De-activation of immune cells through modulation of extracellular matrix metalloproteinase inducer (EMMPRIN) by statins

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I TABLE OF CONTENTS

II LIST OF FIGURES

III LIST OF TABLES

IV LIST OF ABBREVIATIONS

AMOG	Adhesion molecule on glia
APC	Antigen presenting cell
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
CNS	Central nervous system
CSF	Cerebrospinal fluid
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein Barr virus
ECD	Extracellular domain
EDTA	Eythelenediaminotetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol bis (2-aminoethyl ether)-*N,N,N′N′*
EMMPRIN	Extracellular matrix metalloproteinase inducer
ERK	Extracellular signal regulated kinases
FACS	Florescence assisted cell sorting
FCS	Fetal calf serum
FPP	Farnesyl pyrophosphate
FRET	Fluorescence resonance energy transfer
FTI	Farnesyl transferase inhibitor
GA	Glateramer acetate
GGPP	Geranylgeranyl pyrophosphate
GGTI	Geranylgeranyl transferase inhibitor
GTP	Guanosine triphosphate
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
LFA-1	Lymphocyte function-associated antigen 1

MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MCT	Monocarboxylate transporter
MHC	Major histocompatability complex
MMP	Matrix metallo proteinase
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NCAM	Neural cell adhesion molecule
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PNS	Peripheral nervous system
PP-MS	Primary progressive multiple sclerosis
RA	Rheumatoid arthritis
RR-MS	Relapsing-remitting multiple sclerosis
SDS	Sodium dodecyl sulphate
SP-MS	Secondary progressive multiple sclerosis
TCR	T cell receptor
TCSF	Tumor cell-derived collagenase stimulatory
TEMED	*N,N,N',N'*-tetramethyl ethylene diamine tetra acetic acid
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen 4

SUMMARY

Statins are widely used hypocholesterolemic drugs that inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme of the mevalonate pathway whose end product is cholesterol. Statins exert varied functions including anti-proliferative, immuno-suppressive, anti-inflammatory and neuroprotective actions. Immunologically, extracellular matrix metalloproteinase inducer (EMMPRIN) is an important glycoprotein that regulates matrix metalloproteinase (MMP) secretion and activation, immune cell activation and cellular differentiation. It also acts as a chaperone for monocarboxylate transporters. The structure, function and activity of EMMPRIN, depend on the metabolic intermediate derivatives of cholesterol pathway. Targeting EMMPRIN would be an important strategy to regulate inflammation and immunosuppression. If statins could impose immunosuppression and exhibit antiproliferative properties on activated cells, a part of this activity could be by regulating activation molecules like EMMPRIN. Thus, it was hypothesised that some of the pleiotropic effects exerted by statins are EMMPRIN mediated.

Therefore, two immune cell types, namely monocytes and T cells, were tested for EMMPRIN-mediated immunosuppressive effect of statins.

Statin studies in monocytes:

In the monocytic cell line THP-1, statins downregulated the surface expression of EMMPRIN, which exists as an activation complex on the cell surface. Statins also inhibited MMP-9 and MMP-2 activation and secretion in these cells. Inhibition of glycosylation by fluvastatin resulted in the formation of low glycosylated EMMPRIN (an antagonist of matrix metalloproteinase activation), while it inhibited the formation of high glycosylated EMMPRIN (an agonistic of matrix metalloproteinase activation). Furthermore, intracellular permeabilisation and cell surface biotinylation assays demonstrated the cellular location and glycosylation status of EMMPRIN respectively. Down regulated EMMPRIN on these accessory cells inhibited the secondary co-stimulatory signal for T lymphocyte activation and proliferation and directed the T cell towards anergy. This effect could be mimicked by EMMPRIN antagonists.

Statin studies in T cells:

In primary T lymphocytes, statins downregulated the expression of EMMPRIN along with other molecules like CD98, MCT1 and CD29 on the cell surface. EMMPRIN being the chaperone for monocarboxylate transporter 1 (MCT1), its intracellular entrapment in-turn accumulated MCT1

as assessed by FACS analysis. This intracellular accumulation of MCT1 caused cellular acidification and metabolic starvation of activated T cells as evident by decreased intracellular pH and impaired transport of nutrients into the cell respectively. These cellular effects rendered T cells inactive as evident by their decreased proliferation. Polyclonally activated T cells were able to cause neuronal apoptosis in a contact dependent and MHC-I independent manner, while statin-treated activated T cells incurred neuroprotection and abolished neurodegeneration by activated immune cells. Antagonists of EMMPRIN mimicked the neuroprotective effect of statins, implying EMMPRIN mediated neuroprotection. In conclusion, statins as therapeutic drugs might be promising candidates in the treatment of autoimmune diseases like multiple sclerosis, where de-activation of autoactivated cells is of prime importance.

ZUSAMMENFASSUNG

Statine sind vielfach verwendete cholesterinsenkende Medikamente, die das Enzym 3-Hydroxy-3-methylglutaryl Coenzym A (HMG-CoA) Reduktase hemmen. HMG-CoA Reduktase ist das limitierende Enzym des Mevalonat-Stoffwechselweges, dessen Endprodukt Cholesterin ist. Statine weisen eine Reihe von Eigenschaften auf einschließlich anti-proliferativer, immunsuppressiver, anti-inflammatorischer und neuroprotektiver Fähigkeiten. Der extrazelluläre Matrixmetalloproteinase-Aktivator (extracellular matrix metalloproteinase inducer; EMMPRIN) ist ein immunologisch wichtiges Glykoprotein, das die Matrixmetalloproteinase (MMP)-Ausschüttung und -Aktivierung, Immunzellaktivierung und zelluläre Differenzierung reguliert. Es dient auch als Chaperon für Monocarboxylat-Transporter. Struktur, Funktion und Aktivität von EMMPRIN werden von den Zwischenderivaten des Cholesterinsynthesewegs beeinflusst. EMMPRIN könnte ein wichtiger Angriffspunkt sein, um Entzündung und Immunsuppression zu regulieren. Da Statine immunsupprimierend und antiproliferativ auf aktivierte Zellen wirken, könnte ein Teil dieses Effektes auf der Regulation von Aktivatoren wie EMMPRIN beruhen. So wäre es möglich, dass einige der pleiotropischen Effekte, die durch Statine verursacht werden, über EMMPRIN vermittelt werden.

Aufgrund dieser Hypothese wurden verschiedene Immunzellen, nämlich Monozyten und T-Lymphozyten, auf die durch EMMPRIN vermittelten immunsupprimierenden Effekte von Statinen untersucht.

Statin-Studien in Monozyten:

In der Monozyten-Zelllinie THP-1 regulieren Statine die Expression von EMMPRIN, welches sich als Aktivierungskomplex auf der Zelloberfläche befindet, herunter. Statine hemmen auch die Aktivierung von MMP-9 und MMP-2 sowie deren Ausschüttung durch diese Zellen. Die Hemmung der Glykosylierung durch Fluvastatin ergab eine Akkumulation niedrigglykosylierten EMMPRINs (eines Antagonisten der MMP-Aktivierung), während es die Expression hochglykosylierten EMMPRINs hemmte (eines Agonisten der MMP-Aktivierung). Außerdem zeigten intrazelluläre Permeabilisierungs- und Zelloberflächenbiotinylierungsexperimente die zelluläre Expression und den Glykosylierungsstatus von EMMPRIN auf. In Monozyten-T-Zell-Kokulturen hemmte ein verringerter EMMPRIN-Level in den Monozyten das kostimulierende Sekundärsignal für die Aktivierung und Proliferation von T-Lymphozyten und verursachte eine Inaktivierung der T Zellen. Dieser Effekt konnte auch mit EMMPRIN-Antagonisten nachgeahmt werden.

Statin-Studien in T-Lymphozyten:

In primären T-Lymphozyten regulieren Statine nicht nur die Zelloberflächenexpression von EMMPRIN herunter, sondern zusätzlich auch die Expression anderer Moleküle, wie CD98, MCT1 und CD29. Die verringerte Expression von EMMPRIN, welches ein Chaperon für den Monocarboxylat-Transporter 1 (MCT1) darstellt, verursacht eine intrazelluläre Anhäufung von MCT1. Diese intrazelluläre Ansammlung von MCT1 bewirkt eine Ansäuerung der Zellen und verursacht so einen „metabolischen Hungerzustand" der aktivierten T Zellen, der auf einem behinderten Transport von Nährstoffen in die Zelle beruht. Diese Effekte inaktivieren die T-Lymphozyten, was sich durch eine verringerte Zellteilungsrate widerspiegelt. Polyklonal aktivierte T Zellen sind in der Lage, neuronale Apoptose in einer Kontakt-abhängigen und Haupthistokompatibilitätskomplex Klasse I-unabhängigen Weise zu verursachen, während mit Statinen behandelte T-Zellen neuroprotektiv wirken und die Neurodegeneration durch aktivierte Immunzellen verhindern. Antagonisten von EMMPRIN ahmten den neuroprotektiven Effekt der Statine nach und lassen EMMPRIN-vermittelte Neuroprotektion vermuten.

Schlussfolgernd lässt sich sagen, dass Statine als therapeutische Wirkstoffe vielversprechende Anwärter in der Behandlung von Autoimmunerkrankungen, wie der Multiplen Sklerose, sind, wo die Deaktivierung autoreaktiver Zellen von entscheidender Wichtigkeit ist.

1 INTRODUCTION

1.1 IMMUNOLOGY OF MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an inflammatory disease that affects the central nervous system (CNS), i.e., the brain and spinal cord (Giovannoni et al., 2006). It develops with a complex predisposing genetic trait and probably requires an inciting environmental insult such as viral infection to trigger the disease (Flachenecker, 2006). The activation of $CD4^+$ autoreactive T cells and their differentiation into a Th1 (pro-inflammatory) phenotype are crucial events in the initial steps, and these cells are probably the most important players in the long-term evolution of the disease (Charil and Filippi, 2007). Damage of the target tissue - the CNS, is however, most likely mediated by other components of the immune system, such as antibodies, complement, $CD8^+$ T cells and factors produced by the innate immune cells (Antel and Bar-Or, 2006). Perturbations in immunomodulatory networks that include Th2 cells (anti-inflammatory), regulatory $CD4^+$ T cells, natural killer (NK) cells and others may in part be responsible for the relapsing-remitting or chronic progressive nature of the disease (Lassmann et al., 2007).

Monocytes/macrophages play an important role in the progression of disease, and the number of monocytes infiltrating into the CNS environment corresponds to the magnitude of lesion progression (He and Sun, 2007). At the blood brain barrier (BBB) junction, macrophages together with fibroblasts and endothelial cells are primary sources of matrix metalloproteinases (MMPs). Thus, rather than the primary effecter of tissue destruction, macrophages may act as amplifiers of the pathogenic cascade, especially via activation of fibroblasts by molecules such as interleukin (IL)-1 and tumour necrosis factor alpha (TNF-α) (Engelhardt, 2006). Other molecules, such as extracellular matrix metalloproteinase inducer (EMMPRIN)/CD147, may also participate in the process and might play a role in disease pathogenesis (Berger and Reindl, 2007). MS is primarily a $CD4^+$-mediated disease, based on cellular composition of cerebrospinal fluid (CSF), brain biopsies of patients and the data from the experimental autoimmune encephalomyelitis (EAE) model (Hemmer et al., 2006). In the EAE model, addition of myelin components to susceptible animals leads to $CD4^+$ mediated autoimmune disease that is similar with MS in humans. Thus, in the above inflammatory scenario anti-inflammatory and immunomodulatory therapies would best combat relapsing-remitting (RR) MS, while myelin repair strategy would best combat secondary progressive (SP) MS (McQualter and Bernard, 2007). Immunologically, elevated CSF immunoglobulins, the presence of oligoclonal immunoglobulin bands and elevated MMP-9 levels have been reported in MS patients.

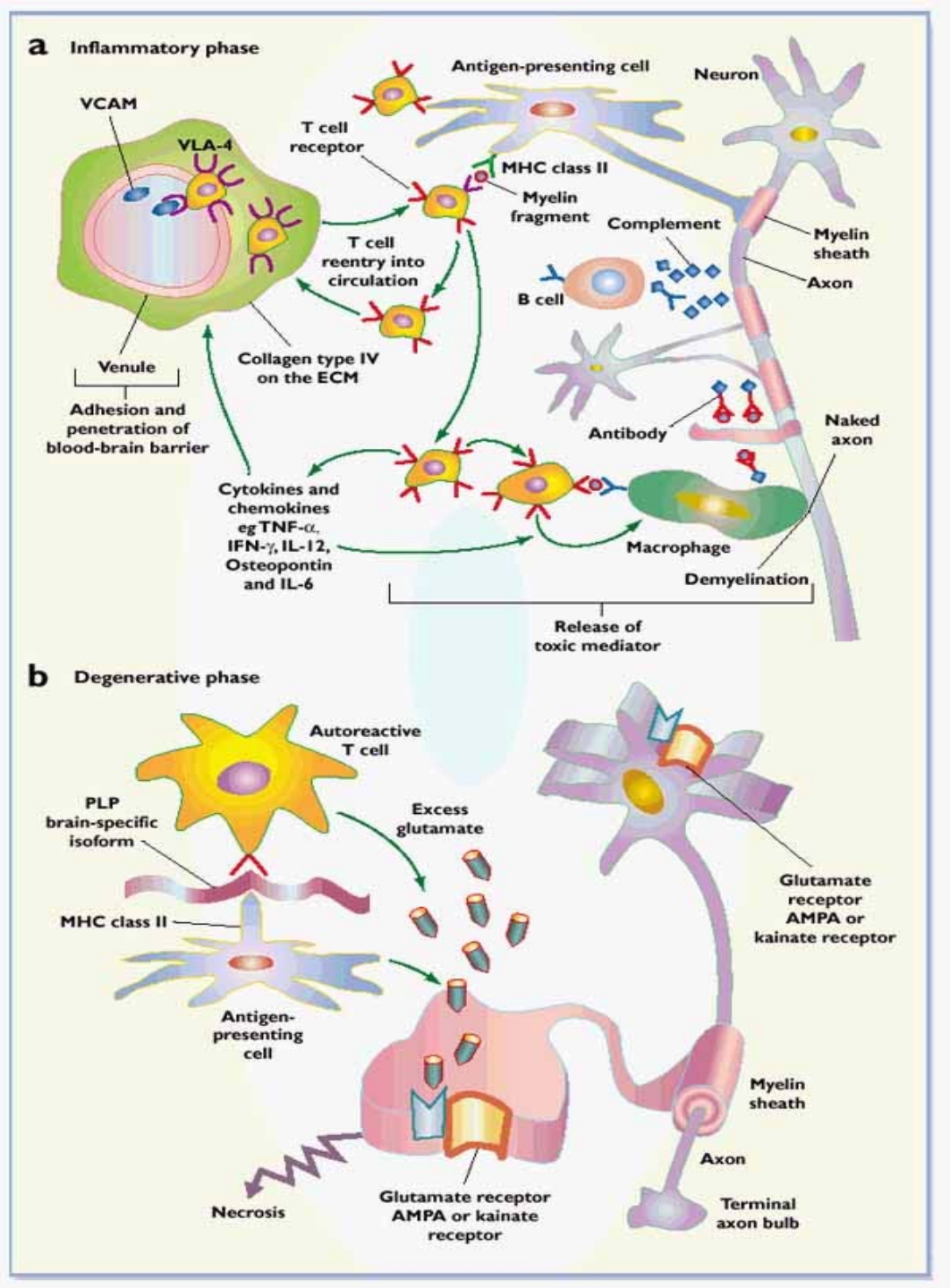


Figure 1.1: Two staged disease progression of MS. MS primarily occurs in two phases - an inflammatory phase, where the T cells get autoactivated in the peripheral environment, produce inflammatory mediators like IL-1β, TNF-α and MMPs. These mediators disturb the blood brain barrier, allow activated T cells to move into the CNS environment and get reactivated to some unknown CNS antigens leading to axonal degradation. $CD8^+$ T cells, macrophages, B cells and complement further add up to the cause of demyelination. The second phase is the degenerative phase where the autoreactive T cells in presence of antigen presenting cells (APCs) and CNS antigens (phospholipid protein, myelin oligodendrocyte protein (MOG), myelin basic protein (MBP) get activated and produce excessive glutamate which activates the glutamate receptor on the axon to cause glutamate mediated toxicity (Hemmer et al., 2006).

1.1.1 MS Etiology

The etiology of MS remains obscure, but data points out that this disease/syndrome develops in genetically susceptible individuals and further may require additional environmental triggers (Olsson et al., 2006). Similar to other T cell-mediated autoimmune diseases, in MS, the specific genes that confer risk are the human leuckocyte antigen HLA-DR and -DQ genes, which are major histocompatability complex (MHC)-linked (Brodkin and Wszolek, 2006). However, there is no doubt that HLA-DR and -DQ molecules are by far the strongest genetic risk factors in MS. HLA class I may act independent of class II in some patients, either through similar mechanisms or by modulation of NK cellular activity. Viral and bacterial infections are also possible candidates as environmental triggers of MS (Giovannoni et al., 2006). The viral etiology of a number of human de-myelinating diseases caused by papova viruses (Laplaud and Confavreux, 2006) or herpes simplex virus (HSV) explains the continued interest as triggers for MS. Epstein Barr virus (EBV) is also linked to MS.

1.1.2 Mechanisms of induction of MS by infectious agents

Two major mechanisms have been proposed to explain how infectious agents might induce MS: a) molecular mimickry (activation of autoreactive cells by cross-reactivity between self-antigens and foreign antigens) (Rebeles et al., 2006); and b) bystander activation, which assumes that autoreactive cells are activated because of non-specific inflammatory events that occur during infections (Dutta and Trapp, 2006). A third proposal emphasises a combination of the above two mechanisms i.e., that molecular mimickry and bystander activation act together in cohort to induce MS.

1.1.2.1 Molecular mimickry

It involves reactivity of T and B cells with either peptides or antigenic determinants commonly shared by infectious and self-antigens (Lassmann et al., 2007). The recognition of self-antigens at intermediate levels of affinity by T cells during thymic selection leads to positive selection of T cells and further causes export of these T cells to the periphery (Charil and Filippi, 2007). Cross-reactivity of these self-reactive cells with foreign antigens can lead to activation during infection, migration across the blood-brain barrier (BBB), CNS infiltration, and, if they recognise antigens expressed in the brain, leads to pathogenesis and progression of MS (Hemmer et al., 2006).

1.1.2.2 Bystander activation

This includes two categories; the first one involves TCR-independent bystander activation of autoreactive T cells by inflammatory cytokines, super antigens and molecular pattern recognition e.g. Toll-like receptor (TLR) activation (Smith, 2006). The second category involves the unveiling of host antigens and the adjuvant effect of infectious agents on antigen presenting cells (APCs) (Correale and Villa, 2007).

1.1.3 Pathogenetic stages in the disease process of MS: lesion pathology

Figure 1 summarises the most accepted inflammatory mechanism of MS. Autoreactive $CD4^+$ T cells are activated in the peripheral blood component to many exogenous and endogenous antigenic peptides in the context of costimulatory and other less-defined signals of APCs. Factors that contribute to a pro-inflammatory environment include a number of mediators (cytokines, chemokines and matrix metalloproteinases) released from T cells and APCs (Manuel et al., 2007). Activated autoreactive T cells adhere to the BBB endothelium via upregulated adhesion molecules such as LFA-1 and VLA-4, and migrate into brain parenchyma through cerebro-vascular endothelial cells (Dutta and Trapp, 2006).

Many unanswered questions still exist regarding the guidance of autoreactive $CD4^+$ T cells to the CNS. Whether a chemokine gradient exists from brain parenchyma to the blood during the initial events is unknown. However, the experimental EAE model reveals that adoptively transferred encephalitogenic T cells (locally reactivated in the CNS) are evident in the deep cervical lymph nodes (Hemmer et al., 2006).

The next step in the pathogenetic cascade of MS involves upregulation of pro-inflammatory cytokines such as (IFN-γ, IL-23, TNF-α) (Martin Mdel and Monson, 2007) and chemokines such as RANTES, IP-10 (Rebenko-Moll et al., 2006) leading to activation of resident microglia and astrocytes as well as recruitment of other immune active cells e.g. monocytes, $CD8^+$ T cells, B cells and mast cells from peripheral blood (Smith, 2006). These cells further orchestrate the formation of inflammatory lesions paralleled by upregulation of inflammatory mediators. Inflammatory mediators (pro-inflammatory molecules, oxygen and nitrogen radicals) lead to BBB disruption with tissue edema after protease release from mast cells, monocytes and T cells (Giovannoni et al., 2006). Damage of CNS tissue already occurs at this inflammatory stage. $CD4^+$ autoreactive T cells are likely driving the disease

pathogenesis, whereas their role in the effector phase is probably secondary (Rebeles et al., 2006).

