts to worsen the disease condition (*Choy E, 2012*).

2.3.2 Multiple Sclerosis (MS). Multiple sclerosis is an organ-specific chronic autoimmune disease that affects the central nervous system. A Th17 cell mediated autoreactive immune response targets proteins of the myelin sheath (e.g., MOG, MBP) insulating the neurons and destroys the myelin sheath resulting in loss of insulation. Loss of insulation of neurons interrupts the signal to and from the brain and ultimately impairs movement, muscle control, vision, and cognition (*Mazumder R et al., 2014; Piradov MA and Suponeva NA, 2015*). The most affected nerve in MS is reported to be the optic nerve which is responsible for blurry vision in MS patients (*Kale N, 2016*). As the disease progresses, it leads to nerve cell damage and shrinking of the cerebral cortex (cortical atrophy) (*Cagol A et al., 2022*). Anti-MOG antibodies produced by autoreactive B cells contribute to the disease pathology by T cell activation and complement-mediated damage to myelin sheath (*Kinzel S et al., 2016*).

The cause, onset, duration and severity of the diseases varies drastically between individuals making it more difficult to predict, diagnose or treat. Based on the disease progression patterns; MS is categorized into relapsing-remitting MS, Primary-progressive MS, Secondary-progressive MS and progressive relapsing MS (*Baecher-Allan C et al., 2018; Lublin FD and Reingold SC, 1996).* Marburg variant and Balò's concentric sclerosis are recently identified acute variant of MS that develops rapidly and causes significant disability and sometimes death (*Capello E and Mancardi GL, 2004*).

Experimental autoimmune encephalomyelitis (EAE) is an animal model sharing pathological and clinical features similar to multiple sclerosis (MS) (*Constantinescu CS et al., 2011*). Therefore, this model is used widely to study MS mechanisms and for testing or developing drugs. EAE can be induced in susceptible strains of animals by using whole or one of the myelin components emulsified in CFA. Pathogenesis of EAE is caused by myelin-specific T cells (Th17 cells, Th1 cells, CD8 T cells). These myelin-reactive T cells are activated in the periphery and move to the CNS crossing blood blood-brain barrier. These T cells are once again activated in the CNS by the local and infiltrating APCs through presenting myelin epitopes. Following this inflammatory process begins rendering damage to the myelin sheath.

2.4 Immune cells in autoimmune diseases. The immune response in autoimmunity primarily involves Th1 cells, Th17 cells and B cells. These cells play a central role by releasing a variety of proinflammatory cytokines and chemokines, which can either attract other inflammatory cells or inflict damage on the cells and tissues containing self-antigens. Key molecules responsible for tissue damage include IFN-γ and IL-17, secreted by autoreactive Th1 cells and Th17 cells, respectively. Additionally, during inflammatory conditions like Multiple Sclerosis (MS), autoreactive T cells upregulate integrin levels, enabling them to breach the blood-brain barrier and accumulate in the central nervous system (CNS) (*Nourshargh S and Alon R, 2014*). To counterbalance the effects of these cells, regulatory T cells (Tregs) come into play. Tregs employ various mechanisms to suppress the immune response, such as secreting immunosuppressive molecules, ectoenzymes, immune checkpoint inhibitors, and inhibiting the proliferation of Th1 cells and Th17 cells. One distinguishing feature of memory T cells is their ability to generate antigen-specific memory, rapidly expanding upon subsequent encounters with the same auto-antigen. Th17 memory cells are notorious for causing remitting relapses in autoimmune diseases (*McGeachy MJ, 2013*). Recent studies have identified the generation of regulatory memory T cells in target tissues after controlling the autoimmune reaction. These cells play a role in resolving inflammation upon future encounters with self-antigens (*Rosenblum MD et al., 2011*). B cells produce antibodies against autoantigens and contribute to tissue damage through complement-mediated lysis. B cells isolated from MS patients have shown significant upregulation in the secretion of inflammatory cytokines like IL-6 and TNF-α, along with downregulation in the secretion of anti-inflammatory cytokines IL-10 and TGF-β (*Duddy M et al., 2007*).

In the case of autoimmune diseases, macrophages play a significant role. The microenvironment of autoimmune disease hampers the phagocytic ability of macrophages. Inhibition of phagocytosis by macrophages lead to less clearance of apoptotic cells. Consequently, resulting in the accumulation of autoantigens which is responsible for further activation of autoreactive T cells and aggravated and more robust immune response. Further, imbalance between M1/M2 macrophages contributes to pathogenesis of autoimmune diseases. Elevated numbers of M1 macrophages and a low level of M2 macrophages results in high level of proinflammatory cytokine and recruitment of other inflammatory cells (Th1/Th17). Low M2 macrophages results in less polarization of Tregs and less tissue repair

by anti-inflammatory cytokines (*Yang S., 2023*). RANKL in combination with cytokines viz TNF-α, IL-6 and IL-1β secreted by M1 macrophages activates osteoclasts and worsen the disease condition of RA (*McInnes IB and Schett G, 2007*).

The activation of T cells hinges on the interaction between antigens complexed with MHC molecules displayed on the surface of dendritic cells. These dendritic cells constitutively express co-stimulatory molecules like CD80, CD86, and CD40, further amplifying T cell activation. A third vital signal, delivered by dendritic cells in the form of cytokines, induces the proliferation and differentiation of T cells. To maintain immunological tolerance, dendritic cells found in the thymus execute the critical task of promoting the negative selection of autoreactive T cells while simultaneously fostering the positive selection of Tregs. Additionally, tolerogenic dendritic cells play a pivotal role in maintaining peripheral tolerance through various mechanisms. These mechanisms include the induction of apoptosis in autoreactive T cells via cognate interactions, the induction of a state of unresponsiveness, the polarization of Tregs, and the secretion of anti-inflammatory cytokines, among others (*Sato K, 2003; Steinbrink K et al., 2002*). However, it is noteworthy that the aberrant activation of dendritic cells has been documented in modulating the severity of autoimmune diseases (*Seitz HM and Matsushima GK, 2010; Silva MA et al., 2004; Serafini B et al., 2006; Sarkar S and Fox DA, 2005*). Thus, as elucidated by scientific studies, dendritic cells exhibit a dual role in the context of autoimmunity (*Amodio G and Gregori S, 2012*). On one hand, they serve as pivotal instigators, initiating autoreactive immune responses. On the other hand, dendritic cells play a crucial role in fostering both central and peripheral tolerance (*Santiago-Schwarz F, 2004*).

NK cells have been reported to promote autoimmune diseases particularly type 1 diabetes (T1D) due to their cytotoxic effect on pancreatic β cells. Apart from their cytotoxic activity NK cells also secrete various cytokines/chemokines that inturn activates other inflammatory cells. Recent studies have also indicated the regulatory role of NK cells in various autoimmune diseases (*Jiao G and Wang B, 2016; Takeda K and Dennert G, 1993; Schuster IS et al., 2014; Lee I et al., 2004*). Although the evidence of regulatory role is unclear but targeting NK cell for controlling autoimmune pathology may be a potential tool.

Myeloid derived suppressor cells (MDSC) are highly inhibitory cells. They were first identified in the cancer microenvironment. Recent studies have implicated their role in

regulating the immune response in autoimmune diseases by suppressing autoreactive B cells, T cells and NK cells (*Knier B et al., 2018; Elliott DM et al., 2018; Nishimura K et al., 2015*). Immunoregulatory function by MDSCis carried out through various mechanisms. MDSC produce key immunosuppressive cytokines like TGF-β and IL-10 leading to downregulation of NKG2D mediated stimulation of NK cells and autoreactive CD8 T cells, inhibition in activation and proliferation of NK cells and effector T cells, inhibition in maturation, migration and activity of DC, promotion of Treg differentiation and generation of M2 macrophages (*Lee YS et al., 2021; Imai K et al., 2012; Huang B et al., 2006*). MDSC also produce immunosuppressive metabolites (arginase1, IDO, adenosine, PGE2) responsible for TCR downregulation and inhibition in autoreactive T cell/NK cell activation and proliferation. Another mechanism through which MDSC perform their inhibitory function is through cognate interaction with autoreactive cells via inhibitory molecules (Tim3/Galectin9, PDL1, FasL) expressed on the surface. These molecules suppress the activated autoreactive T cells and induces apoptosis in those cells (*Crook KR and Liu P, 2014; Xu D et al., 2022*). A recent study has demonstrated the suppressive role of MDSC in allergic airway inflammation and asthma by suppressing Th2/ILCs through cyclo-oxygenase 2 (*Cao Y et al., 2019*). Surprisingly, novel pro-inflammatory role of MDSC has recently been reported in arthritis by producing IL-1β and TNF-α(*Guo C et al., 2016*). After that a series of studies have reported the Th17 cells promoting MDSC role in severity of autoimmune diseases (*Glenn JD et al., 2019; Xue F et al., 2020; Pang B et al., 2020*). Further, the inhibition of Th17 cells promoting MDSC could control the disease severity of inflammatory arthritis confirming the pathogenic role of MDSC in autoimmunity (*Geng Z et al., 2019*). These findings suggest careful consideration before targeting MDSC for the management of autoimmunity.

2.5 Therapies against autoimmune diseases. The underlying causes of different autoimmune diseases are highly dynamic making the management of AIDs very difficult. The current strategies for the management/treatment of autoimmune diseases basically reside on symptomatic relief or immunosuppressive medication (*Chandrashekara S, 2012*). Symptomatic relief is achieved through modifying the physiological changes caused by the disease (disease-modifying therapies) for example modulation of thyroxine level in the case of autoimmune thyroiditis (*Singer PA et al., 1995*). But, in the case of systemic autoimmunity where the function of multiple organs is affected, immunosuppressive therapy is used to counteract the hyperactive immune response. The drawback of current therapies is that

disease-modifying therapies have no effect on remission or cure of the disease and immunosuppressive drugs cause systemic immunosuppression leading to opportunistic infections. Some of the strategies used for the management of autoimmunity are discussed below.

2.5.1 Anti-inflammatory drugs. Inflammation is the hallmark cause of most of the autoimmune diseases. Anti-inflammatory drugs are pharmacological interventions that are used to reduce inflammation and associated complications to provide relief in an ongoing autoimmune disease. These drugs can inhibit the production of different inflammatory mediators (e.g., cyclo-oxygenase) resulting in the relief of inflammation.

2.5.2 Disease modifying therapies. Disease-modifying therapies as discussed above focus on treating complications of a particular disease. Since different diseases possess different physiological complications, these therapies may vary in different diseases. Type 1 diabetes is characterized by hampered insulin production due to immune-mediated attacks on β cells. To modify the symptoms of the disease in case of insulin injections are used in the case of type 1 diabetes. Similarly, hypothyroidism results in less thyroxin production due to an immunological attack on the thyroid gland and thyroxin supplement is used to provide symptomatic relief.

2.5.3 Immunosuppressive drugs. As discussed earlier, a hyperactive immune response is the main culprit behind every autoimmune disease. Immunosuppressive medication has served as a primary tool to inhibit the activity of the immune system primarily by generating regulatory T cells (e.g., cyclosporin, rapamycin etc.). Although, the use of such drugs was limited to the late stages of the disease, for early management of the disease use of immune system-suppressing drugs has been tried recently and the chance of a cure for some of these conditions seems possible. Although, advancement in the management of the infectious disease has encouraged the use of immunosuppressive medication current studies have reported an increase in the incidence of cancer on long-term immunosuppressive therapies (*Gutierrez-Dalmau A and Campistol JM, 2007; Reyes A et al., 2022*).

2.5.4 Corticosteroids. Corticosteroids are another class of anti-inflammatory medication e.g., cortisone, prednisone. These drugs are considered rescue medications upon acute remission of the disease as the desired effect can be achieved within hours of administration (*Hanauer SB and Reddy S, 2023*). Corticosteroids can be given systemically (oral, i.v.) or locally at the

site of inflammation to reduce inflammation e.g., injection in the joint in case of RA. This class of drugs are more effective in comparison to other anti-inflammatory drugs as these medication work directly on a variety of immune cells and inhibit their activation and function (*Chatham WW and Kimberly RP, 2001*). Further, corticosteroids can deplete the number of circulating inflammatory cells through induction of apoptosis, impaired migration of cells from lymphoid tissue and inhibition of T cell growth factors. Another mechanism through which corticosteroids are through the production of Tregs (*Azab NA et al., 2008*). Although, the use of corticosteroids in the management of the disease is very useful but long-term use of corticosteroids has been reported to have severe side effects (*Saag KG et al., 1994; Luo JC et al., 2002; Yoon BK et al., 2020*).

2.5.5 Pain-killing medication. The main problem arising from inflammatory disease is significant chronic pain. Chronic pain is another unpleasant and debilitating condition associated with autoimmune diseases. The mechanism behind pathological pain associated with AIDs has been linked to T cell and B cell-mediated cytokine and antibody production. T and B cell-induced damage to different tissues leads to the development of pain (*Mifflin KA and Kerr BJ, 2017)*. Further, autoantibodies have been reported to modulate the nociceptive neurons and sensory neurons expressing Fc receptors to induce persistent pain (*Lacagnina MJ et al., 2021*). Based on the severity of pain analgesics (pain-killing medications) that are being used can be of steroidal or non-steroidal origin. Non-steroidal anti-inflammatory drugs (Ibuprofen, Diclofenac sodium) are the most commonly used anti-pain medication to manage chronic pain but steroidal analgesics (Dexamethasone) and opiates such as codeine are used for acute severe pain.

2.5.6 Cell-based therapies. Previously discussed therapies have certain drawbacks that limit their long-term use for the management of autoimmune diseases. Recent advancement in the field is focused on targeted therapies to minimize the side effects. One such group of therapy that is being explored is cell-based therapies. Most exploited cell-based therapies for the treatment of AIDs are stem cell-based (autologous HSC, mesenchymal stem cells) and Treg-based therapies (*Dazzi F et al., 2007; Bluestone JA et al., 2015*). The most recent advancement is the use of autoantigen-targeted engineered Tregs known as CAR-Treg is heavily exploited to provide an antigen specific immunosuppression (*Elinav E et al., 2009*). Although, induction of antigen-specific tolerance can be the future of cell-based therapies but

the problem with antigen-specific Tregs is phenotypic plasticity in an inflammatory microenvironment (*Hovhannisyan Z et al., 2011; Hwang SM et al., 2018*). Future strategies should be focused on generating antigen-specific Tregs that are highly stable and can retain their suppressive function even in inflammatory microenvironments.

2.5.7 Nanotherapeutics. The last few decades have witnessed a surge in the use of nano-particle-based therapies for targeted approaches in case of cancer and other diseases. More recently, nanotechnology has been exploited to induce antigen-specific immunological tolerance in autoimmune diseases (*Serra P and Santamaria P, 2018*). The nanoparticle-based therapies majorly target dendritic cells to deliver tolerogenic cargos or target autoreactive T cells for in-vivo reprogramming of these cells (*Triantafyllakou I et al., 2022; Kishimoto TK, Maldonado RA, 2018; Yuan B et al., 2014; Kenison JE et al., 2020; Yeste A et al., 2012*). Some of the nano-therapeutics have been used to induce/expand regulatory T cells directly/indirectly and could suppress the disease severity effectively (*Nguyen TL et al., 2022; Tseveleki V et al., 2015; Horwitz DA et al., 2021; Carey ST et al., 2023*). These formulations have performed very well in pre-clinical studiesin mitigating the disease symptoms and delaying the onset of the disease. However,none of these studies have been tested to prevent the disease upon future encounters.

Above mentioned therapies that are being utilized to solve the mystery of autoimmune inflammation are successful up to some extent but they carry many disadvantages due to overwhelming immunological complexity. Another drawback of current therapies is that they do not provide a durable cure or preventive potential. The need of the hour is to develop therapies that not only can treat the disease effectively but can also prevent the disease development.

1.6 Autophagy in immunity. Autophagy is a cellular phenomenon where the rouge components of the cell are digested into specified cell organelles known as lysosome and autophagosome. It plays a pivotal role in maintaining cell survival and cellular homeostasis by recycling of nutrients into the cell and by degrading cellular debris and foreign molecules (*Zheng YT et al., 2009*). Mechanistically, the target moiety is delivered into the lysosome with/without the help of an intermediate organelle/substrate. Depending on the mode of delivery to a lysosome, autophagy is categorized into three different categories viz. microautophagy, macroautophagy and chaperon-mediated autophagy (*Yang Z and Klionsky*

DJ, 2009) (*Figure 5*). Autophagy hasbeen reported to be interrelated with various important cellular processes e.g., gluconeogenesis, cellular renovation, inflammation and tumor-suppression (*Ezaki J et al., 2011; Mizushima N and Komatsu M, 2011; Takamura A et al., 2011; Mathew R et al., 2009; Lapaquette P et al., 2015; DeNicola GM et al., 2011*). Due to its involvement in cellular processes, it may affect the phenotype, activity and fate of the cells and ultimately plays a role in disease progression and control.

The role of autophagy has been vividly studied in relation to immunity, as the process plays a key role in degrading intracellular pathogens along with damaged components and aggregated proteins by phagosome formation. It is now well established that autophagy has the ability to modulate both the arms of the immune system viz. innate immunity and adaptive immunity. Studies have now established that autophagy can influence survival, homeostasis, activation, differentiation and proliferation of the cells of the immune system like NK cells, macrophages, dendritic cells, MDSC and T & B cells (*Jiang GM et al., 2019*).

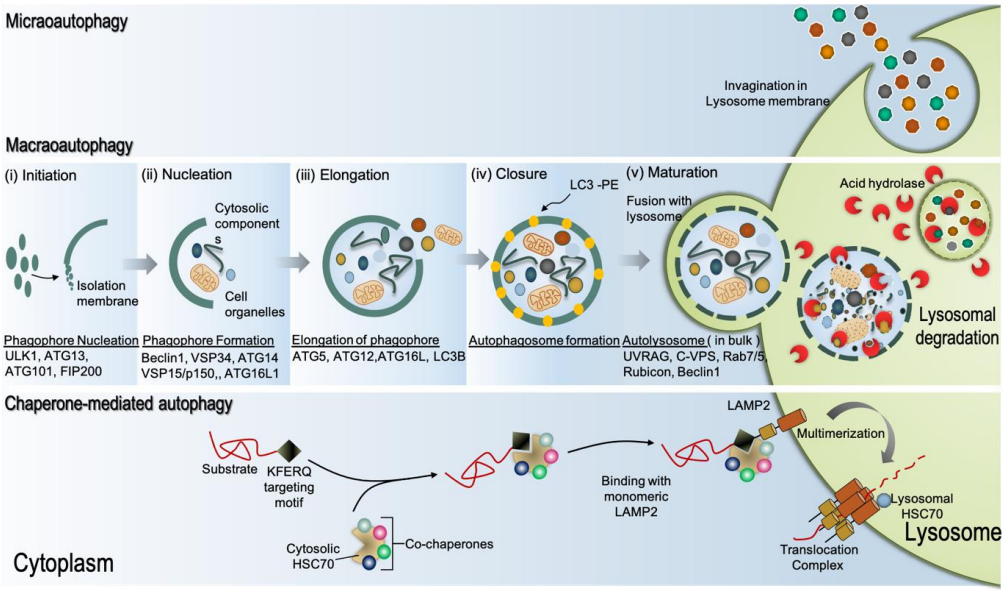


Figure 5. Different pathways are involved in the induction of autophagy. Microautophagy, Macroautophagy and chaperone-mediated autophagy differ from each other based on cargo delivery mode to the lysosome. Microautophagy delivers the cargo through invagination in the lysosome membrane, Macroautophagy delivers the cargo by forming a specialized vesicle

around the cargo and chaperone-mediated autophagy and utilizes certain substrates to form a complex to be recognized by the lysosome.

Different studies have also shown that autophagy can modulate the immune cells in terms of inflammatory processes like phagocytosis, antigen processing and antigen presentation. Additionally, autophagy can regulate the secretion of various cytokines and vice versa (*Harris J, 2011; Singh SB et al., 2006; Zhou HQ et al., 2023*).

In antigen-presenting cells autophagy meets innate immunity downstream to pattern recognition receptors e.g. TLRs and NLRs (*Jiang GM et al., 2019*). Various TLRs like TLR2, TLR4 and TLR7 are reported to stimulate autophagy through downstream molecules (*Delgado MA et al., 2008; Fang L et al., 2014; Pahari S et al., 2020*). TLR signaling-induced autophagy in macrophages promotes phagocytosis while NOD2 receptor stimulation-induced autophagy promotes the ability of dendritic cells to process and present antigens (*Sanjuan MA et al., 2007; Cooney R et al., 2010; Lee HK et al., 2010*). Antigen presentation to CD4 T cells by dendritic cells through MHCII is also regulated by autophagy (*Schmid D et al., 2007*). Alternatively, the regulatory function of myeloid-derived suppressor cells is orchestrated by autophagy (*Alissafi T et al., 2018*).

The other arm of the immune system *i.e.* adaptive immune system is majorly comprised of cells namely B cell and T cell. As discussed above these cells play a major role in the progression of inflammatory autoimmune diseases. An interesting report confirmed the induction of cell death by autophagy in CD4 T cells through induction of apoptosis which was controlled through cytokine-induced inhibition of autophagy (*Feng CG et al., 2008*). Further, induction of autophagy in DC enhanced their ability to cross-presentation of antigens to CD8 T cells (*Li Y et al., 2008*). Additionally, different autophagy-related genes (ATG) have been reported to promote the development and survival of B cells and T cells (*Miller BC et al., 2008*).

Recent studies in the field of autophagy have provided compelling evidence of its significant role in various aspects of Tregs generation, stability, and function, as supported by multiple references (*Wei J et al., 2016; Kabat AM et al., 2016; Zhang J et al., 2019*). One study, in particular, sought to assess the impact of autophagy on Treg generation and found that the suppressive capacity of regulatory T cells is critically reliant on autophagy. This is notably evident in cases where the disruption of autophagy leads to severe autoimmune responses

(*Yin H et al., 2018*). Furthermore, the differentiation of Tregs from naive CD4 T cells necessitates the presence of TGF-β. Recent investigations have demonstrated that impaired autophagy also results in defective Treg polarization in response to TGF-β (*Mandatori S et al., 2020*). Dendritic cells, on the other hand, possess the capability to present antigens to naive CD4 T cells and direct their differentiation into various helper T cell subsets, including Tregs (*Maldonado RA and von Andrian UH, 2010*). Significantly, recent research has unveiled the impact of macroautophagy in dendritic cells on the functionality, homeostasis, and stability of Tregs (*Niven J et al., 2019*).

In light of the aforementioned findings, it becomes apparent that autophagy plays a pivotal role in modulating the immune response. While autophagy is known to contribute to the initiation of inflammatory immune responses, targeting specific molecules within the autophagic pathways that regulate the immune system's regulatory arm, including Tregs and MDSC, can potentially reverse this effect. Moreover, the newfound role of autophagy in maintaining the lineage stability of Tregs and its involvement in generating memory T cells could be harnessed to create antigen-specific, stable memory Tregs. As a result, the targeting of autophagy for the development of next-generation therapies aimed at promoting immune tolerance and preventing autoimmunity emerges as a promising avenue of exploration.

CHAPTER 3
MATERIALS AND METHODS

3.1 Chemicals and reagents.All the chemicals were purchased from Sigma Aldrich (St. Louis, MO)unless otherwise mentioned. Fluorochrome conjugated Abs and FoxP3 staining kit was procured from Biolegend (San Diego, CA). MOG_{35-55} and recombinant cytokines were purchased from GenScript (Piscataway, NJ). MAP LC3α/β siRNA was acquired from Santa Cruz (Dallas, TX). TRIzol and lipofectamine RNAiMAX transfection reagents were purchased from Thermo Fisher Scientific (Waltham, MA). iScript cDNA synthesis and SYBER green were bought from Biorad (Hercules, CA). IL-10, IL-17A and TGF-β ELISA kits were purchased from BD Biosciences (Franklin Lakes, NJ).

3.2 Animals. Female C57BL/6J, FoxP3-GFP-reporter, OT-II and BALB/c mice (20±2 gm) were procured from a small animal facility for experimentation (SAFE), Indian Institute of Science Education and Research (IISER) Mohali, India and zebrafish from Marine Dreams, SAS Nagar, India.The experiments were approved by the Institutional Animal Ethics Committee (IAEC) (IISERM/IAEC/2020/018, IISERM/SAFE/PRT/2022/014). Animals were kept at SAFE, IISER Mohali in individually ventilated cages (IVC) with 12 h day/night cycle, temperature between 20-24°C and humidity from 45-60%. The experiments were performed according to the guidelinesof the Committee for Control and Supervision of Experiments on Animals (CCSEA).

3.3 Medium and culture conditions. The cells were cultured in a complete medium RPMI-1640/DMEM supplemented with FBS (10%), penicillin (100 U/ml), L-glutamine (100 mM) and streptomycin (100 mg/ml)in a humidified CO_2(5%) incubator at 37°C.

3.4 Isolation of naïve CD4 T cells and CD8 T cells. Spleen/LN/Peyer's patches were aseptically isolated from the mice. RBC were lysed and a single-cell suspension was prepared by passing through a 70 μm cell strainer (Sigma-Aldrich, St. Louis, MO). Naïve CD4 T cells and CD8 T cells were isolated using magnetic-activated cell sorting (MACS) through negative selection according to the manufacturer's instruction (BD Biosciences, San Diego, CA). Briefly, a naïve CD4/CD8 T cells enrichment cocktail was added (5 μl/10^6 cells) for 15 min on the ice. The cells were washed 3x with RPMI-FBS 1% by centrifugation at 300×g for 7 min.

Streptavidin-Fe DM particles (5 µl/10^6 cells) were added in 1 ml medium to the pellet for 30 min on ice. Antibody-bound cells were removed by placing them under a magnetic field for 7 min and the negative fraction containing naïve CD4/CD8 T cells was collected. This step was repeated 3x. The purity of naive CD4 T cells/CD8 T cells was more than94%, as demonstrated by $CD44^{lo}CD62L^{hi}$ by flowcytometry.

3.5 Polarization of naïve CD4 T cells towards Tregs and Th17 cells. Naïve CD4 T cells were polarized to Tregs and Th17 cells in the presence of rapamycin, as described elsewhere. The control cells were cultured in the absence of rapamycin (*Gurram RK et al., 2014*). Briefly, anti-CD3 (2 µg/ml) and CD28 (1 µg/ml) Abs stimulated naive CD4 T cells (1.5 x 10^5) were cultured with TGF-β (5ng/ml), IL-2 (100units/ml) for Tregs and IL-6 (40 ng/ml) and TGF-β (2.5 ng/ml) for Th17 cells for 5 days with or without rapamycin.

3.6 Generation of $MDSC^{rapa}$. The RBCwere lysedfrom thebone marrow cells (2 x 10^6) obtained from femur bones and cultured in 6 well plates with GM-CSF (2 ng/ml), IL-4 (2 ng/ml) and rapamycin (10 ng/ml) for 7 days (*Lutz MB et al., 1999*). Fresh medium containing GM-CSF (2 ng/ml), IL-4 (2ng/ml) and rapamycin (10 ng/ml)was replenished on day +3. Control cells were cultured without rapamycin. At day +7, the cells were either used for different experiments or scrapped for flow cytometric/western blotting/qRT-PCR analysis. The MDSC obtained expressed $CD11b^{hi}$and $Gr1^{hi}$, as demonstrated by flow cytometric analysis.

*3.7 Flow cytometric analysis.*MDSC and CD4 T cells were harvested and washed with FACS buffer (PBS + 2% FBS). Fc receptor was blocked using Fc block (Biolegend, San Diego, CA). Cells were then stained using fluorochrome-conjugatedAbs with different markers of Tregs [CD4-PE (0.25µg/million cells), FoxP3-GFP/FoxP3-APC (5µl/million cells)], Th17 [CD4-PE (0.25µg/million cells), IL-17-PerCP/Cy5.5 (5µl/million cells)], MDSC [CD11b-FITC (0.25µg/million cells), Gr1-APC (0.25µg/million cells)], co-stimulatory molecules [CD80-APC (1µg/million cells), CD86-PE (0.25µg/million cells), CD40-APC (1µg/million cells)], memory Tregs and tissue-resident memory [CD44-PerCP/Cy5.5 (0.25µg/million cells), CD62L-APC) (0.25µg/million cells), CD69-APC (1µg/million cells) , CD103-APC

(0.25 μg/million cells)], chemokine markers [CCR5-PerCP/Cy5.5 (0.25 μg/million cells), CXCR5-APC (0.25 μg/million cells)], inhibitory molecules [Tim3-PerCP/Cy5.5 (0.25 μg/million cells), PD1-PE (1 μg/million cells), CD39-PE/Cy7 (1 μg/million cells), CD73-APC (0.25 μg/million cells)] and others [CD8-PE (0.25μg/million cells), LFA 1-APC (0.25μg/million cells), FAS L-APC (0.25μg/million cells),TCRαβ-PerCP/Cy5.5 (0.25μg/million cells),TCRγδ-APC(0.25μg/million cells)] with appropriate dilutions for 45 min on ice. Excess Abs were washed using the FACS buffer. For IL-17/FoxP3 staining, cells were stimulated with MOG or PMA (40 nM) and ionomycin (1 μM) for 2 h and then cytokine secretion was blocked with brefeldin A (1x) for 3 h. Cells were surface stained with anti-CD4-PE for 30 min and fixed with fixation buffer (1x) for 30 min on ice in the dark. Cells were permeabilized using permeabilization buffer (1x) (BD Biosciences, San Diego, CA) for 30 min on ice in the dark. Anti-FoxP3-APC and anti-IL-17-PerCP/Cy5.5 Abs diluted in permeabilization buffer were added to the cells for 1 h on ice. Cells were washed 3x with FACS buffer and acquired using BD Accuri (BD Biosciences, San Diego, CA). Data were analyzed using FlowJo software (BD, Biosciences, San Diego, CA).

3.8 Antigen uptake assay by MDSC. The $MDSC^{rapa}$ and control MDSC (5×10^4) were cultured with dextran-FITC (100 μg/ml) for 30 min. Later the cells were scrapped, washed and acquired through flowcytometry and data were analyzed by FlowJo software for uptake of dextran-FITC.

3.9 Proliferation of CD4 T cells by $MDSC^{rapa}$. MDSC (2×10^5) were generated in the presence ofdifferent doses of rapamycin(1-100 ng/ml) ($MDSC^{rapa}$). Later, $MDSC^{rapa}$(5×10^4) were pulsed with $OVA_{323-339}$ (50 μg/ml)for 4 h and co-cultured for 72 h with CFSE-labelled naïve CD4 T cells (1:5 ratio) isolated from OT-II mice. The proliferation of CD4 T cells was enumerated by CFSE-dye dilution assay by flow cytometry.

3.10Proliferation of CD4 T cells by syngeneic/allogeneic $MDSC^{rapa}$. $MDSC^{rapa}$(C57BL/6) (5×10^4) were cultured for 72 h with CFSE-labelled naïve CD4 T cells (1:5 ratio) isolated from syngeneic (C57BL/6J) or allogeneic (BALB/c) mice. The proliferation of CD4 T cells was examined by CFSE-dye dilution assay by flow cytometry.

*3.11*Demonstration of autophagy. Naive CD4 T cells (1.5 x 10^5) and/or $MDSC^{rapa}$ (2 x 10^5) were subjected to treatment with wortmannin (500 nM) for 4 hours. Subsequently, naive CD4 T cells were harvested, and flow cytometry was employed to assess the expression of FoxP3 and IL-17.To achieve transient knockdown of MAP1LC3A via siRNA, adherent cells were cultivated until they reached 60-80% confluency. A mixture of MAP LC3α/β SiRNA (10 nM), comprising four distinct target-specific siRNAs (Santa Cruz, Dallas, TX) with sequences ranging from 19 to 25 nucleotides in length was combined with lipofectamine RNAiMAX transfection reagents (9 µl) in serum-free media for 5 min. Subsequently, this mixture was introduced into the designated wells and incubated for 24 h. Following this, cells were subjected to a washing step, and the degree of autophagy was assessed using qRT-PCR.Furthermore, MDSC were co-cultured with naive CD4 T cells for 72 h. Subsequent to co-culture, the expression levels of FoxP3 and IL-17 were evaluated via flow cytometry.

3.12 Western blotting. $MDSC^{rapa}$ /splenocytes were lysed with RIPA buffer containing PMSF in the presence of a 1X protease inhibitor cocktail (Halt™ protease inhibitor cocktail; ThermoFisher Scientific, Waltham, MA). The amount of protein was quantified in lysate supernatant using BCA assay (QuantiPro™ BCA Assay Kit; Sigma Aldrich, St. Louis, MO). Next, different proteins in the samples were separated by SDS-PAGE gel electrophoresis. Separated proteins were transferred on the PVDF membrane (0.2 microns) and non-specific sites on the membrane were blocked using freshly prepared 5% BSA. The membrane was probed with Abs against LC3, FoxP3, LAMP1, beclin-1, Atg12 and loading control β–Actin. Blots were incubated with HRP-tagged secondary either anti-mouse, rabbit or rat Abs. Regular washing was done after each step with TBST buffer). Blots were developed using Pierce ECL western blotting substrate™ (ThermoFisher Scientific, Waltham, MA) and visualized using the ChemiDoc imaging system (Bio-rad, Hercules, CA). Blot analysis and quantification were done by ImageJ software (National Institutes of Health).

3.13 Histopathology and immunohistochemistry. Sections were prepared offrozen spinal cord tissues obtained from control, EAE (MOG), and vaccinated (MOG^{rapa}) animals using a cryotome (Leica, Wetzlar, Germany). Sections were analyzed by H&E staining for inflammation. Briefly, tissue samples were fixed with formalin overnight and dehydrated with a sucrose gradient. The tissue was then embedded in an OCT poly freeze medium.

Sections (15 µm) were collected using cryotome on precoated microscope slides. Sections were rehydrated using ethanol gradient (100%, 95%, 80%, 70%, 50%) and washed with tap water. Hematoxylin was added to the sections for 30 seconds, washed with water (2x) and dipped into 80% ethanol (2x). Eosin was added to the sections for 10 seconds and washed with 95% ethanol (2X) followed by 100% ethanol. Slides were dipped into a clearing agent (xylene), air-dried and mounted by using a DPX medium analyzed under a microscope. For luxol fast blue staining sections were dipped into chloroform/ethanol (1:1) for de-fat for 6 hours and hydrated back using 95% ethanol. Sections were left in luxol fast blue stain (0.1%) for overnight at 56°C. The excess stain was rinsed off with 95% ethanol and the dH_2O. The section was differentiated (between grey matter and white matter) by using lithium carbonate solution (0.05%) for 30 seconds followed by 70% ethanol for 30 seconds and washed with dH_2O. Counterstain was added to the sections for 30 seconds, washed with 70% ethanol and dipped into 95% ethanol for 5 minutes followed by 100% ethanol. Slides were dipped into a clearing agent (xylene), air-dried and mounted by using a resinous mounting medium. Sections were also analyzed by immunohistochemistry for FoxP3 expression. Briefly, slides containing sections were dipped into chilled methanol for 5 minutes followed by washing with PBST (0.025%). Sections were permeabilized with 0.25% triton-X-100 for 15 minutes. The BSA (1%) was used to block the off-target sites for 1 h. Anti-FoxP3 Ab was added to the section for 1 h at room temperature followed by washing (3X). Alexa fluor 488 conjugated secondary Ab was incubated for 1 h at room temperature followed by 3x washings. Finally, the sections were stained with DAPI and mounted using DPX media.Imaging was done using a Leica DMi8 fluorescence microscope and analysed with LAS X software (Leica Biosystems, Mumbai, India).

3.14 Microscopy. $MDSC^{rapa}$ were cultured on pre-coated poly-L-Lysine coverslips. After washing, cells were fixed with paraformaldehyde (4%) for 10 min. Later, cells were permeabilized with triton X-100 (0.05 %) for 10 min and incubated in 1% BSA for 1 h at 22 ^{o}C. Abs to LC3 and LAMP1 were added for 2 h at 22 ^{o}C. Later, cells were incubated with secondary Abs Alexa Fluor 594 conjugated anti-rabbit/Alexa Fluor 488 conjugated anti-rat Absfor 45 min/22 ^{o}C. The cells were also stained with DAPI and lysotracker dyes. Regular incubation and washing steps were followed after every phase (0.025% PBST). The cells were mounted on glass slides using SlowFade™ Diamond Antifade Mountant (Thermo Fisher Scientific,

Waltham, MA). Imaging was done using a Leica DMi8 fluorescence microscope and analysed with LAS X software (Leica Biosystems, Mumbai, India).

3.15 qRT-PCR analysis. Total RNA was isolated from splenocytes/ naïve CD4 T cells/MDSC (1.5-2 x 10^5) cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. Briefly, cells were lysed using TRIzol reagent for 5 min at room temperature. Lysate was mixed with chloroform (5:1). Aqueous layer was aspirated after centrifugation (13000 rpm, 15 min, 4°C). RNA was precipitated using isopropanol (2:1) for 10 min/ 22 ^oC at room temperature. RNA was washed with 75% ethanol (7500 rpm, 15 min, 4°C) and air dried at 22 ^oC. Contaminating DNA was removed by using DNase I (Sigma-Aldrich,St. Louis, Missouri, United States). The purity and quantity of RNA were measured using NanoDrop (Thermo Fisher Scientific, Waltham, MA). RNA (1000 ng) was used to synthesize cDNA by using an iScript cDNA synthesis kit, according to the manufacturer's instructions (Biorad Laboratories, Hercules, CA). Briefly, RNA was mixed with 5x reaction mixture, reverse transcriptase enzyme and incubated for 5 min at 25 °C, 20 min at 46 °C and 1 min at 95 °C. Synthesized cDNA was amplified using primers for different genes. Amplified DNA was quantified by SYBR green on CFX96™ (Biorad Laboratories, Hercules, CA), according to the manufacturer's instructions. Initially, denaturation and enzyme activation were done at 95°C for 2 min followed by denaturing (0:10 min at 95°C), annealing (0:30 min at 60°C) and extension (0:30 min at 72°C) was done for 40 cycles. The comparative Ct method was used to analyze the results. Data are presented as fold change in comparison to control.

3.16 Cytokine ELISA. Cytokine were estimated by sandwich ELISA (*Das DK et al., 2022*). Briefly, the ELISA plates were coated with Abs against IFN-γ, IL-6, IL-12, TGF-β and IL-10 overnight at 4 °C. The next day, the plates were washed with PBS-Tween-0.05% to remove unbound Abs. Further, wells were blocked with BSA (1%). Subsequently, serum/culture SN were added along with the respective standards for the cytokine. Following 4 h incubation, the samples were treated with biotinylated capture Abs against their respective cytokine for 2 h. Later, unbound Abs were removed by 3x washing the wells and incubated with HRP-avidin for 1 h at 37 ^oC. The colour was developed using a TMB substrate. The reaction was stopped with 0.14 M H_2SO_4. The absorbance was measured at 450 nm. Usual steps of

washings and incubations were performed at each stage. The quantification of cytokine was done using the standard plot generated through the recombinant cytokine. The results were expressed as pg/ml.

3.17 EAE induction and vaccination studies.

3.17.1 Murine model of EAE. Female C57BL/6J FoxP3-GFP reporter mice were immunized with MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) (50 μg) and rapamycin (20 μg) emulsified in 100 μl of CFA (1:1) s.c. at the flanks on day 0. In addition, 200 ng pertussis toxin was injected through the i.p. route at day 0 and day +2. Additional doses of rapamycin (1 mg/kg/bwt) were injectedi.p.daily from day -2 to day +10 (MOG^{rapa}). Animals were re-challenged with MOG_{35-55} (100 μg) emulsified in IFA (1:1) at day +30. The control group was not injected with rapamycin (MOG). Another control group was also kept that was injected with PBS only and no MOG or rapamycin (Control). Body weight change and the clinical score wereperiodically observed up to day +45. Clinical score was evaluated as reported earlier 1: distal tail limpness; 2: complete tail limpness; 3: partial hind limb paralysis; 4: complete hind limb paralysis in one leg; 5: fore limb and hind limb paralysis; 6: mortality (*Kujur W et al., 2017*).

3.17.2 Zebrafish model of EAE. Zebrafish (*Danio rerio*) were immunized with MOG_{35-55} and rapamycin (0.5 μg) emulsified in CFA (0.6 mg/kg/bwt) [MOG^{rapa}].Additional doses of rapamycin (1.0mg/kg/bwt) were injected daily from day -1 to day +3 through the i.p.route (MOG^{rapa}). The control groups were not injected with rapamycin (MOG). Another control group was also kept that was injected with PBS only (control). Clinical score was evaluated as reported earlier 0: normal gait; 1: loss of gait; 2: mild paralysis; 3: total paralysis; 4: mortality (*Kulkarni P et al., 2017*).

3.18 Statistical analysis. Statistical analysis was performed using “one-way analysis of variance (one-way ANOVA) and between the groups was compared by Tukey’s test” or “unpaired Students t-test” using GraphPad Prism software (GraphPad Software Inc, San Diego, CA). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

List of oligonucleotide primers		
Genes	**Forward (5 --> 3)**	**Reverse (5 --> 3)**
Actb	AGCCATGTACGTAGCCATCC	ACCCTCATAGATGGGCACAG
Il10	TGAATTCCCTGGGTGAGAAGC	AGACACCTTGGTCTTGGAGCTTATT
Tgfb1	CATGACATGAACCGGCCCTT	GAAGTTGGCATGGTAGCCCT
Il17a	CAGGGAGAGCTTCATCTGTGTCTC	GGACACGCTGAGCTTTGAGG
Ifng	CTGGAGGAACTGGCAAAAGGATG	GCTGATGGCCTGATTGTCTTT
Tnf	ATGGCCTCCCTCTCATCAGT	ACCCTGAGCCATAATCCCCT
Cd69	TCCAGCTACATCTCTCCGTGG	GCAGACCCAGTGGAAGTTTG
Sell	GCAGACCCAGTGGAAGTTTG	TGCGCAAGGAGTCTGAGTTT
Gata3	CCTCCTCCTCTACGCTCCTT	GATACCTCTGCACCGTAGCC
Icos	CCGTACTTCTGCCGTGTCTT	ATCAAGTCAGGTGCCCTGTG
Il6	GCCTTCTTGGGACTGATGCT	GACAGGTCTGTTGGGAGTGG
Ccr4	GGTGTTGGGACAATCCCTTCT	GAAAGCCAAACTGCACGGAC
Ccr5	TCCTAGCCAGAGGAGGTGAG	GAGCTGAGCCGCAATTTGTT
Ccr7	ATCCACCGAATTGCTGCTGA	GGCAGCCTCTCACTTTCACT
Ccr8	TGTTTGGGACTGCGATGTGT	TGATGGCATAGACAGCGTGG
Cxcl10	GGTCTGAGTCCTCGCTCAAG	GTCGCACCTCCACATAGCTT
Atg12	TTCTGGTTCTTGCGCCTCAT	TAGGAGTGCTGCCATGTGTG
Icam1	CAGTGGGTCGAAGGTGGTTC	CAGCCGAGGACCATACAGCA
Foxp3	CCTCTAGCAGTCCACTTCACC	AGCTTTCTTCTGTCTGGAGTG
Itgae	ATGACAGTCCTTCCCAGCAGA	CGAAGTAGTGGAGTCCCGAG
Il1r2	TGATGTCTGGGCATCTGCTTT	CCCTTGGAGCCCAATGCT
Ccr6	CAGCTTGGAGCAGAATAGCAAG	GGAGAGCAGAGGTGAAGCAATAA
Rps6kb1	GAGCTGGAGGAGGGGG	CACAATGTTCCATGCCAAGTTC
Ulk1	CACCTTCTCCCCAAGCCAC	CACATCAGCTCCTTGTGGGG
Rorc	CTACCGCACAACGCACTTTC	CCAGGGCCTTGACCGGG
Pdcd1	GCAAGGACGACACTCTGAAGG	AGCTCTGGTGTCTTCTCTCGT
Havcr2	TTGCAGGCAGTTCTGGGATG	CTCCGTGGTTAGGGTTCTTGG
Entpd1	GAGAGGAAGACCAAGAGGCAC	TCCAACACAATCCCATACTTAACA
Nt5e	CAGCATTCCTGAAGATGCGAC	CGATCGTTCTCCCGAGTTCC
Nrp1	GCTGTGAAGTGGAAGCACCT	CACTGTGGCAGTTGGCCTG
Tbx21	TGGAGCCCACAAGCCATTAC	CATATAAGCGGTTCCCTGGC

Mtor	TTCCTGAACAGCGAGCACAA	GTAGCGGATATCAGGGTCAGG
Atg13	GGCCTTTTCCCATCAACTCTC	GAGTTACCCCGCCTTCCTTC
Map1lc3a	GTCACCCAGGCGAGTTACC	TTACAGCGGTCGGCGAAG
Il1b	TGCCACCTTTTGACAGTGATG	ATGTGCTGCTGCGAGATTTG
Il12b	TGGGAGTACCCTGACTCCTG	AGGAACGCACCTTTCTGGTT
Becn1	CAGGAACTCACAGGAGCCATT	CTCCCCGATCAGAGTGAAGC
Lamp1	GAGCGTTCAACATCAGCCCA	GGCTAGAGCTGGCATTCATC
Kitl	GAAGACACAAACTTGGATTATCACT	TGGAAGATTTGCCACCAGTTT
Wipi2	CCTGGACTGGCTACTTTGGG	ACGTCTGTGTTGCTAAGGGG
Ambra1	TTCCTGCACATGCTGTCCTC	GAAGGGCTCGGTCTGGATG
Rb1cc1	CCAGGTGAGGGAGCTTCAGG	CCATTACTTTCCCAAGGACCCA
Arg1	ATTGGCTTGCGAGACGTAGA	CGGCCTTTTCTTCCTTCCCAG
Ccl5	CTCCAATCTTGCAGTCGTGTTT	CACACACTTGGCGGTTCCTT
Cox2	AGCAGATGACTGCCCAACTC	AGGAAGCTCCTTATTTCCCTTCA
foxp3	CATGCATCCGACACTTGCAC	TGGCCATCTGCATTGTCCTT
il17a/f1	TCAGCGTTAAACCTCCGCTT	TCCACATAAGGACGAACGCA
actb1	ACTCAGGATGCGGAAACTGG	GAAGTCCTCTCGGGGAAAGC

4.1 Rapamycin promoted in vivo differentiation of MOG-specific CD4 T cells into regulatory T cells (Tregs). Immunization of FoxP3-GFP mice with varying doses of rapamycin yielded a dose-dependent generation of Tregs (Figure 1a). Subsequently, we explored rapamycin's potential to induce MOG-specific Tregs by supplementing MOG_{35-55} with rapamycin (Figure 1b). Remarkably, a noteworthy increase ($P<0.0001$) in MOG-specific Tregs ($Tregs^{rapa}$) was a higher percentage ($P<0.0001$) of MOG-reactive Th17 cells was detected, coupled with a diminished frequency of Tregs (Figure 1c-d).