Numerous processes, like direct complement deposition, antibody-mediated complement activation (Duddy and Bar-Or, 2006) and direct lysis of axons by cytolytic $CD8^+$ cells (Engelhardt, 2006), might all lead to CNS damage. Furthermore, CNS damage is also due to increased production of glutamate by astrocytes via calcium influx in oliogodendrocyte apoptosis (Hemmer et al., 2006).

The above inflammatory phase lasts from days to weeks resulting in demyelinated axons, apoptotic oligodendrocytes and T cells. Besides clearing debris, lesion resolution further induces a relative dominance of Th2/Th3 cytokines such as IL-10, transforming growth factor beta (TGF-β) and various neurotrophins (Hemmer et al., 2006). Oligodendrocyte precursors that are still present in the CNS are activated, and the surviving oligodendrocytes begin to remyelinate denuded internodal areas, although the original thickness might not be reached (Lassmann et al., 2007). Inhibitory signals between axonal and myelin structures are physiologically relevant during shaping and maintainance of the intricate cyto-architecture of the CNS.

The cellular composition and involved pathogenic molecular pathways vary among patients. Investigators have identified four pathologic MS subtypes based on relative contribution of different immune cells, antibody and complement deposition, myelin loss and oligodendrocyte death. The following pathologic subtypes are described (Lassmann et al., 2007):

- Pattern I - This is dominated by T cells, macrophages and effector molecules including TNF-α, IFN-γ and radical species.
- Pattern II - Antibody and complement deposition predominates involving MOG and MBP specific antibodies. This pattern has a similarity sharing with Guillain Barre syndrome, an acute inflammatory demyelinating disease of the peripheral nervous system (PNS).
- Pattern III - This is characterised by preferential loss of myelin-associated glycoprotein (MAG) and oligodendropathy. Furthermore, the vulnerability of oligodendrocytes is increased by immune responses against heat shock proteins in this pattern.

- Pattern IV - Non-apoptotic oligodendrocyte degeneration is a common pattern and occurs primarily in primary progressive (PP) MS.

The overall extent of inflammation is highest in relapsing-remitting (RR) MS and decreases into secondary progressive (SP) MS. Other common pathologic feature includes axonal loss even in the earliest stages of the disease. Cytolytic $CD8^+$ and antiganglioside antibodies were suggested to be probably involved in this process (Duddy and Bar-Or, 2006). Finally, a number of other findings indicate that contributors to tissue destruction and aberrant repair include the vulnerability of the CNS tissue, the local deregulation of apoptotic mechanisms such as elevated expression of bcl-2 on oligodendrocytes during RR-MS, glutamate-mediated excitotoxicity and the re-expression of developmentally important recognition molecules (Smith, 2006).

1.1.4 Lessons from therapies

Multiple sclerosis as an autoimmune inflammatory disease is supported by the positive response to immunomodulatory and suppressive treatments (Weber et al., 2005). Glucocorticoids, at high doses during clinical exacerbations of MS, act as anti-inflammatory agents by reducing edema and arachidonic acid metabolites and by decreasing pro-inflammatory cytokines and other inflammatory mechanisms (Stuve and Bennett, 2007). A number of chemotherapeutic agents with broad immunosuppressive activities are used at more advanced stages of the disease, i.e., the transition from RR-MS to SP-MS or in patients with aggressive disease who do not respond or who incompletely respond to the approved agents (Freedman, 2006). Immunosuppressants include mitoxantrone (Neuhaus et al., 2006), cyclophosphamide and mycophenolate (Hemmer et al., 2006). The mechanisms of action of the immunosuppressants are finally understood.

Interferon beta (IFN-β) as treatment for RR-MS and SP-MS is currently the most approved agent (Freedman, 2006). It was originally used as an antiviral agent but is also reported to have immunomodulatory activities including upregulation and shedding of adhesion molecules, induction of IL-10 and neurotrophic factors, blocking BBB opening via inhibition of MMP-2 and -9 and reduction of cell adhesion to the BBB. It reduces exacerbations by 30% and has modest impact on disease progression. IFN-β is a step forward in MS therapy, but the frequency of subcutaneous or intra-muscular injections, the flu-like symptoms that occur at the beginning of therapy, the modest activity required of patients, and the treatment failures are all reasons to search for better agents (Rebeles et al., 2006).

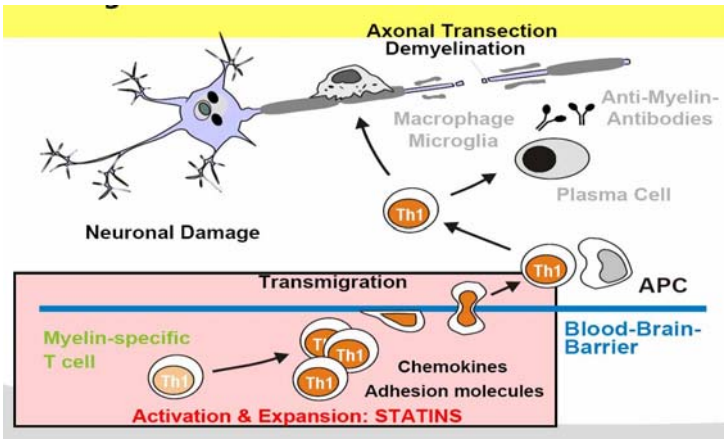


Figure 1.2: Therapeutic intervention of MS pathogenesis. The most accepted model of immunosuppressive action is that these agents decrease the activation of T cells and bias the T cells towards a Th2 environment. Further these cells are unable to cross the BBB and cause greater harm in the CNS environment (Antel and Arnold, 2007).

Glatiramer-acetate (GA) (Ziemssen et al., 2001) is yet another approved therapy for RR-MS. GA is a random copolymer of the four amino acids - alanine, lysine, glutamine and tyrosine, with various lengths and fixed molar ratios of (4.5):(3.6):(1.5):(1) (Ziemssen et al., 2002b). This was originally developed as a mimick of MBP and to induce EAE. GA blocks experimental disease primarily by displacing autoantigenic peptides from HLA class II binding grooves i.e., via competition for binding, polyclonal T cell stimulation, partial agonist effects (Ziemssen et al., 2001), Th2 activation and cross-reactivity with myelin peptides, shift of antibody response towards IgG4, interference with dendritic cell (DC) differentiation and induction of neurotrophins (Ziemssen et al., 2002a).

Other promising therapeutic strategies include humanised monoclonal antibodies against very late antigen-4 (VLA-4) such as natalizumab, which blocks BBB migration of T cells, their activation, reduces brain inflammation and acts against CD25 (interleukin-2 receptor), needed for sustainment of T cell activation (Wiendl et al., 2003).

Further strategies include the modulators of cAMP levels e.g. phosphodiesterase type 4 inhibitor, pentoxyfylline and β-adrenergic agents, inhibitors of chemokine receptors (CCR2 antagonists), blocking agents of CD4, retinoic acid, vitamin A and D derivatives and others. Promising results were observed with estriols and the cholesterin-lowering statins (Weber et al., 2006).

1.2 EMMPRIN

Extracellular matrix metalloproteinase inducer (EMMPRIN), also known as basigin; CD147 (Zucker et al., 2001), CE9, HT7, M6, neurothelin, OX-47 and gp42, is a widely distributed cell surface glycoprotein that belongs to the Ig superfamily and is enriched in

malignant tumour cells (Zucker et al., 2001). It is a cell surface glycoprotein that belongs to the immunoglobulin superfamily and is highly expressed on the surface of tumour cells (Yan et al., 2005). It stimulates adjacent fibroblasts or tumour cells to produce matrix metalloproteinases. EMMPRIN exists in both a high glycosylated form - HG-EMMPRIN (40-60 kDa) and low glycosylated form - LG-EMMPRIN (32 kDa) (Yu et al., 2006), as a result of its heterogeneous N-glycosylation. It has been recently shown that EMMPRIN also stimulates expression of vascular endothelial growth factor (VEGF) and hyaluronan, which leads to angiogenesis and anchorage-independent growth/multidrug resistance, respectively (Zheng et al., 2006). EMMPRIN also regulates the activation of T cells (Pistol et al., 2007). Furthermore, it acts as a chaperone for monocarboxylate transporters (Kirk et al., 2000), as a receptor for cyclophilin A (Manoharan et al., 2006) and as a neural recognition molecule. It plays an important role in general immune homeostasis and pathologic sustainment of inflammation (Korn et al., 2005).

Membrane-associated cofactors, including caveolin-1 and annexin II, regulate EMMPRIN activity (Yan et al., 2005). EMMPRIN induces angiogenesis via stimulation of VEGF production (Zucker et al., 2001), invasiveness via stimulation of matrix metalloproteinase production (Yoon et al., 2005), and multidrug resistance via hyaluronan-mediated upregulation of ErbB2 signalling and cell survival pathway activities (Zucker et al., 2001).

1.2.1 EMMPRIN structure

EMMPRIN is a highly glycosylated member of immunoglobulin superfamily (IgSF) and is composed of two extracellular Ig domains, a single transmembrane domain, and a short cytoplasmic domain of 39 amino acids (Yurchenko et al., 2005). The extracellular region contains three aspargine glycosylation sites, but the glycan portion of the molecule differs according to the source of EMMPRIN (Yoshida et al., 2000). The different glycosylation pattern accounts for its variable molecular weight ranging between 30-66 kDa (Fadool and Linser, 1993). The extracellular region contains three N-linked glycosylation sites associated with 5-35 kDa glycan content (Tang et al., 2004a). The first Ig domain of EMMPRIN is required for counter receptor binding activity, involved in MMP induction and oligomerisation (Yurchenko et al., 2005). The second Ig domain of EMMPRIN was shown to associate with caveolin-1 (Zucker et al., 2001). A splice variant with three Ig domains was recently identified in the retina (Ochrietor et al., 2003). The overall amino acid sequence identity between mouse and rat EMMPRIN is 94%, between human, mouse EMMPRIN is 58%, while between mouse, and chicken EMMPRIN is 45%. Most of the differences exist within the extracellular domains. The

transmembrane domain is almost totally conserved among species (human, mouse and chick), while the cytoplasmic domain is only moderately conserved (Yan et al., 2005).

Similar to other members of the Ig superfamily, EMMPRIN forms oligomers in a cis-dependent manner, probably through hydrophobic interactions (Yoshida et al., 2000). The N-terminal Ig-like domain is necessary and sufficient for oligomerisation. A subset of EMMPRIN molecules, separate from those associating with integrin, associate with caveolin-1 in lipid rafts (Tang et al., 2004a). The second Ig domain is required for lipid raft association. Association with caveolin-1 thereby contributes to the onco-suppressive effects of caveolin-1.

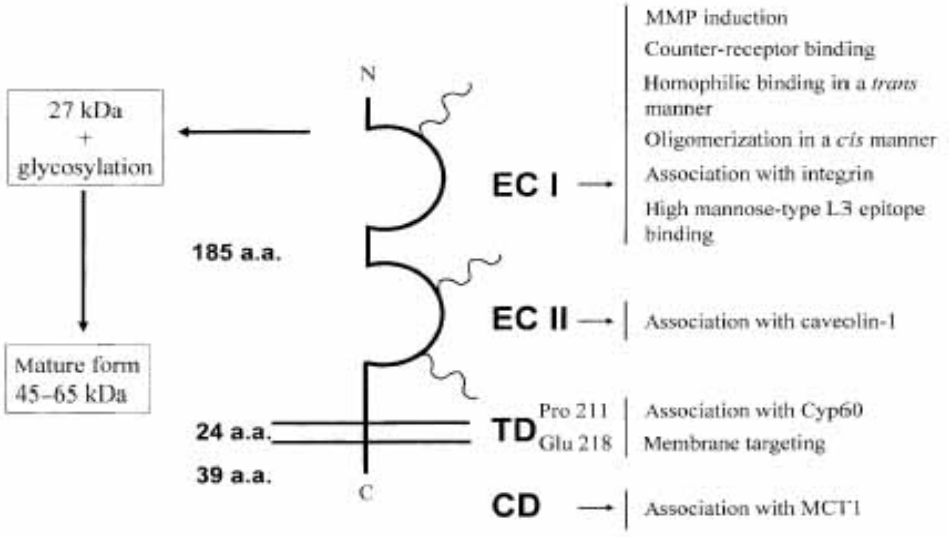


Figure 1.3: Scheme of EMMPRIN molecule. EC I - first extracellular Ig domain; EC II - second extracellular Ig domain; TD - transmembrane domain; CD - cytoplasmic domain. Three N-linked oligosaccharides are shown in helixes. EC I is involved in matrix metalloproteinase induction, binding to counter-receptors, EMMPRIN in trans and cis manners and high -mannose-type L3 epitope, and association with integrins. EC II is required for association with caveolin-1. Prp211 and Glu218 of TD are involved in association with Cyp60 and membrane targeting of EMMPRIN respectively. CD is required for association with monocarboxylate transporter 1 (MCT1) (Nabeshima et al., 2006).

1.2.2 Cellular expression

EMMPRIN expression in normal tissue cells is relatively weak. It is upregulated in relation to a physiological and pathological role, which is associated with increased MMP expression (Zhou et al., 2005a). EMMPRIN was shown to be expressed in the monocytes of peripheral blood and synovial fluid in rheumatoid arthritis (Yoon et al., 2005), corneas, human placenta, fetal membranes and mouse ovary (Zhou et al., 2005b). EMMPRIN is also highly expressed on the cell surface of various tumours and paracrinally stimulates tumour cells themselves or stromal fibroblasts to produce MMP (Suzuki et al., 2004). EMMPRIN plays a role in oesophageal squamous cell carcinoma invasion and MMP-2 production and is

overexpressed by T lymphoma cells (Nabeshima et al., 2004), thereby stimulating adjacent fibroblasts to produce MMP-2 (Riethdorf et al., 2006).

Cellular expression analysis using the monoclonal antibodies from an international workshop on HLA indicates that EMMPRIN is broadly expressed on haemopoietic and non-haemopoietic cell lines (Zucker et al., 2001).

The expression levels of EMMPRIN are upregulated in urinary bladder, breast, lung and oral carcinomas, and high-grade gliomas (Seulberger et al., 1990), and EMMPRIN has been implicated in progression and invasion of these tumours (Zheng et al., 2006).

1.2.2.1 EMMPRIN expression in monocytes

Monocytes and macrophages were known to play an important role in the pathogenesis of inflammatory diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA) (Nabeshima et al., 2006). In MS, macrophage differentiation and activation has been demonstrated to induce demyelination (Lassmann et al., 2007). The number of monocytes infiltrating into rheumatoid synovium correlates with the extent of the inflammation in synovial tissues. At the cartilage-pannus junction, macrophages, together with fibroblasts and endothelial cells, are important sources of MMPs, which had been demonstrated to be involved in the process of cartilage and subchondral bone degradation (Yan et al., 2005).

Thus, therapeutic strategies aim to counteract macrophage localisation, activation and differentiation in inflammatory diseases (Yan et al., 2005). Macrophages, together with fibroblasts and endothelial cells are important sources of matrix metalloproteinases (MMPs) (Yoon et al., 2005), which in MS have been demonstrated to be involved in the process of demyelination. Although the potential of macrophages to directly degrade extracellular matrix is modest, macrophages as the primary effectors of tissue destruction may act as an amplifier of the pathogenic cascade, especially via activation of fibroblasts by pro-inflammatory molecules such as IL-1 and TNF-α (Zheng et al., 2006). Other molecules such as EMMPRIN also participate in this process and may play an important role in MS or RA. Thus, therapeutic intervention for EMMPRIN modulation would be of major importance (Ge et al., 2006).

Activated macrophages are known to play an important role in the degradation process of normal and abnormal matrix (Hlavcak et al., 1999). Upon differentiation, the response of macrophages to pathogens is markedly enhanced allowing them to participate in the inflammatory and immune response (Haug et al., 2004). The differentiation process is a complex one and is controlled by the expression or activation of several factors.

1.2.2.2 EMMPRIN expression in the CNS environment

EMMPRIN was shown to induce neuronal differentiation including glial cells and also to maintain BBB function (Papoutsi et al., 2000). The BBB is a regulatory interface maintaining the homeostasis of internal environment of the brain. In coherence with other organs, BBB controls the import of intravascular substances and of physical and chemical changes in blood circulating through it (Schlosshauer, 1993). A crucial function of BBB is to ensure that the microenvironment of neurons is tightly regulated, in order to guarantee unhampered synaptic activity as a means of neuronal conservation (Rosenberg et al., 2001). The interaction between endothelial cells and astroglial cells via EMMPRIN might be involved in induction of tight junctions. High levels of monocarboxylate transporter (MCT)-1 and EMMPRIN are also found in rat retinal pigment epithelium (Clamp et al., 2004), the barrier between retina and choridial blood supply (Hori et al., 2000), as the primary function of EMMPRIN being to chaperone MCT1 to cell surface which maintains lactate influx and cell-cell interactions (Koho et al., 2005).

1.2.2.3 EMMPRIN expression in T cell lymphomas and T cells

Recently, EMMPRIN expression in Hodgkins lymphoma and anaplastic large cell lymphoma has also been reported (Lee et al., 2003). T cell lymphomas frequently show extranodal organ involvement and skin invasion. When compared to normal counterparts, EMMPRIN expression on HTLV-1 transformed T cells stimulated MMP-2 production and activation in cocultures with fibroblasts (Huang et al., 2005). These lines of evidence suggest that EMMPRIN expressed on neoplastic T cells may be involved in their invasion via interactions with stromal fibroblasts (Suzuki et al., 2004). EMMPRIN may also be involved in the extravasation process of T lymphoma cells, possibly via interactions with endothelial cells. This extravasation is also an important event in migration of autoimmune T cells from periphery to the CNS environment in MS (Correale and Villa, 2007). Adhesion of T cells to the brain endothelium is the major rate-limiting step of this infiltration process (Hauser and Oksenberg, 2006). Activity stimulating mAbs against EMMPRIN induced homotypic aggregation of U937 cells, which were inhibited by anti-LFA-1 and ICAM-1 mAbs (Stonehouse et al., 1999). Thus, it has been suggested that EMMPRIN might generate intracellular signals that activate the LFA-1/ICAM-1 intercellular adhesion pathway. The binding of EMMPRIN mAb was reported to mimick binding of the natural ligand (Kasinrerk et al., 1999).

EMMPRIN expression has also been reported on human non-neoplastic haematopoietic cells (Sameshima et al., 2000a). The expression level is higher in immunologically activated cells than in resting cells (Pushkarsky et al., 2005). Further phtoheamagglutinin (PHA)-activated T cells show an elevated expression of EMMPRIN than resting T cells. Thus, elevated expression of EMMPRIN is not only specific for transformation, but also depends on T cell activation. Moreover, several reports have shown the involvement of EMMPRIN in sustainment of T cell activation (Koch et al., 1999).

Cylophilin A and B act as potent lymphotactic agents by virtue of their interactions with EMMPRIN on immune cells (Yurchenko et al., 2001). Recently EMMPRIN has been identified as a higher affinity receptor for cylophilin A (Zhu et al., 2005). EMMPRIN is responsible for cyclophilin signalling cascade that culminates in ERK activation and chemotaxis. Interestingly, the signalling cascade initiated by interaction between cyclophilin A and EMMPRIN appears to be involved with residues in the known cyclosporine binding domain of cyclophilin A, emphasising the importance of this domain in cyclophilin-dependent chemotaxis. Thus, agents targeting either EMMPRIN or cyclophilins act as anti-inflammatory therapeutic agents (Yurchenko et al., 2002).

1.2.3 General EMMPRIN functions

Carcinoma cells stimulate fibroblasts to produce interstitial collagenase via a glycoprotein expression on tumorogenic cancer cells (Nabeshima et al., 2004). This protein was first christened as tumour cell-derived collagenase stimulatory factor (TCSF) and later renamed as EMMPRIN to indicate its role in extracellular matrix metalloproteinase induction via normal as well as pathological cellular interactions (Pizzi and Crowe, 2006). EMMPRIN has been reported to be involved in MMP stimulation and activation, including collagenase (MMP-1) (Tang et al., 2004b), gelatinase A (MMP-2), stromelysin (MMP-3), gelatinase B (MMP-9) (Vigneswaran et al., 2006), membrane type (MT)-1 MMP and MT2-MMP (MMP-15).