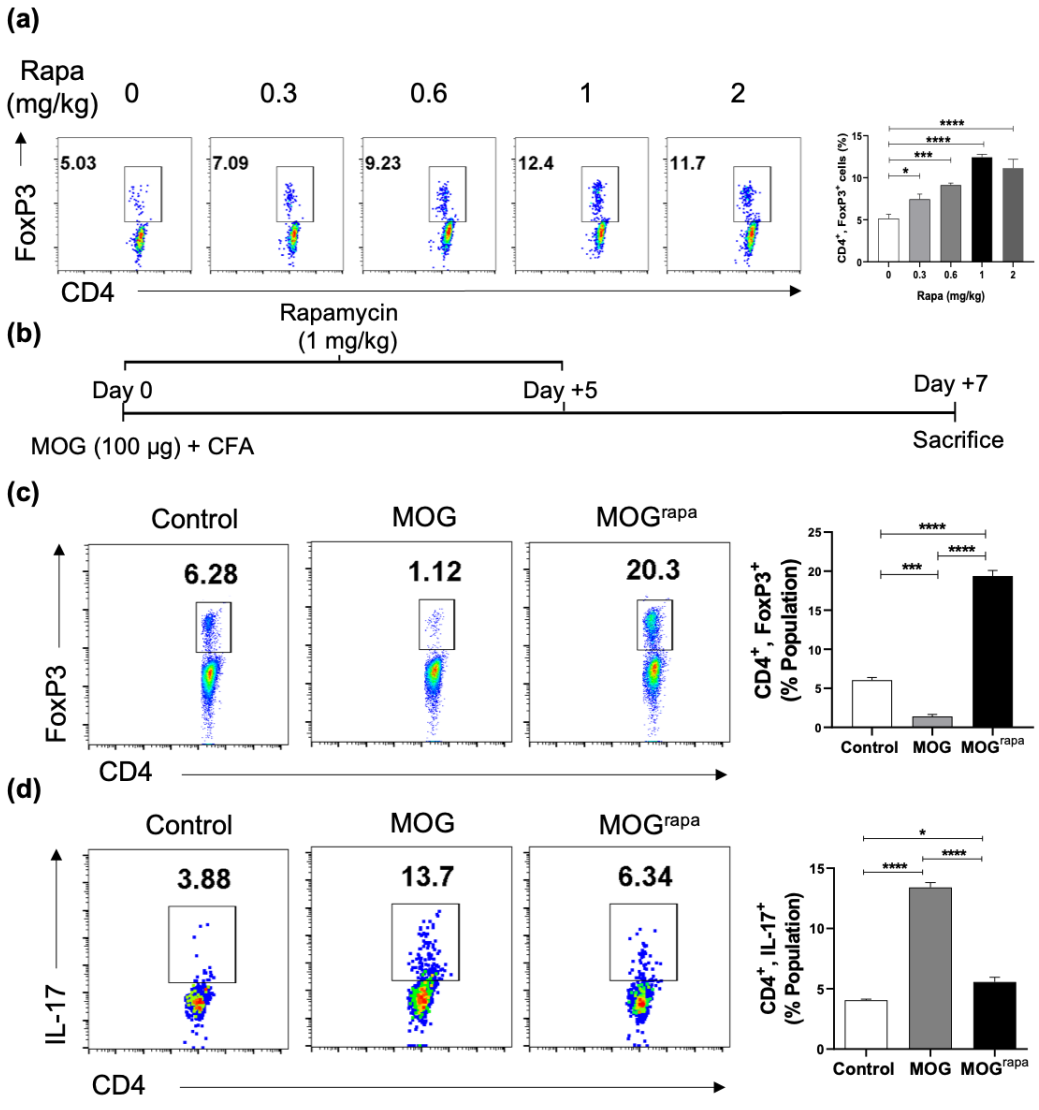
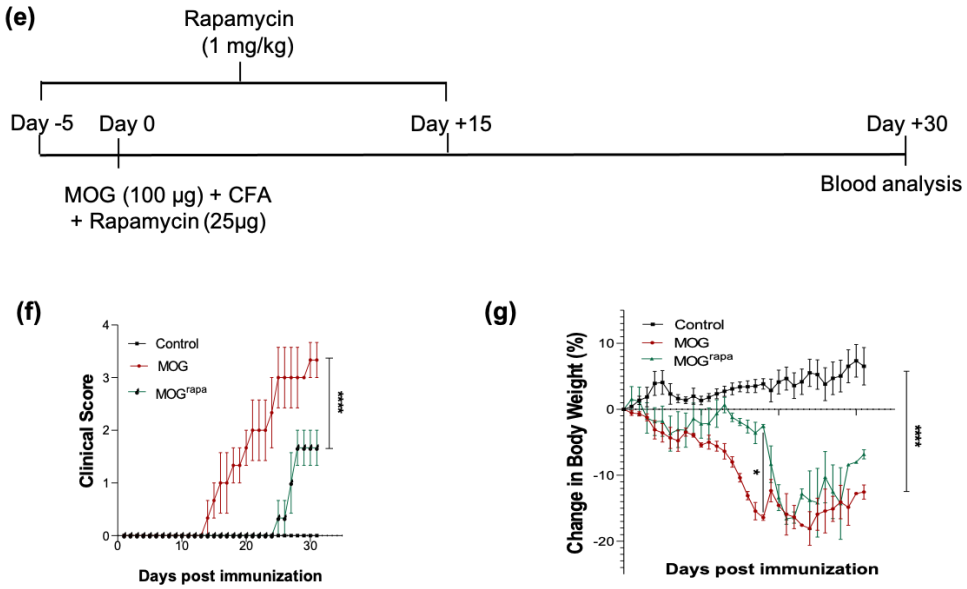


Figure 1: *Rapamycin induced in vivo differentiation of naive CD4 T cells specific to myelin oligodendrocyte glycoprotein (MOG) into regulatory T cells (Tregs).* (a) Weight and age-matched FoxP3^{GFP} mice were immunized with different doses of rapamycin (0.3, 0.6, 1, 2 mg/kg/bwt) for 5 days and animals were sacrificed at day +7. The splenocytes were *in vitro* cultured with rapamycin. The level of Tregs (CD4^{+}, FoxP3^{+}) was estimated in the splenocytes through a flowcytometer. (b) FoxP3^{GFP} mice were injected with MOG+CFA. Additionally, rapamycin (1 mg/kg/bwt) was administered for 5 days. The animals were sacrificed at day +7 and splenocytes were isolated and (c) Tregs (CD4^{+}, FoxP3^{+}); (d) Th17 cells (CD4^{+}, IL-17^{+}) were measured through a flowcytometer. (e) Mice were administered rapamycin for 5 days prior to administering MOG + CFA + rapamycin. The animals continued receiving rapamycin for the next 15 days. On day 30 of post-immunization, animals were monitored for (f) clinical score; (g) change in body weight. (h) MOG (100 μg) and rapamycin (25μg) emulsified in CFA were injected in Foxp3^{GFP} mice. Rapamycin (1 mg/kg/bwt) was administered from day -5 to day +15. The control groups were injected with MOG+CFA or PBS. On day +45, mice were re-challenged with MOG (100 μg) emulsified in IFA. (i-k) The animals were sacrificed on day +60 and (i) CD4^{+}FoxP3^{+} cells; (j) Th17 cells; (k) effector CD4 T cells and CD8 T cells were computed in the spleen through flowcytometry. (l, m) Clinical scores and body weight change were recorded up to day +60. (n-o) Zebrafish were injected low (Rapa^{lo}: 0.3 mg/kg/bwt) and high (Rapa^{hi}: 1.0 mg/kg/bwt) doses of rapamycin for 5 days. The fish were sacrificed on day +7. The splenocytes were harvested and qRT-PCR was performed for *foxp3 and il-17a/f1*. (p) Zebrafish were injected with MOG (0.6 mg) emulsified in CFA at the mid-spine region on day 0. Rapamycin was injected from day -1 to day +3 through the intra-peritoneal route. The control groups were injected with MOG and PBS (placebo). The fish were sacrificed at day+7 as depicted in the scheme. (q-s) Clinical score, per cent survival and change in body weight were recorded throughout the study. (t, u) The *foxp3* and *il-17a/f1* were assessed in the splenocytes by qRT-PCR The data were analysed using one-way ANOVA, with comparisons between groups by Tukey's test. The results expressed as mean ± SEM are representative of 3 independent experiments (n=3 mice/group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



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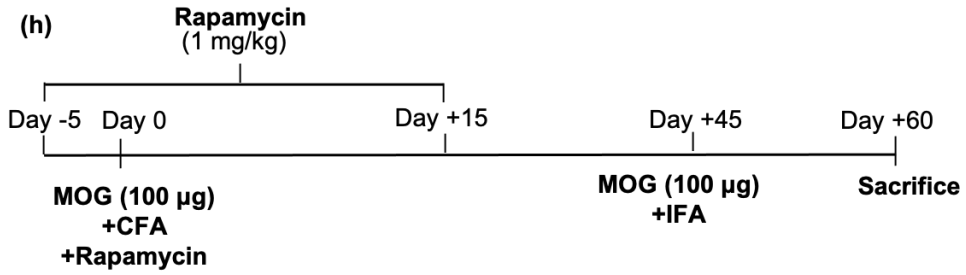
Moreover, mice vaccinated with MOG (100 µg) along with rapamycin (MOG^{rapa}) exhibited a significantly delayed onset ($p<0.01$) and reduced severity ($P<0.0001$) of disease symptoms (Figure 1f, g). Additionally, they exhibited less body weight loss initially (up to day+18) ($p<0.05$) and had a higher Tregs population ($P<0.05$) (Figure 1g-i). Conversely, the control group, which received MOG alone exhibited a sudden increase in the clinical score ($P<0.01$) and body weight loss ($P<0.05$), along with activation of Th17 cells ($P<0.0001$) (Figure 1j).



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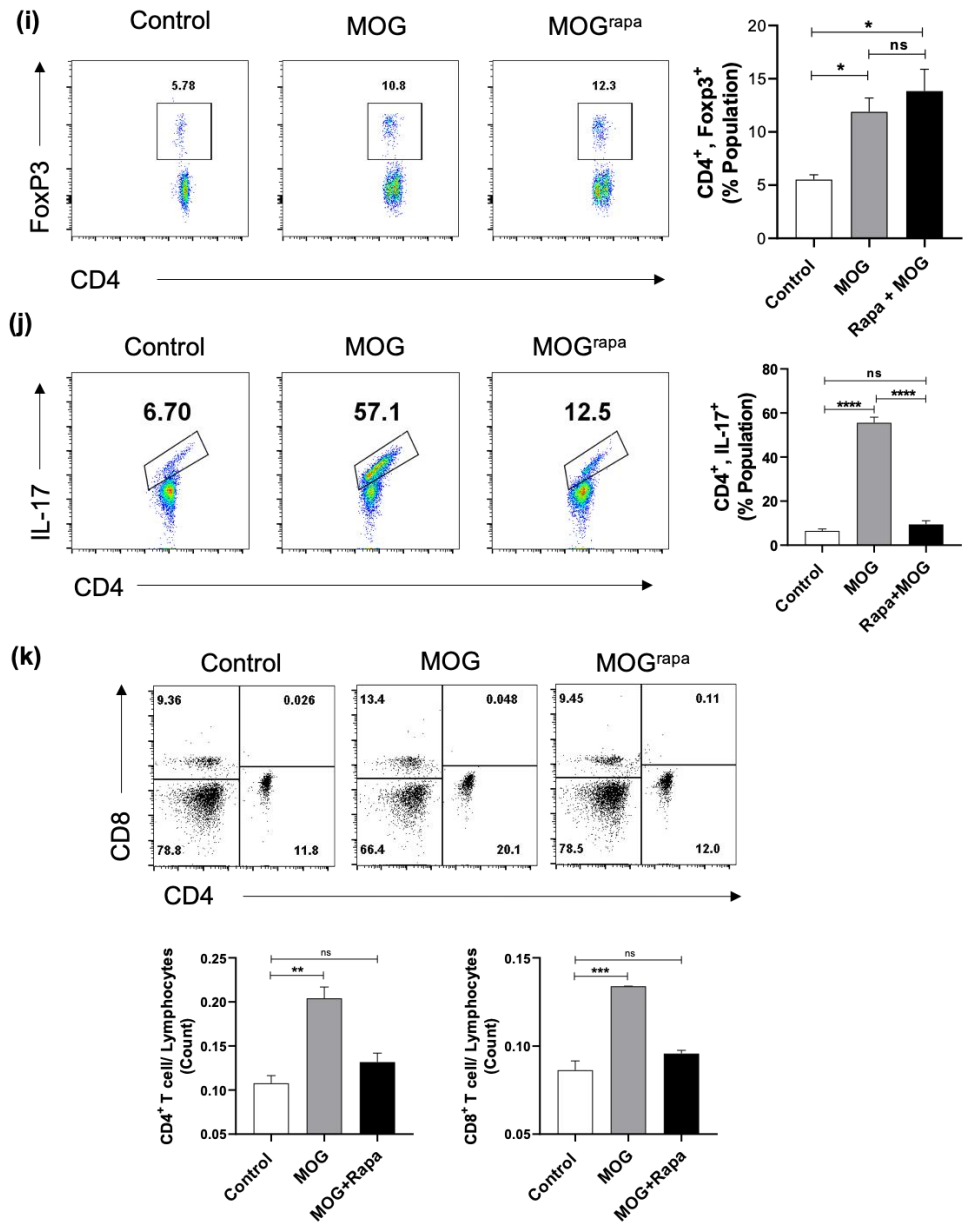

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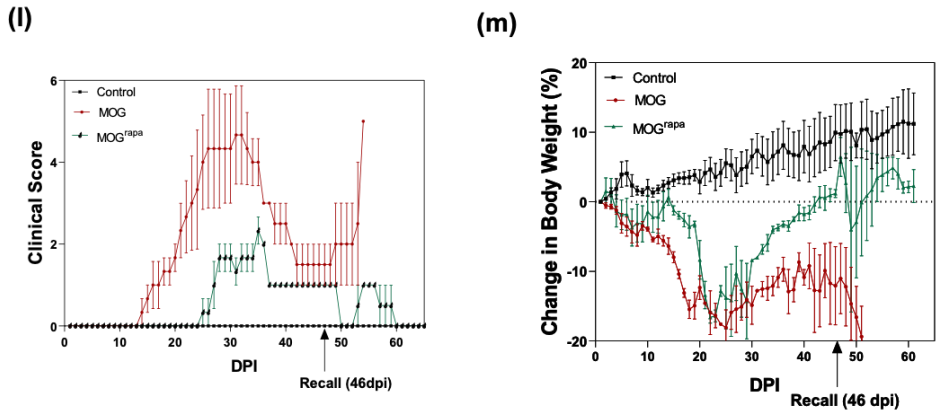
Additionally, there was a significant decrease in the accumulation of effector CD4/CD8 T cells in the spleen of the MOG-rapa immunized mice (Figure 1k). The MOG^{rapa} vaccinated animals remained relatively healthy, with mild disease symptoms appearing on 8 days after rechallenge (day 46 post-immunization) but mice were normalised within 7 days (Figure 1l, m).



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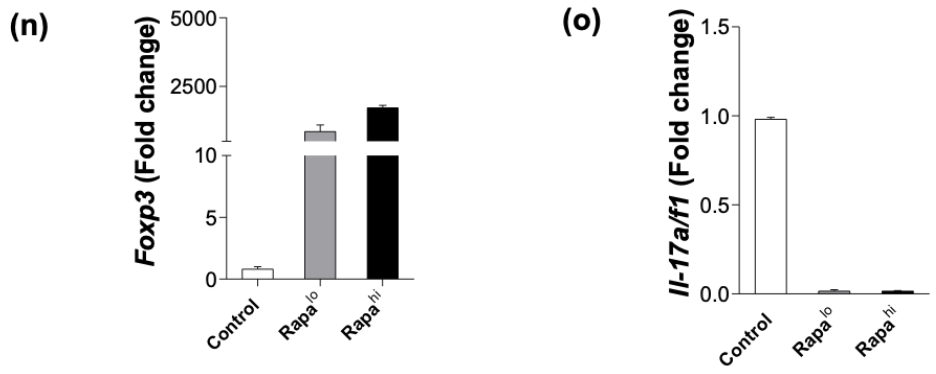
Furthermore, these findings were validated in a zebrafish model of EAE (Figure 1n-u). These results suggested that rapamycin effectively expands the pool of MOG-reactive naive CD4 T cells towards Tregs ($Tregs^{rapa}$) while inhibiting Th17 cells. Tregs play a protective role in suppressing autoimmune diseases (AIDs), whereas Th17 cells can exacerbate the disease with their pathogenic effects.



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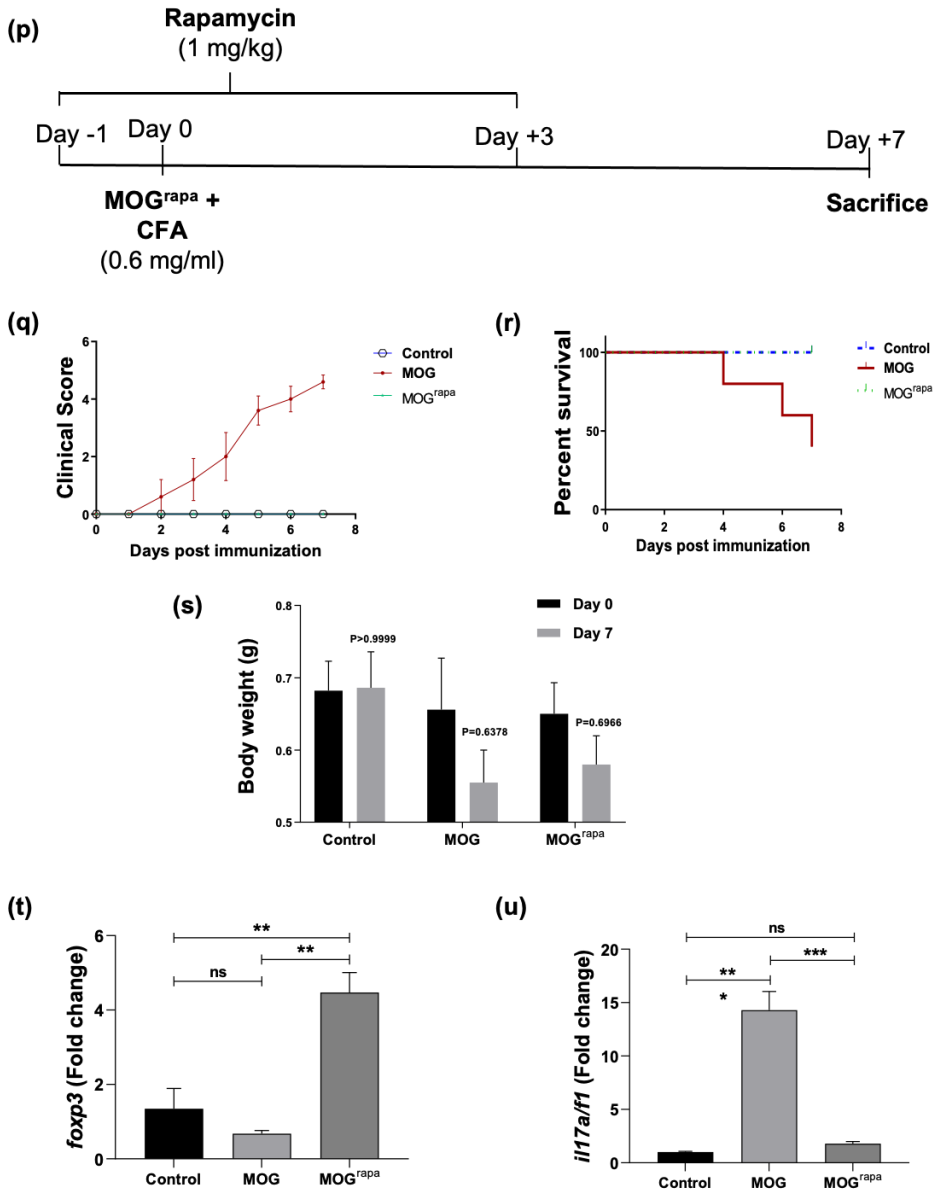

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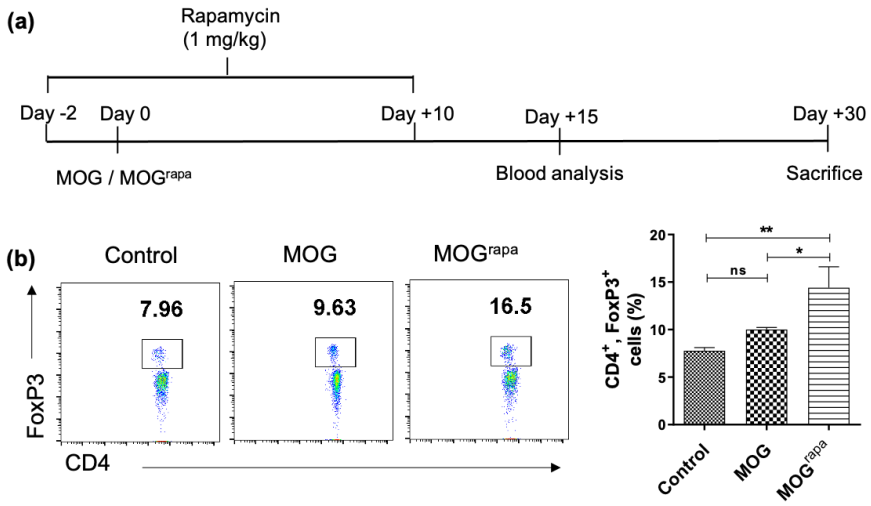
Our findings provided valuable insights into the generation of autoantigen-specific Tregs on exposure of animals to autoreactive antigens supplemented with rapamycin. This novel strategy mightpotentially protect against AIDs.

4.2. Immunization with MOG^{rapa} resulted in the generation of central and resident memory CD4 T cells. Currently, there is no vaccine available for multiple sclerosis (MS) or any other AIDs. Various prophylactic strategies, such as T cell-based vaccines, T-cell peptide receptor vaccines, DNA vaccines, and altered peptide ligand (APL) based vaccines, have been attempted to prevent MS (*Correale J et al., 2008*). Unfortunately, none of these approaches have yielded satisfactory results. In this study, we have judiciously developed a novel vaccination strategy of exploiting the immunosuppressive drug rapamycin to generate autoantigen-specific Tregs for preventing EAE. Rapamycin is known for its efficacy to treat AIDs.

To improve vaccine efficiency and avoid toxicity, we modified the immunizationprotocol based on the observation that mice exhibited mild symptoms of EAE when inoculated with MOG (100 μg) and rapamycin (25 μg) (Figure 1f). Consequently, the dose of MOG was reduced to 50 μg. Mice were vaccinated with MOG (50 μg) complemented withrapamycin (25 μg) [MOG^{rapa}] (Figure 2a). Flow cytometric analysis revealed a significantly higher percentage ($P<0.01$) of MOG-specific-Tregs in the MOG^{rapa} group compared to the control animals (Figure. 2b). Animals immunized with MOG alone mainly exhibited Th17 cells and a lower frequency of Tregs (Figure. 2b, c). Additionally, a significant increase ($P<0.001$) in the number of Gr1^{+}/CD11b^{+} myeloid-derived suppressor cells (MDSC) was also detected (Figure 3d).Gene expression analysis showed downregulation of pro-inflammatory molecules (*Il-17, Rorc, Ifng, and Tnf-α*) and upregulation of anti-inflammatory molecules (*Havcr2, Pdcd1, Nt5e*) in the MOG^{rapa} group (Figure 2e-k).