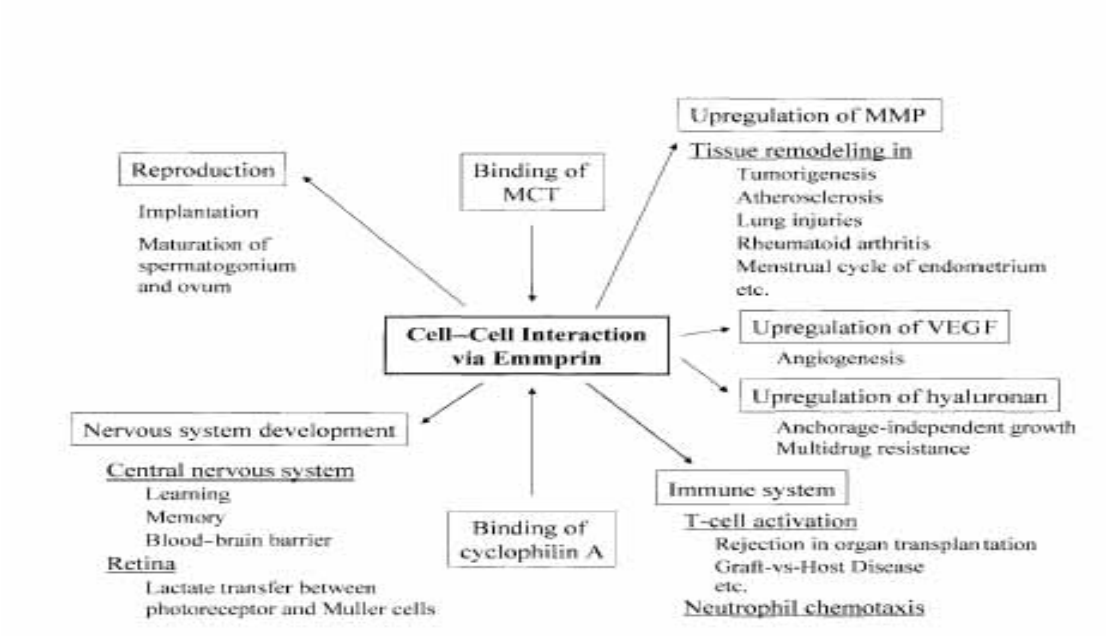


Figure 1.4: Multiple functions of EMMPRIN. Cell-cell interactions via EMMPRIN upregulate production of MMP, vascular endothelial growth factor (VEGF) and hyaluronan. They activate T cells and are involved in the development of nervous system and maturation of reproductive cells. Cell-cell interactions through binding of EMMPRIN with cylophilin A or monocarboxylate transporters also plays a role in chemotaxis of inflammatory cells and lactate transfer between glial cells and neurons respectively (Nabeshima et al., 2006).

The presence of different names for EMMPRIN suggests variable functions for this molecule, other than MMP induction. It modulates the activation and thymic development of T cells, chaperones monocarboxylate transporters to the plasma membrane, acts as a receptor to cyclophilin A through heparins as an intermediate and is associated with BBB function of cerebral endothelial cells (Fig 1.4). Deletion of EMMPRIN/basigin gene in mice leads to defects in spermatogenesis, female fertilisation, retinal development, and learning (Yan et al., 2005). The above diverse functions suggest that this molecule mediates diverse molecular events in intercellular interactions that are critical for many pathological and physiological processes.

1.2.3.1 MMP induction and activation

EMMPRIN plays an essential role in stimulating MMP production, not only in heterotypic cell interactions between tumours and fibroblasts (Zhu et al., 2006), but also in homotypic cell interactions among tumour cells, monocytes and lymphomas (Riethdorf et al., 2006). Overexpression of EMMPRIN either in fibroblasts or in tumour cells results in increased MMP production within the same population of cells. In heterotypic cell interactions, the first Ig domain and N-glycosylation are responsible for MMP-stimulating activity. This MMP induction activity is confirmed by two reports. According to one report, in an EMMPRIN mutant, the first Ig domain lost the reactivity to the activity-blocking monoclonal antibody (mAb) E11F4,

indicating that the epitope for the antibody exists in this region (Schmidt et al., 2006). In the second report recombinant EMMPRIN produced by bacteria was without glycosylation and was inactive (Smedts et al., 2006). This was also the case in homotypic cell interactions, where the extracellular (EC) domain 1 and N-linked glycosylation were involved in bidirectional signalling.

Glycosylation of EMMPRIN is not important for homophilic binding but essential for MMP induction (Sameshima et al., 2000a; Tang et al., 2004a). U251 (a glioblastoma cell line) (Sameshima et al., 2000b) and FU-EPS-1 (an epithelioid sarcoma cell line) express high levels of EMMPRIN but low levels of MMP-2 which is upregulated upon coculture with fibroblasts. EMMPRIN activity-blocking peptide interferes with EMMPRIN activity in cocultures, when preincubated with tumour cells but not with fibroblasts. These lines of evidence suggest the heterophilic interaction of EMMPRIN with undetermined molecules in a trans manner (Papoutsi et al., 2000). Thus, counter-receptor interactions between EMMPRIN and an unidentified cell surface molecule(s) cannot be excluded.

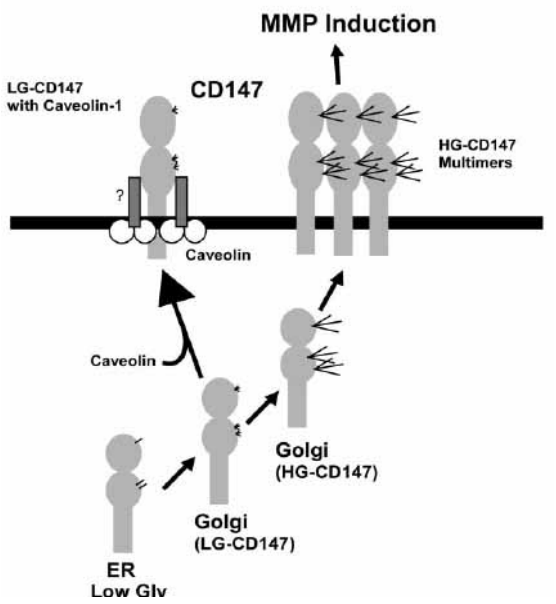


Figure 1.5: Schematic diagram of MMP regulation by EMMPRIN. EMMPRIN maturation is regulated by caveolin-1. EMMPRIN enters the golgi complex; it can mature via two possible pathways. Initially, it associates with caveolin-1, fails to acquire polylactosamines, and is escorted to the cell surface while still at least partly associated with caveolin-1, in the context of larger, caveolin-1 containing complex. This LG-EMMPRIN does not self-aggregate and does not induce MMP production. (Tang et al., 2004a)

Recent studies have identified the mechanism that regulates EMMPRIN activity through modulation of its N-glycosylation (Yu et al., 2006). In immune cells, EMMPRIN exists in various forms (Fadool and Linser, 1993), including a high glycosylated (HG 45-65 kDa) and low glycosylated (LG 32-44 kDa) as well as the core protein (approximately 27 kDa; Fig 1.3). The HG form of EMMPRIN contains N-acetylglucosaminyltransferase V-catalysed, β1,6-branched

polylactosamine-type sugars. Caveolin-1, which is a principal component of plasma membrane and caveolae, associates with LG-forms of EMMPRIN and inhibits the formation of HG-forms and cell surface clustering of EMMPRIN, leading to impairment of MMP induction (Tang et al., 2004a). It is hypothesised that caveolin-1 associated with LG-EMMPRIN during biosynthesis in the golgi complex and escorts LG-EMMPRIN to the cell surface preventing addition of polylactosamine, conversion to HG-EMMPRIN and cell surface clustering of EMMPRIN, leading to impairment of MMP induction. The second Ig domain (EC II) of EMMPRIN, but not its glycosylation is required for this association with caveolin-1 (Fig 1.3).

The negative effect of caveolin-1 on HG-EMMPRIN not only explains how caveolin-1 can suppress EMMPRIN-dependent MMP induction but also shows how caveolin-1 decreases its self-association (Jia et al., 2006a). In this regard, HG-EMMPRIN (and not LG-EMMPRIN) is covalently cross-linked confirming that HG-EMMPRIN is the one undergoing self-association. When caveolin-1 removes HG-EMMPRIN, it simultaneously impairs its self-association. In summary, EMMPRIN self-aggregation, MMP induction and its higher glycosylation form are all linked because they are all simultaneously downregulated by caveolin-1 upregulation and it is the HG-EMMPRIN that triggers MMP induction and undergoes self-aggregation as detected by mAb AAA6. Conversely, LG-EMMPRIN enhanced by caveolin-1 does not bear the AAA6 epitope, and neither triggers MMP induction nor its self-aggregation (Gabison et al., 2005).

1.2.3.2 Chaperone function for monocarboxylate transporters

Studies show that EMMPRIN is an important chaperone for the assembly of MCT1 and MCT4 into membranes (Sepponen et al., 2006), which catalyze the proton-linked transport of short chain monocarboxylates, of which lactate is metabolically the most important (Koho et al., 2002; Wilson et al., 2002). As a product of glycolysis, and as a substrate for oxidative metabolism and gluconeogenesis, lactate must be transported very rapidly across the plasma membrane of most cells. Failure of MCT expression on the cell surface leads to loss of communication between two types of cells, namely the muller cells and photoreceptor cells (Clamp et al., 2004). Fluoroscence resonance energy transfer (FRET) studies have shown that homodimer of EMMPRIN associates with two MCT1 monomers, such that the ‘C’ termini of EMMPRIN in the cytosol are close to the ‘C’ and ‘N’ termini of an associated MCT1 molecule respectively (Chen et al., 2001) (Fig 1.3).

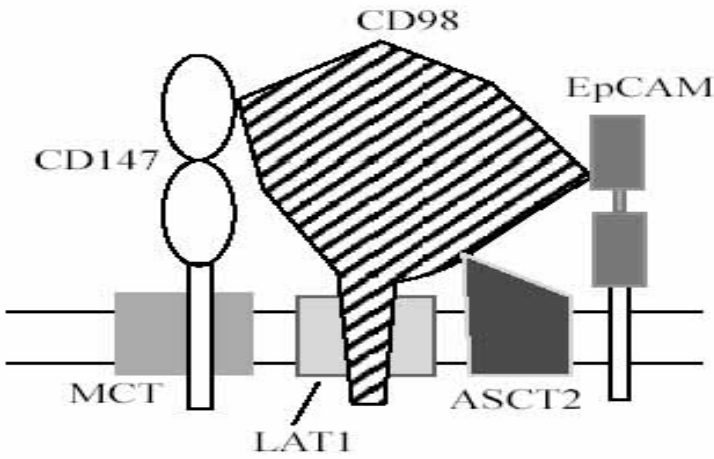


Figure 1.6: EMMPRIN and associated transporters. EMMPRIN associates directly with MCT1 and -4. CD98 heavy chain (hc) associates directly with LAT1(amino acid transporter) and epCAM (epithelial cell proliferation inducer) associates directly with CD98hc (Xu and Hemler, 2005).

Cross-linking studies demonstrated the homo-oligomer formation of basigin/EMMPRIN. EMMPRIN formed homodimers even in the cells that had been transfected with a mutant EMMPRIN, in which all the N-glycosylation sites were mutated (Jia et al., 2006b). Thus, it has been evident that for the oligomer formation in a cis manner, the first Ig domain is necessary and sufficient, while the N-glycosylation is not required (Yoshida et al., 2000).

1.2.3.3 Lectin activity

Basing on the homology between the first Ig domain of EMMPRIN and the fourth Ig domain of neural cell adhesion molecule (NCAM), shown to bind oligomannosidic glycans and the lectin domain of the mannose receptors (Miyauchi et al., 1990), EMMPRIN was shown to be an oligomannose-binding lectin. Conceivably, oligomannosidic carbohydrate and its recognition molecules are mediators of cis and trans interactions between cell surface glycoprotein in the nervous system (Kanekura et al., 1991). Indeed EMMPRIN interacts with the β2 subunit of Na^+/K^+-ATPase originally called adhesion molecule on glia (AMOG), through biding to its L3 epitope, possibly leading to EMMPRIN-dependent astrocytic outgrowth (Hunt et al., 2001).

1.2.3.4 Monocyte-dependent T cell de-activation through EMMPRIN and its associated CD98

The activation of T cells in response to antigen requires at least two types of signals to be provided by an antigen-presenting cell (APC) (Cho et al., 2001). The first of these is

mediated via the interaction of the antigen-specific T cell receptor (TCR) with a peptide that is associated with major histocompatibility complex (MHC) molecules on the surface of the APC. As a result of this interaction, the TCR-associated CD3 complex mediates intracellular signals that are necessary but not sufficient for T lymphocyte clonal proliferation (Mori et al., 2004). The second critical signal is mediated either by proteins on the T lymphocytes that interact with co-stimulatory molecules on the surface of the APC or by soluble cytokines produced by the APC acting on the T cell. Unlike TCR-mediated signals, these co-stimulatory signals are not antigen specific. The type of co-stimulatory signal that is delivered depends on several factors, including not only the type and activation status of the APC, but also the type of T cell ($CD4^+$ or $CD8^+$), and whether the T cells are of naive or memory phenotype (Woodhead et al., 1998).

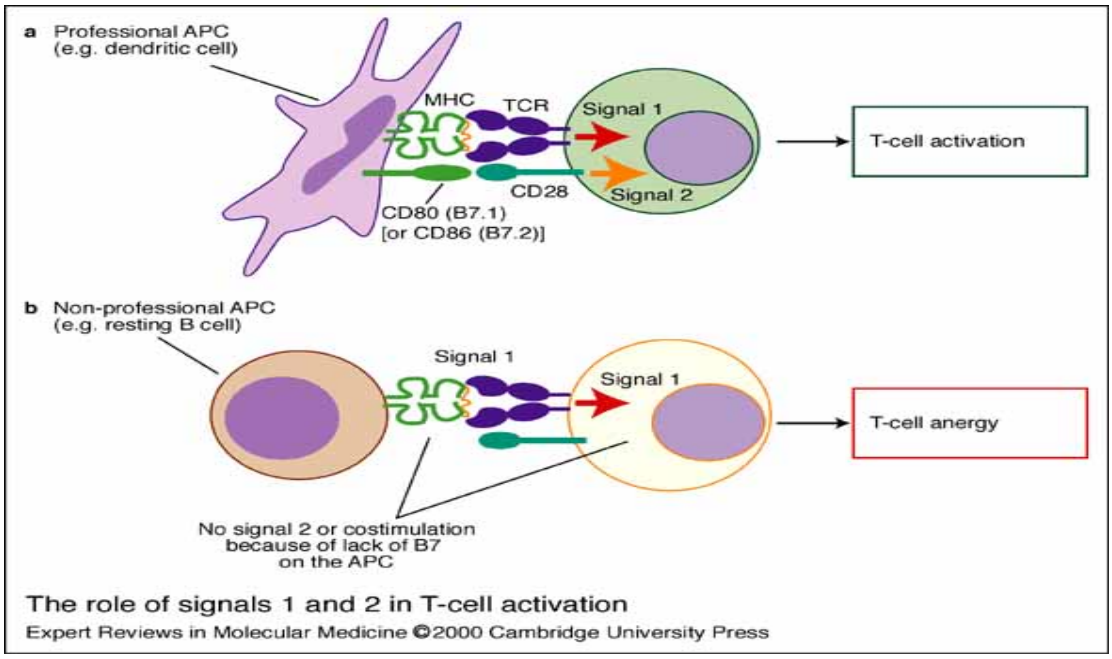


Figure 1.7: T cell activation and proliferation needs two signals. Signal-1 is through TCR in context of MHC class I and II. Signal 2 is mediated via co-stimulatory signals on APCs. Established co-stimulatory signals are via CD80 family. Novel co-stimulatory signals include EMMPRIN, CD98 and EMMPRIN (David Wraith et al., 2000).

Several cell surface co-stimulatory interactions have been implicated in T lymphocyte activation (Gaglia et al., 2001). The most thoroughly investigated are those between CD80 (B7.1)/CD86 (B7.2) on the APC, and CD28/CTLA-4 on T cells, between CD70 and CD27, between CD2 and CD58, CD59, or CD48, and between the β_2 integrins, especially α_1/β_2 (lymphocyte function-associated antigen-1; LFA-1; CD11a/CD18) and the intracellular adhesion molecules (ICAM) (Stonehouse et al., 1999). However, there have been several suggestions that there may well be other additional co-stimulatory molecules. An example of this is the previous study in which Johnson and Jenkins demonstrated that the monoblastoid U937 cell can act as an accessory cell providing co-stimulatory signals for T cell activation induced by antibody against

CD3. This costimulatory activity was independent of the CD80/CD86-CD28 pathway, as U937 cells do not express CD80, and express very low levels of CD86 (Johnson and Jenkins, 1994).

Two molecules newly identified here as being implicated in co-stimulation of both $CD4^+$ and $CD8^+$ T cells in this model are CD98 and EMMPRIN (Mori et al., 2004; Stonehouse et al., 1999). CD98 is a type II transmembrane glycoprotein which is important in cell survival during haemopoietic cell development, in the formation of human immunodeficiency virus-induced multinucleate giant cells, and has been shown to mediate a calcium influx when bound by galectin 3 (Woodhead et al., 1998). Although CD98 co-precipitation with CD3 in extracts derived from a human thymoma cell line suggests a role at the T cell level, pre-pulsing and T cell subpopulation experiments suggest that the effect is via APC. Further evidence that suggests CD98 co-stimulatory role is that it is expressed at much higher levels on U937 and on dendritic cells than on resting T cells (Stonehouse et al., 1999). Detailed phenotypic analysis showed that one CD98 mAb stained T cells more than U937 cells, and there were also different patterns of expression between U937 and dendritic cells with the same CD98 mAb, therefore raising the possibility that different isoforms of CD98 exist (Ge et al., 2007). These results are consistent with the reported role for CD98 as a control mechanism for integrin-mediated adhesion, and in co-stimulation in xenoactivation (Cho et al., 2001).

EMMPRIN is however widely distributed, including expression on mitogen-activated T cells, and EMMPRIN knockout mice have an increased mitogenic response in mixed leukocyte reactions (Lee et al., 2003; Schernthaner et al., 2005). CD98Ab pre-pulsing and T cell subset experiments suggest that EMMPRIN is more important as a costimulatory signal on an APC rather than on a T cell due to its association with CD98 (Khunkeawla et al., 2001).

It is not yet clear from these studies how the inhibitory effect of the antibodies on U937 function is mediated. One mechanism might be by blocking signalling into the APC, and hence production of a key mediator for sustaining proliferation or by altering a critical pattern of surface expression of known co-stimulatory molecules or by blocking direct receptor-ligand interaction with a molecule on the T cell surface (Xu et al., 1998).

Both the molecules CD98 and EMMPRIN appear at the site of inflammation and endothelial outgrowth. Their contribution may therefore be to play a parallel role in the induction of innate and adaptive immunity (Gwinn et al., 2006).

1.2.3.5 Monocyte-independent T cell de-activation through EMMPRIN and its associated proteins

EMMPRIN expressed on the plasmamembrane contains two Ig like domains and a single charge containing transmembrane domain (Chiampanichayakul et al., 2006). It is proved by chemical crosslinking that EMMPRIN associates with proton-coupled monocarboxylate transporter family members, MCT1 and MCT4 (Sepponen et al., 2006). MCT1 Interacts with GP70 (embigin), another member of basigin family (Kirat et al., 2006). Evidence for a close physical association between EMMPRIN and a specific monocarboxylate transporter is also provided by their colocalisation at the cell surface (Juel and Halestrap, 1999; Koho et al., 2002). The expression pattern of EMMPRIN in different tissues is compatible with its role in lactate transport (Juel and Halestrap, 1999). In highly glycolytic cells such as activated lymphocytes, an excess of lactic acid is produced which has to be exported from the cell to avoid a drop in intracellular pH. In these cells, the predominant monocarboxylate transporter is MCT4. Conversely, cells with high oxidative capacity such as heart and red skeletal muscle are rich in mitochondria and may utilize lactate as an energy source (Hashimoto et al., 2006). In these cells the net influx of lactic acid by MCT1 predominates.

EMMPRIN is a chaperone for monocarboxylate transporters (MCT1 and MCT4) and enables insertion of MCT1 and -4 into cell membranes, facilitating import and/or export of lactate and pyruvate (Korn et al., 2005). Like EMMPRIN, CD98hc (CD98 heavy chain, 4F2, FRP-1) also is a multifunctional glycoprotein with a single transmembrane domain, and is highly expressed on proliferating cells and functions as a chaperone for transporters. Furthermore, RNAi depletion of either EMMPRIN or CD98hc diminished cell surface expression of both molecules and decreased cell proliferation (Deora et al., 2005).

The increase in glycolytic rate observed in activated T cells is essential both for providing ATP for ATP-consuming processes and for supplying metabolic intermediates that are channeled through pentose-phosphate pathway to feed *de novo* nucleotide biosynthesis (Halestrap and Price, 1999). Significant reduction in glycolytic flux results from MCT1 blockade and thus the rapid phase of cell division cannot be sustained. T cells might be more sensitive than other cells to MCT1 inhibition owing to their reliance on aerobic glycolysis during lymphocyte activation to supply the high energy requirement for cell growth and proliferation, resulting in a large increase in lactate production (Xu and Hemler, 2005). Further due to intracellular entrapment of MCT1, acidification may occur resulting in metabolic starvation (Liebetanz et al., 2003).

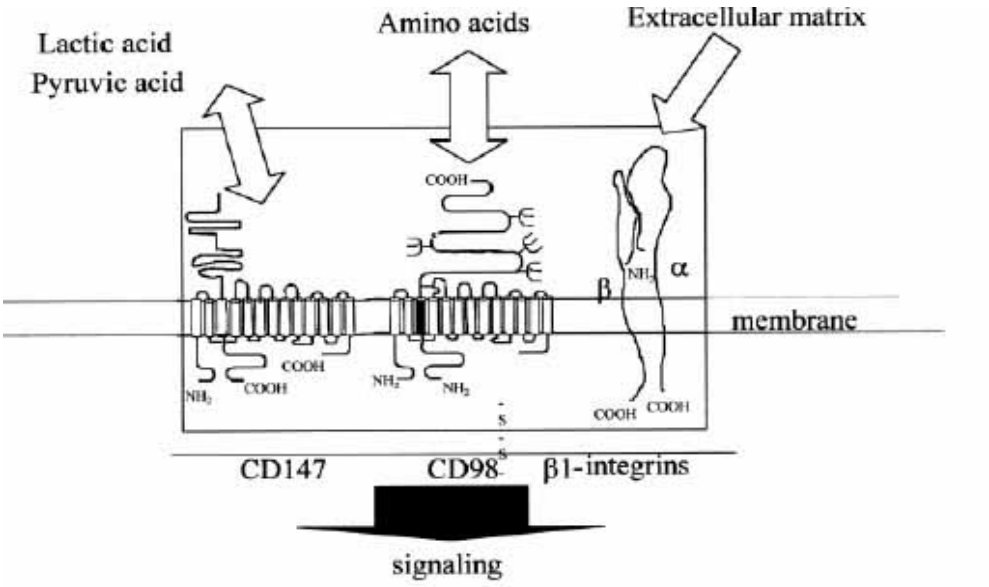


Figure 1.8: Regulation of T cell activation through EMMPRIN. EMMPRIN is associated with MCT1 which regulates lactic acid influx into the cell and CD98 which is associated with LAT1 regulates amino acid shuffle and β1 integrins which sustains adhesion of the cells (Cho et al., 2001).

1.2.4 Membrane translocation of EMMPRIN

Membrane targeting of EMMPRIN plays a critical role in exerting its multiple functions. However, the mechanism behind it has not been fully elucidated. Recent studies have outlined three possible mechanisms of membrane targeting -

- A glutamic residue 218 within the transmembrane domain of EMMPRIN, which shows a higher degree of cross-species conservation, is responsible for the association with MCT1 (Philp et al., 2003). Mutation of this gluatamate to arginine resulted in impaired translocation of EMMPRIN and MCT1 to the cell surface (Fig 1.3).

- A single leucine 252 was identified as the basolateral targeting motif in the cytoplasmic tail of EMMPRIN in madin-darby canine kidney cells (Wilson et al., 2005). However, this was not the same case in retinal pigment epithelium showing apical distribution of EMMPRIN. Thus the signal is individual and cell type specific suggesting its association with some cell-specific regulator of protein trafficking.

- Cyp60, a member of the cyclophilin family, was shown to be a regulator of the membrane EMMPRIN and is involved with proline 211 at the end of the transmembrane domain adjacent to the extracellular domain (Li et al., 2006b) (Fig 1.3).

1.2.5 EMMPRIN and signal transduction

Higher levels of EMMPRIN in cancer cells have recently been attributed to deregulation of epithelial growth factor receptor (EGFR) signalling (Menashi et al., 2003; Yurchenko et al., 2002). Amphiregulin, acting through interaction with EGFR promotes tumour progression through EMMPRIN-induced increases in production of MMPs by fibroblasts and endothelial cells (Wautier et al., 2007). EMMPRIN exists in both soluble and membrane bound forms and it signals via both membrane and soluble forms. Thus, stromal cells or fibroblasts that are far from source of tumour cells still signal through EMMPRIN via shed EMMPRIN (Haseneen et al., 2003). There are two different mechanisms reported for the shedding of EMMPRIN (Zucker et al., 2001):

- Metalloproteinase-dependent generation of a proteolytic cleavage of EMMPRIN lacking the carboxy terminus.
- Release of full-length EMMPRIN from the cell surface via micro vesicle shedding (Baj-Krzyworzeka et al., 2006).

Annexin II has been recently described as a binding partner and as a requisite for the activity of EMMPRIN in stimulating MMP production (Zucker et al., 2001). Thus, annexin II has been suggested to have a co-ordinating function in assembling proteases at the cell surface. Intercellular signalling pathways leading to MMP induction via EMMPRIN have been not so far clear. MMP-1 induction is through a MAPK p38-dependendent manner, while MMP-2 stimulation is through activation of phopholipase A2 and 5-lipoxygenase (Yan et al., 2005). Further investigation revealed that EMMPRIN stimulated cell survival pathway signalling, including phosphorylation of Akt, Erk and FAK. These effects of EMMPRIN were shown to be dependent on stimulation of hyaluronan production, a pericellular polysaccharide. The increase in anti-apoptotic signalling in-turn leads to increased multidrug resistance and is dependent on hyaluronan-induced ErbB2 and cell survival signalling pathways (Zucker et al., 2001).

1.2.6 Molecular regulation of EMMPRIN expression

EMMPRIN gene consists of seven exons and six introns spanning 7.5 kb. The 5´ upstream sequence of the EMMPRIN gene contains no TATA or CAAT box but has a CpG-rich island. A 470 bp fragment upstream of the coding region of EMMPRIN was shown to promote its

transcription. A 30 bp element of this sequence (-142 to -112 bp) which contains a binding site for Sp1, was also demonstrated to be important for EMMPRIN transcription (Vigneswaran et al., 2006). It has been recently reported that EMMPRIN upregulates its own expression through a positive feedback mechanism. Tumour cell surface EMMPRIN upregulates EMMPRIN expression on fibroblasts (Suzuki et al., 2004). This EMMPRIN dependent induction of EMMPRIN in cocultures of EMMPRIN positive MDAMB 231 (human breast cancer carcinoma) cells and fibroblasts was further enhanced or reduced by either stimulation or antisense suppression of EMMPRIN expression in tumour cells, respectively (Ge et al., 2006). In the lung carcinoma cell line NCI-H460, Phorbol-12-myristate-13 acetate (PMA)-treatment upregulated EMMPRIN expression and its pathway included protein kinase C, calcium mobilization and mitogen-activated protein kinase (MEK1/2) (Li et al., 2006a). Amphiregulin and Epidermal growth factor (EGF) were shown to upregulate EMMPRIN both at mRNA and protein levels in NS2T2A1 (human breast tumour) cells and this was downregulated by amphiregulin and EGFR antisense cDNAs. The nuclear protein, pinin, a nuclear and cell adhesion-related protein and a putative tumour suppressor (Ge et al., 2006), was shown to be a potential negative regulator of EMMPRIN as its induction caused downregulation of EMMPRIN.

1.2.7 EMMPRIN-associated cell surface molecules

The molecules that interact with EMMPRIN include caveolin-1 (Tang et al., 2004a), annexin II, MCT1, MCT4 the β2-sbunit of Na^+/K^+-ATPase, Cyp60 and CypA (Yan et al., 2005). In addition, EMMPRIN also interacts with integrins and MMP-1. Integrins - α3β1 and α6β1 associate with EMMPRIN possibly in a lateral fashion via the first Ig domain (EC I; Fig 1.3). EMMPRIN targets MMP-1 to invasive domains of the tumour cell probably by providing a docking site. Thus, EMMPRIN localized in the membrane of the tumour cell, might promote invasion by modifying the pericellular matrix of the tumour cell (Yan et al., 2005).

1.2.8 Therapeutic targeting of EMMPRIN

MMP induction and activation plays an important role in cancer invasion (Baj-Krzyworzeka et al., 2006), metastasis and migration of autoimmune cells (Hagemann et al., 2005) from peripheral compartment to the CNS environment. Blocking EMMPRIN would prevent migration of autoimmune T cells and cancer cells but the strategy of preventing MMP induction and activation by inhibitors did not yield positive results due to side effects (Zuckler

etal., 2001). Another strategy is to prevent overproduction of MMP for which EMMPRIN could be a correct molecule to target at as it is involved in homophilic and heterotyphic cell interactions (Jia et al., 2006a). Further the fact that EMMPRIN stimulates angiogenesis of tumours makes it an attractive target (Tang et al., 2005). Further, its role as a chaperone for MCT1 and MCT4 makes it an attractive target in any de-activation mechanism (Yan et al., 2005). The fact that the non-glycosylated ectodomain is incapable of stimulating MMP activity (Yu et al., 2006) in fibroblast-monocyte coculture clarifies the role of glycosylation in EMMPRIN function. Further MCT1, which is associated with EMMPRIN, causes entrapment of MCT1 in the cell (Xu and Hemler, 2005), which results in lactate accumulation leading to acidosis and metabolic starvation of cell. T cell therapy with anti-EMMPRIN monoclonal antibody ABX-CBL has been applied to 51 patients with steroid-refractory acute graft-versus-host disease after allogeneioc hemopoietic stem cell transplantation (Zucker et al., 2001). The results were encouraging: among 51 patients, 26 patients (51%) responded and 21 patients (41%) had extended partial responses.

Further, as blocking EMMPRIN completely will cause negative effects, it is essential to clarify the mechanisms involved in EMMPRIN downregulation, with special reference to its domains or structures responsible for each function, and to identify and function-specific counter receptors or cofactors. Glycosylation may be a key factor regulating MMP-inducing activity (Tang et al., 2004a).

Further EMMPRIN induces several malignant properties associated with cancer, including invasiveness, angiogenesis, anchorage-independent growth and chemoresistance. Consequently, the development of effective therapeutic interventions targeted to EMMPRIN would provide a novel and potentially powerful alternative to current treatments (Gwinn et al., 2006).

1.3 NEUROPROTECTION VIA DE-ACTIVATED T CELLS

Multiple sclerosis as an autoimmune disease of the central nervous system is characterised by demyelination and axonal loss (Diem et al., 2007). Neurological disability has been attributed to atrophy of spinal cord, cerebellum and cortex (Halder et al., 2007).

Axonal injury occurs early in the course of MS and correlates with the extent of inflammation within the CNS (Hemmer et al., 2006). This injury leads to transection of axons and to formation of axon spheroids at their proximal end. Recently neuronal death in cortical and thalamic MS lesions was observed (Laplaud and Confavreux, 2006). The morphology of

MS lesions suggests infiltration of perivascular T lymphocytes (Diem et al., 2007). In conjunction with the prevailing hypothesis of MS pathogenesis, preactivated T cells to autoantigens (myelin and no myelin antigens) (Hemmer et al., 2006) produce inflammatory mediators (cytokines and metalloproteinases) (Rebenko-Moll et al., 2006) that weaken the blood brain barrier and undergo clonal proliferation in the CNS due to antigen restimulation. This results in an inflammatory cascade leading to activation of microglia, infiltrating macrophages and B cells leading to axonal and neural damage through either bystander mechanism or Ag specific mechanism (Rebeles et al., 2006).

Evidence of neural toxicity due to activated T cells was well documented (Hendrix and Nitsch, 2007). The first evidence of susceptibility of neurons cell cytotoxicity was shown using mouse peripheral nervous system *in vitro*; only allogenic T cells were cytotoxic. More recently, syngenic T cells were shown to be toxic to neurons; however, this required expression of MHC class I on neurons with prior treatment of IFNγ and tetrodotoxin (Kotter et al., 2001). Cell contact mediated neuronal cytotoxicity to fetal neurons was reported (Lu et al., 2001). Most of neurons in the CNS do not express considerable amount of MHC-I. Most of the infiltrating T cells are non antigen specific, thus other means of neuronal cytotoxcity play a major role in the neuronal apoptosis (Ryu et al., 2003). Polyclonally activated T cells have a cytotoxic effect on neurons in an allogenic and syngenic system in the absence of added antigen. The activation of T cells was mediated through contact dependent non-MHC-I mechanisms.

1.4 STATINS

Statins (3-hydroxy-3-methylglutaryl CoA reductase inhibitors) are orally administered cholesterol-lowering agents and are effective in treatment of cardiovascular diseases (Kurian et al., 2006). Some of their immunosuppressive actions may be independent of cholesterol reduction including potential anti-proliferative, anti-inflammatory and immunomodulatory actions (Endres, 2006).

1.4.1 Mechanism of statin action

These drugs bind the 3-hydroxy-3-methylglutaryl CoA reductase leading to displacement of HMG-CoA, thereby inhibiting production of L-mevalonate (Wolfovitz, 2005). Further downstream inhibition of mevalonate also results in inhibition of biosynthesis of dolichol phosphate and the rate of N-linked glycosylation (Nath et al., 2004).

Statins as the inhibitors of (HMG-CoA) reductase, the rate limiting enzyme of the cholesterol biosynthesis pathway, have been shown to promote immunomodulatory effects *in vitro* and in animal models in vivo and if proven effective in controlled trials, may serve as a future treatment option in MS or RA (Mach, 2002). Statins reduce mevalonic acid, the product of HMG-CoA reductase and thus disrupt cholesterol-processing mechanisms. Mevalonate is a precursor not only to cholesterol synthesis but also to intermediate lipid donors that are important for the isoprenylation of guanosine triphosphate (GTP)-binding proteins, such as Ras, Rap1a, RhoA and RhoB (Peng et al., 2006). These small GTPases from the Ras superfamily require lipid modifications for membrane tethering and subsequent interaction with downstream effector cascades (Steffens and Mach, 2004). Only then are they capable of regulating their diverse cellular functions, which include cell survival proliferation, differentiation and cytoskeletal organisation. In this process, statins deplete cells of isoprenoids and dolichol. Isoprenoids derived from farnesyl pyrophosphate or geranylgeranyl pyrophosphate form important lipid attachments for membrane translocation of small guanosinetriphosphate (GTP) binding proteins such as Ras or Rho (Neuhaus et al., 2005b). Only if isoprenylated, these GTP binding proteins are able to promote major cell functions vital for cellular activation. For example, farnesyl pyrophosphate is necessary for the prenylation of Ras, and consequently, the association of Ras with the cell membrane. In contrast, if isoprenylation is inhibited by statins, these GTP binding proteins accumulate inactive in the cytosol (Dunn et al., 2006). Consistently, treatment with farnesyl transferase inhibitors results in cytosolic Ras accumulation and decreased signalling in response to ligand. Dolichol is required for correct N-glycosylation. Reduction of mevalonic acid synthesis results in the depletion of dolichol phosphate. Dolichol phosphate acts as a carbohydrate donor during N-linked glycosylation of membrane-targeted protein.

1.4.2 Pathways inhibited due to statin action

Depletion of mevalonic acid by statins inhibits the formation of mevalonic acid thereby disrupting many pathways (Kurian et al., 2006). Mevalonate, a key intermediate of the cholesterol pathway is a major product blocked by statins. Further farnesyl pyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) are substrates for the posttranslational isoprenylation of various proteins involved in cell signalling. The major products that were formed from mevalonate are:

1. Two molecules of FPP condense into ‘’head to head” fashion to form squalene.

2. FPP extends “head to tail “with isopentenyl pyrophosphate groups or short-chain polyprenosyl to form long chain isoprenyls which results in formation of ubiquinone.

3. Repeated addition of farnesyl pyrophosphate can be converted into the long-chain polyisoprenyl alcohol, dolichol.

4. A fourth product of mevalonate metabolism is formation of isopentenyl adenosine, a constituent of transfer RNA.

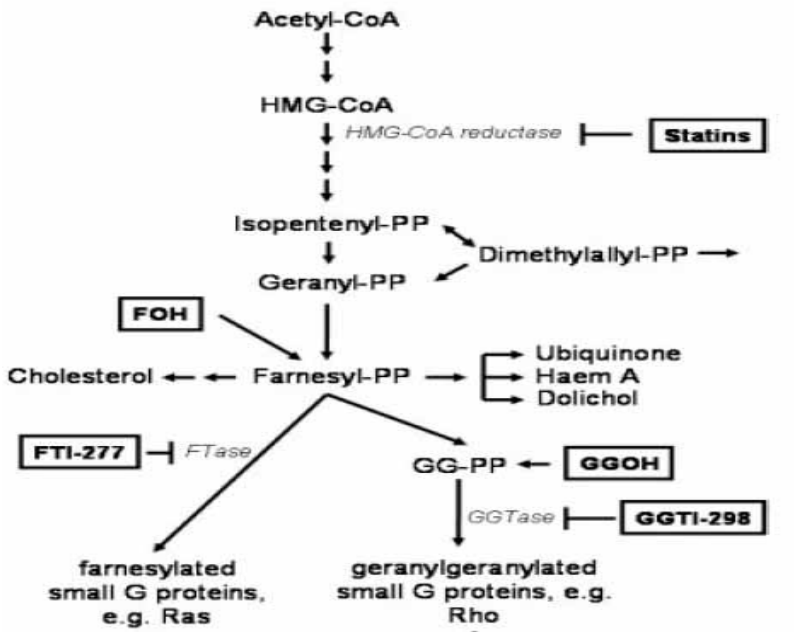


Figure 1.9: Cholesterol pathway. Acetyl-CoA converts to HMG-CoA, which is then acted by HMG-CoA reductase to convert to mevalonate. Mevalonate then results in formation of farneysl pyrophosphate, which is the main branch point for generation of farnesylated proteins, Ras proteins and there are side chains leading to generation of ubiquinone, dolichol and haem A. A side pathway of farnesyl pyrophosphate leads to generation of Geranylgeranyl pyrophosphate, which generates Rho proteins. Squalene is formed from FPP to form cholesterol (Kurian et al., 2006).

1.4.3 Immunosuppression by statins

Statins were reported to have immunosuppressive activities and the effects were believed to be mediated through either isoprenylation or depletion of cholesterol intermediates. Following are the important effects of statins in important disease conditions.

1.4.3.1 Anti-inflammatory actions of statins in atherosclerosis

Hypercholesterolemia is an established risk factor for atherosclerosis and coronary artery disease (Endres, 2006). Patients with atherosclerotic coronary artery disease also exhibit elevated plasma levels of inflammatory cytokines, enhanced activation of circulating leukocytes and extensive infiltration of macrophages and T cells into atherosclerotic plaques, reflecting the important role of inflammation in atherogenesis (Kurian et al., 2006). Although the clinical benefits of statins in cardiovascular disease have been predominantly attributed to their lipid-lowering properties, new data indicate that other properties, especially immunomodulatory actions, might also exert beneficial effects. At sites of atherosclerotic lesions (Mitsdoerffer et al., 2005), endothelial cells and leukocytes increase their expression of numerous adhesion molecules and counter receptors, such as the intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) (Mach, 2002), triggering the migration of leukocytes into the vascular vessel wall. Statins inhibit expression of both ICAM1 and its ligand, lymphocyte function-associated antigen 1 (LFA1) (Weitz Schimidt et al., 2001).

1.4.4 Statins in multiple sclerosis and rheumatoid arthritis

The potent immunomodulatory activity of statins indicates that these drugs might have therapeutic potential in immune disorders other than atherosclerosis (Sena et al., 2003). In support of this hypothesis, a beneficial effect of statins was observed in experimental mouse models of multiple sclerosis and rheumatoid arthritis. In multiple sclerosis, acute inflammatory injury of the central nervous system (CNS) followed by partial repair and recovery of function produces a clinical course of relapsing-remitting neurological symptoms (Vollmer et al., 2004). In EAE atorvastatin not only prevented, but also reversed, chronic and relapsing paralysis. This effect was associated with pleiotropic immunomodulatory effects in atorvastatin-treated mice, including inhibition of major histocompatibility complex (MHC) class II upregulation, and secretion of T helper type 2 (Th2) cytokines and transforming growth factor β (TGF-β) (Weber et al., 2006). Atorvastatin also induced phosphorylation of signal transducer and activator of transcription (STAT) 6, which is required for commitment to Th2 lineage (Stanislaus et al., 2002). Conversely, phosphorylation of STAT4 - a key player in commitment to T helper type 1 (Th1) lineage and secretion of Th1 cytokines were inhibited. Similarly, in an RA model, statin treatment markedly inhibited collagen-induced arthritis via suppression of Th1 humoral and cellular responses (Busso N., et al., 2004).