(a)
Rapamycin
(1 mg/kg)
Day -2
Day 0
Day +10
Day +15
Day +30
MOG / MOGrapa
Blood analysis
Sacrifice
(b)
Control
MOG
MOGrapa
7.96
9.63
16.5
FoxP3
CD4
CD4+, FoxP3+ cells (%)
**
*
ns
20
15
10
5
0
Control
MOG
MOGrapa

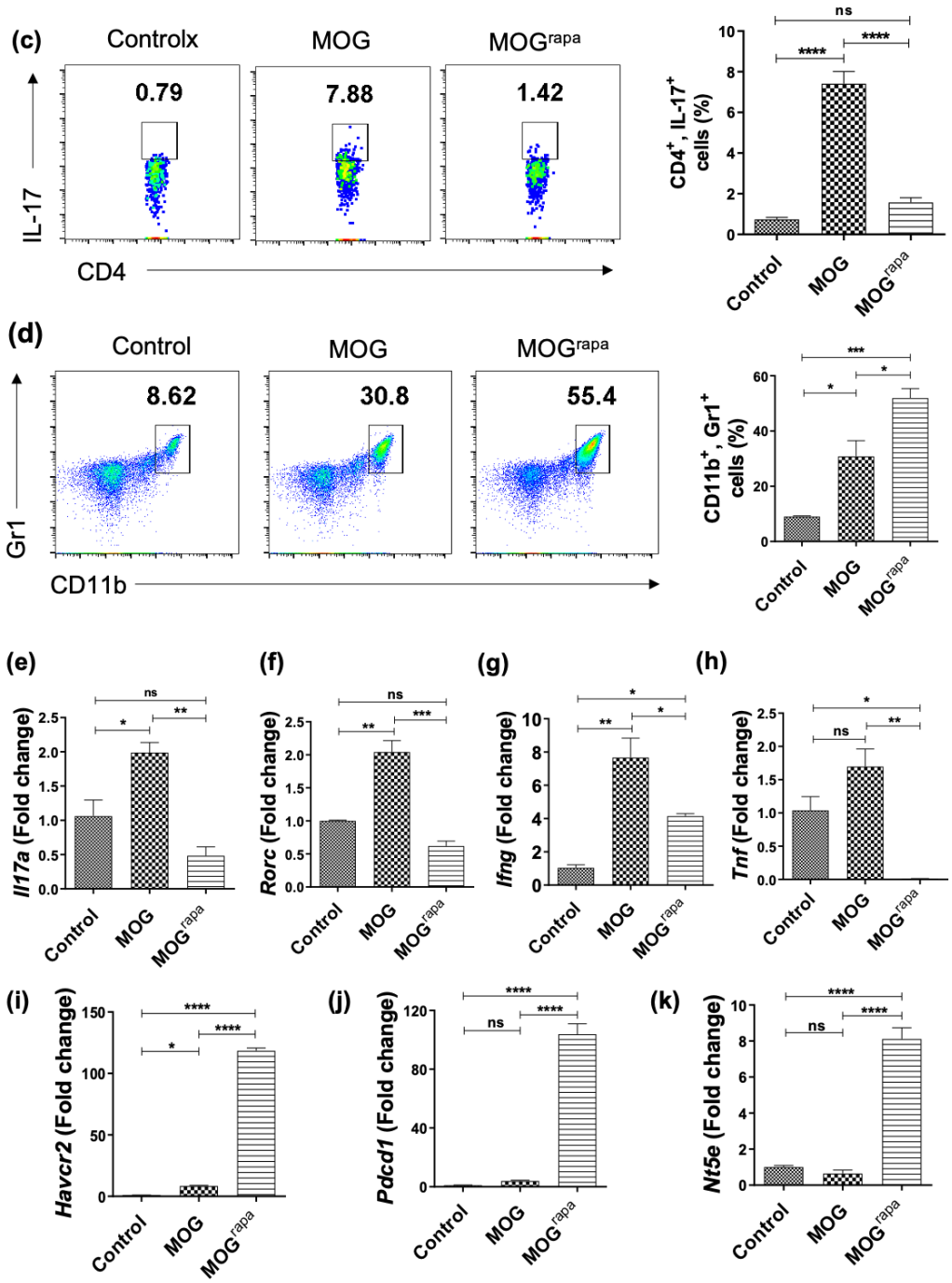

(c)
Controlx
MOG
MOGrapa
0.79
7.88
1.42
IL-17
CD4
CD4+, IL-17+ cells (%)
(d)
Control
MOG
MOGrapa
8.62
30.8
55.4
Gr1
CD11b
CD11b+, Gr1+ cells (%)
(e)
Il17a (Fold change)
(f)
Rorc (Fold change)
(g)
Ifng (Fold change)
(h)
Tnf (Fold change)
(i)
Havcr2 (Fold change)
(j)
Pdcd1 (Fold change)
(k)
Nt5e (Fold change)
ns

Figure 2: *Immunization with MOG+rapamycin (MOG^{rapa}) resulted in the generation of central and resident memory Tregs.* (a) The schematic diagram depicts the study plan of FoxP3^{GFP} mice administered rapamycin (1 mg/kg bwt) for 2 days prior to MOG (50 µg) and rapamycin (25 µg) [MOG^{rapa}] inoculation. The animals continued receiving rapamycin (1 mg/kg/bwt) till day +10.The control groups were injected with either MOG or PBS (placebo). At day +15, blood was drawn and analysed for (b) Tregs (CD4^{+}, FoxP3^{+}); (c) Th17 cells (CD4^{+}, IL-17^{+}) (d) MDSC (CD11b^{+}Gr1^{+}) through flowcytometer; (d) On day +15 expression was assesed of (e-k) pro-inflammatory (e) *Il17;* (f) *Rorc;* (g) *Ifng;* (h) *Tnfα*; anti-inflammatory (i) *Havcr2;* (j) *Pdcd1;* (k) *Nt5e* genes by qRT-PCR in the RNA isolated from the blood. The animals were sacrificed at day +30 post-immunizations. The splenocytes were isolated and CD4^{+}/FoxP3^{hi} gated population was used to enumerate (l) effector memory; (m, n) resident memory CD103, CD69 by flowcytometer. (o-q) The cells were harvested from the spinal cord and expression of *CD69*, *Itgae*, and *Sell* was assessed by qRT-PCR. The flowcytometer data are also expressed as bar diagrams (b, c, k-m). The data were analysed using one-way ANOVA, with comparisons between groups by Tukey's test. The results expressed as mean ± SEM are representative of 3 independent experiments (n=3 mice/group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

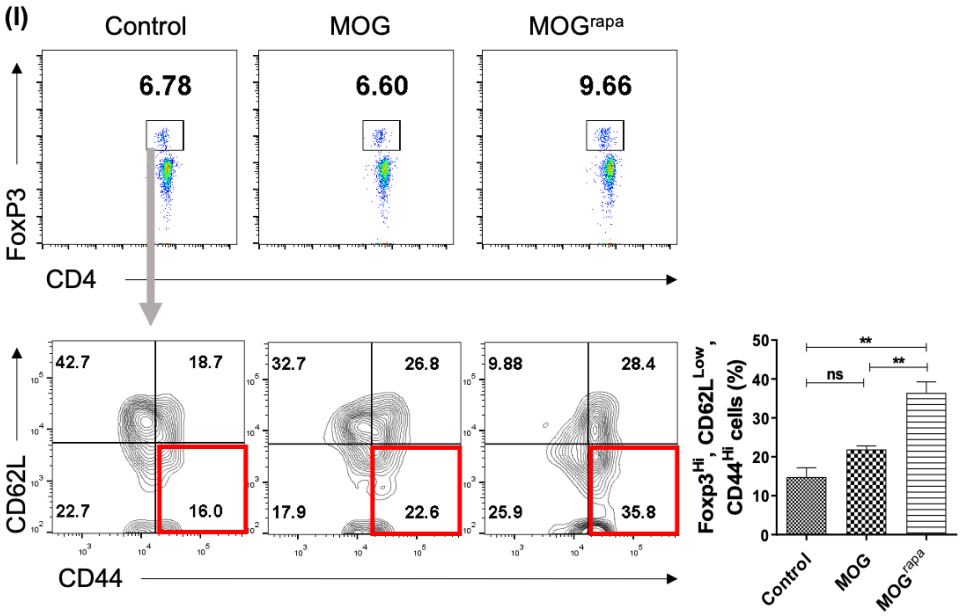


Fig. 2 Continued.

Memory CD4 Tregs, characterized by the expression of CD4, FoxP3^{hi}, CD62L^{lo}, and CD44^{hi}, play a crucial role in rapidly generating effector T cells upon subsequent encounters with the same antigen; thus forming the basis of vaccination (*Rosenblum MD etal., 2016*). At day 30 after the MOG^{rapa} vaccination, a significantly higher induction of memory Tregs was observed (Figure 2l).

CD103^{+}CD69^{+} memory T cells are known as tissue-resident memory T cells (TRM). They exist in the tissues and provide a rapid response against local tissue infection or injury, making them of considerable interest in vaccine development (*Schenkel JM and Masopust D, 2014; Hassert M and Harty JT, 2022*). Interestingly, CD4^{+}FoxP3^{+} Tregs of the MOG^{rapa} vaccinated group showed significantly higher expression of CD69 and CD103 molecules (Figure 2m, n). Further analysis revealed upregulated gene expression of TRM markers (CD69, CD103, CD62L) in the cells isolated from the spinal cord (Figure 2o-q).

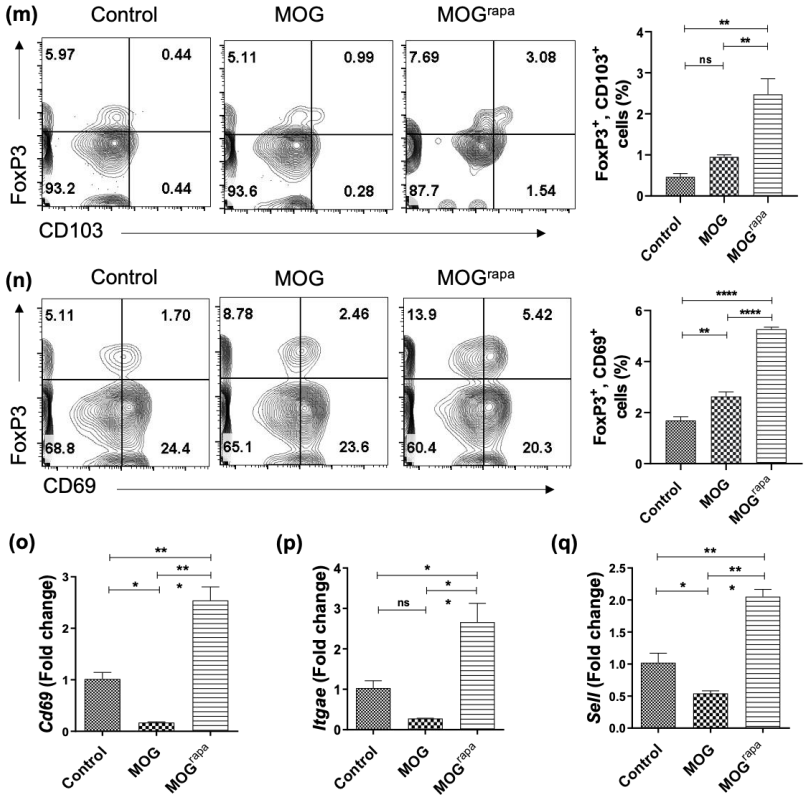
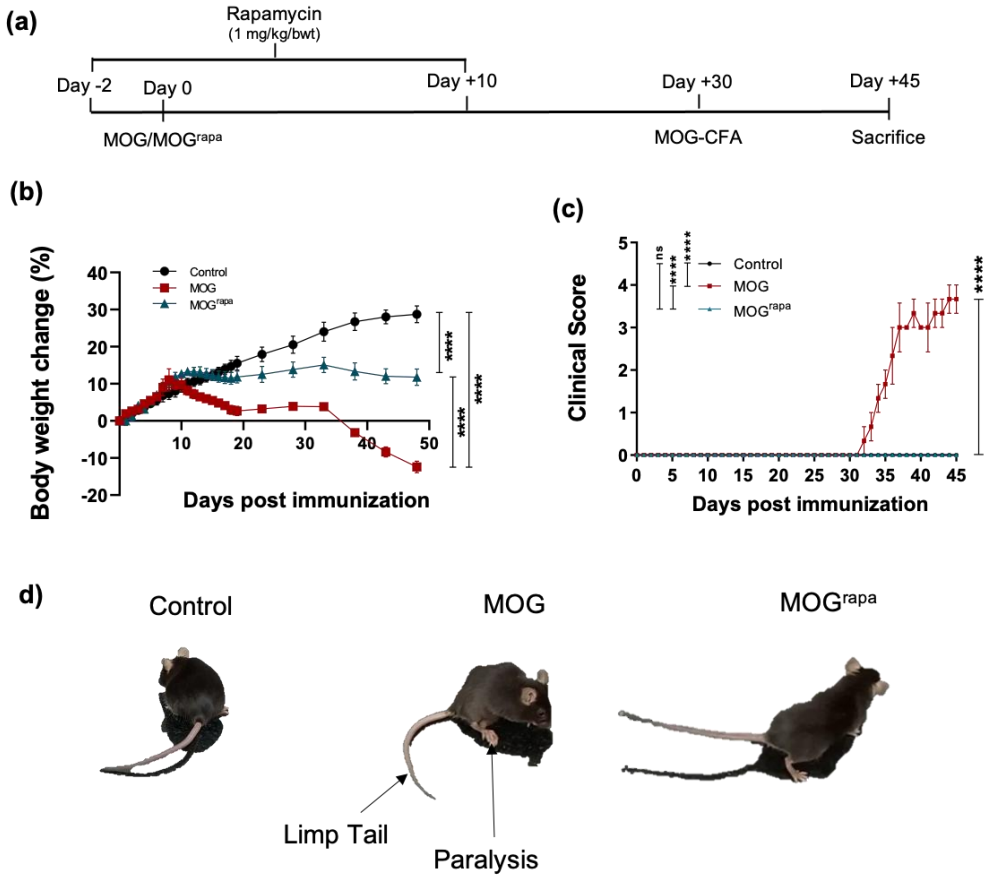


Fig. 2 Continued.

4.3. The animals vaccinated with MOG^{rapa} induced Tregs and MDSC and protected animals from EAE following exposure to a lethal dose of MOG. To assess the protective efficacy of memory Tregs induced by MOG^{rapa} against EAE, mice were vaccinated with MOG^{rapa}, while control received either MOG or PBS. After 30 days, the animals were challenged with a lethal dose of MOG (100 µg) emulsified in CFA. The mice were monitored regularly until day 45 post-immunization for clinical score and body weight changes (Figure 3a). Interestingly, the MOG^{rapa} vaccinated group remained healthy, showing no clinical signs or abnormalities (Figure 3b-d). In contrast, from day 32 post-immunization, the MOG-treated group exhibited a sudden spike in clinical score and loss of body weight (Figure 3 b-c).



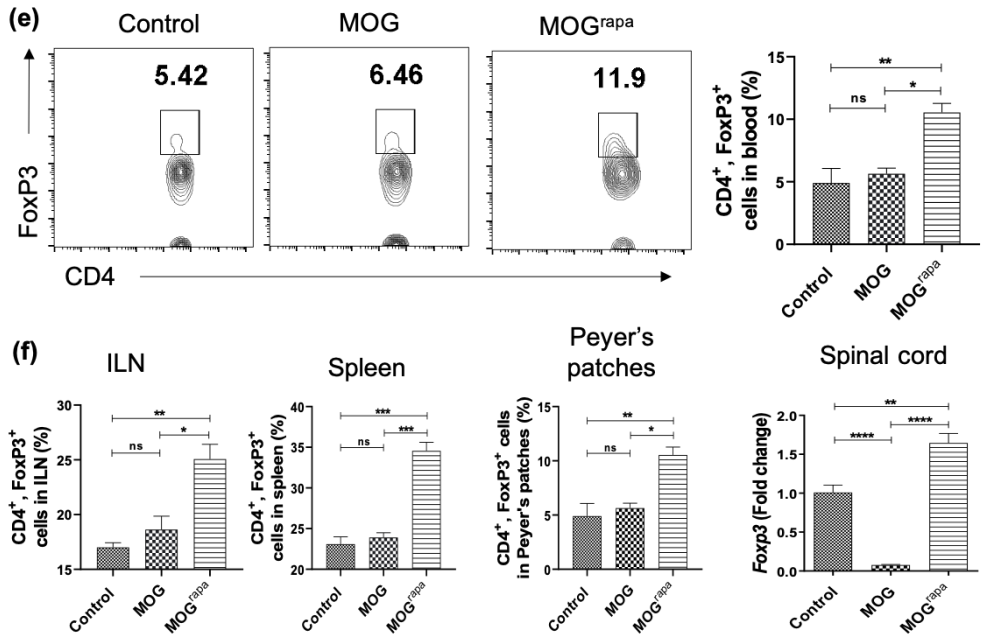


Figure 3: *The animals vaccinated with MOG^{rapa} generate Tregs and MDSC and protected animals from EAE on exposure to a lethal dose of MOG.* (a) FoxP3^{GFP} mice were exposed to rapamycin from 2 days prior to vaccination with MOG^{rapa} till day +10.The control groups were inoculated with either MOG or PBS. On day +30, the mice were challenged with MOG (100 μg) emulsified in CFA. From day 0 to day 45, the animals were examined for the (b) change in body weight; (c, d) clinical score. (e, f) At day +45 cells from different group were analyzed to enumerate the presence of Tregs in the (e) blood; (f) ILN, spleen, Peyer patches; spinal cord. The cells isolated from the ILN were *in vitro* cultured with MOG-Rapa and controls with MOG or PBS for 72h. The cells were harvested, gated on CD4/FoxP3^{hi} were examined for the presence of (g) memory Tregs (CD44^{hi}CD62L^{hi}); (h) CD44^{hi}; (i) CCR5 by flowcytometer. Cells were also stained for the presence of (j) CD4^{+}, IL-17^{+}. (k) Plots and their representative bar diagrams depict a level of MDSC (CD11b^{+}, Gr1^{+}) in the blood. (l-o) Presence of immunosuppressive markers (l) CD39; (m) CD73; (n) PD1; (o) Tim3 on CD4/FoxP3^{hi} cells by flowcytometer. (p-s) Concentration of (p) IL-10; (q) TGF-β1; (r) IL-17A; (s) IFN-γ in the serum was estimated by ELISA. Heat map of qRT-PCR depicts the expression of genes (t) *Tgf-β, Il-10, FoxP3, Havcr2, Pdcd1, Il1r1, Nrp1, Itgae, Il2ra, Icos*; (u) *Il6, Rorc, Il17, Ifng, Tbx21, Tnfα, Nos2, IL1β, Il12β, Ox40*); (v) *Ccr4, Ccr8, Cxcl10, Ccr5,*

Ccr7. (w) Flowcytometer-sorted $CD4^{+}FoxP3^{GFP+}$ Tregs from ILN were analysed for the genes related to *Tbx21* through qRT-PCR. (x) MNCs infiltrating CNS were isolated and CD4 T cells were enumerated by a flowcytometer. (y) Spinal cord sections were stained for the presence of $FoxP3^{+}$ cells through fluorescence microscopy, H&E for inflammation and luxol fast blue for demyelination. (z) At day +45, serum was isolated and anti-MOG Abs were estimated by ELISA.The data were analysed using one-way ANOVA, with comparisons between groups by Tukey's test. The results expressed as mean ± SEM are representative of 3 independent experiments (n=3 mice/group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

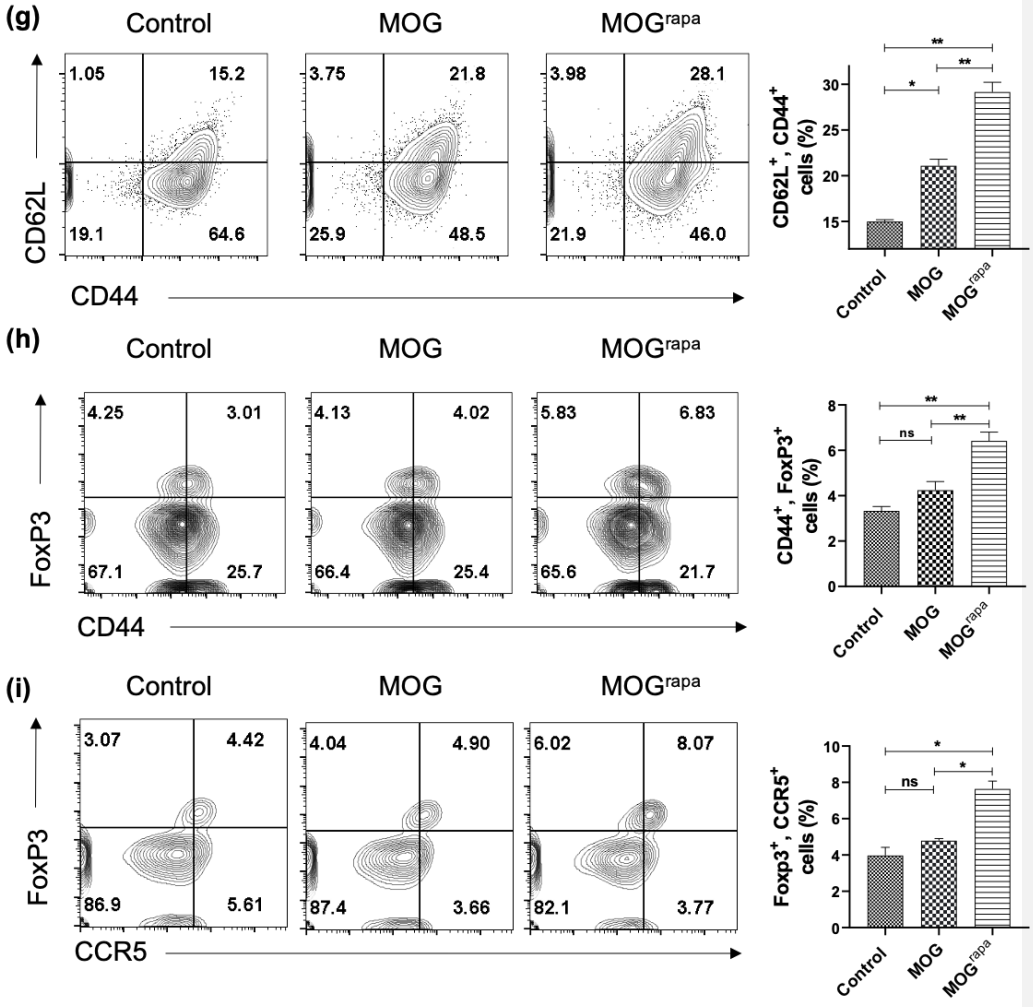
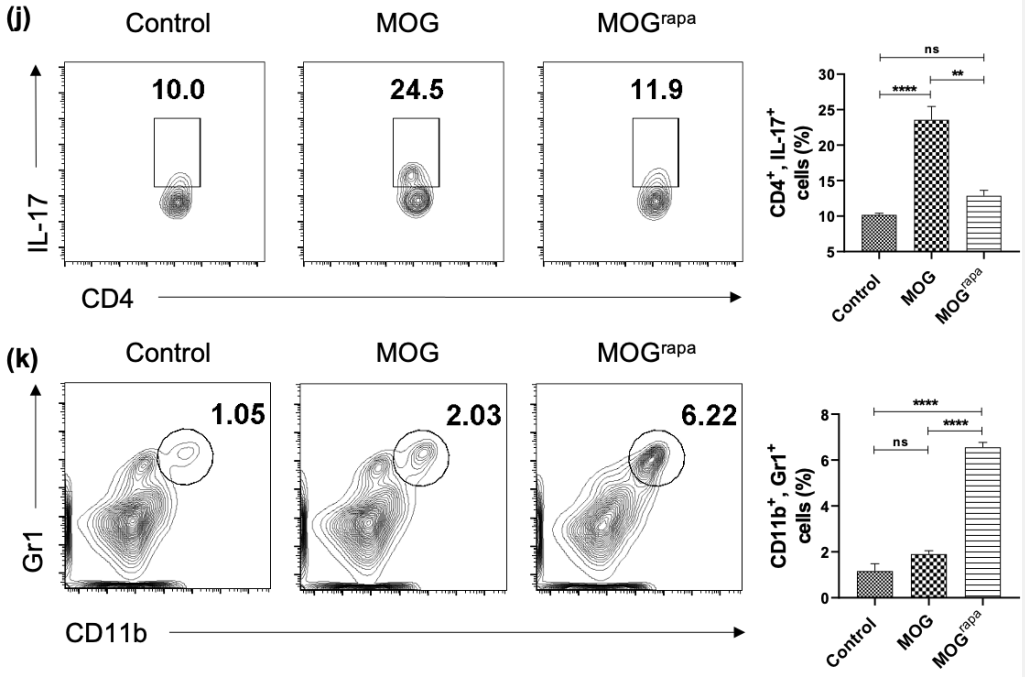
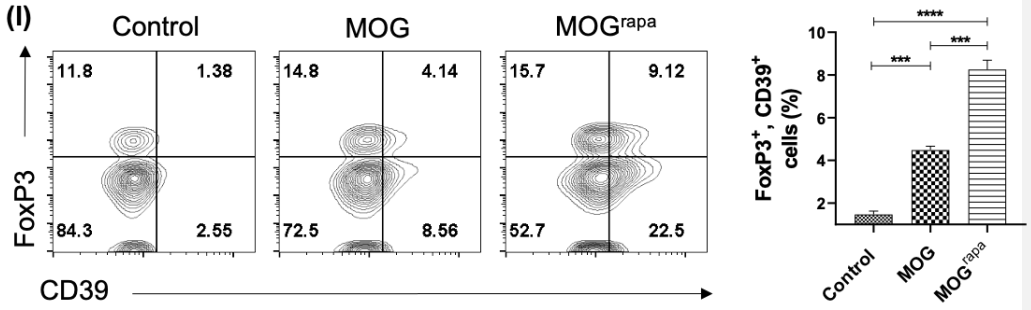


Fig. 3 Continued.