A key characteristic of neuroinflammatory responses in the CNS is infiltration of leukocytes from the vasculature to the neural parenchyma (Stanislaus et al., 1999). Rho proteins, which must undergo post-translational prenylation to become functional, have been shown to be essential for transendothelial migration in the CNS. Lovastatin treatment of CNS endothelial cells substantially attenuated lymphocyte migration *in vitro* (Greenwood et al., 2003).

2 AIM OF THE STUDY

Activated encephalotogenetic T cells are the primary cause in the progression of pathogenesis of MS. These autoimmune T cells activate monocytic cells, macrophages and dendritic cells producing pro-inflammatory mediators, leading to disruption of BBB. Once in the CNS environment, autoreactive T cells get reactivated by a number of mechanisms finally causing neurodegeneration. Statins are therapeutic agents against cardio-vascular complications, with positive effects in EAE.

The primary aim of the thesis is to delineate the de-activation mechanisms of statins in immune cells, namely monocytes and T cells via EMMPRIN generated signals. The study was broadly categorised into three parts

1) In the first part of the thesis, anti-inflammatory effects of statins on MMP activation and their regulation in monocytes was explored.

2) In the second part of the thesis, de-activation of T cells through inhibition of EMMPRIN and MCT1 expression and function was studied.

3) In the last part of thesis, statin modulated T cell-neuroprotection was analysed.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 General chemicals

Table 1: General laboratory chemicals

Compounds	Manufacturer
Atorvastatin	Pfizer
Alpha-cyano-4 hydroxycinnamate (CHC)	Sigma
β-Mercaptoethanol	Sigma
3MM Whatman paper	Schleicher and Schuell
Ammonium persulfate (APS)	Promega
Bradford reagent	Bio-Rad
Bromophenol blue	Sigma
Complete™ (Protease inhibitor cocktail tablets)	Roche
Cytofix/Cytoperm®	Becton Dickinson
Dimethyl sulfoxide (DMSO)	Sigma
Dithiothreiotol (DTT)	Promega
Dolichol	Larodan
Dual colour precision protein standards	Bio-Rad
ECL western blotting detection reagents	Pierce
Eythelene diamino tetra acetic acid (EDTA)	Promega
Farnesyl pyrophosphate (FPP)	Sigma
Farnesyl transferase Inhibitor-277 (FTI)	Calbiochem
Ficoll-hypaque	Pharmacia
Fluvastatin	Sigma
Glass fiber filters (Filter mat 11731)	Skatron Instruments
Geranylgeranyl pyrophosphate (GG –PP)	Sigma
Geranylgeranyl transferase inhibitor-296 (GGTI)	Calbiochem
Isopropanol	Merck
Methanol	Fluka

Mevalonate	Sigma
Pravastatin	Sigma
Phloretin	Sigma
Rotipherose® gel 30	Roth
Sodium dodecyl sulfate (SDS)	Promega
Sodium ortho vanadate	Sigma
Squalene	Sigma
Nitro-cellulose membrane	Bio-Rad
Igepal (Nonidet P-40)	Sigma
Phosphate-buffered saline (PBS)	PAA
TEMED	Bio-Rad
Tris	Roth
Triton X-100	Sigma
Tunicamycin	Sigma
Tween-20	Sigma
Water-soluble cholesterol	Sigma

3.1.2 Other materials

Table 2: Fluorescent/radioactive probes

Compounds	Manufacturer
CFSE	Molecular probes
Cholera-toxin FITC	Sigma
DioC18	Sigma
BCECF	Molecular probes
$^{3}H^{1}$ Thymidine	Amersham Life Sciences
Propidium iodide	Sigma

3.1.3 Cell culture reagents

Table 3: Cell culture reagents

Compounds	Manufacturer
Trypsin-EDTA	PAA
Dulbecco's modified eagle medium (DMEM)	Gibco
RPMI	Gibco
Fetal calf serum (FCS)	PAA
Glutamine	PAA
Pencillin-streptomycin	PAA
phorbol-12-myristate-13 acetate (PMA)	Sigma
Concavalin A (Con A)	Sigma
Phytoheamagglutinin (PHA)	Sigma
Pokeweed mitogen (PWM)	Sigma
Staphylococcus enterotoxin A (SEA)	Sigma

3.1.4 Peptides

AP-9 is a EMMPRIN-antagonistic peptide (Zhinan Chen, 2005) with the amino acid sequence YKLPGHHHHYRP and a molecular weight of 1541.09 Da. CB-2 is a negative control for AP-9 (Zhinan Chen, 2005) with the amino acid sequence LHRHSHGHSYTS and a molecular weight of 1389.50 Da. The purity of the peptides synthesised by standard methods was >95 % as assessed by high-pressure liquid chromatography. The peptides were synthesised in the BMFZ (Düsseldorf).

3.1.5 Cell lines and media

Two different monocytic cell lines THP-1 (kind gift from Dr Angelika Bierhaus, University of Heidelberg, Germany) and U937 from American Type Cell Culture (ATCC) were used as a source of monocytic cell lines. All these cell lines were maintained in 5% FCS complete RPMI medium.

Peripheral blood mononuclear cells (PBMCs) were used as a primary T cells in monocyte-T cell costimulation assays. Both the cell types were maintained in complete RPMI media.

SH-SY5Y (ATCC) is a human neuronal cell line cultured in 1:1 RPMI medium and F12 medium supplemented with glutamine and pencillin. LN-18(ATCC), an astrocyte cell line used as a comparitor for neuronal cell death was cultured in RPMI and DMEM medium (1:1) supplemented with glutamine, penicillin, MEM sodium pyruvate and sodium carbonate.

3.1.6 Antibodies

Table 4: Primary and neutralising antibodies

Description	Supplier
EMMPRIN	Immunotools
EMMPRIN FITC	Immunotools
CD14 PE	Immunotools
CD29 FITC	Immunotools
CD 98 PE	Immunotools
MCT1	Biogenesis
Neutralising Antibodies	
EMMPRIN (MEM6/6)	Immunotools
EMMPRIN (H2K)	Biolegend
MHC -HLADR	Serotec
MHC-HLA ABC	Serotec
CD11b (MEM174)	Immunotools

Table 5: Secondary antibodies

Description	Supplier
Goat anti mouse FITC	Jackson Immunoresearch
Goat anti rabbit FITC	Immunotools
Rabbit anti mouse HRP	Pierce
Mouse anti rabbit HRP	Pierce

3.2 METHODS

3.2.1 Monocyte activation

3.2.1.1 Cell culture

All experiments were performed in the human premonocytic cell line THP-1 and other monocytic cell lines K562, monomac and U937 (Zhou et al., 2005). Cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 1% penicillin/streptomycin and 2% L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2. For induction of THP-1 differentiation, cells (2-3 x 10^6) were seeded and activated with 200 nM (PMA) for 24 h (Zhou et al., 2005). After incubation, non-attached cells were aspirated, and the adherent cells were washed three times with medium. THP-1 cells without PMA (undifferentiated cells) were used as a control. For statin and other rescue experiments, cells were treated with statins and/or inhibitors as indicated in the figures.

3.2.1.2 Cytofluorometric analysis

The expression of EMMPRIN and CD14 for monocyte de-activation and other cell surface antigens for T cell de-activation was determined by flow cytometry. THP-1 cells and PBMCs were washed with FACS buffer (PBS with 2% FCS) and incubated with respective antibodies and CD14PE for 45 min. For experiments involving unconjugated antibodies, cells were first treated with primary antibody, incubated for 30 min on ice, washed 3 times in FACS buffer and then incubated with secondary antibodies for another 30 min. Unless otherwise mentioned all the antibody dilutions were 1:100 of the primary stock. After washing three times with staining buffer, cells were analysed on FACS Scan (Becton Dickinson) using Cell Quest® software. Permeabilisation of cells was achieved by intially fixing in a buffer containing paraformaldehyde and saponin (cytofix and cytoperm). Cells were then analysed for intracellular and extracellular expression of antigens by flow cytometry.

3.2.1.3 Gelatin zymography

THP-1 cell supernatants were used to monitor the cellular MMP production. To assess MMP secretion, supernatants were collected after incubation with statins or controls, and MMP activity was determined by SDS-polyacrylamide gel zymography. Samples were centrifuged to remove cellular debris, and supernatants were collected and stored at -20 °C. Ten µl of

supernatant was mixed with 10 µl of SDS loading buffer and pipetted into 10% polyacrylamide gel containing 0.1% gelatin (Sigma). Positive controls for MMP-2 and -9 were used as standards. After electrophoresis at 125 V for 150 min, the gel was renatured in a renaturating buffer containing 25% Triton X-100 for 30 min. After equilibrium in developing buffer for 30 min, fresh developing buffer was added, and the gelatin containing gel was allowed to develop overnight at 37 °C. The gelatin gels were stained with 0.5% coomassie blue (Sigma) and destained with buffer containing of 10 % acetic acid, 50% methanol and 40% distilled water for 30 min, to visualise the zymogen bands produced by MMP digestion. An image of each gel was scanned after drying. To validate that the zymogram bands represented MMP activity, 20 mM EDTA (Sigma) was added to the developing buffer to abolish catalytic activity and 0.1 µM P-aminophenylmercuric acetate (known to convert latent zymogens to their active forms), was also added to the developing buffer.

3.2.1.4 Western blotting

3.2.1.4.1 Protein isolation from cells

Medium was aspirated from the cells and the cells were washed twice with ice-cold 1× PBS. Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% (v/v) nonidet P-40 containing a mixture of protease and phosphatase inhibitors. The lysates were vortexed every 5 min for a total span of 40 min and centrifuged at 13,000 rpm for 15 min. The supernatant protein was measured and stored at -80 °C.

3.2.1.4.2 Protein separation by poly acrylamide gel electrophoresis (PAGE)

The separating gel mixture was poured between two glass plates with spacers between and allowed to polymerise. The stacking gel mixture was poured on the top of separating gel and the comb was inserted in the gel to form the wells (table 6). The gel was run in 1× laemmli-running buffer (25 mM Tris-Cl, pH 8.3, 0.2 M glycine and 0.1% (v/v) SDS) until the desired distance had been reached.

3.2.1.4.3 Immunoblot

After gel electrophoresis, proteins were transferred to a nitrocellulose membrane using a Bio-Rad transfer chamber containing transfer buffer (24 mM Tris base, 193 mM glycine, 10% v/v) methanol). Transfer was performed at 4 °C overnight at 12 V.

Blots were incubated for 1 h in blocking buffer (5% (m/v) non-fat dry milk powder in PBS with 0.1% (v/v) tween-20 at room temperature. Incubation with the respective primary antibodies (1 µg/ml) was in blocking buffer for 1 h at room temperature. After 3 × 10 min wash with wash buffer (PBS - 0.1% (v/v) tween-20), blots were incubated with the respective HRP-labeled secondary antibodies at room temperature for 1 h. After 3 × 10 min washing with wash buffer, the blots were developed by enhanced chemi-luminescence and visualised with hyperfilm ECL.

To re-probe with another antibody, the blots were stripped with stripping buffer (0.063 M Tris-Cl pH 6.8, 2% (m/v) SDS, 0.8% DTT) at 50 °C for 30 min, and then probed as described above.

Table 6: Composition of SDS-PAGE gels

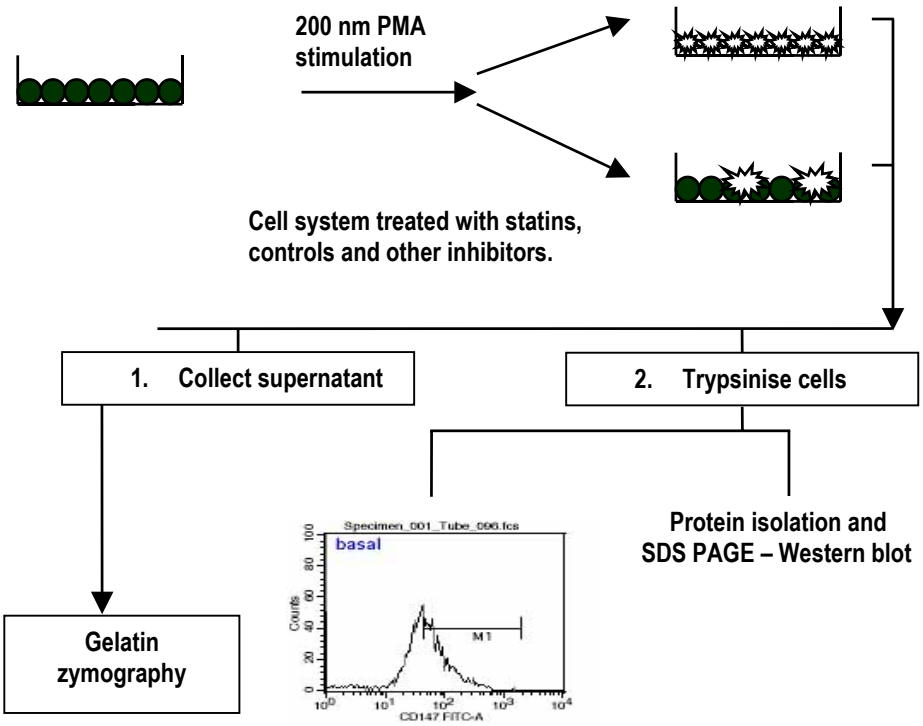
Composition of 10% resolving gels (40 ml)		**Composition of 5% stacking gels (20 ml)**	
Component	**Volume**	**Component**	**Volume**
Distilled water	15.9 ml	Distilled water	13.6 ml
30% acrylamide mix	13.3 ml	30% acrylamide mix	3.4 ml
1.5 M Tris-Cl (pH 8.8)	10 ml	1 M Tris-Cl (pH 6.8)	2.5 ml
10% (m/v)SDS	400 µl	10% (m/v) SDS	200 µl
10% (m/v)APS	400 µl	10% (m/v) APS	200 µl
TEMED	16 µl	TEMED	20 µl

3.2.1.5 Cell surface biotinylation

To assess the proportion of EMMPRIN that reached the cell surface, THP-1 cells were seeded at 5 x10^6 in a 40 mm dish, differentiated with PMA and treated with 10 µM of statins or tunicamycin, respectively. Cells were then washed three times with cold PBS and slowly agitated with 1 mg/ml biotin (Sulfo-NHS-Biotin; Pierce) for 30 min on ice. Cells were then lysed in modified radioimmunoprecipitation buffer (mRIPA; 50 mM Tris-HCl, [pH 8], 150 mM NaCl, 1% NP-40, and 1% sodium deoxycholate, containing 1 mM Na_3VO_4 including Complete™

protease inhibitor cocktail [Roche]). Aliquots (100 µg of protein) were incubated at 4 °C for 4 h with end-over-end shaking in the presence of streptavidin beads. The beads were thoroughly washed and then resuspended in 30 µl of Laemmli sample buffer. Proteins were analysed by SDS-PAGE and western blotting.

3.2.1.6 Experimental design - monocyte de-activation



3.2.2 T CELL ACTIVATION

3.2.2.1 Isolation of T cells

Peripheral blood was isolated from healthy donors with informed consent and PBMCs were isolated by density gradient centrifugation using ficoll-hypaque. Isolated PBMCs were suspended in complete RPMI media and incubated at 37 °C for 2 h. The adherent cells were removed to enrich the T cell population. The non-adherent cell population was further treated with 5 mM methyl leucine ester for 45 min to deplete remaining monocytes and APCs. The obtained lymphocyte T cell population was assessed by flow cytometry for CD3 positive cells, which were usually greater than 90%.

The obtained T cell population was activated by anti-CD3 antibody for specific activation through T cell receptor (TCR) and polyclonal activation was achieved by Con A.

3.2.2.2 T cell proliferation - radio(methyl-^{3}H)-thymidineincorporation assay

Lymphocyte proliferation assays were performed in triplicates. After incubation at 37 °C, 5% CO_2, the cells were pulsed with 1 µCi per well of (methyl-^{3}H)-thymidine complete RPMI. ^{3}H-thymidine incorporation was measured in a liquid scintillation counter (Wallac MicroBeta Trilux) after harvesting the cells onto glass fibre filters. The results were expressed as mean counts per minute/culture ±SD. Results were expressed as raw data or as 'lymphocyte stimulation index', which is the geometric mean cpm of the cells plus mitogen or anti-human CD3 antibody divided by the geometric mean cpm of the cells without mitogen (medium alone).

3.2.2.3 CFSE - proliferation assay

The succinimidyl ester of carboxy fluorescein diacetate is commonly referred to as CFSE. Stocks of 10 mM in DMSO were supplied with the kit. T cells free of monocyte population were incubated with CFSE such that the final concentration of CFSE was 2 µM in 0.8% FCS RPMI medium. The cells were prewarmed to 37 °C and the incubation was terminated by adding 5% FCS RPMI media. The cells were washed three times and finally the cells were loaded with CFSE. As the cell divides in response to stimuli, the dye also divides among daughter generations. FACS gating of the dilutions can thus assess the number of generations the cell has passed. Later, the cell populations were analysed by FACS and the proliferation was assessed as the total percentage of cells in dividing phase in relation to the control (no stimulation).

3.2.2.4 Lactate estimation

3.2.2.4.1 Intracellular pH and metabolic starvation assay

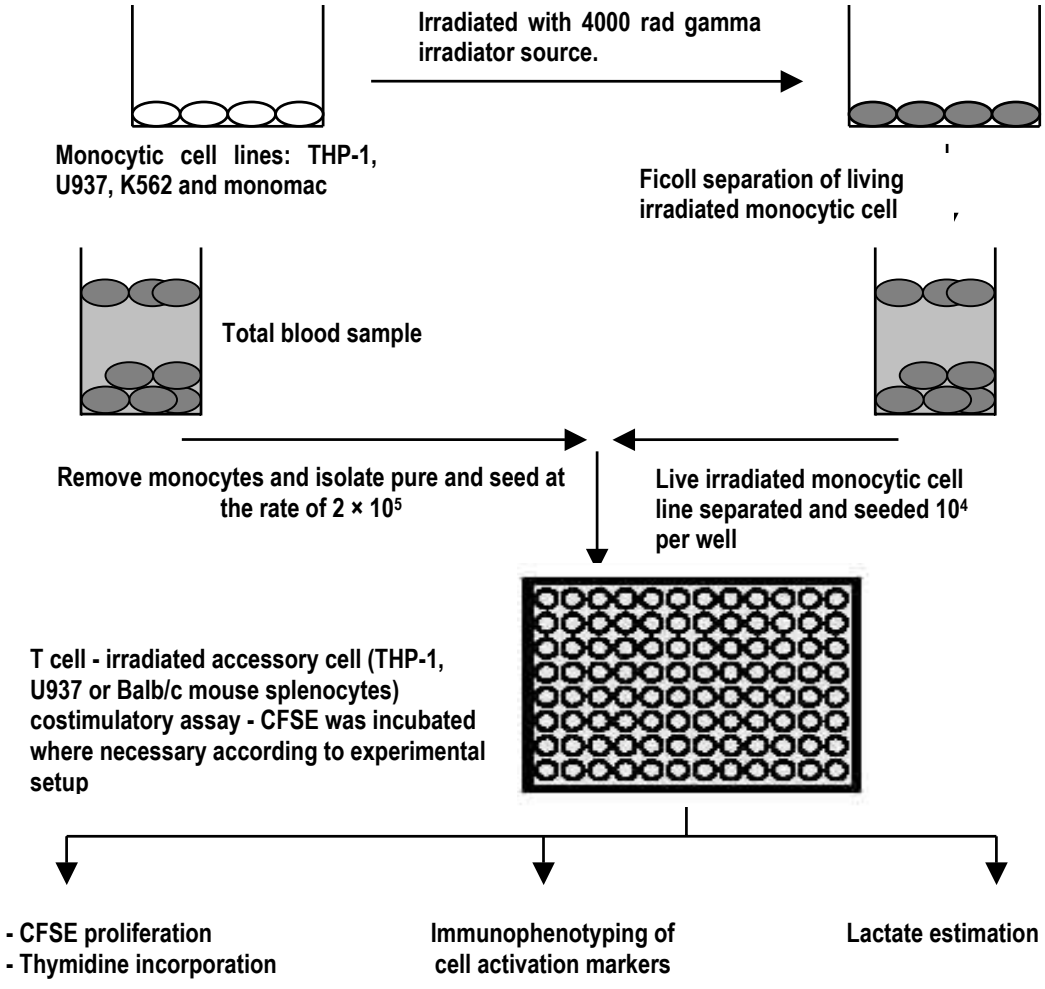
Cells (1 x 10^6 cells) were loaded with 1 µM BCECF (Molecular probes) after pre-treatment with statins and/or other inhibitors and incubated for 1 h in tyrode buffer, pH 7.4. The ratio of fluorescence obtained at 535 nm(FL1) after excitation at 490 nm(FL2) was measured at basal level to estimate intracellular pH after statin treatment. Further, an exogenous source of lactic acid (L-lactic acid) was added in increasing concentrations in a time

mode and intracellular pH_i (intracellular pH was estimated as the ratio of fluorescence in a time mode using BD FACS Scan. The change of pH_i was determined from a calibration curve, using buffer containing nigercin to equilibrate internal and external pH. Phloretin and alpha-cyano-4 hydroxycinnamate (CHC) were used as positive controls for MCT1 inhibition.

3.2.2.4.2 Lactate assay

Lactate reagent is used for the quantitative, enzymatic determination of lactate in solution at 540 nm. The principle of the assay is that the lactic acid derived from cell metabolism is converted to pyruvate and hydrogen peroxide (H_2O_2) by lactate oxidase. In the presence of the H_2O_2 formed, peroxidase catalyses the oxidative condensation of chromogen precursors to produce a coloured dye with absorption maximum at 540 nm. The increase in absorbance at 540 nm is directly proportional to lactate concentration in the sample.

3.2.3 Experimental design for T cell activation - costimulatory assay



3.2.4 NEUROPROTECTION

3.2.4.1 Human neuronal and astrocyte cell line cultures

SH-SY5Y is a human neuronal cell line cultured in 1:1 RPMI medium and F12 medium supplemented with glutamine and pencillin. NB-1 was cultured in RPMI and DMEM medium supplemented with glutamine, penicillin, MEM sodium pyurvate and sodium carbonate. Both neuronal cultures were seeded at a density of 30,000 cells/well in 24 well plates and 10,000/well in 96 well plates. LN-18 is a human astrocyte cell line incubated in RPMI supplemented with glutamine and penicillin.

3.2.4.2 Neuronal apoptosis coculture assay

Activated T cells were coincubated with 30,000 neuronal and astrocyte cell lines at different ratios. Cell-cell contact-mediated neuronal apoptosis was monitored through phase contrast microscopy and live imaging microscopy (Nikon TE 200) used to monitor cell-cell mediated contact.

3.2.4.3 Coculture assay with statin treated T cells

T cells isolated as described above were incubated with 10 μM statins (pravastatin, fluvastatin and atorvastatin) and with 100 μM mevalonate to prove statin specific effects. T cells were then activated with anti-CD3 mAb and mitogens, to activate the cells. Immunomodulated T cells treated with statins were then incubated with the neuronal cultures.

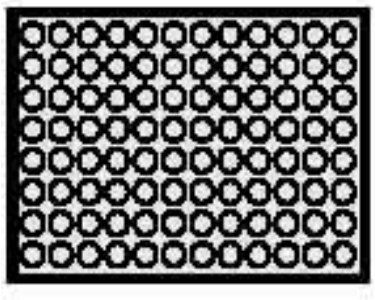
3.2.4.4 Antibodies used to attenuate T cell cytotoxicity via monocytes

T cell inactivation was also achieved by incubating T cells with neutralising EMMPRIN (MEM6/6), CD98 and CD11b antibodies to inhibit T cell activation. Neutralising antibodies against MHC are MHC-HLADR and MHC-II is used to block MHC antigens on T cells. Neutralising antibody against cell adhesion (CD11b) was used to block expression on both neuronal cells and T cells.

3.2.4.5 MTT assay

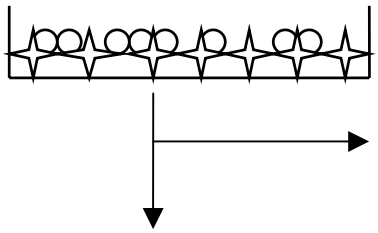
Neuronal toxicity was evaluated using the [3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide)] MTT spectrophotometric assay after exposure of neurons to activated T cells. 15 µl of 5 mg/ml MTT was used per well and incubated for further 2 h. 200 µl of dimethyl sulfoxide (DMSO) was added to each well and the solubilised product was measured at 550 nm after 10 min incubation in an ELISA reader (Multiscan ES, ThermoElectro corporation).

3.2.5 Neuroprotection model



Day 0: 100,000 PBMCs were preincubated with statins and activated with mitogens for 3 days.

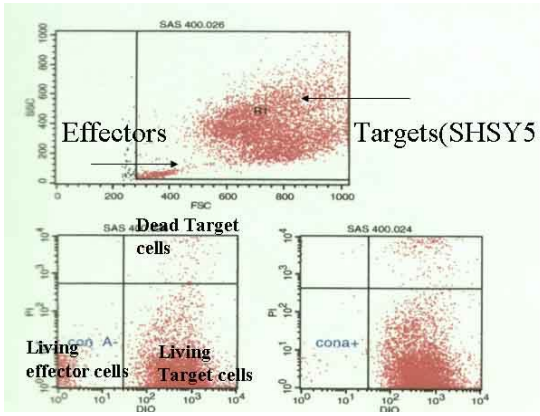
Day 2: 30,000 SHSY5Y were labeled with/without DioC18 fluorescent dye and incubated in a 24 well plate.



Day 3: statin treated PBMCs were activated and coincubated with SHSY5Y cell line

MTTassay

Flow cytometric estimation of neuronal cell death in a coculture assay.



4 RESULTS

4.1 MONOCYTE DE-ACTIVATION

4.1.1 Alteration of morphology of PMA-differentiated THP-1 cells by statin treatment

Phenotypically, naive THP-1 cells are round in shape, non-adherent and free floating in culture plates (Fig 4.1a). Differentiation with 200 nM PMA for 24 h resulted in change of morphological phenotype of naive cells to adherent differentiated cells. Differentiated THP-1 cells became flat, elongated, amoeboid, and adherent with extended processes (Fig 4.1b).

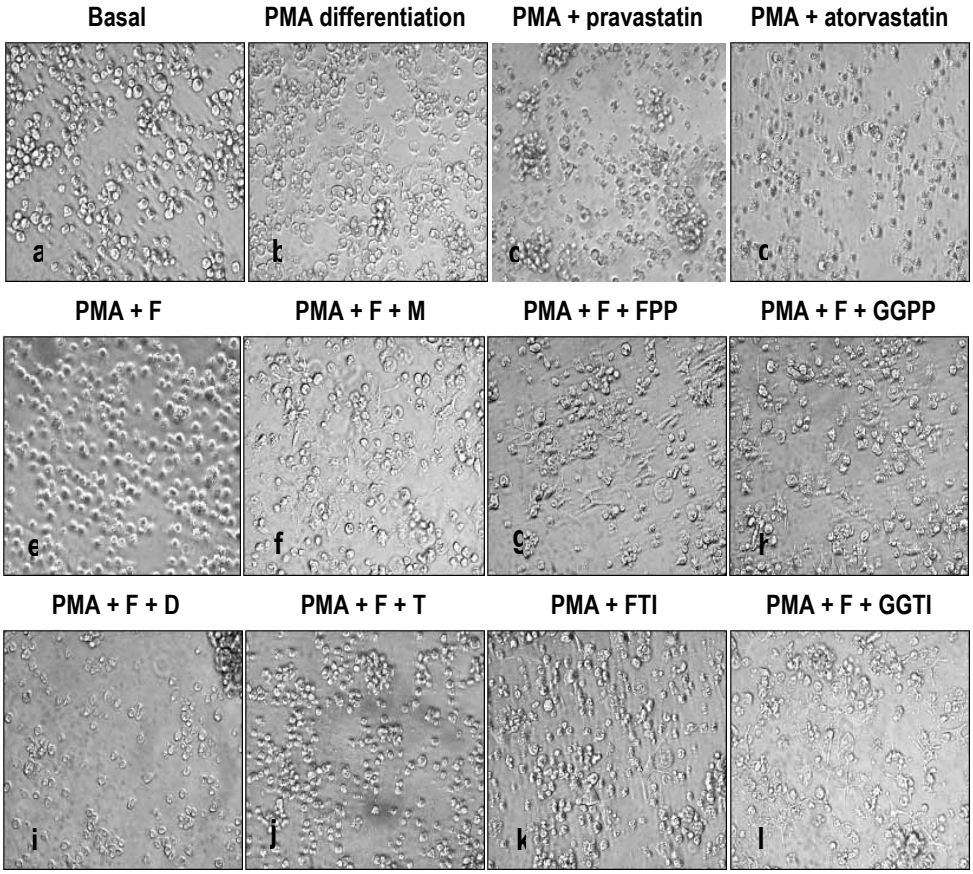


Figure 4.1: De-activation of THP-1 cells by statins. THP-1 cells were either activated or not with PMA (200 nM) and then incubated with statins (10 µM) or the relevant rescues as indicated. F-fluvastatin; M-mevalonate; FPP-farnesyl pyrophosphate (10 µM); GGPP-geranylgeranyl pyrophosphate (10 µM); D-dolichol (10 µM); T-tunicamycin (10 µM); FTI-farnesyl transferase inhibitor (10 µM); GGTI-geranylgeranyl transferase inhibitor (10 µM). Cell morphology was assessed by light microscopy. (Magnification 20x). Results are from one representative of three experiments.

In contrast, treatment with different statins, pravastatin (hydrophilic), atorvastatin and fluvastatin (hydrophobic), followed by differentiation with PMA resulted in a phenotype similar to undifferentiated THP-1 cells with additional bunched clusters, which were most pronounced with fluvastatin and pravastatin (Fig 4.1, c-e). We next tried to dissect the cholesterol pathway

responsible for the inhibition of differentiation. Therefore, rescue experiments (addition of different intermediate components of the cholesterol pathway namely (FPP, GGPP, mevalonate and dolichol) to inhibit statin inhibited differentiation. Mevalonate, FPP, GGPP as well as dolichol reversed the effects of fluvastatin by decreasing degree respectively and induced a morphology similar to differentiated THP-1 cells (Fig 4.1, f-i). Similar to statins, tunicamycin (an inhibitor of N-linked glycosylation, which mimicks the depletion of the intermediate component - dolichol, of the cholesterol pathway) and the isoprenylation inhibitors FTI and GGTI prevented differentiation of THP-1 cells (Fig 4.1, j-l).

4.1.2 Alteration of cell surface markers in PMA-differentiated THP-1 cells by statin treatment

Next, in order to assess the activation status of the cell, the cell surface expression of various markers was tested. Out of them EMMPRIN (activation marker) and CD14 (differentiation marker) displayed pronounced differences (Fig 4.2 & 4.3) and this change in expression correlated with the morphological changes as shown in figure 4.1.

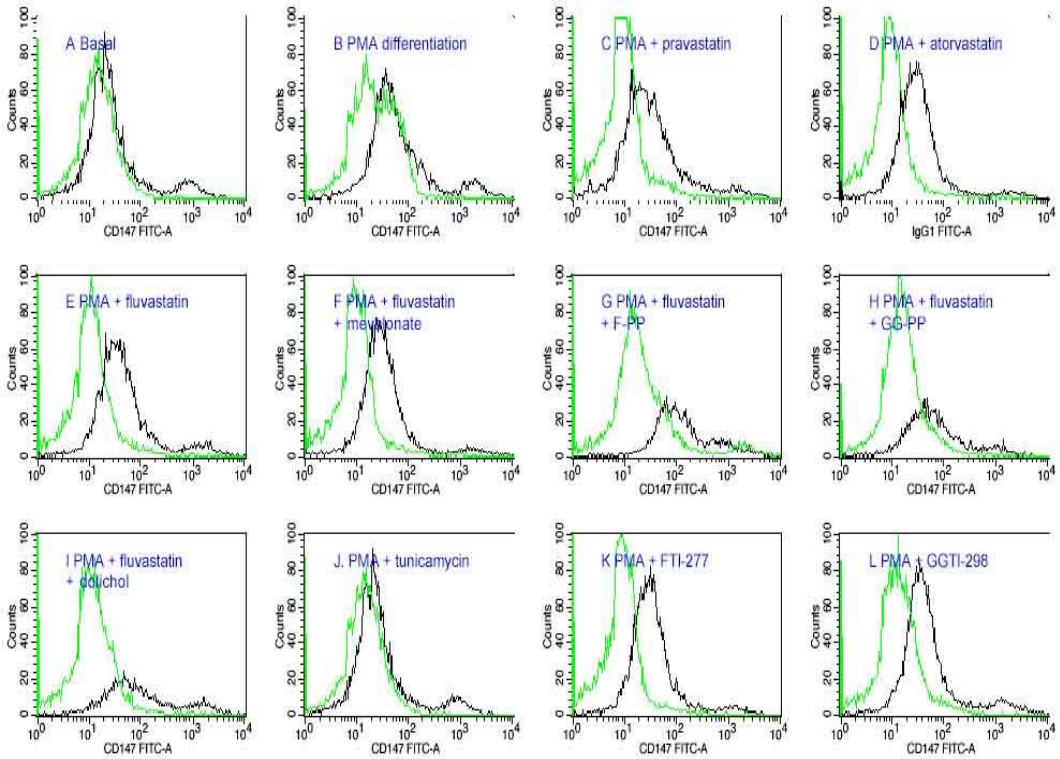


Figure 4.2: Alteration of cell surface EMMPRIN (CD147) in PMA-activated monocytic cells by statins. THP-1 cells were either activated or not with PMA (200 nM) and then incubated with statins (10 μM) or the relevant rescues as indicated. Cells were then analysed by flow cytometry to assess the expression of EMMPRIN (black histograms). Green histograms represent isotype controls. Results are one representative of three independent experiments.

Flow cytometric analysis revealed that PMA-differentiated THP-1 cells upregulated EMMPRIN compared to the basal condition of the cell (Fig 4.2, A and B). Treatment of cells with either pravastatin, atorvastatin or fluvastatin for 24 h prior to stimulation with PMA resulted in the downregulation of EMMPRIN (Fig 4.2, C-E). In order to dissect the influence of different intermediates of the cholesterol biosynthesis pathway on the surface expression of EMMPRIN, we treated the cells with rescues of cholesterol pathway. The expression of EMMPRIN was rescued by pretreatment with mevalonate, FPP, GGPP, and dolichol (Fig 4.2, F-I). Tunicamycin treatment did not alter the surface expression of EMMPRIN, while FTI and GGTI decreased its surface expression (Fig.4.2, J-L).

The next molecule which displayed change in its expression was CD14, which is a marker for cellular differentiation. The expression of the macrophage-specific differentiation antigen CD14 was similarly increased in PMA-differentiated THP-1 cells, and inhibited by all three statins tested (Fig 4.3, M-Q). CD14 was similarly downregulated in statin treated cells, which specifies that the cell has aborted differentiation and de-activation was achieved. Mevalonate completely rescued downregulation of CD14, implying that differentiation is regulated mevalonate generated pathways (Fig 4.3, R).

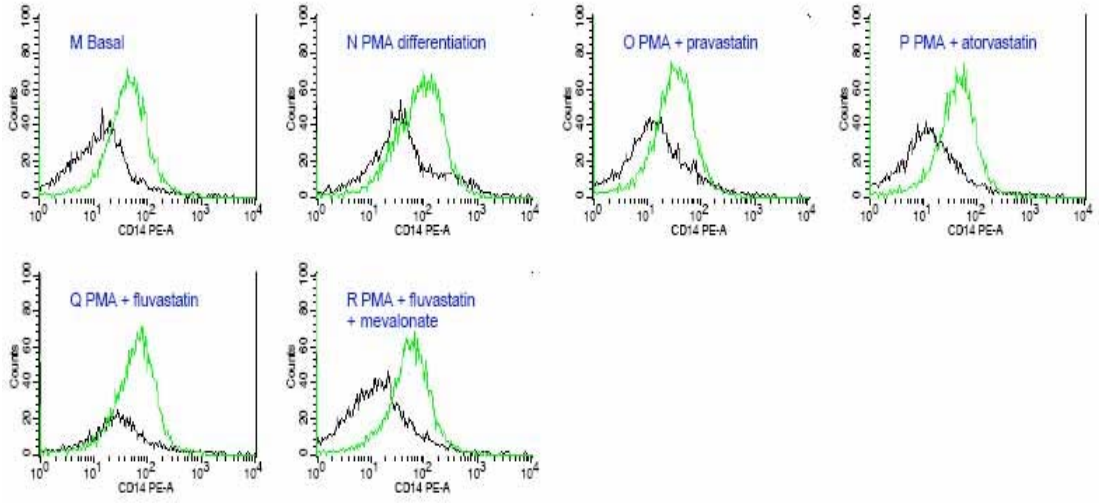


Figure 4.3: De-activation of THP-1 by surface expression of CD14, differentiation marker. THP-1 cells were either activated or not with PMA (200 nM) and then incubated with statins (10 µM) and/or mevalonate (10 µM). Cells were then fixed and analysed by flow cytometry to assess the expression of CD14 (black histograms). Green histograms represent isotype controls. Results are one representative of three independent experiments.

4.1.3 Intracellular entrapment of EMMPRIN

Next, inorder to evaluate if the downregulated expression of EMMPRIN was due to decreased expression of EMMPRIN in general or due to its intracellular entrapment, we

conducted permeabilsation studies on THP-1 cells treated with fluvastatin. Thus, to monitor the changes in the surface versus intracellular expression patterns of EMMPRIN, part of the cells were permeabilised prior to flow cytometry by cytofix cytoperm containing formaldehyde and saponin. Fluvastatin reduced the cell surface expression of EMMPRIN as inferred from non-permeabilised cells, whereas in permeabilised cells, there was a difference in the ratio of cell surface to intracellular expression of EMMPRIN (Fig 4.4, S-X). This clearly indicated a statin-mediated inhibition of EMMPRIN translocation from the cytosol to the cell surface. Thus, we concluded that statins cause EMMPRIN entrapment inside the cell due to defective translocation to the surface, owing to combined inhibition effects of isoprenylation and glycosylation.

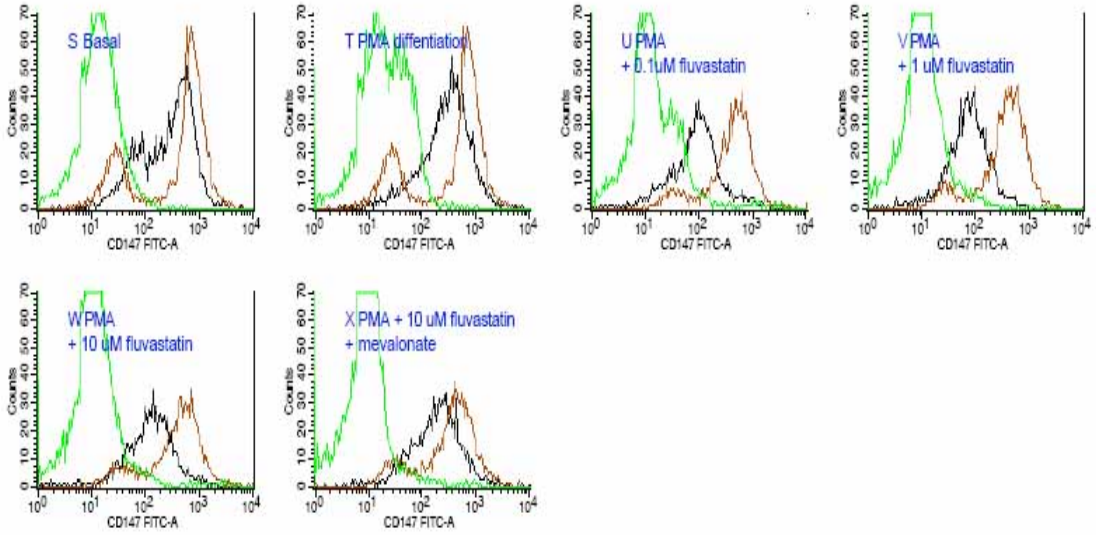


Figure 4.4: Cellular entrapment of EMMPRIN by fluvastatin. THP-1 cells were either activated or not with PMA (200 nM) and then incubated with fluvastatin and/or mevalonate (10 µM) as indicated. Cells were then fixed and either permeabilised (red histograms) with Triton-X-100 or not (black histograms) and analysed by flow cytometry to assess the expression of CD14. Green histograms represent isotype controls. Results are one representative of three independent experiments.

4.1.4 Downregulation of MMP-2 and -9 activity by statins

We elucidated the impact of inhibition of cell differentiation and EMMPRIN by monitoring the MMP secretion and activation as it was already known that EMMPRIN regulated MMP-2 secretion and activation (Zhou et al., 2005). Cell supernatants from the above experiments were subjected to gelatin zymography. PMA-differentiated THP-1 cells upregulated activation of MMP-2 and -9, as indicated by MMP zymograms. There was downregulation of active MMP-2 and -9 predominantly induced by fluvastatin as compared to pravastatin and atorvastatin, which was restored by co-incubation with mevalonate, FPP or dolichol and to a lower extent with GG -PP, suggesting that both isoprenylation (predominantly

farnesylation) and/or N-glycosylation are involved in MMP activation. We then tried to confirm the above findings by directly inhibiting the specific pathways of isoprenylation by using isoprenylation inhibitors FTI, GGTI and the N-glycosylation inhibitor tunicamycin. MMP inhibition was most pronounced with tunicamycin and GGTI, indicating that not only N-glycosylation, but also isoprenylation (geranylgeranylation) plays an important role in MMP activation (Fig 4.5). Further we observed different bands of MMP -9 i.e., heterodimer at a molecular range of 220 kDa and homodimer at 132 kDa. Pro and active forms of MMP-9 were also observed at a molecular range of 92 and 82 kDa. Pro and active forms of MMP-2 were visualised at the molecular range 72 and 62 kDa respectively.