The MOG^{rapa} vaccinated group showed a significantly greater pool of Tregs in the blood (P<0.05), inguinal lymph nodes (ILN) (P<0.05), spleen (P<0.001), Peyer's patches (P<0.05) and spinal cord (P<0.0001) compared to the MOG-inoculated group (Figure 3e, f). CD4^{+}/FoxP3^{+} cells exhibited substantial expansion (P<0.01) in the central memory (CD62L^{hi}CD44^{hi}) phenotype. Additionally, they expressed higher levels of activation CD44 (P<0.01) and migratory markers CD44^{hi}/CCR5^{hi} (P<0.05) (Figure 3g-i). In contrast, the MOG^{rapa} group showed a considerable decline (P<0.01) in the CD4^{+}/IL-17^{+} Th17 cells (Figure 3 j). Intriguingly, a significantly higher (P<0.0001) population of MDSC in the MOG^{rapa} group was noticed (Figure 3k).




(I)
Control
MOG
MOGrapa
11.8
1.38
84.3
2.55
14.8
4.14
72.5
8.56
15.7
9.12
52.7
22.5
FoxP3
CD39
FoxP3+, CD39+ cells (%)
****
***
***
Control
MOG
MOGrapa

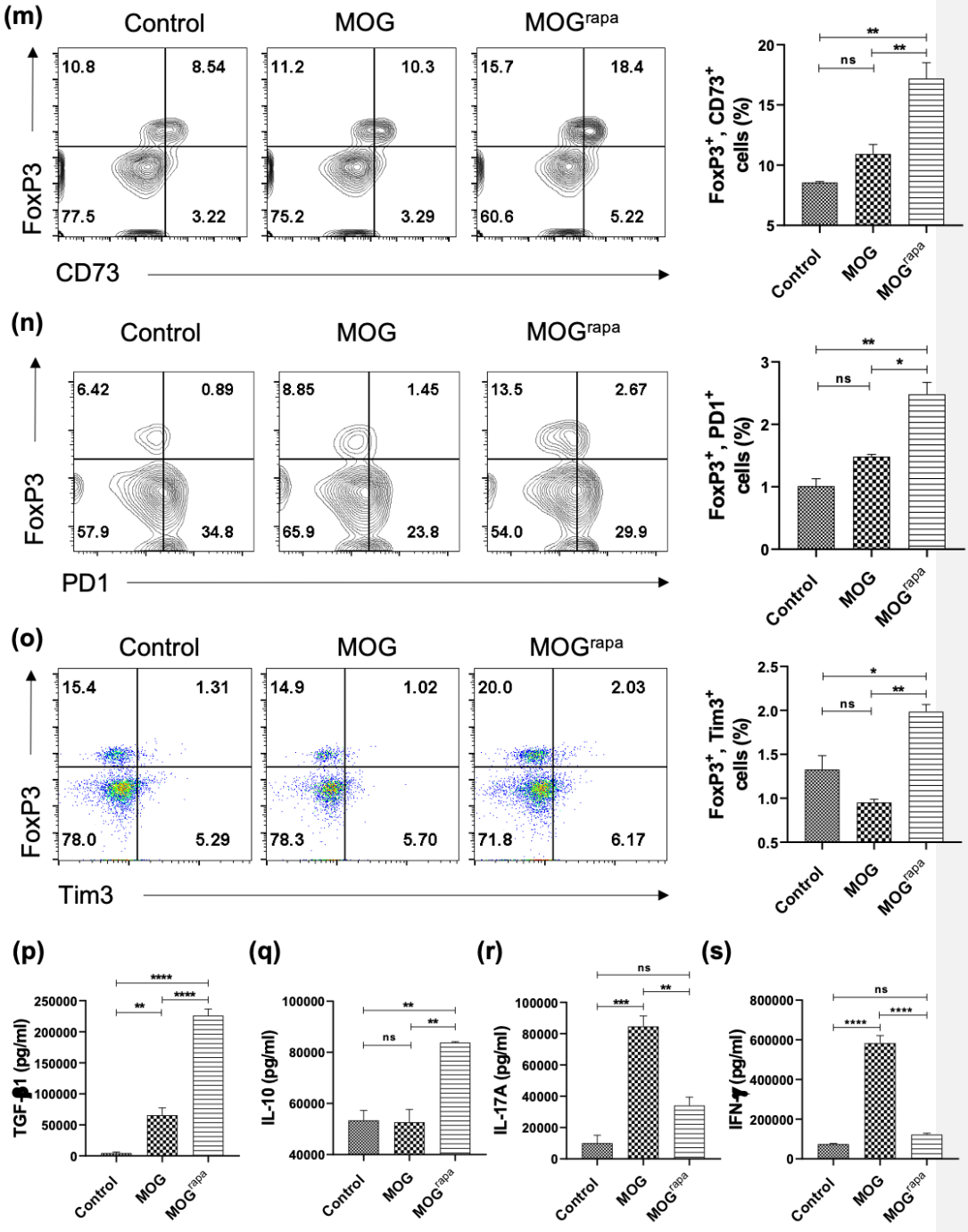


Fig. 3 Continued.

The central memory Tregs expressed significantly higher levels of inhibitory markers, viz CD39 (P<0.001), CD73 (P<0.01), PD1 (P<0.01), and Tim3 (P<0.01) (Figure 3l-o). Moreover, these cells secreted considerably higher levels of TGF-β1 (P<0.0001), IL-10 (P<0.01) and lower concentrations of IL-17A (P<0.01) and IFN-γ (P<0.0001) cytokine (Figure 3p-s).

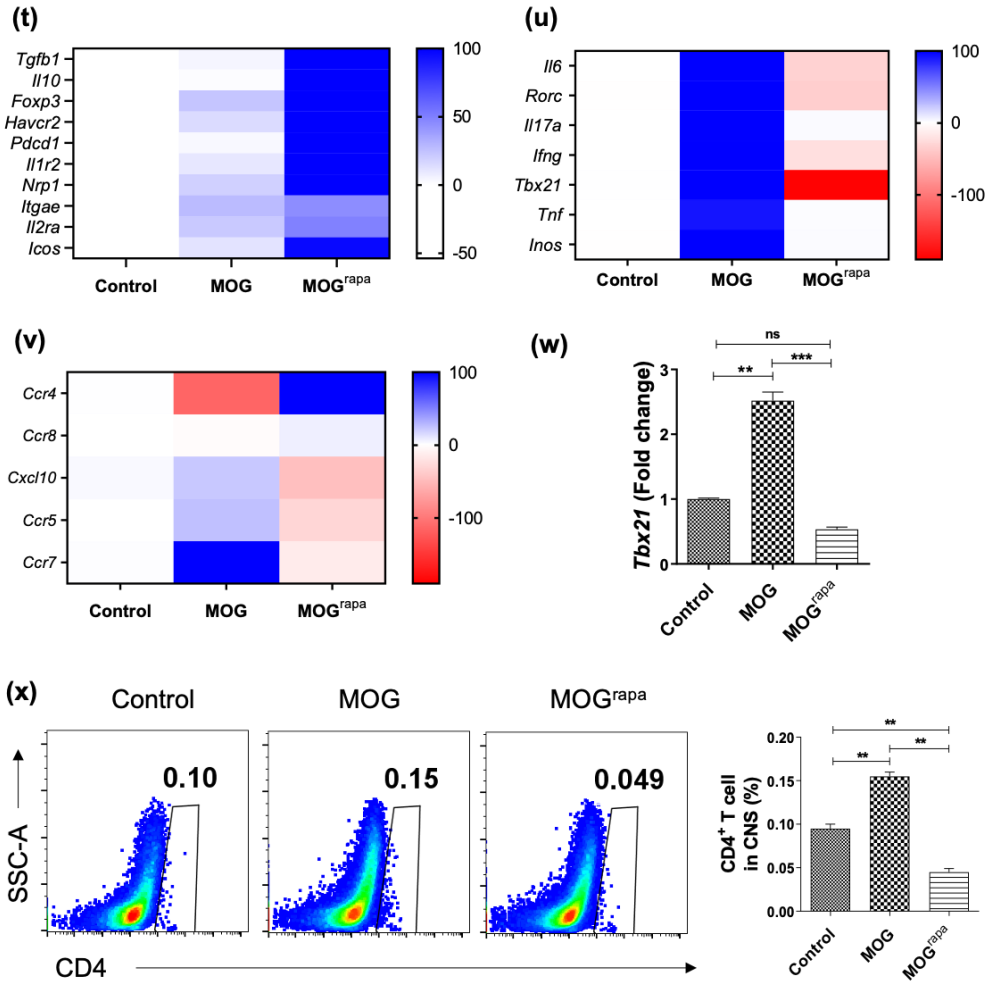
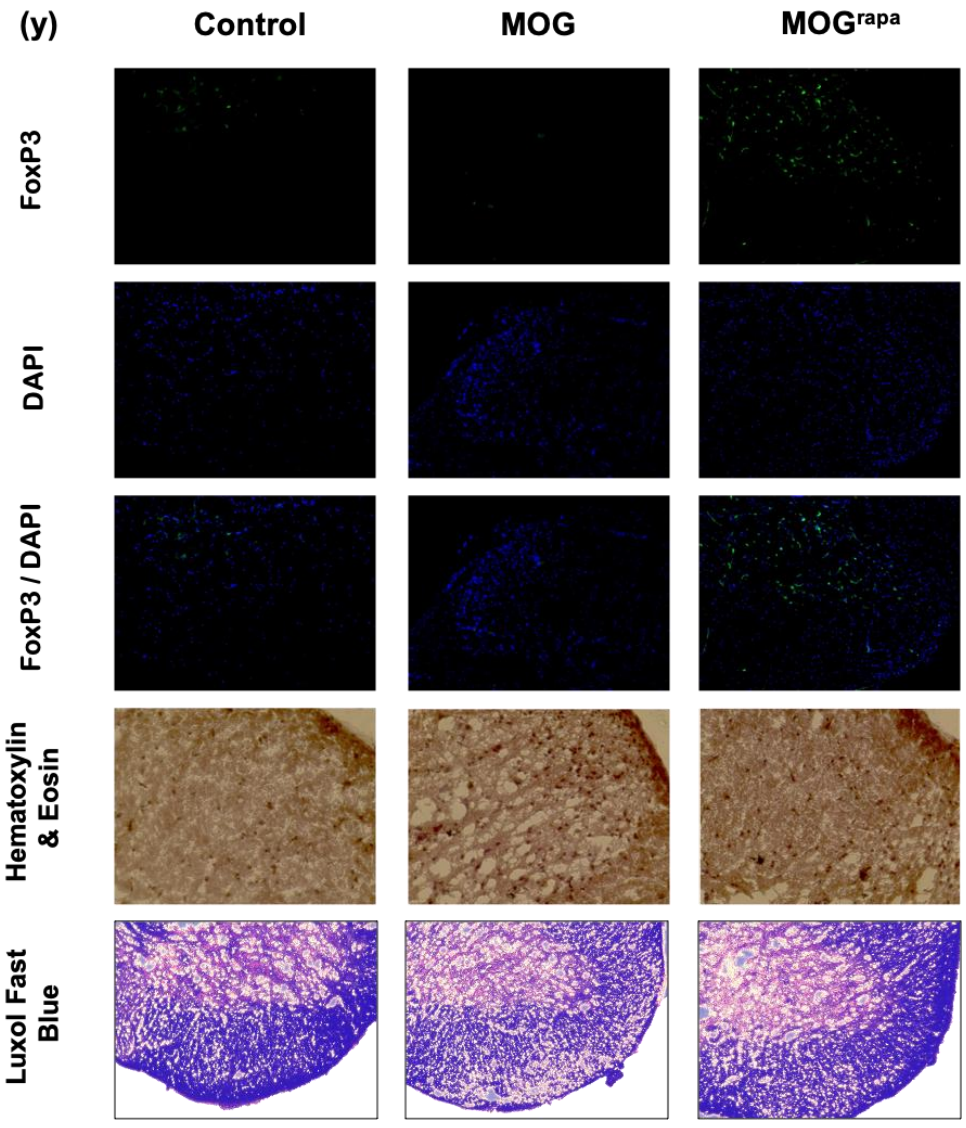


Fig. 3 Continued.

These results were further supported by examining the modulation of gene expression. Like flow cytometry and ELISA data, the manifestation of inhibitory genes *Tgf-β, Il-10, Foxp3, Havcr2, Pdcd1, Il1r1, Nrp1, Itgae, Il2ra, Icos* was highly upregulated in MOG^{rapa} inoculated

mice (Figure 3t). In contrast, a downregulation in the expression of pro-inflammatory genes *Il6, Rorc, Il17, Ifng, Tbx21, Tnf* and *Nos2* was observed (Figure 3u). Further, higher expression of markers responsible for the migration of the cells*Ccr4* (P<0.0001) and *Ccr8* (P<0.01) and a decrease in *Cxcl10* (P<0.01), *Ccr5* (P<0.0001), and *Ccr7* (P<0.0001) was noted in the MOG^{rapa}group (Figure 3 v). These results suggest that the cells obtained from the MOG^{rapa}group acquireda higher migratory potential and may preferably utilise *Ccr4* and *Ccr8* for their movements.

Comment [a1]:



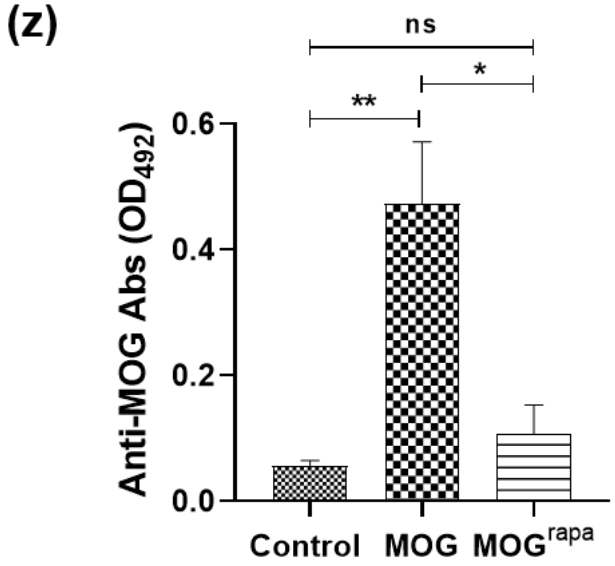
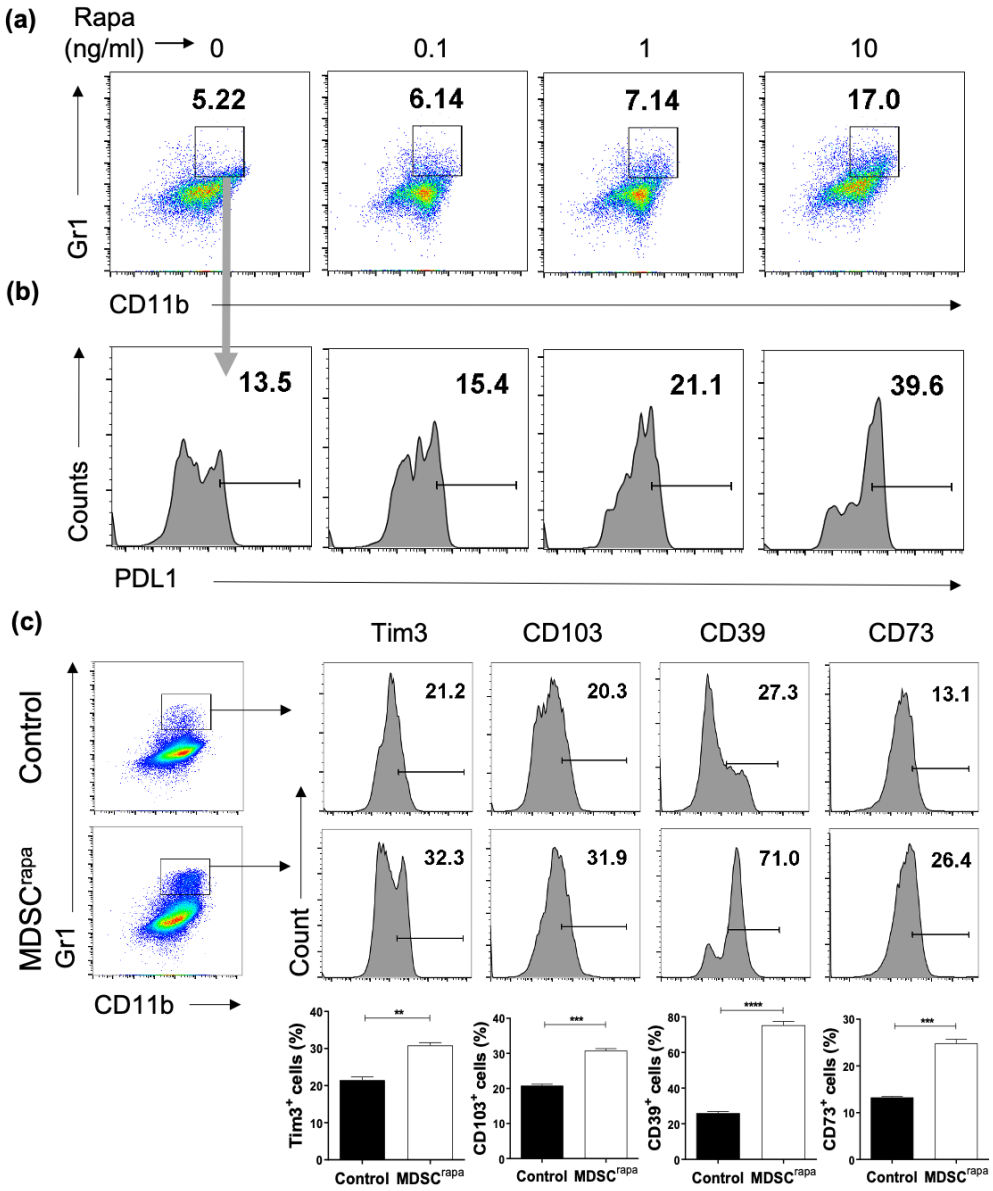


Fig. 3 Continued.

Importantly, we observed a significant decline in *Tbx21* ($P<0.001$) expression in the $CD4^{+}FoxP3^{+}$ Tregs sorted from the LN of MOG^{rapa}-inoculated mice, compared to MOG alone. Further, significant ($P<0.01$) plasticity was observed in these cells(Figure 3w). These results corroborated with the data shown in Figure 3u.The CNS infiltrating CD4 T cells were significantly less ($P<0.01$) in the MOG^{rapa} group (Figure 3x). Furthermore, the immunohistochemistry data revealed a greater accumulation of $FoxP3^{+}$ cells in the spinal cord of the MOG^{rapa}-treated group (Figure 3y, upper panel), which resulted in lesser pathology and demyelination (Figure 3y, lower panel) and no clinical symptoms (Figure 3c, d).

Additionally, the serum level of anti-MOG Abs was reduced ($P<0.05$) in the MOG^{rapa}vaccinated mice, as compared to the MOG group (Figure 3z). This also contributed toless damage to the myelin sheath. These results suggest that supplementing rapamycin with autoantigens may have future prospects in producing vaccines against autoimmune diseases.

1.4. In addition to Tregs, rapamycin also generated MDSC with a tolerogenic phenotype. We demonstrated that MOG^{rapa}protected mice from EAE primarily by promoting Treg production (*Figure 3*). Additionally, we observed a significantly higher population of MDSC, as indicated by the expression of Gr1 and CD11b markers (Figure 4a).



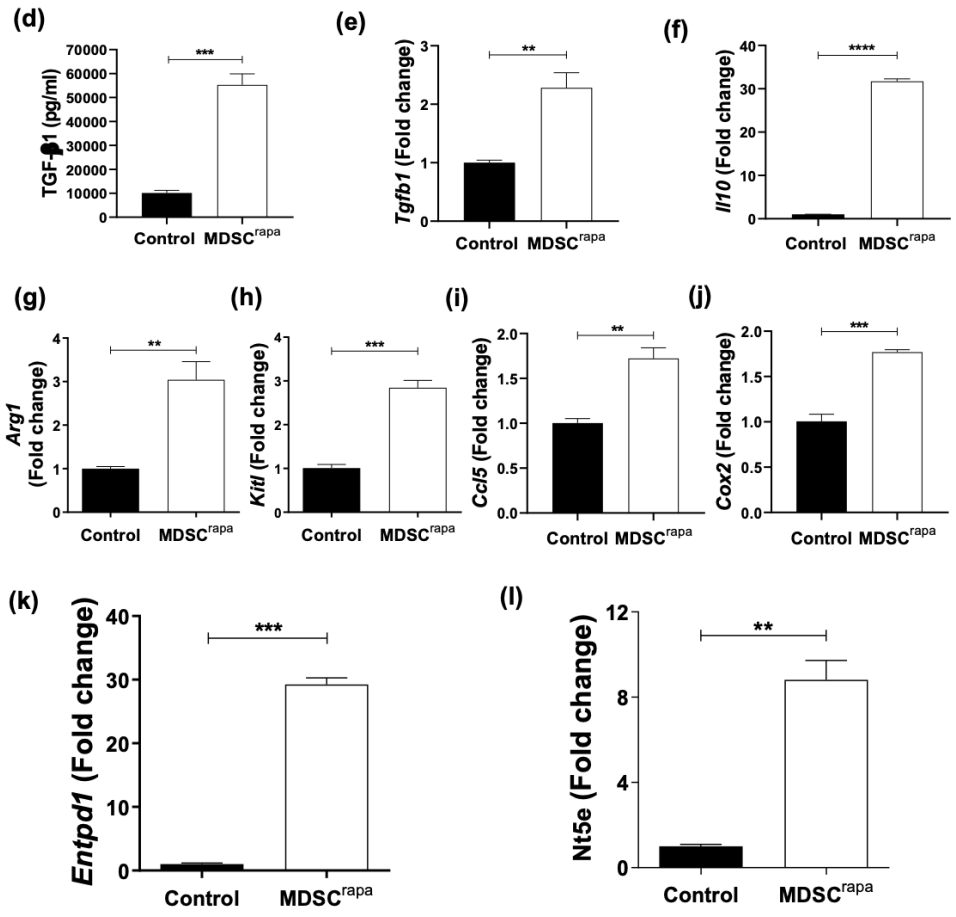


Figure 4. *MOG^{rapa} immunization elicited MDSC in addition to Tregs.* Bone marrow cells (2 × 10^6) were cultured with GM-CSF (2 ng/ml), and IL-4 (2 ng/ml) in the presence or absence of rapamycin (0-10 ng/ml) for 7 days. Fresh media containing GM-CSF (2 ng/ml) and IL-4 (2ng/ml) was replenished at day +3. At day +7, cells were scrapped and assessed for the presence of (a) $CD11b^+/Gr1^+$ MDSC.The cells were gated on $CD11b^+/Gr1^+$ MDSC to examine the molecules associated with the (b-c) immunosuppression PDL1; Tim3, CD103, CD39, CD73 by flowcytometer. The corresponding bar diagrams of flowcytometer data are also illustrated. (d) TGF-β was estimated in the culture SN of MDSC through ELISA. (e-l) The data of flowcytometer and ELISA was further substantiated by qRT-PCR by monitoring the gene responsible for suppression: (e) *Tgfb1; (f) Il10; (g) Arg1; (h) Kitl; (i) Ccl5; (j) Cox;* and(k, l) ectoenzymes (*Entpd1, Nt5e*). (m) MDSC were used to monitor the migration and

adhesion markers CCR5, CXCR5, and LFA-1 by flowcytometer. The data were analysed using one-way ANOVA, with comparisons between groups by Tukey's test. The results expressed as mean ± SEM are representative of 3 independent experiments (n=3 mice/group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The differentiation of DC from BMCs can be influenced by their exposure to various immunomodulators (*Siddiqui KF et al., 2014*). We generated MDSC by exposing BMCs to rapamycin, IL-4, and GM-CSF ($MDSC^{rapa}$). The rapamycin showed a dose-dependent increase in the production of $CD11b^{+}Gr1^{+}$ $MDSC^{rapa}$ (Figure 4a). $MDSC^{rapa}$ displayed upregulated levels of PDL1, Tim3, CD103, CD39 and CD73 (Figure 4b, c).MDSCsexerted suppression by expressing inhibitory molecules such as PDL-1, TGF-β1, IL-10, NO, arginase1, SCF, CCL5, COX2, and CD103 (*Yang Y et al., 2020*). $MDSC^{rapa}$ showed significantly higher secretion of TGF-β1 (Figure 4d). Further, we observed that $MDSC^{rapa}$ exhibited considerably greater expression of *Tgfb1 (P<0.01), Il10 (P<0.0001), Arg1 (P<0.01), Kitl (P<0.001), Ccl5 (P<0.01), Cox2 (P<0.001), Nt5e (P<0.01), Entpd1* (P<0.001) compared to control MDSC(Figure 4e-l). CD39 and CD73 induce T cell anergy and regulate adenosine metabolism, which is crucial for T cell priming (*Groth C et al., 2019*). Upregulated level of CD39 and CD73 expression was observed by $MDSC^{rapa}$ (Figure 4c, k, l).