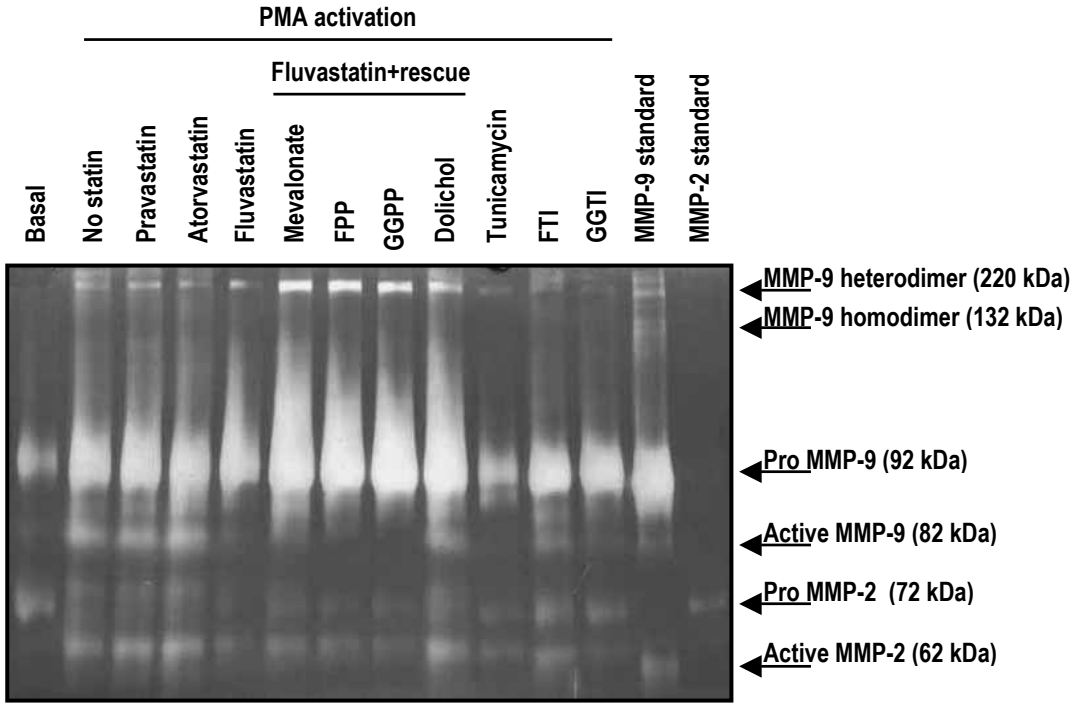


Figure 4.5: Gelatin zymography of MMP-2 and MMP-9. THP-1 cells were treated appropriately, as indicated and the supernatants were collected and analysed for MMP activity by gelatin zymography (8% gelatine). MMPs were identified according to their size (Waas et al., 2002) and reference standards. Note that the MMP-9 standard contained small degradation products mimickking a band at the active MMP-2 level. Results are from one of five independent experiments.

4.1.5 Reduction of total cellular glycoprotein content by statins

As observed from figure 4.1 and 4.2, tunicamycin had a pronounced effect on both cellular morphology and EMMPRIN expression, indicating a role of glycosylation in the expressional regulation of EMMPRIN. Therefore, we speculated a change in the glycoprotein content of the cell upon statin treatment. The total protein content of PMA-differentiated THP-1 cells, as assessed by colloidal coomassie blue staining was reduced by pravastatin, atorvastatin, and fluvastatin (Fig 4.6B). Moreover, the content of total glycoprotein as assessed

by enhanced alcian blue/silver staining was downregulated by a larger extent upon statin and tunicamycin treatment (Fig 4.6A). Some glycoprotein bands were apparently recovered by isoprenylation rescues GGPP more than by FPP (Fig 4.6A).

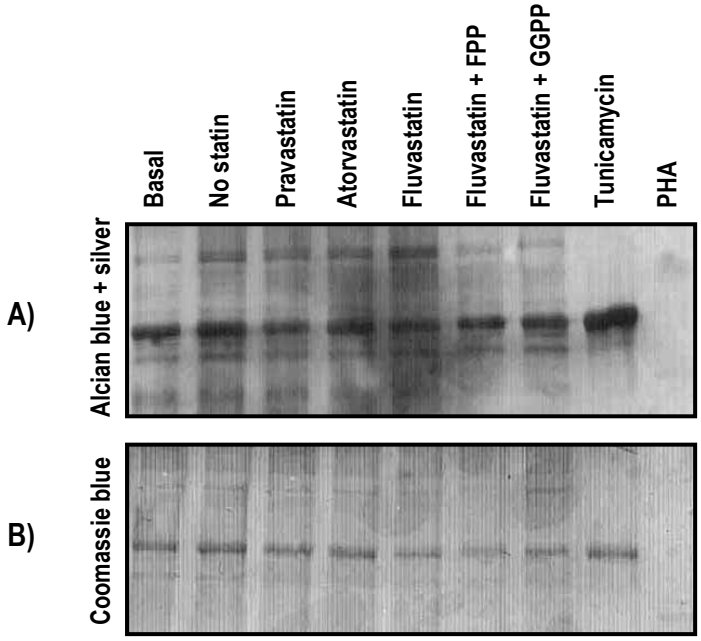


Figure 4.6: Total protein versus glycoprotein content of statin-treated THP-1 cells. THP-1 cells were treated appropriately, as indicated and total protein was extracted using NP-40 buffer and visualised by colloidal blue staining (A) and the glycoprotein content was visualized by combined alcian blue/silver staining. Phytohemagglutinin (PHA) served as a glycoprotein control. Results are one representative of three independent experiments.

4.1.6 Induction of lower glycosylated EMMPRIN forms by statins

Above experiments confirmed that statins inhibited THP-1 differentiation as observed by phase contrast microscopy (Fig 4.1), and downregulated the surface expression of EMMPRIN and CD14 (Fig 4.2 and 4.3). It was also evident from figure 4.4 that EMMPRIN was entrapped in the cell. Moreover, statins changed the pattern of total glycoproteins in the cell. As such, we next proceeded to evaluate if stains played a role in the glycosylation pattern of EMMPRIN itself, as EMMPRIN was known to exist in both low and high glycosylated forms. Therefore, statin treated THP-1 cells were subjected to immunoblotting. As illustrated in figure 4.7, upon differentiation with PMA, the expression of lower glycosylated forms of EMMPRIN decreased whereas that of higher glycosylated forms increased or remained unchanged. Treatment of THP-1 cells with fluvastatin induced lower glycosylated forms, with the highest expression of lower glycosylated forms at the highest concentration (10 μM) which can be seen by a further decrease of lower form of EMMPRIN by 5-7 kDa. Mevalonate, squalene, and water-soluble cholesterol were able to reduce the promotion of lower forms by

high-dose fluvastatin. Inhibition of isoprenylation by FTI, GGTI, and FPT1 as well as treatment with the antagonistic peptide to EMMPRIN, AP-9, also resulted in lower forms. Treatment of THP-1 with tunicamycin, the inhibitor of N-linked glycosylation of newly synthesised proteins, affected both lower glycosylated and higher glycosylated forms. The lower glycosylated form (33 kDa) almost completely disappeared and the expression of the higher glycosylated form (51 kDa) was greatly diminished and resulted in formation of core EMMPRIN at 27 kDa. The 27 kDa form that appeared is consistent with the non-glycosylated core protein. The fraction of higher glycosylated EMMPRIN remaining at 51 kDa was likely synthesised before tunicamycin treatment (Fig 4.7). Taken together, statins induced the formation of low glycosylated EMMPRIN and inhibited the formation of the high glycosylated form of EMMPRIN.

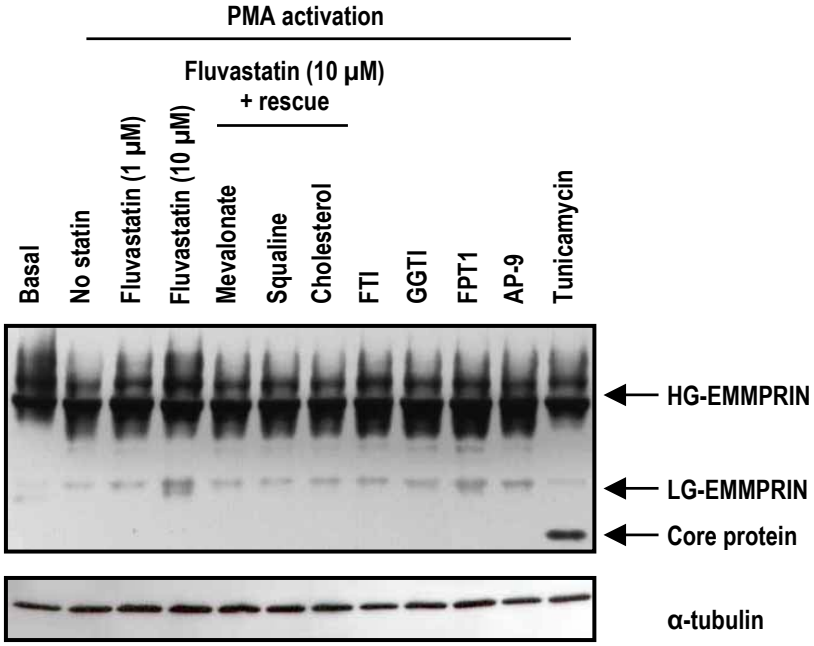


Figure 4.7: Deglycosylation of EMMPRIN by fluvastatin. THP-1 cells were treated accordingly as indicated and the total protein was extracted in NP-40 buffer and then subjected to SDS-PAGE electrophoresis and probed with EMMPRIN antibody to assess its glycosylation status. Alpha-tubulin served as an equal protein loading control. The blot is one representative of three independent experiments.

4.1.7 Downregulation of the expression of de novo synthesised EMMPRIN glycoprotein at the cell surface by statins

Downregulation of EMMPRIN by statins (Fig 4.2), impaired transport of EMMPRIN to the cell surface due to its entrapment EMMPRIN in the cell (Fig 4.4), glycosylation bias towards LG-EMMPRIN (Fig 4.6), all of the above reasons led us to further investigate the nature of glycosylated EMMPRIN reaching the cell surface. Therefore, in order to investigate whether a lowered HMG-CoA reductase activity affected the N-linked glycosylation and translocation of

EMMPRIN to the cell surface, immunoblot analysis of biotinylated surface proteins was performed on cells treated with pravastatin, fluvastatin, and atorvastatin. There was a marked downregulation of EMMPRIN translocated to the surface by all three statins (Fig 4.8A). As a control, tunicamycin even more markedly decreased the number of EMMPRIN molecules translocating to the cell surface. Comparison of the total protein content in whole cell lysates (lower panel) and the total protein translocated to the cell surface (upper panel), illustrates that lower glycosylated forms were not obtained in the immunoblot (lower forms visible in the lower panel are absent in the upper panel) with surface proteins. This suggests that the lower forms were not translocated to the cell surface in contrast to their higher forms (Fig 4.8B).

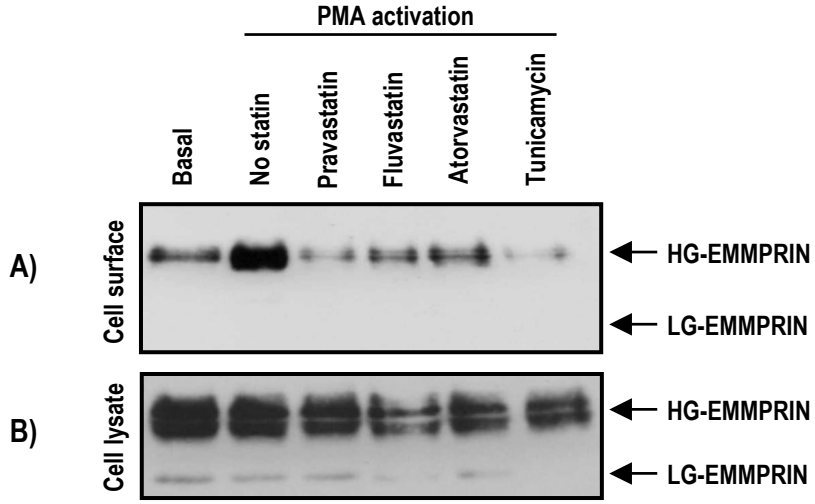


Figure 4.8: LG-EMMPRIN is not translocated to the cell surface. THP-1 cells were treated accordingly as indicated, the cells were surface biotinylated, and the bitoinylated proteins were pulled-down with streptavidin beads. The isolated protein (15 µg each) was then subjected to SDS-PAGE electrophoresis and probed with EMMPRIN antibodies. Note that the lower glycosylated (LG-EMMPRIN) has not reached the cell surface. Results are one representative of three independent experiments.

4.2 T CELL DE-ACTIVATION

4.2.1 Accessory cells are necessary for mitogen stimulated T and B cell responses in human peripheral blood

T cell activation needs two major signals, the primary signal is through T cell receptor (TCR), while the secondary costimulatory signal is through accessory cells like monocytes, macrophages, dendritic cells, and B cells. In order to prove that accessory cells provide secondary costimulatory signals. We depleted the peripheral blood of accessory cells by using leucine methyl ester. Leucine ester has been previously demonstrated to diffuse freely into cells and cause lysosomal degradation and eventual lysis of the organelles. Preincubation of PBMCs with leucine methyl ester, exhibited profound decrease in their response (proliferation)

to the mitogens PHA, Con A and poke weed mitogen (PWM), indicating that T cells were unable to receive any secondary costimuli from accessory cells due to their lysis by leucine ester. It is to be noted here that Leu-OMe incubation did not harm the lymphocytes themselves as assessed by their viability (>95 %) with trypan blue staining after ester incubation.

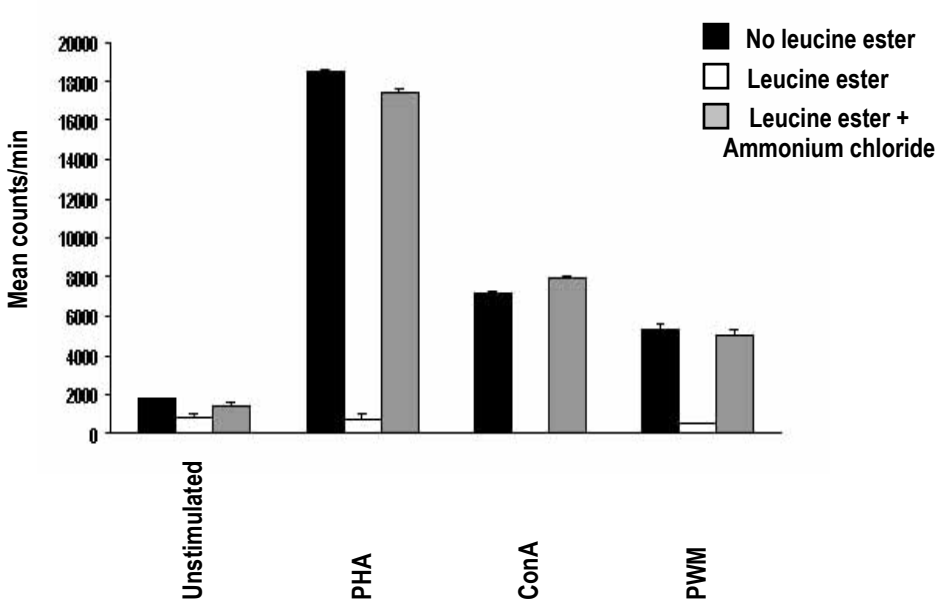


Figure 4.9: Accessory cell-dependent T cell proliferation. : PBMCs isolated from blood were depleted of accessory cells by treating them with 5 mM leucine ester for 45 min in complete RPMI medium. These cells were then washed and treated with different mitogens namely PHA, Con A and PWM (1 µgm/ml each) for three days. To neutralise the effect of leucine ester where necessary, cells were pre-incubated with ammonium chloride for 40 min. The extent of T cell response to the mitogens was determined by (methyl-^{3}H)-thymidine incorporation assay as counts per minute (±SE). Data represents one mean of three independent experiments.

Ammonium chloride (NH_4Cl) has been shown to raise intra-lysosomal pH and inhibit its degradative processes, thus antagonising leucine ester. Mitogenic stimulation of PBMCs by PHA, Con A and PWM promoted PBMC proliferation, while their preincubation with leucine ester inhibited proliferation. However, pretreatment of PBMCs with NH_4Cl prior to leucine ester incubation reversed the inhibition of proliferation (Fig 4.9). Thus, it can be concluded that accessory cells are of prime importance for sustainment of T cell activation.

4.2.2 Biphasic effect of fluvastatin on T cell proliferation through accessory cell costimulation

Monocytic cell lines (THP-1 and U937) are able to provide secondary signals for co-stimulation of T cells (Stonehouse, 1992). In order to prove that statins regulate T cell responses by modulating secondary costimulative signals through accessory cells, T cell

proliferation was assessed upon incubation with statins in T cell-monocytic cocultures. Primary stimulus was provided through soluble anti-CD3 (0.1 µg/ml). The secondary costimulatory signal was provided through THP-1. The THP-1 cell line is unable to provide the regular costimulatory signalling through CD28-B7 due to impaired expression of CD80 and CD86 antigens (Stonehouse et al,1999). The ability of monocytic cell lines to costimulate proliferative responses in resting T cells was used in the costimulatory assay to characterise the effect of statins on T cell proliferation through costimulation dependent mechanisms. The response was dependent on the numbers of both monocyte cell lines and primary T cells. Based on our initial studies all the experiments were conducted with 10^4 monocytic cell lines and $2x10^5$ T cells per well.

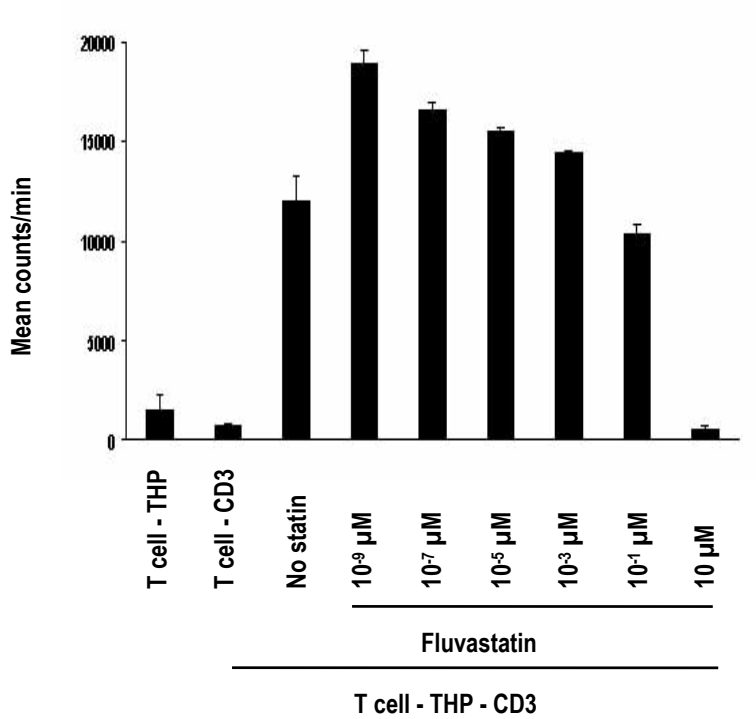


Figure 4.10: Biphasic effect of fluvastatin on T cell costimulation. Accessory cell-dependent T cell proliferation was assessed. The primary stimulus was provided by anti-CD3 antibody (0.1 µg/ml), while THP-1 cells were used as secondary signal mediators. PBMCs were depleted of accessory cells by incubating them with 5 mM leucine ester for 45 min in complete RPMI medium. Cells were washed and coincubated with THP-1 cells and fluvastatin (0.0000001 - 10 µM) was added. These cocultures were then stimulated with CD3 for three days.(methyl-^{3}H)-thymidinewas added overnight and proliferation was estimated as counts per minute of thymidine (±SE). Data represents one mean of 25 independent experiments.