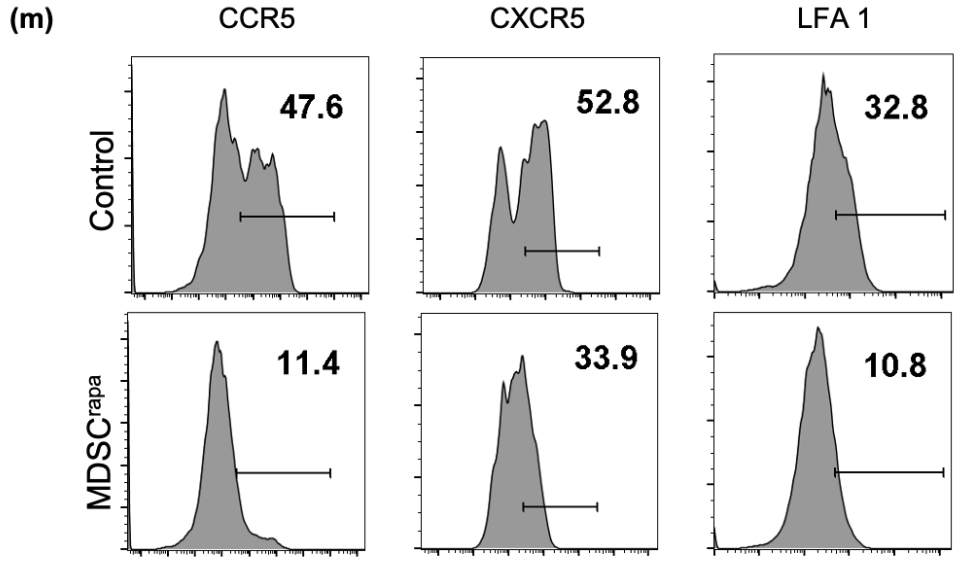


Fig. 4 Continued.

Furthermore, MDSC^{rapa} displayed downregulated expression of chemokines CCR5 and CXCR5, as well as the adhesion molecule LFA-1 (Figure 4m). MDSC^{rapa} showed a higher manifestationof immunosuppressive molecules and lower migration markers compared to control MDSC. This suggests the reduced migratory ability of MDSC^{rapa} from the site of inflammation to secondary lymphoid organs, thus resulting in lesser T cell priming against autoantigens.

1.5. The induction of the generation of protective Tregs by MDSC^{rapa}. Optimal activation of T cells requires the presentation of antigen complexed with MHC and the expression of costimulatory molecules (*Sharpe AH, 2009*). MDSC^{rapa} revealed significantly lower expression of CD11c, MHC II, CD80, CD86, and CD40, indicating their diminished capacity to activate autoreactive T cells (Figure 5a).

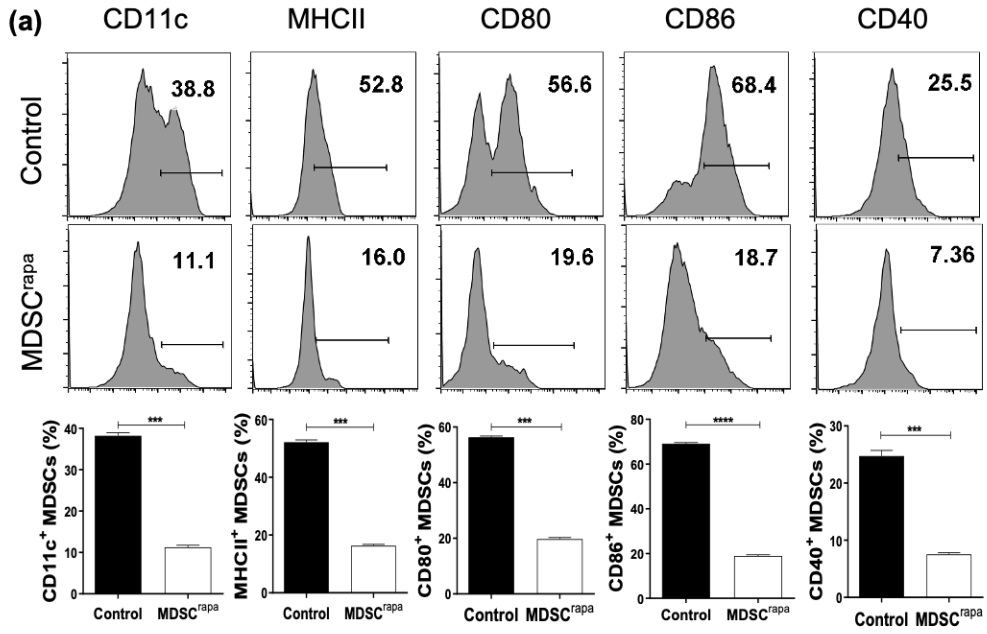
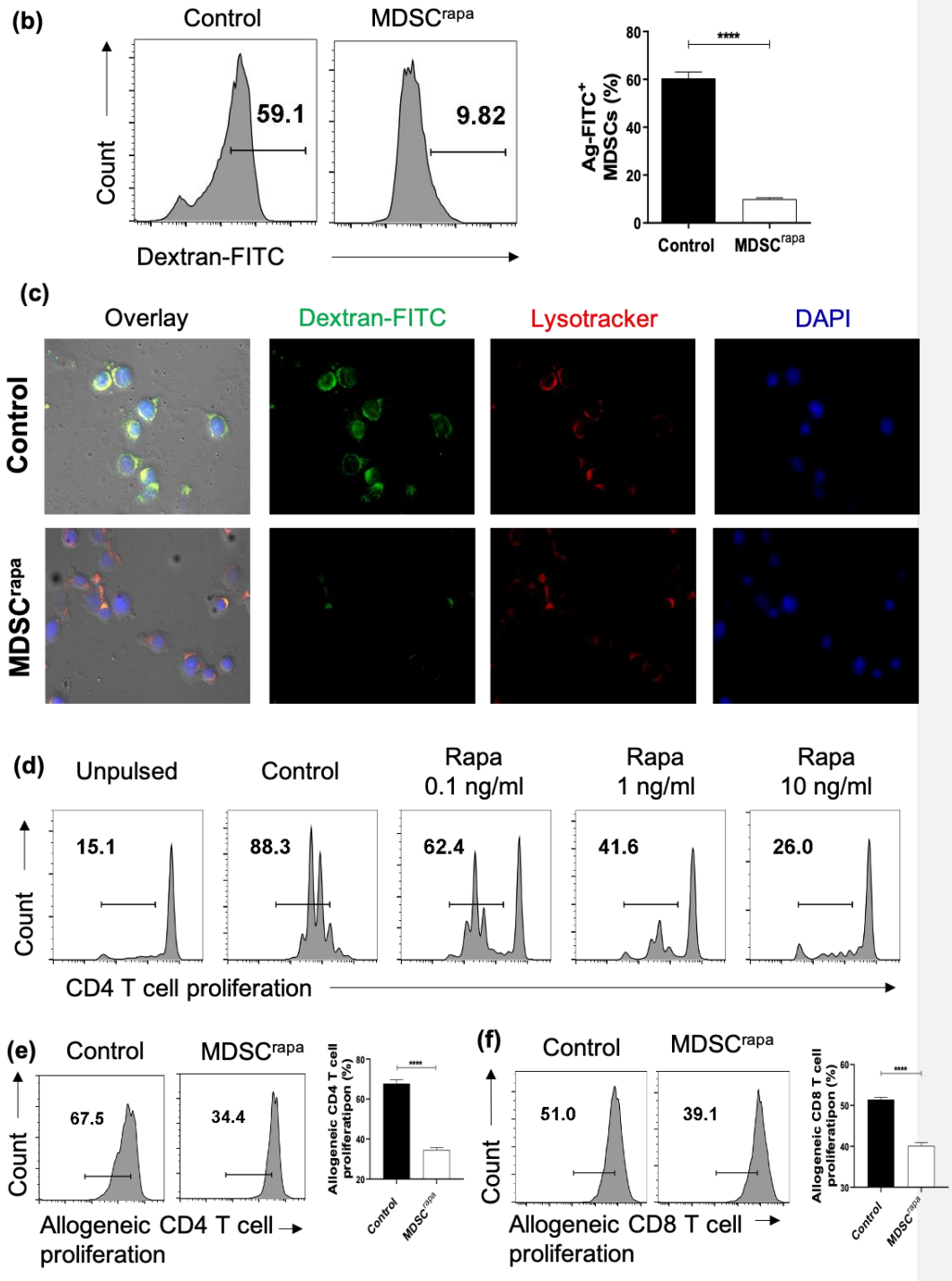


***Figure 5**. MDSC^{rapa} induced the generation of Tregs and suppressed the proliferation of effector CD4 T cells and Th17 cells.* The Gr1^{+}/CD11b^{+} MDSC generated in the presence or absence of rapamycin as mentioned in Figure 4 were stained for the expression of (a) CD11c, MHCII and co-stimulatory molecules CD80, CD86, and CD40 by flowcytometer. (b, c) MDSC were assessed for antigen uptake by (b) flowcytometer; (c) fluorescence microscopy.

(d) $MDSC^{rapa}$ (C57BL/6) were generated in the presence of different doses of rapamycin (0-10 ng/ml). The $OVA_{323-339}$ (50 μg/ml) pulsed $MDSC^{rapa}$ were co-cultured with naïve CD4 T cells (1:5 ratio) isolated from OT-II mice. (e, f) $MDSC^{rapa}$ of C57BL/6 mice were co-cultured with allogenic (e) naïve CD4 T cells (f) naïve CD8 T cells of BALB/c mice. (g, h) $MDSC^{rapa}$ were co-cultured with anti-CD3/CD28 Abs stimulated naïve (g) CD4 T cells (h) CD8 T cells. (i-k) Naïve CD4 T cells isolated from the spleen of mice immunized with MOG + rapamycin (MOG^{rapa}) controls (MOG, rapamycin, PBS) were stimulated with anti-CD3/anti-CD28 Abs to evaluate the (i) proliferation of CD4 T cells; (j) $CD4^{+}FoxP3^{+}$ (k) $CD4^{+}IL\text{-}17^{+}$ T cells. The cultures were set for 48h to 72h and proliferation was assessed by CFSE-dye dilution assay by flowcytometer. Data are expressed as histograms and their representative bar diagrams. (l-o) The proinflammatory cytokine (l) IL-6; (m) TNF-α; (n) IL-12; (o) IFN-γ were estimated in the culture supernatants by ELISA. (p) $MDSC^{rapa}$ treated with 2-NBDG (10 μM) in the glucose-free medium for 30 min were analyzed for glucose uptake by flowcytometer. (q) FasL on MDSC was assessed through a flowcytometer. Naïve CD4 T cells stimulated with anti-CD3/CD28 Abs were co-cultured with $MDSC^{rapa}$ (5:1 ratio) in the presence of rapamycin and control MDSC without rapamycin for 48 h and (r) apoptosis; (s) TCRαβ; (t) TCRγδ expression was measured by flowcytometer. The data were analysed using one-way ANOVA, with comparisons between groups by Tukey's test. The results expressed as mean ± SEM are representative of 3 independent experiments (n=3 mice/group). *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

We also observed reduced uptake of antigen by $MDSC^{rapa}$, as demonstrated by flow cytometry and fluorescence microscopy (Figure 5b, c). $OVA_{323-339}$-pulsed $MDSC^{rapa}$ inhibited the proliferation of naïve CD4 T cells obtained from OT-II mice (Figure 5d). These findings were further validated as $MDSC^{rapa}$ retarded the expansion of allogeneic and anti-CD3/CD28 Ab-stimulated syngeneic CD4 T cells and CD8 T cells (Figure 5e-h).


(b)
Control
MDSCrapa
59.1
9.82
Count
Dextran-FITC
Ag-FITC+ MDSCs (%)
****
Control
MDSCrapa
(c)
Overlay
Dextran-FITC
Lysotracker
DAPI
Control
MDSCrapa
(d)
Unpulsed
Control
Rapa 0.1 ng/ml
Rapa 1 ng/ml
Rapa 10 ng/ml
15.1
88.3
62.4
41.6
26.0
Count
CD4 T cell proliferation
(e)
Control
MDSCrapa
67.5
34.4
Count
Allogeneic CD4 T cell proliferation
Allogeneic CD4 T cell proliferatipon (%)
(f)
Control
MDSCrapa
51.0
39.1
Count
Allogeneic CD8 T cell proliferation
Allogeneic CD8 T cell proliferatipon (%)

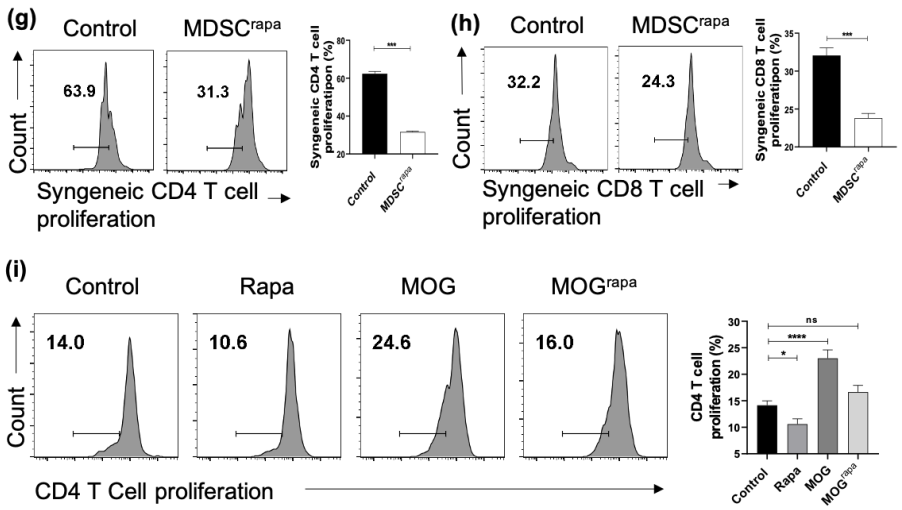


Fig. 5 Continued.

Additionally, MDSC^{rapa} retarded the proliferation (P<0.001) of MOG-specific CD4 T cells isolated from the mice injected with MOG^{rapa} (Figure 5i). MDSC^{rapa} cultured with MOG-reactive CD4 T cells showed a significantly higher frequency of Tregs (P<0.0001) anda reduction in Th17 cells (P<0.0001) (Figure 5j, k). Furthermore, the culture supernatants of MOG-specific CD4 T cells cultured with MDSC^{rapa} showed decreased yield of proinflammatory cytokine IL-6 (P<0.001), TNF-α (P<0.0001), IL-12 (P<0.0001), and IFN-γ (P<0.001) (Figure 5l-o).

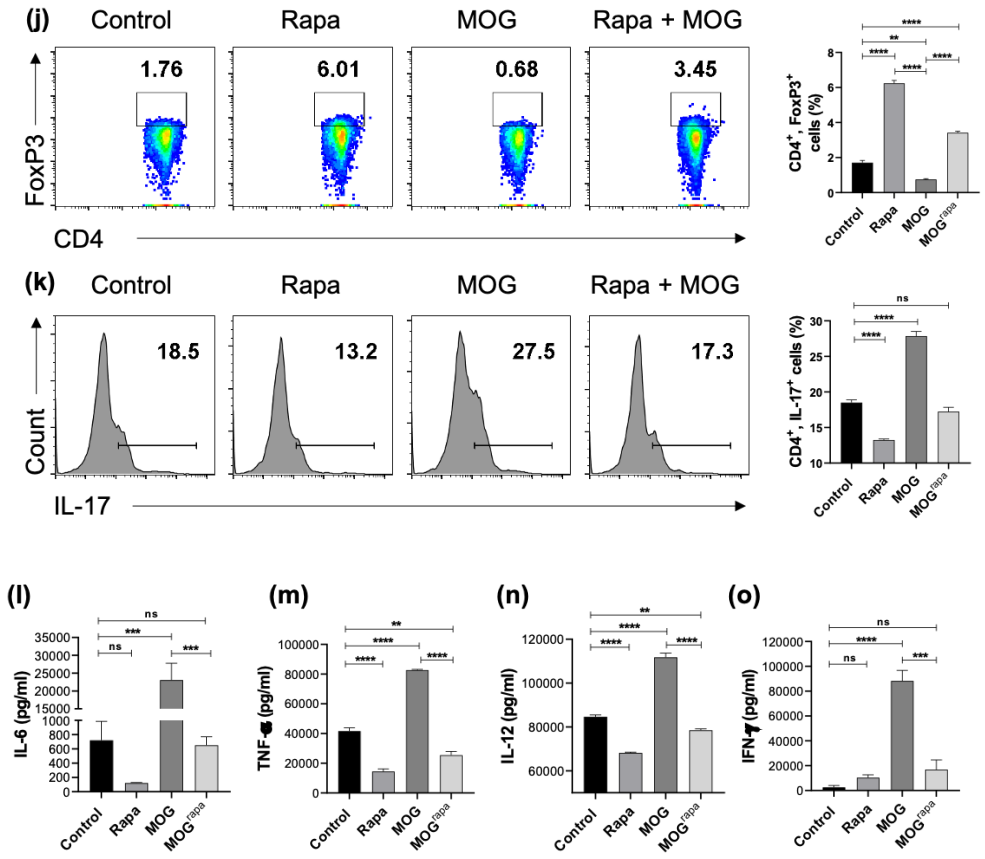
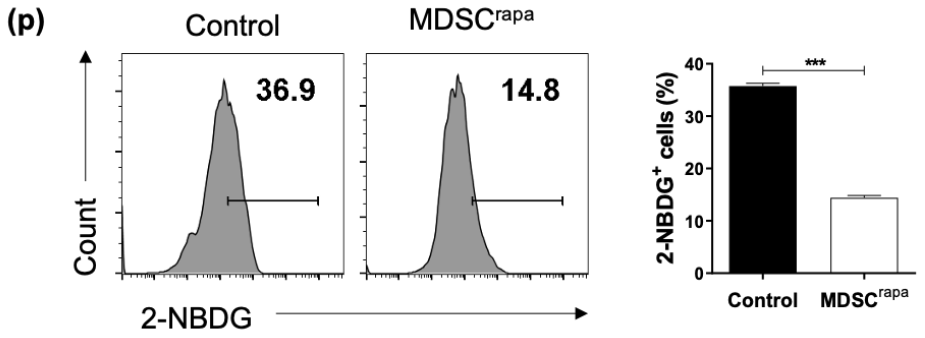


Fig. 5 Continued.

In addition to their tolerogenic phenotype, MDSC^{rapa}also suppressed the activation of T cells. This may be due to the metabolic inertness of MDSC^{rapa}, as evidenced by a substantial decrease ($P<0.0001$) in the uptake of 2-NBDG (Figure 5p). Furthermore, it may be due to the lower ($P<0.0001$) expression of LFA-1, hence not providing enough adhesion time for the optimal T cell activation throughcognate interaction. Furthermore, MDSC^{rapa} showed a higher level of FasL compared to MDSC (Figure 5q). FasL is responsible for immune regulation, tolerance maintenance, and elimination of autoreactive T cells (*Yamada A et al., 2017*). Engagement of FasL with the Fas receptor induces apoptosis in the interacting cell (*Groth C et al., 2019*). Increased uptake of annexin V by CD4 T cells indicates the induction of apoptosis by MDSC^{rapa} (Figure 5r). Production of arginase1 by MDSC depletes extracellular L-arginine leading to downregulation of T cell receptors (*Rodriguez PC et al., 2004*).

MDSC^{rapa} exhibited higher expression of arginase1 and downregulation of TCRαβ and TCRγδ expression by co-cultured T cells (Figure 5s-t).



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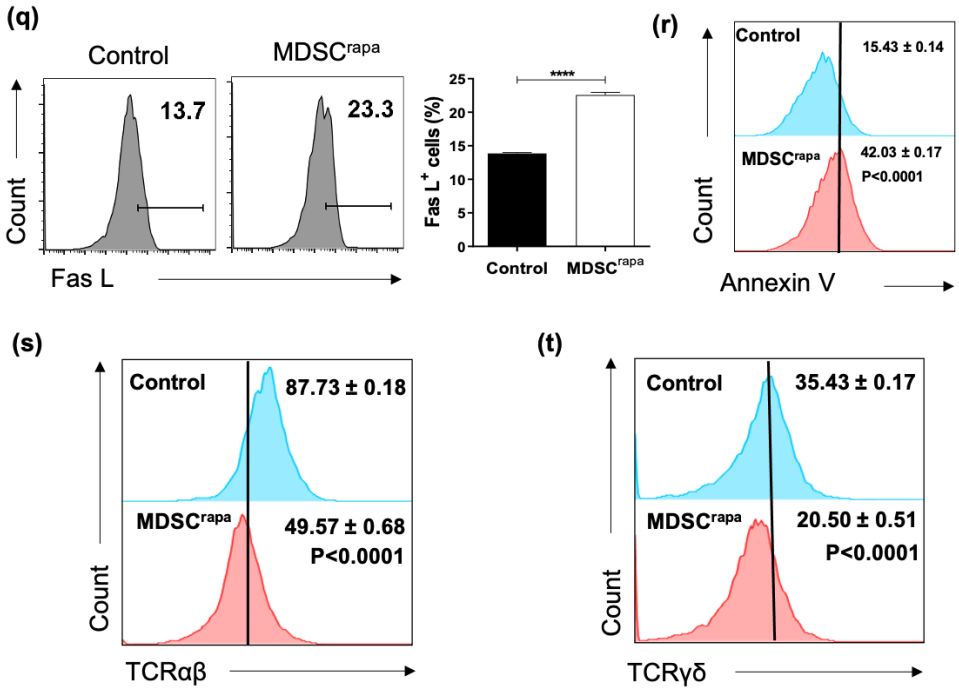
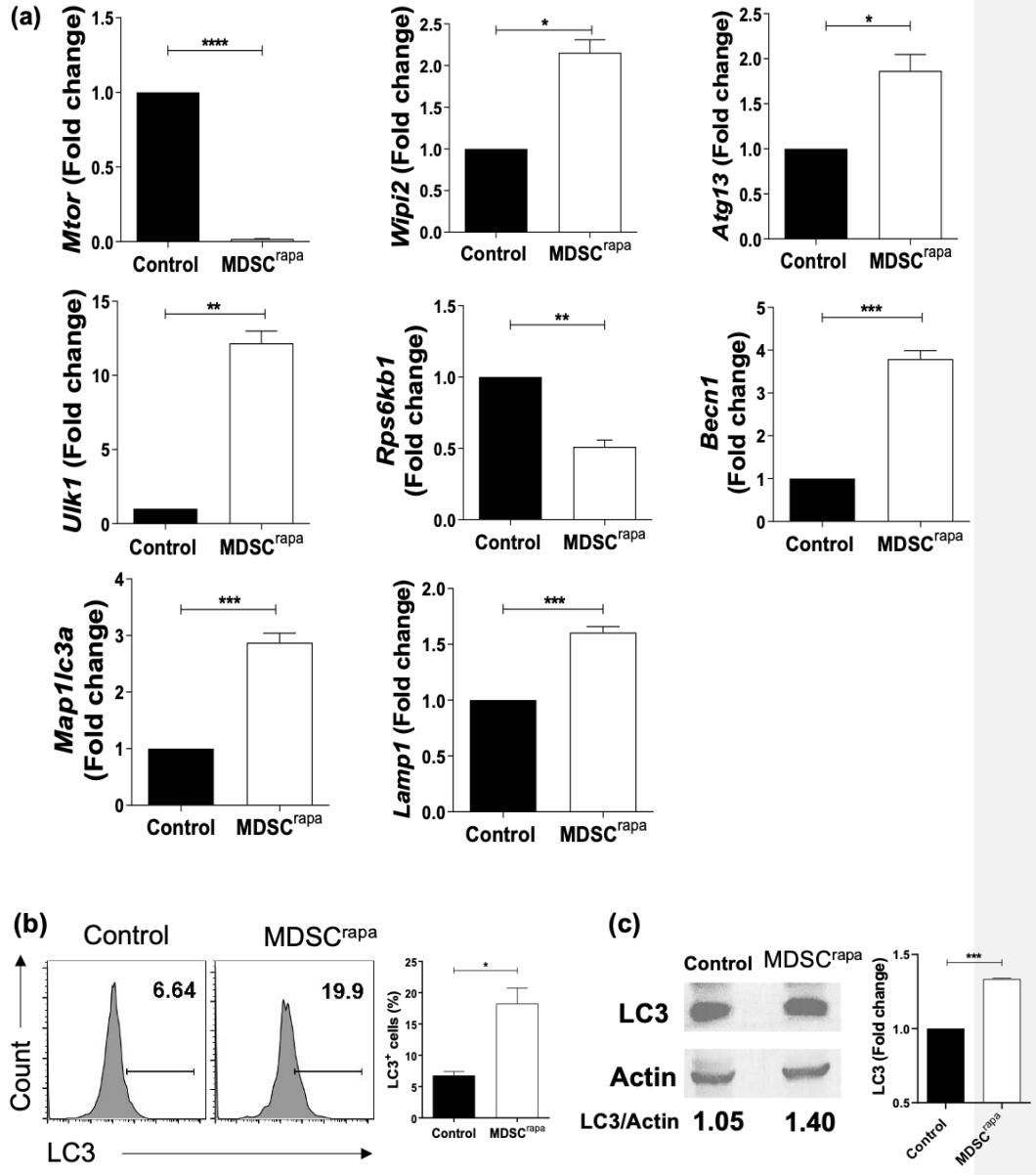


Fig. 5 Continued.

Therefore, based on these results, it can be inferred that the protection induced by MOG^{rapa} vaccination against EAE is attributed not only to the induction of Tregs but also due to the induction of the activation of tolerogenic MDSC.

1.6. The mechanism of protection against EAE by rapamycin-induced Tregs and MDSC is through autophagy. To elucidate this mechanism, we investigated the modulation of the expression of key molecules associated with autophagy (*Kim YC, Guan KL, 2015*). Rapamycin was found to induce autophagy in MDSCs ($MDSC^{rapa}$), as evidenced by significant modulation of autophagy genes such as *Mtor (P<0.0001), Wipi2 (P<0.05), Atg13 (P<0.05), Ulk1 (P<0.01), Rps6kb1 (P<0.01), Becn1 (P=0.0001), Map1lc3a (P<0.001),* and *Lamp1 (P<0.001)* compared to control DC without rapamycin (Figure 6a). The upregulation of LC3 was further confirmed by flow cytometry (P<0.05), Western blotting (P<0.001) and fluorescence microscopy (Figure 6b-d), while LAMP1 expression was validated through fluorescence microscopy, as well (Figure 6d, e). Additionally, we confirmed the involvement of autophagy by silencing the MAP1LC3A (P<0.01) gene with siRNA (Figure 6f).


(a)
Mtor (Fold change)
Wipi2 (Fold change)
Atg13 (Fold change)
Ulk1 (Fold change)
Rps6kb1 (Fold change)
Becn1 (Fold change)
Map1lc3a (Fold change)
Lamp1 (Fold change)
Control
MDSCrapa
(b)
Control
MDSCrapa
6.64
19.9
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LC3
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Actin
LC3/Actin 1.05 1.40
LC3 (Fold change)

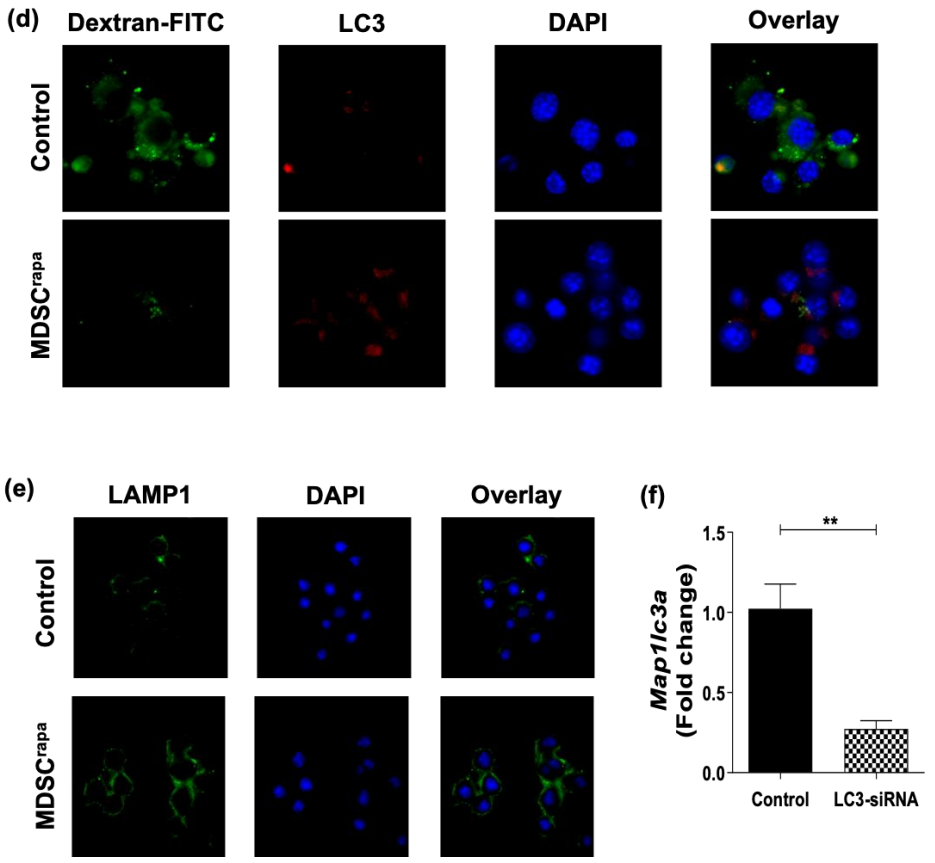

Figure 6. *The mechanism underlying the protection against EAE by rapamycin-induced Tregs and MDSC is through autophagy.* Anti-CD3/CD28 Abs stimulated naïve CD4 T cells were cultured with Tregs polarizing conditions with or without rapamycin and monitored for the expression of (a) an array of genes associated with autophagy *Mtor, Wipi2, Atg13, Ulk1, Rps6kb1, Becn1, Map1lc3a, Lamp1 by qRT-PCR*; (b-d) LC3 protein through flow cytometry, Western blotting and fluorescence microscopy;(e) LAMP1 by microscopy. The specificity of autophagy markers was confirmed by impairing the gene expression of (f) LC3 by knocking down by siRNA; (g-j) by using inhibitor wortmannin for (g) *Foxp3*; (h) *Mtor*; (i) *Map1lc3a*; (j) *Ambra1* by qRT-PCR. (k, l) *Map1lc3a* was knockdown by siRNA and inhibited through wortmannin and expression of (k) FoxP3; (l) IL-17 was demonstrated by flowcytometer. The SN were collected from the cultures for the estimation of (m) TGF-β1; (n) IL-10 by ELISA. (o) Bone marrow cells treated with wortmannin were cultured as described in Figure 4 in the

presence/absence of rapamycin and assessed for the presence of $CD11b^{+}/Gr1^{+}$ cells by flowcytometry. (p) Anti-CD3/CD28 Ab stimulated naïve CD4 T cells were cultured with siRNA knockdown MAP1LC3A-$MDSC^{rapa}$ ($LC3^{KD}$-$MDSC^{rapa}$) or$MDSC^{rapa}$ treated with wortmannin and evaluated for $CD4^{+}FoxP3^{+}$ Tregs by flowcytometer. (q) Anti-CD3/CD28 Ab stimulated $LC3^{KD}$- CD4 T cells were cultured with $LC3^{KD}$-$MDSC^{rapa}$ in the presence of rapamycin and the percent of $CD4^{+}/FoxP3^{+}$ Tregs was measured by flowcytometer. (r, s) Splenocytes from MOG^{rapa} immunized animals were isolated and the expression of (r) *Mtor, Atg13, Ulk1, Fip200, Becn1, Atg12, Map1lc3a, Lamp1* genes by qRT-PCR; (s) LC3, Atg12, beclin1 and lamp1 by western blotting was evaluated. (t-w) $CD4^{+}FoxP3^{+}$ cells were sorted from MOG^{rapa} immunized animals and assessed for the expression of (t)*Mtor*, (u) *Ulk1*, (v) *Fip200,* (w) *Map1lc3a* by qRT-PCR. The data were analysed using one-way ANOVA, with comparisons between groups by Tukey's test. The results expressed as mean ± SEM are representative of 3 independent experiments (n=3 mice/group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Different subsets of T helper cells undergo polarization through the influence of signature cytokine secreted by dendritic cells (DC) during their interaction with naïve T cells. Notably, DC treated with rapamycin have the capacity to amplify and activate regulatory T cells (Tregs)(*Fischer R et al., 2009*). Furthermore, autophagy plays a pivotal role in maintaining the lineage stability and survival fitness of Tregs (*Wei J et al., 2016*). Consequently, our investigation sought to elucidate whether rapamycin induces Treg polarization via autophagy modulation. Remarkably, we observed that rapamycin-induced augmentation (P<0.0001) of Foxp3 expression in Tregs was suppressed (P<0.0001) when exposed to wortmannin. However, this suppression was restored upon the reintroduction of rapamycin (Figure 6g).

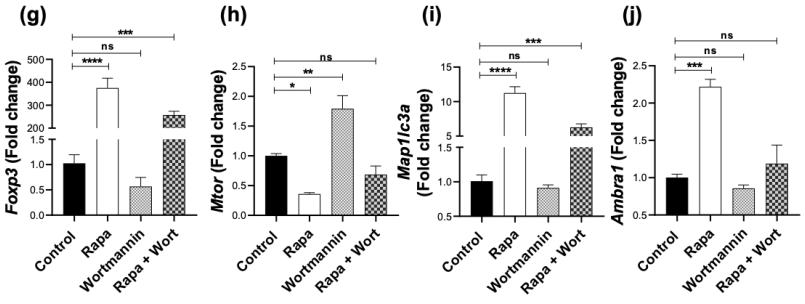

Fig. 6 Continued.

Importantly, rapamycin inhibited the expression of *mTOR*, while wortmannin restored it in Tregs (Figure 6h). Wortmannin inhibits autophagy by suppressing the PI3K pathway, which is upstream of mTOR. mTOR is the mechanistic target of rapamycin (*Blommaart EF et al., 1997*). Thereforethe effect of wortmannin may be due to PI3K inhibition. Furthermore, rapamycin-induced significant upregulation of *Map1lc3a* (P<0.0001) and *Ambra1* (P<0.0001) in Tregs, which was inhibited by the addition of wortmannin to the cultures (Figure 6i, j). The qRT-PCR data (Figure 6g-j) were further confirmed by flow cytometric analysis using siRNA to silence *Map1lc3a* in Tregs (Figure 6k).

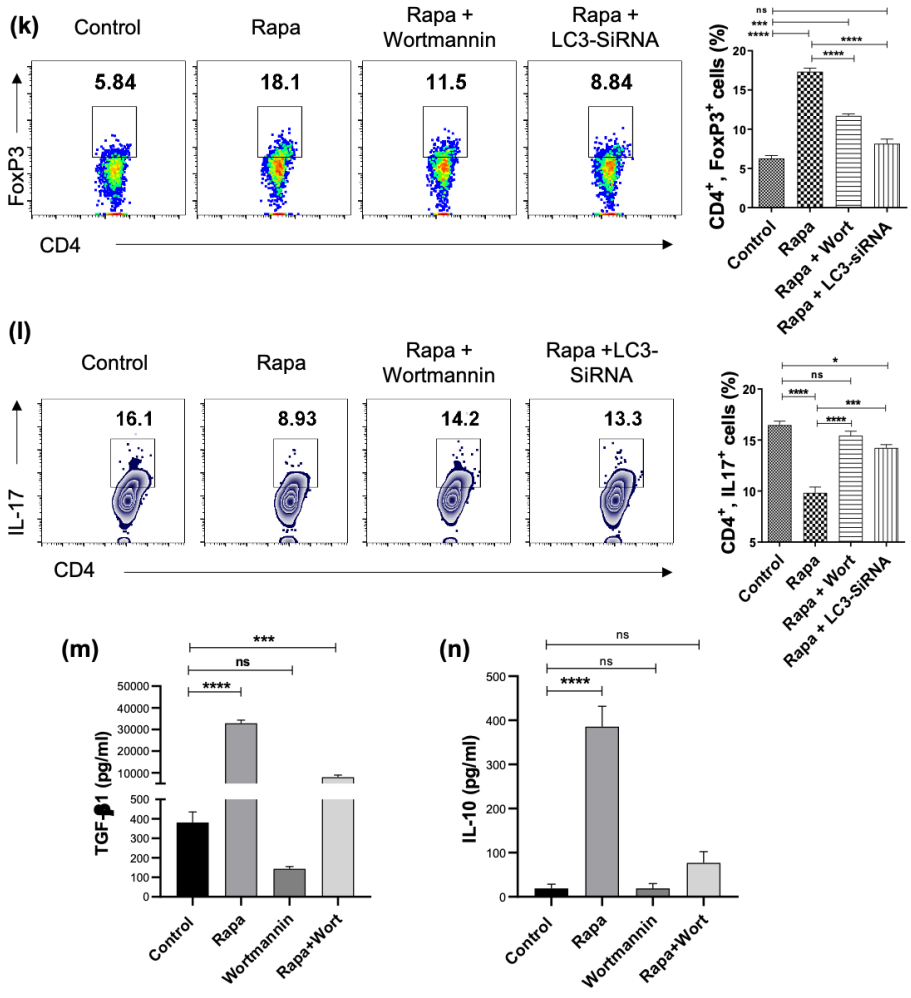

Fig. 6 Continued.

The significant increase (P<0.0001) in FoxP3 expression induced by rapamycin was hindered by wortmannin (P<0.0001), a finding further supported by *Map1lc3a* knockdown using siRNA (P<0.0001) (Figure 6k). In contrast to the outcomes observed in Tregs, our research revealed that the inhibition of Th17 cells by rapamycin was amplified by wortmannin (P<0.0001) or by knocking down *Map1lc3a* with siRNA (Figure 6l).Additionally, the authenticity of our findings was accentuated by the reduced release of TGF-β and IL-10 by Tregs on the addition of wortmannin in the cultures (Figure 6m, n). Consequently, the inhibition of *Map1lc3*a using LC3α/β siRNA corroborated the major role of rapamycin-induced autophagy in Treg generation. Tregs are pivotal for maintaining cellular tolerance and suppressing Th17-mediated autoimmune diseases (*Othy S et al., 2020*). Therefore, we can deduce from our results that the protective effects conferred by rapamycin-induced Tregs against EAE are mediated through autophagy.

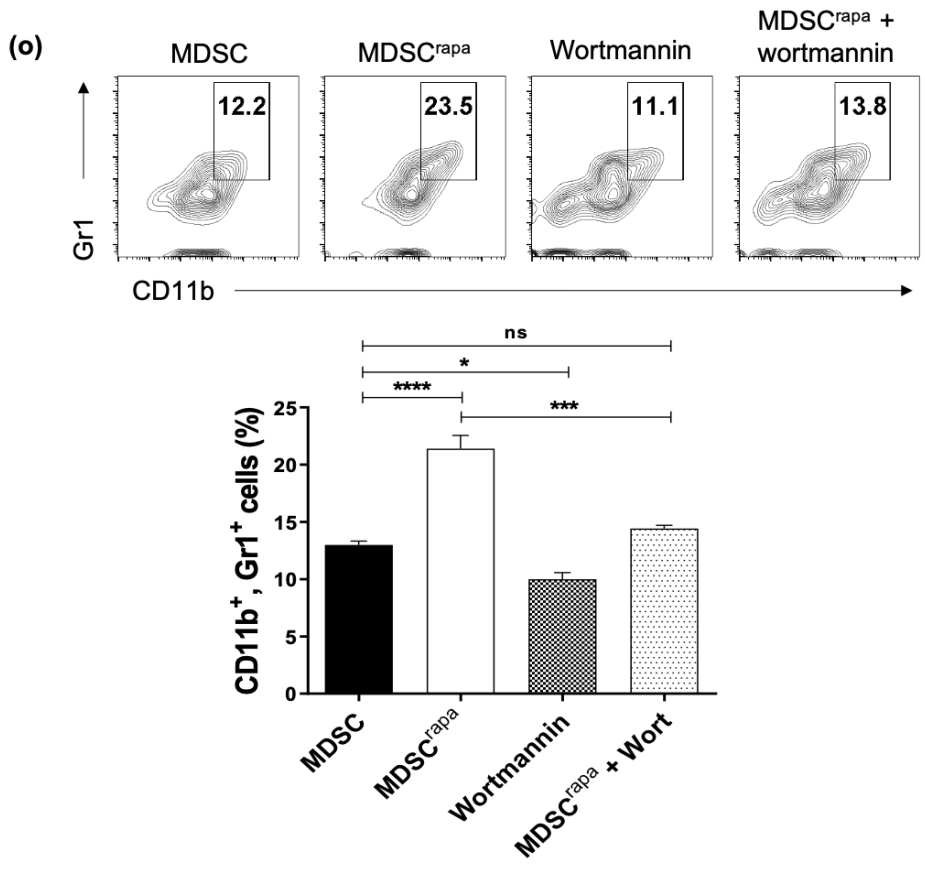

Fig. 6 Continued.

Next, we examined the role of autophagy in influencing the production and function of MDSC. Interestingly, there was a significant ($P<0.0001$) reduction in the frequency of rapamycin-induced MDSC upon the addition of wortmannin to the cultures, suggesting the role of rapamycin-induced autophagy in MDSC generation (Figure 6o). Furthermore, we investigated the involvement of autophagy on the $MDSC^{rapa}$ in polarizing naïve CD4 T cells to Tregs. The addition of wortmannin to the cultures resulted in a significant decline ($P<0.001$) in the pool of $CD4^{+}FoxP3^{+}$ Tregs. These results were further established by *Map1lc3a* knockdown using siRNA (Figure 6p).

(p)

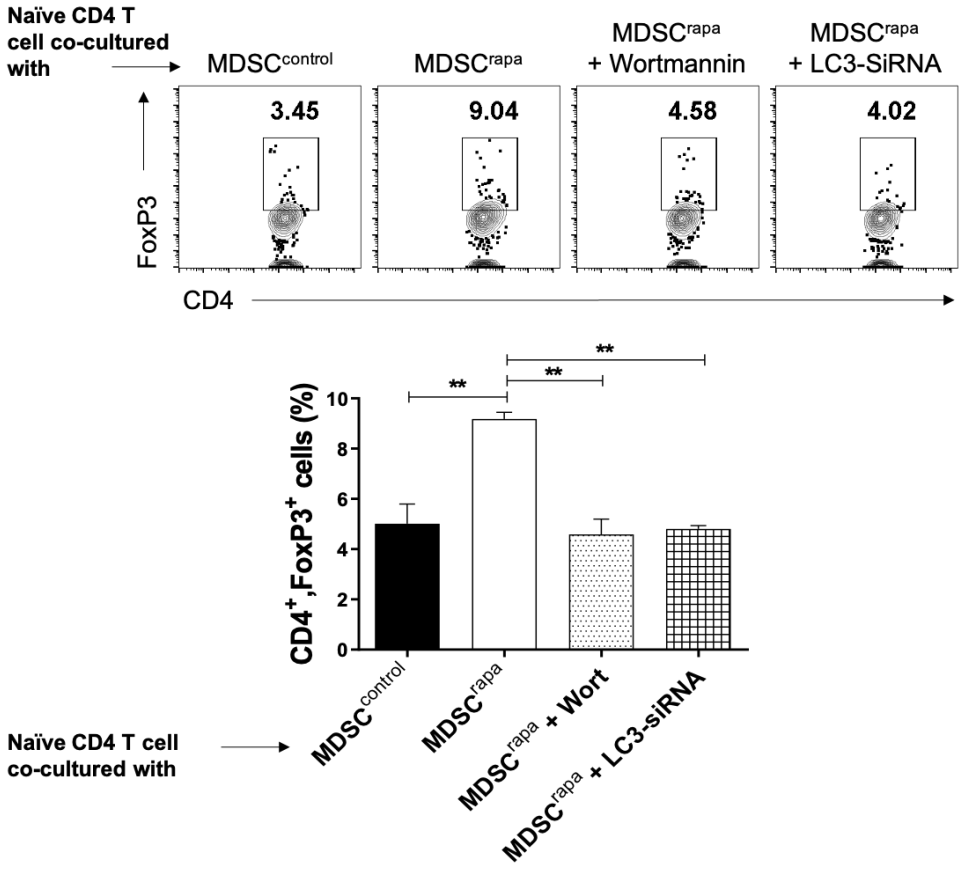

Fig. 6 Continued.

To confirm the involvement of autophagy in Tregs lineage stability, we cultured i) siRNA *Map1lc3a* knockdown (KD) MDSC generated in the presence of rapamycin ($LC3^{KD}$-$MDSC^{rapa}$) with CD4 T cells; ii) $MDSC^{rapa}$and $LC3^{KD}$- CD4 T cells; iii) $LC3^{KD}$-$MDSC^{rapa}$and $LC3^{KD}$- CD4 T cells. As compared to control cultures $MDSC^{rapa}$and CD4 T cells. All three cultures mentioned above (i-iii) showed a significant ($P<0.0001$) decline in the percentage of $CD4^{+}FoxP3^{+}$ T cells (Figure 6q).

We further confirmed the *in vitro* data by examining the expression of autophagy in the splenocytes of mice vaccinated with MOG^{rapa}. Remarkably, the autophagy genes, *Atg13, Ulk1, Fip200, Becn1, Atg12, Map1lc3a, and Lamp1* were highly upregulated, and *Mtor* was downregulated compared to the groups inoculated with MOG (Figure 6r). The qRT-PCR data were validated by observing decreased expression of autophagy proteins LC3, Atg12, beclin1, and LAMP1 through Western blotting (Figure 6s).

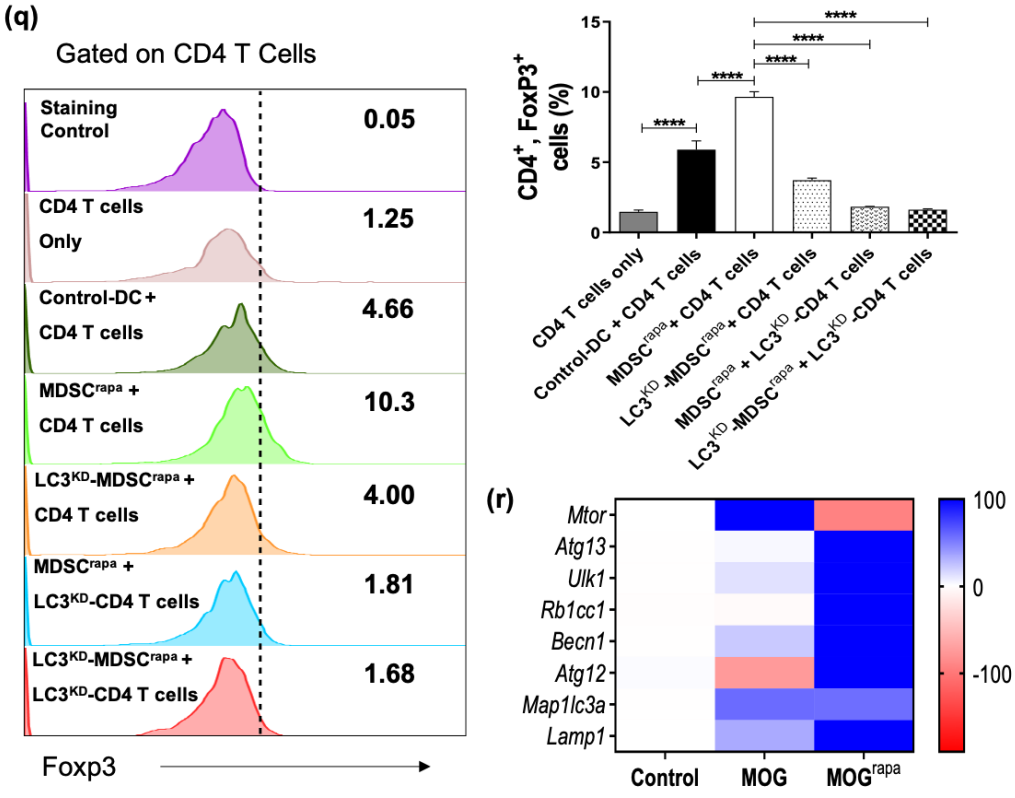

Fig. 6 Continued.

To further confirm the involvement of autophagy in lineage stability during EAE, we measured the expression levels of autophagy genes in $CD4^{+}/FoxP3^{+}$ sorted Tregs from MOG^{rapa} vaccinated animals and control groups inoculated with MOG or PBS (placebo). We observed significant modulation in the autophagy genes *Ulk1, Fip200, Map1lc3a* and *Mtor* in mice immunized with MOG^{rapa}(Figure 6t-w).

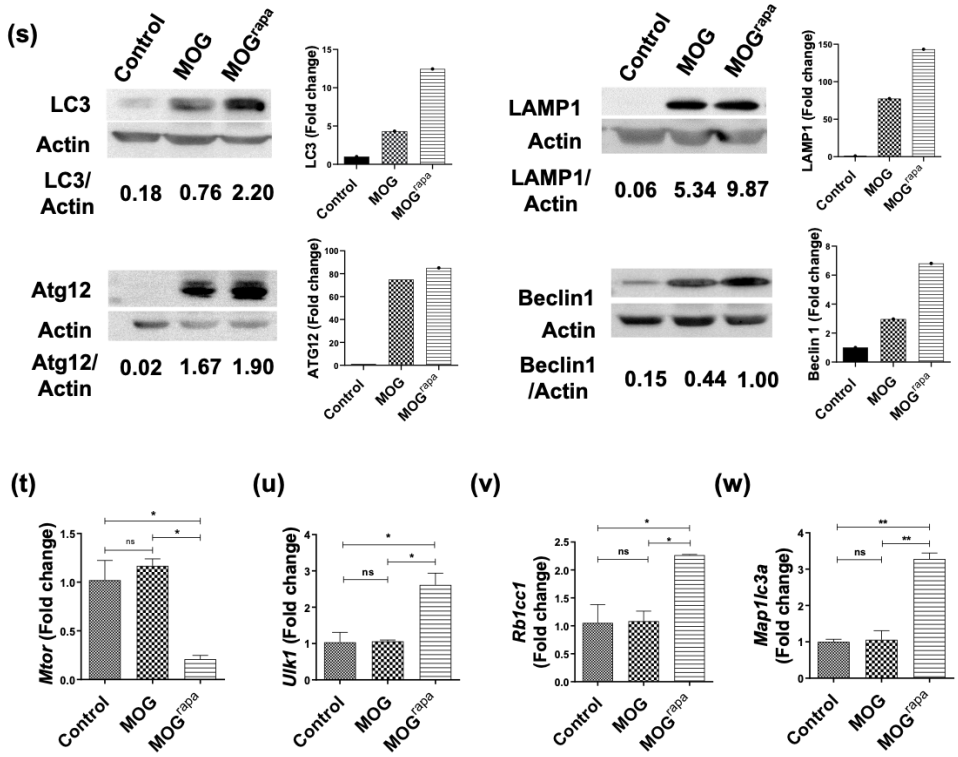

Fig. 6 Continued.

Overall, the results suggested that the mechanism involved in protection against EAE by rapamycin-induced MOG-reactive Tregs and MDSC is mediated through the induction of autophagy.

CHAPTER 5
DISCUSSION

Multiple sclerosis (MS) is characterized by the immune system fallaciously attacking the myelin sheath, which covers nerve fibres (*Bartsch U, 1996*). MOG is a protein that maintains the integrity of the myelin sheath. Autoreactive MOG-specific Th17 cells initiate an attack against MOG, leading to the destruction of the myelin sheath and the progression of the disease (*Babaloo Z et al., 2015*). In addition, Th17 cells secrete pro-inflammatory cytokine, recruit other immune cells to the site of inflammation, and contribute to the disruption of the blood-brain barrier, allowing immune cells to enter the central nervous system (*Balasa R et al., 2020*).Thus, averting the development of autoantigen-reactive Th17 cells to Tregs could be a potential remedial measure for MS.

Tregs play a vital role in maintaining immune homeostasis by suppressing misguided attacks on the body's tissues by autoreactive-Th17 cells (*Fletcher JM et al., 2009*). Tregs can inhibit the differentiation and function of Th17 cells by secreting anti-inflammatory cytokine such as IL-10 and TGF-β, thereby reducing their pro-inflammatory activity (*Chaudhry A et al., 2011*). Rapamycin is an immunosuppressive drug used to manage autoimmune diseases (*Zhang Z et al., 2012*). It promotes the differentiation of naive CD4 T cells into Tregs by enhancing the expression of FoxP3, a master regulator of Tregs' suppressive function (*Battaglia M et al., 2005*). Thus, in the current study, we aimed to skew pathogenic autoantigen-reactive Th17 cells to protective autoantigen-specific Tregs and restrict the development of EAE. The role of rapamycin is established in generating Tregs (*Qu Y et al., 2007*). To test this hypothesis, we immunized mice with MOG emulsifiedwith rapamycin (MOG^{rapa}), and noted the following interesting observations: 1] emergence of protective MOG-specific Tregs, suppression of MOG-reactive Th17 cells; 2] Tregs exerted inhibitory effect and expressed elevated levels of immunosuppressive molecules; 3] generation of long-lasting MOG-specific-memory Tregs; 4] origination of myeloid-derived suppressor cells (MDSC); 5] inhibition in the formation of anti-MOG Abs; 6] protection of mice from EAE upon subsequent exposure to a lethal dose of MOG; 7] mechanism of EAE protection was attributed to the induction of rapamycin-elicited Tregs and MDSC through autophagy. The valuable findings of this study may provide a ray of hope for the development of a possible vaccine against MS and a permanent cure for many autoimmune diseases. Currently, there is no permanent cure for other autoimmune diseases (*Bebo Jr BF et al., 2022*).

CHAPTER 5
DISCUSSION

Remarkably, mice vaccinated with MOG^{rapa} generated primarily MDSC and MOG-specific Tregs, resulting in protection against subsequent challenges with MOG and thereby EAE. Earlier studies have shown that Tregs generated in the presence of immunosuppressive molecules (iTregs) are highly stable, and their function is not influenced by the presence of either Th1 cells or Th17 cells (*Gurram RK et al., 2014*). Similarly, we observed a decline in the percentage of effector CD4 T cells and CD8 T cells, as well as anti-MOG Abs. Importantly, rapamycin increased the number of MOG-specific Tregs and simultaneously decreased the percentage of MOG-reactive Th17 cells. All these factors support the idea of protection against MS (*Fletcher JM et al., 2010; Marta CB et al., 2005*). Mice vaccinated with MOG^{rapa} exhibited no clinical symptoms of EAE and showed rapid recovery, thus confirming the functionality of rapamycin-induced MOG-specific Tregs. Furthermore, mice immunized with MOG alone displayed a predominance of MOG-reactive Th17 cells and developed chronic EAE disease. Additionally, we confirmed the accumulation of $FoxP3^{+}$ cells and less disease pathology/demyelination in the CNS of the group vaccinated with MOG^{rapa} through immunohistochemistry and histopathology. Additionally, when naive CD4 T cells were exposed to rapamycin, they predominantly differentiated into Tregs. This was demonstrated through experiments involving culturing peptide-pulsed-DC in the presence of rapamycin with antigen-specific naive CD4 T cells obtained from OT-II mice. Intriguingly, we observed an increased number of $FoxP3^{+}$ Tregs and a decreased frequency of Th17 cells. Similarly, we observed *in vitro* an increased number of MOG-specific $FoxP3^{+}$ Tregs and a decreased frequency of MOG-reactive Th17 cells. Importantly, these Tregs expressed a variety of immunosuppressive molecules such as TGF-β, IL-10, Tim3, PD1, CD121b, neuropilin1, CD103, CD25, and ICOS, and exhibited potent suppressive functions.

Another notable observation pertained to the heightened presence of MDSC in mice that received MOG^{rapa}. Additionally, a compelling outcome emerged when bone marrow cells (BMCs) were pre-exposed to rapamycin under conditions conducive to DC differentiation, *viz* IL-4 and GM-CSF. This exposure predominantly led to the generation of MDSC rather than DC.These MDSC were characterized by the expression of immunosuppressive markers while concurrently downregulating the expression of MHCI, MHCII, CD80, CD86, and CD40. This unique profile, characterized by antigen-presenting cells with reduced MHC and costimulatory molecule levels, translated into diminished T cell activation capabilities (*Steinman RM et al.,*

2003). Consequently, it fostered tolerance in autoreactive T cells and a concomitant attenuation of immune responses (*Steinman RM et al., 2003*). Furthermore, these MDSC played a pivotal role in promoting the formation of Tregs and established an immunosuppressive microenvironment. This microenvironment further served to dampen autoreactivity and enhance overall immunosuppression(*Maldonado RA and von Andrian UH, 2010*).

Memory T cell generation is a crucial aspect of a successful vaccine. Our study also revealed the generation of MOG-specific memory Tregs. Importantly, the current strategy was judiciously designed to induce and expand MOG-specific memory Tregs that specifically suppress immune responses against self-antigens involved in autoimmune diseases. Memory Tregs have the ability of long-term protection and persistence in the body and retain their regulatory function upon reactivation. By inducing auto-antigen-specific memory Tregs in this study, a sustained and controlled suppression of autoimmunity can be achieved (*Rosenblum MD et al., 2016*). Furthermore, this can help prevent disease relapses or recurrences by maintaining immune tolerance over an extended period. Rapamycin has been shown to have an impact on the generation and maintenance of memory T cells (*Araki K et al., 2010*).

The mechanism behind the protection against EAE by Tregs and MDSC was elucidated through autophagy. Autophagy plays an important role in maintaining immune tolerance by influencing the generation and function of Tregs (*Wei J et al., 2016*). Therefore, our proposed vaccine against MS aims to induce autophagy, which can potentially activate Tregs, leading to immune regulation and suppression of the autoimmune response. This will prevent harmful immune attacks on self-tissues/cells. Autophagy selectively targets and removes cellular components such as damaged/misfolded proteins, pathogens, and immune complexes (*Levine B et al., 2011*). By inducing autophagy, our vaccine facilitates the clearance of self-antigens that triggered or augmentedautoimmunity. Additionally, we observed reduced expression of pro-inflammatory molecules by CD4 T cells and MDSC exposed to MOG-rapamycin. Autophagy promotes tolerance in CD4 T cells towards self-antigens, dampening the autoimmune response while preserving normal immune functions (*Lünemann JD and Münz C, 2009*). Moreover, a vaccine promoting autophagy may shift the immune response towards an anti-inflammatory state, reducing excessive inflammation associated with autoimmune diseases. Therefore, it will mitigate tissue damage and fostertissue repair (*Figure 1*).

CHAPTER 5
DISCUSSION

In conclusion, this study demonstrates a novel approach leveraging the unique properties of rapamycin to develop a vaccine that generates auto-antigen-specific Tregs and MDSC with protective efficacy against EAE. These results propose an important tool to prevent and cure the occurrence of multiple sclerosis in humans. In the future, this strategy may open avenues for developing vaccines to avert different autoimmune diseases of known autoantigen by inducing the development of MDSCs and auto-antigen-specific Tregs.

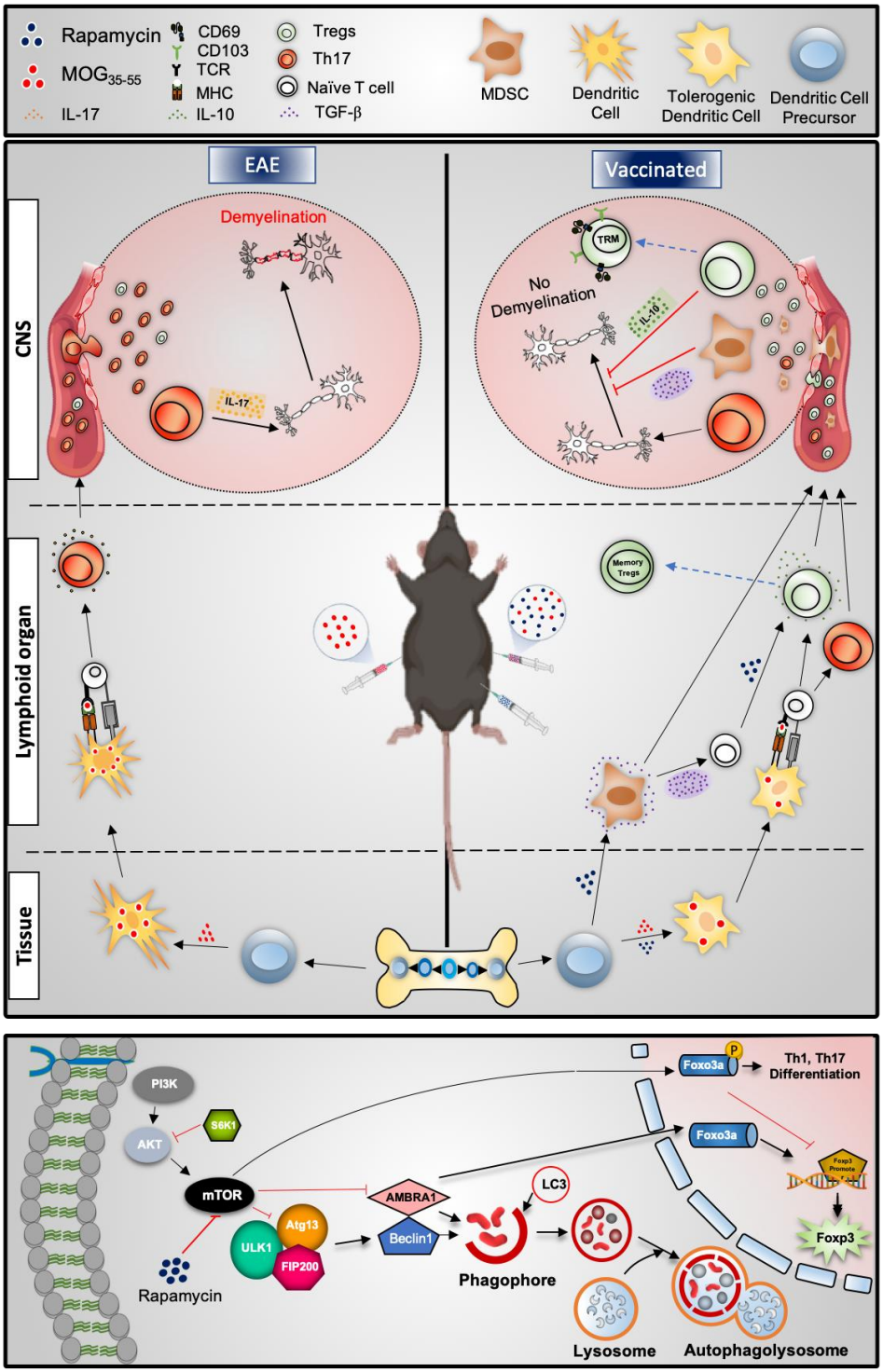
Rapamycin
MOG35-55
IL-17
CD69
CD103
TCR
MHC
IL-10
Tregs
Th17
Naïve T cell
TGF-β
MDSC
Dendritic Cell
Tolerogenic Dendritic Cell
Dendritic Cell Precursor
EAE
Vaccinated
CNS
Demyelination
IL-17
TRM
No Demyelination
IL-10
Lymphoid organ
Memory Tregs
Tissue
PI3K
AKT
S6K1
mTOR
Rapamycin
ULK1
Atg13
FIP200
AMBRA1
Beclin1
LC3
Phagophore
Lysosome
Autophagolysosome
Foxo3a
P
Th1, Th17 Differentiation
Foxo3a
Foxp3

Figure 1*.Vaccination with MOG^{rapa} protected from EAE by autophagy-induced generation of Tregs and MDSC.* The mice injected with MOG^{rapa} generated predominantly MDSC and MOG-specific Tregs. Contrary to this, immunization with MOG mainly elicited MOG-reactive Th17 cells. Tregs obtained from MOG^{rapa} but not MOG-administered mice showed a significantly higher pool of central and resident memory CD4^{+} FoxP3^{+} Tregs in the blood, lymphoid organs and CNS and protected animals from subsequent challenges with MOG. Induction of autophagy triggered the formation of autophagosomes to incorporate harmful antigens *e.g.,* auto-antigens. Autophagosome development is activated by the mTOR-Ulk1-beclin1 pathway. Consequently, the autophagosome fuses with the lysosome to deliver the cargo for degradation. Mechanistically, rapamycin targeted the mTOR-Ambra1-FoxP3 axis to generate Tregs and protected the animals by the induction of autophagy, as evidenced by heightened expression of LC3, Ulk1, FIP200, Atg13 and Beclin-1.

CHAPTER 6
SUMMARY

Autoimmune diseases are on the rise worldwide, with a particular increase among females in Western populations (*Lerner A et al., 2015; Miller FW, 2023*). This increase can be attributed to dietary changes, lifestyle modifications, and environmental factors. It places a substantial socio-economic burden on affected individuals, distressing various age groups, including childhood and adolescence (e.g., type 1 diabetes), early adulthood (e.g., myasthenia gravis, multiple sclerosis), and late adulthood or older adults (e.g., rheumatoid arthritis, primary vasculitis). Current projections indicate that autoimmunity will become a major lifestyle disease in the coming years, necessitating a significant escalation in our efforts.

Current therapies for autoimmune diseases primarily focus on providing immune suppression and thereby symptomatic relief. While these treatments offer substantial relief to patients, there is currently no durable cure for autoimmunity. Different autoimmune diseases manifest differently, which poses challenges in diagnosis and treatment. Nevertheless, recent decades have witnessed advancements in the remedial action against autoimmunity, with researchers working on developing next-generation diagnostic tools and targeted therapies.

Vaccines have the potential to prevent disease, as demonstrated by their success in eradicating devastating diseases such as poliomyelitis, measles, and smallpox. However, some vaccines, like BCG, have failed to provide lifelong immunity. Researchers encountering similar challenges in developing therapies against autoimmunity due to the phenotypic plasticity of suppressive cells during inflammatory conditions are exploring the use of vaccines against autoimmunity. Such vaccines should not only prevent disease occurrence but also offer protection against future encounters.

After careful consideration, we utilized rapamycin to induce auto-antigen-specific regulatory T cells (Tregs). Consequently, we co-administered mice with the autoantigen MOGand rapamycin. Interestingly, rapamycin-induced autophagy that aided in generating antigen-specific memory Tregs with lineage stability, even in inflammatory conditions. Further, generation of MOG-specific suppressive Tregs and a decrease in pathogenic Th17 cells was observed. Furthermore, rapamycin induced myeloid-derived suppressor cells (MDSC), which contributed to creating a suppressive microenvironment conducive to Treg polarization. The Tregs generated in response to vaccination transitioned into memory Tregs expressing

multiple suppressive markers. These memory Tregs in the vaccinated group provided protection against lethal doses of MOG upon rechallenge and retained their phenotype. In contrast, MOG-immunized animals without rapamycin did not demonstrate protection, and the Tregs from that group exhibited phenotypic plasticity toward Th1-like Tregs.

Overall, our study presents a novel strategy of exploiting rapamycin for generating auto-antigen-specific memory Tregs to confer protection against autoimmune diseases. Looking ahead, this pioneering approach holds promise for the future development of vaccines to prevent and treat autoimmune diseases.

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Zhou HQ, Zhang LM, Li X, Huang ZH. Crosstalk Between Autophagy and Inflammation in Chronic Cerebral Ischaemia. Cellular and Molecular Neurobiology. **2023** Mar 23:1-0.

LIST OF PUBLICATIONS

1. Kumar Das D[#], **Zafar MA**[#], Nanda S[#], Singh S, Lamba T, Bashir H, Singh P, Maurya SK, Nadeem S, Sehrawat S, Bhalla V, Agrewala JN. Targeting dendritic cells with TLR-2 ligand-coated nanoparticles loaded with *Mycobacterium tuberculosis* epitope induces antituberculosis immunity. ***J Biol Chem. 2022*** Dec;298(12):102596 **(# Equal contributions**).

2. Malik JA, **Zafar MA**, Lamba T, Nanda S, Khan MA, Agrewala JN; The impact of ageing-induced gut microbiome dysbiosis on dendritic cells and lung diseases. ***Gut Microbes, 2023***(in press).

3. Kaur G, Singh S, Nanda S, **Zafar MA**, Malik JA, Arshi MU, Lamba T, Agrewala JN. Fiction and Facts about BCG Imparting Trained Immunity against COVID-19. ***Vaccines 2022***, 10, 1006.

4. Malik, J. A., Nanda, S., **Zafar, M. A**., Sehrawat, S., & Agrewala, J. N. (2023). Influence of chronic administration of morphine and its withdrawal on the behaviour of zebrafish. ***Journal of Biosciences***, 48(3), 33.

5. **Zafar MA**, Lamba T, Tehseen A,Sarkar R, Nanda S, Prajapati S, Khan MA, Malik JA, Sehrawat S*, Agrewala JN; Rapamycin-induced autophagy transforms MOG-reactive pathogenic Th17 cells to protective Tregs and averts experimental autoimmune encephalomyelitis. ***Autophagy, 2023***(under review).

6. Nanda S, Zafar MA, Lamba T, Malik JA, Khan MA, Kaur G, Ghadi R, BhardwaJ P, Bisht B, Owais M, Bhalla V, Jain S, Agrewala JN. Acr1 nanovaccine displaying morphine and Pam3Cys elicits enduring anti-morphine immunity and protects against addiction. ***Nano letters***, 2023 (under review).

7. Nanda S, **Zafar MA**, Singh S, Gautam R, Ghosh A, Basu D, Agrewala JN; Chronic administration of morphine provokes the generation of anti-morphine antibodies and immunosuppression in individuals with opioid use disorder. ***Indian J Med Res, 2023***(under review).

8. Prajapati S, Lamba T, **Zafar MA,** Khan MA, Malik JA, Sangwan B, Nanda S, Agrewala JN; Mycobacterial and monkeypox homologous epitopes: building blocks of robust monkeypox vaccine. ***Immunology letters***, ***2023*** (under review).

9. Malik JA, Khan MA, Lamba T, Zafar MA, Nanda S, Owais M, Agrewala JN*; Immunosuppressive effects of morphine on macrophage polarization and function. ***Eur J Immunol*** (under review).

10. Pasricha K, Prajapati S, Nanda S, Lamba T, **Zafar MA**, Malik JA, Tripathi NM, Bandyopadhyay A, Agrewala JN, Cross-reactive T cell and B cell epitopes of mycobacteria and SARS-CoV-2: a possible reason for a higher rate of protection of TB-endemic population against COVID-19. ***Amino Acids*** (under review).

11. Lamba T, **Zafar MA**, Sehrawat S, Agrewala JN. Novel role of 16 kDa protein of *Mycobacterium tuberculosis* in curbing experimental autoimmune encephalomyelitis by generating Tregs and myeloid-derived suppressor cells (under preparation).

CONFERENCES

International

2. Presented a posterentitled ‘Rapamycin plays an adjuvanting role in combination with MOG to prevent EAE through autophagy axis’ at International Union of Immunology Congress 2023, held at Cape Town South Africa from Nov 27to Dec 2, 2023.
3. Abstract entitled ‘Rapamycin plays an adjuvanting role in combination with MOG to prevent EAE through autophagy axis’ selected for an oral presentation at British Society of Immunology Congress, 2022 held at Liverpool UK from Dec5 to 8, 2022.

National

2. Presented a posterentitled ‘Rapamycin-induced autophagy transforms MOG-reactive pathogenic Th17 cells to protective Tregs and averts experimental autoimmune encephalomyelitis’ at the 50th Annual Conference of Indian Immunology Society (IMMUNOCON 2023), held at AIIMS New Delhi on October 5 to8, 2023.
3. Presented a poster entitled “Targeting dendritic cells with *Mycobacterium tuberculosis* epitope encapsulated in nanoparticles expressing TLR-2 ligand protects against tuberculosis” at the 49th Annual Conference of the Indian Immunology Society (IMMUNOCON 2022) held at PGIMER, Chandigarh on Nov23-26, 2022.
4. Attended 46th Annual Conference of Indian Immunological Society (IMMUNOCON 2019) on November14 to 16, 2019 held at Bhabha Atomic Research Centre, Mumbai.
5. Presented a poster entitled “ Restricting the development of autoimmune disease by skewing the generation of Th17 cells towards Tregs” in Research Conclave 2019 held at IIT Ropar from April 20 to 21, 2019.