Fluvastatin dose dependently inhibited proliferation of T cells in the costimulatory assay where the primary stimulation was through soluble 0.1 µg/ml CD3 antibody. THP-1 provided the costimulatory signal for sustainment of T cell proliferation. THP-1 in the absence of CD3 antibody was unable to provide proliferative response through costimulatory signals. Clusters

were formed in the absence of CD3 mAb, but they were much smaller and included a much smaller percentage of cells.

Fluvastatin dose dependently decreased proliferation of T cells in the above costimulatory assay (Fig 4.10). Surprisingly, lowest concentrations of statins in femto and pico molars increased proliferation of T cells, which represents a unique biphasic effect of this drug on T cell proliferation. We also observed upregulation of unique T cell subsets at lower concentrations of this drug. In summary, fluvastatin exhibits biphasic effects on T cell proliferation, upregulating T cell proliferation at lower concentrations and inhibiting T cell proliferation at higher concentrations.

4.2.3 Dissecting individual pathways involved in statin inhibition of T cell proliferation

Fluvastatin, a representative of the statin family, dose dependently decreased proliferation of T cells in the T cell costimulatory assay (Fig 4.10). Inhibition of HMG-CoA reductase by statins blocks synthesis of mevalonate, which is the major branch point for synthesis of isoprenylated products and cholesterol. Rescue experiments were conducted by addition of individual rescues of the mevalonate pathway to the culture well. Statin inhibition was directly counteracted by addition of mevalonate, which rescued all the pathways inhibited by fluvastatin. Downstream of mevalonate is FPP, which is responsible for farnesylation of proteins. GGPP governs geranylation and is derived from FPP. Dolichol pyrophosphate, derived from FPP mediates N-linked glycosylation. Individual rescues were added to all fluvastatin treated T cells. The effect of proliferation by statin rescues (Fig 4.11) was completely restored on treatment with mevalonate. GGPP stands next in the restoration of proliferation indicating a role of geranylated proteins in regulating proliferation. Farnesylated and dolichol derivatives also restored proliferation but only to a partial extent. Water-soluble cholesterol did not restore proliferation at all. Thus taken together, statin mediated inhibition of T cell proliferation is reversible with mevalonate and geranylated proteins and can incur a major rescue than farnesylated proteins.

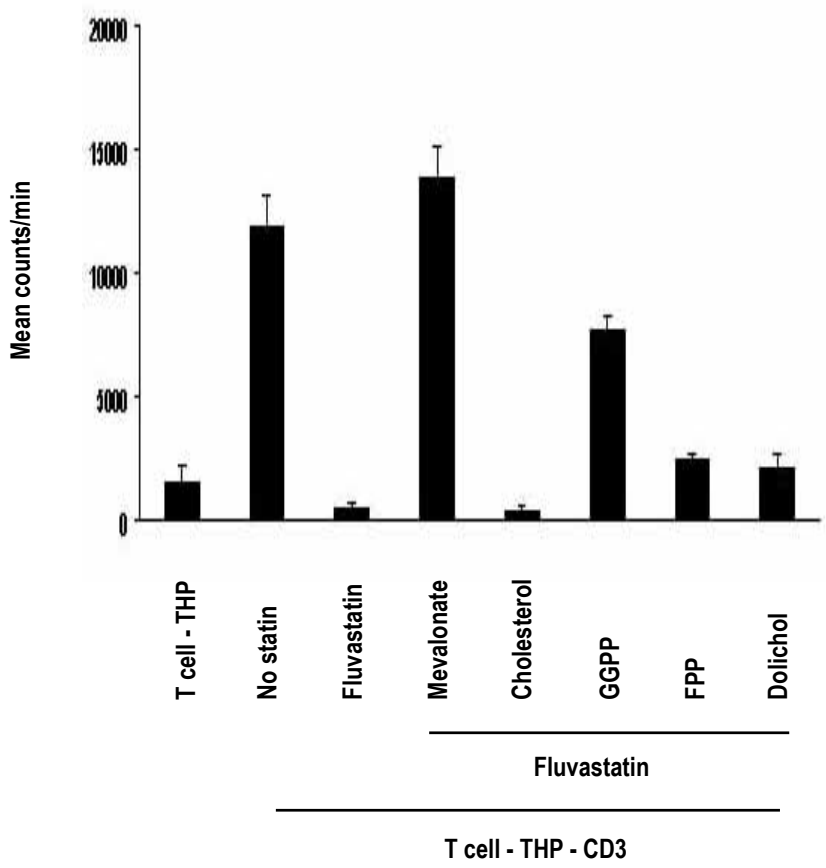


Figure 4.11: Pathway dissection of statin inhibition. Accessory cell-dependent T cell proliferation was assessed. The primary stimuli was provided by anti-CD3 antibody (0.1 µg/ml), while THP-1 cells were used as secondary signal mediators. PBMCs were depleted of accessory cells by incubating them with 5 mM leucine ester for 45 min in complete RPMI medium. Cells were washed and coincubated with THP-1 cells and fluvastatin (10 µM) and rescued with mevalonate (100 µM), or cholesterol (100 µM), or GGPP (5 µM), FPP (5 µM), dolichol (5 µM), was added as indicated. These cocultures were then stimulated with anti-CD3 for three days. (Methyl-^{3}H)-thymidine was added overnight and proliferation was estimated as counts per minute of thymidine (±SE). Data represents one mean of 25 independent experiments.

4.2.4 Inhibition of T cell proliferation by isoprenylation inhibitors

Fluvastatin inhibited proliferation of T cells completely (Fig 4.10). This was reversible by mevalonate and further by geranylated proteins (GGPP) (Fig 4.11). In order to mimick the pathway responsible for inhibition of proliferation, inhibitors of isoprenylation, i.e., FTI (Ras inhibitor) and GGTI (Rho inhibitor) were employed. Both the inhibitors reduced T cell proliferation, but the inhibition did not reach the substantial level as induced by statins (Fig 4.12).

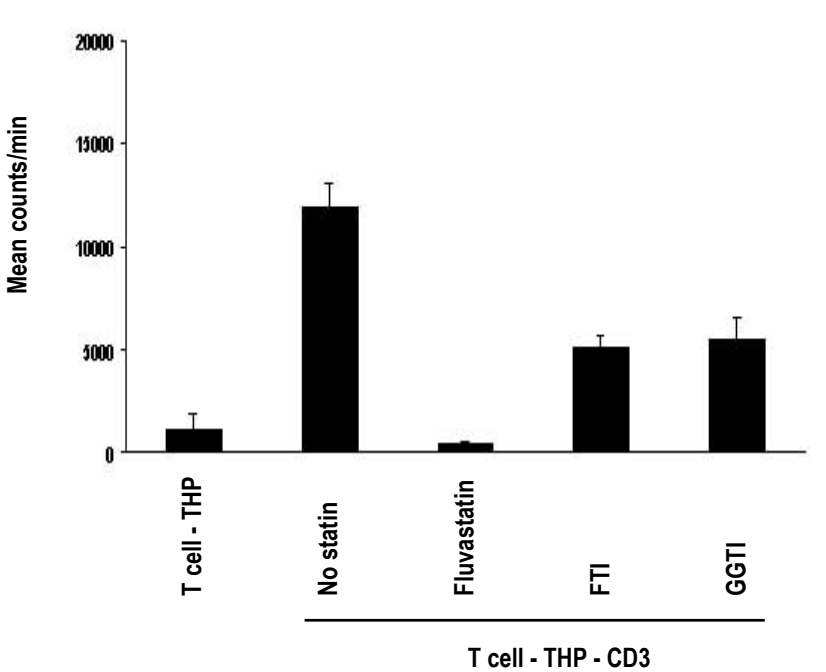


Figure 4.12: Statin inhibition by isoprenyl inhibitors. Accessory cell-dependent T cell proliferation was assessed. The primary stimuli was provided by anti-CD3 antibody (0.1 µg/ml), while THP-1 cells were used as secondary signal mediators. PBMCs were depleted of accessory cells by incubating them with 5 mM leucine ester for 45 min in complete RPMI medium. Cells were washed and coincubated with THP-1 cells and fluvastatin or Ras inhibitor FTI or Rho inhibitor GGTI (10 µM each), was added as indicated. These cocultures were then stimulated with anti-CD3 for three days.(Methyl-^{3}H)-thymidinewas added overnight and proliferation was estimated as counts per minute of thymidine incorporation(±SE). Data represents one mean of 25 independent experiments.

4.2.5 Inhibition of surface expression of EMMPRIN and its associated molecules by fluvastatin

EMMPRIN is upregulated in T cells activated by mitogens and CD3 stimulation (Fig 4.13a). Fluvastatin dose dependently decreased surface expression of EMMPRIN on T cells. This effect was completely reversed by addition of mevalonate. Isoprenylation inhibitors FTI and GGTI did not mimick the effects of inhibition of surface EMMPRIN. Further, there was a slight increase of EMMPRIN expression upon FTI treatment. Isoprenylation rescue GGPP reversed the expression of EMMPRIN on the cell surface to the Con A-activated level. Farnesylation by FPP in contrast was unable to rescue EMMPRIN inhibition caused due to fluvastatin.

In summary isoprenylation, inhibitors were unable to mimick statin-mediated downregulation of EMMPRIN expression in T cells. GGPP and mevalonate rescue could reverse the inhibition of EMMPRIN mediated by fluvastatin. Thus, additional pathways might also play a role in EMMPRIN inhibition by fluvastatin treatment.

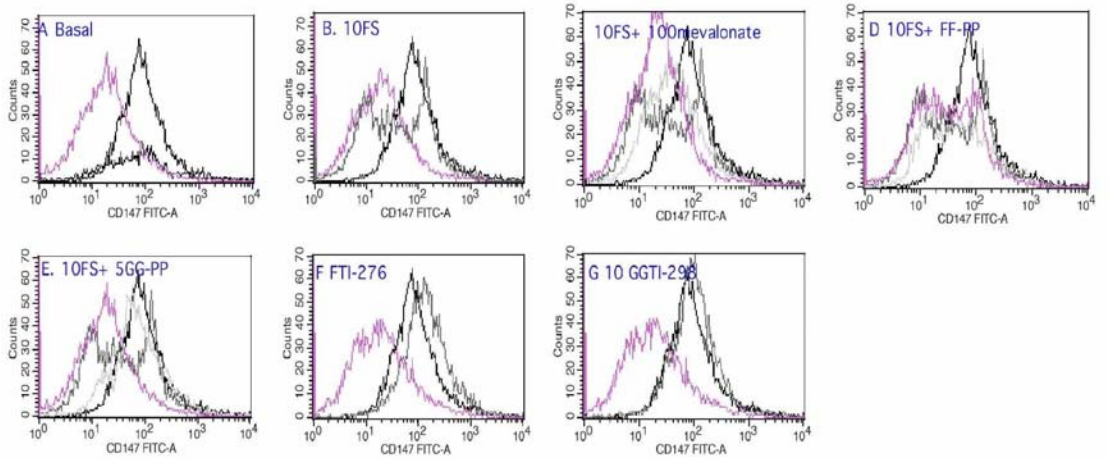


Figure 4.13a: EMMPRIN expression kinetics with statin treatment. 10^5 PBMC were activated with 2 µg/ml Con A for three days. Surface expression of EMMPRIN was quantified with EMMPRIN-FITC antibody at a concentration of 1:100 in FACS buffer. After 30 min of incubation in dark, cells were washed in FACS buffer and acquired on FACS Scan in FL1 channel. Data represents one of five consecutive experiments. Data represents overlay of EMMPRIN mean fluorescent intensity expression of A) Unactivated cells with activated cells, B) 10 µM fluvastatin with Con A, C) 10 µM Fluvastatin with 10 µM fluvastatin coincubated with 100 µM mevalonate, D) 10 µM fluvastatin with 10 µM fluvastatin coincubated with the farnesylation rescue 5 µM FPP, E) 10 µM fluvastatin with 10 µM fluvastatin coincubated with its geranylated rescue 5 µM GG -PP, F) Con A-activated cells with farneyslation inhibitor 10 µM FTI and G) Con A-activated cells with geranylation inhibitor 10 µM GGTI. (pink represents isotype, black represents con A activated and green represents drug treatment).

We also show the downregulation of EMMPRIN on proliferating T cells upon treatment with fluvastatin. ConA activated cells upregulated EMMPRIN to sustatin the energy requirements of the proliferating cells. However, treatment with 10 µM fluvastatin rapidly downregulated EMMPRIN and arrested the cells in a non-proliferating phase.

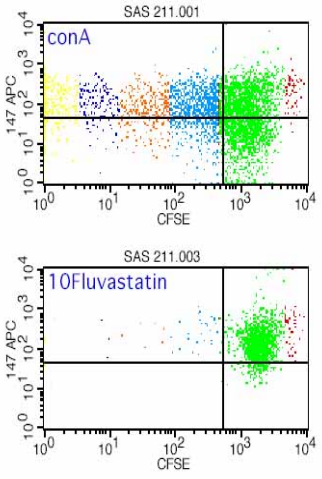


Figure 4.13b: Downregulation of EMMPRIN on proliferating cells. 10^5PBMC are treated with 2 µg/ml Con A for three days along with or without 10 µM fluvastatin. PBMCs were initially loaded with 2 µM CFSE for 8 min in 0.8% FCS-RPMI medium. The reaction was stopped with 10% FCS complete RPMI media and cells were washed and incubated for three days in complete RPMI media with or without fluvastatin as stated before. After three days, PBMCs were stained with primary CD147 antibody 1:100 in FACS buffer for 30 min and later with a secondary allophycocyanin (APC) antibody for another 30 min. Cells were then acquired in FACS Scan with CFSE in FL1 and APC in FL4 and data was analysed with cell quest software. Upper left quandrant represents proliferating cells and upper right quandrant represents non proliferating cells. Data represents one of five individual experiments.

The expression of CD98 and CD29 that were coexpressed along with EMMPRIN was also monitored to study the impact of the EMMPRIN inhibition on its coexpressed molecules. Both CD98 and CD29 were downregulated upon fluvastatin treatment and this was reversed by addition of mevalonate. CD98 was quantified as this molecule chaperones LAT1 (L-amino acid transporter) to the cell surface. In total fluvastatin downregulated expression of EMMPRIN on the cell surface (Fig 4.13) and simultaneously downregulated the expression of CD98 and CD29 (Fig 4.14).

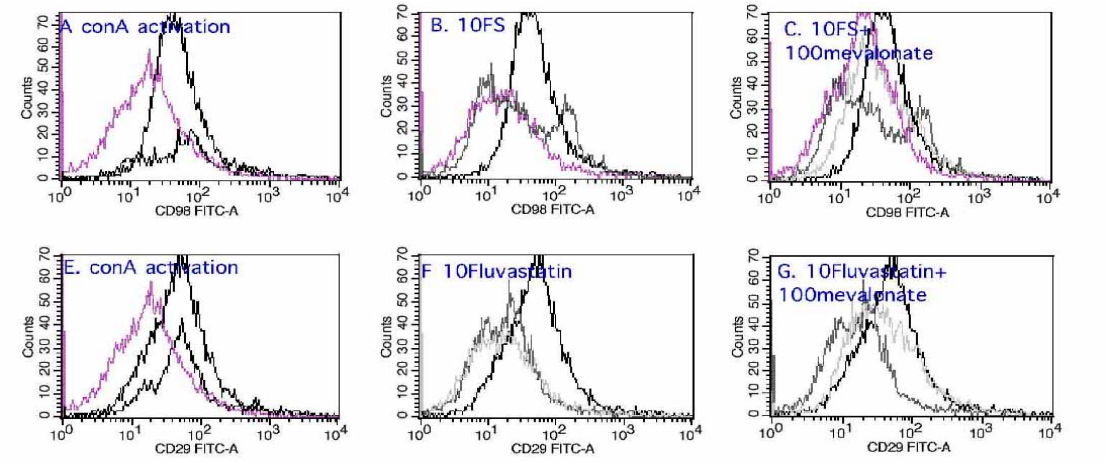


Figure 4.14: CD98 and CD29 expression kinetics upon statin treatment. 10^5 PBMC were activated with 2 µg/ml Con A for three days. Surface expression of CD98 was quantified with CD98FITC (A-C) and CD29FITC (E-G) Antibodies at a concentration of 1:100 in FACS buffer. After 30 min of incubation in dark, cells were washed in FACS buffer and were acquired on FACS Scan in FL1 channel. Data represents overlay of CD98 (A-C) and CD29 (E-G). Overlay of mean fluorescent intensity expression of CD98 a) activated Vs unactivated T cells b) activated Vs 10 µM fluvastatin c) 10 µM fluvastatin Vs 10 mevalonate rescue for fluvastatin treatment. Data represents one of five consecutive experiments. Overlay of mean fluorescent intensity expression of CD29 a) activated Vs unactivated T cells b) activated Vs 10 µM fluvastatin c) 10 µM fluvastatin Vs 100 µM mevalonate rescue for fluvastatin treatment. Data represents one of five consecutive experiments. (pink represents isotype, black represents con A-activated and green represents drug treatment).

4.2.6 Costimulation dependent inhibition of T cell proliferation

Statins inhibited T cell proliferation (Fig 4.10), paralleled by inhibition of EMMPRIN on T cells (Fig 4.13) and monocytes (Fig 4.2) and its associated molecules. Both APC and T cells express EMMPRIN and it has been reported that EMMPRIN acts as a costimulatory molecule for T cell proliferation. The next question we addressed was the impact of EMMPRIN downregulation on APC induced T cell proliferation. We used a unique costimulatory assay in which the costimulatory signal was provided by irradiated mouse splenocytes (Balb/c) which were used as accessory cells.

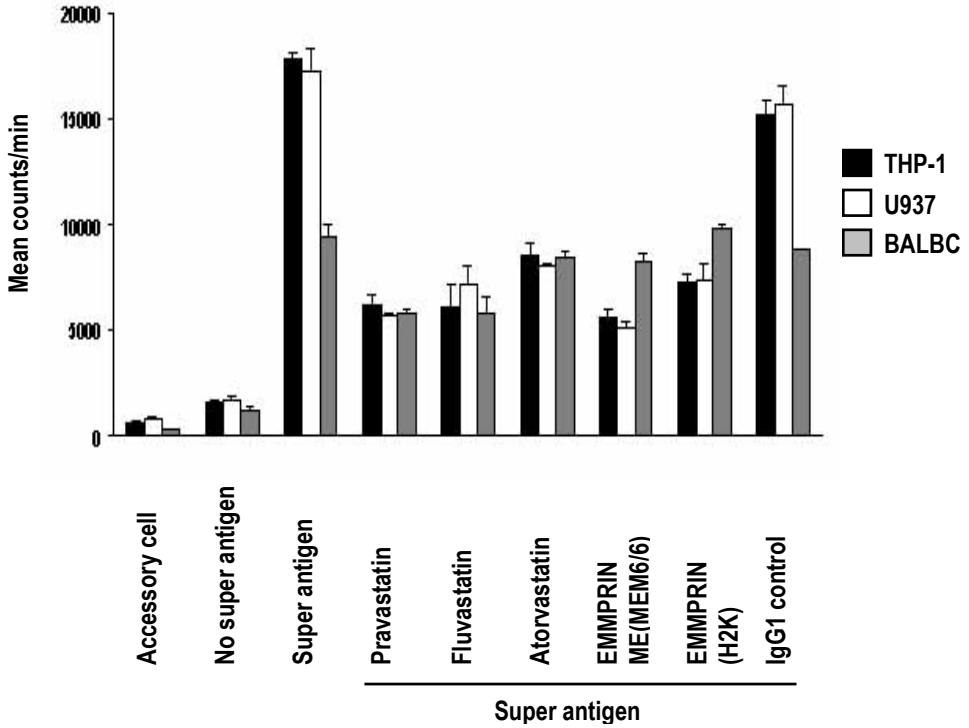


Figure 4.15: Monocyte-dependent regulation of T lymphocyte activation through xenogeneic system. Accessory cell-dependent T cell proliferation was assessed. The primary stimulus was provided by superantigen SEA (1 µg/ml), while irradiated THP-1 or U937 or Balb/c mice thymocytes were used as secondary signal mediators. THP-1 or U937 or Balb/c mice thymocytes were treated with statins (10 µM each) or neutralising antibodies (1 µg/ml) overnight. The following day, freshly isolated PBMCs were depleted of accessory cells by incubating them with 5 mM leucine ester for 45 min in complete RPMI medium. PBMCs were then washed and coincubated with either washed THP-1 cells or U937 or Balb/c mice thymocytes from the previous day. The cocultures were then activated with superantigen SEA for three days. (Methyl-^{3}H)-thymidinewas then incubated overnight and proliferation was assessed as counts per minute of thymidine (±SE). Data represents one mean of three independent experiments.

Mouse splenocytes were used as human T cells lack xenoreactivity against mouse splenocytes (Cho, 2004). Despite the absence of a direct xenoresponse to murine spleen cells, human T cells were activated by super antigen (SEA) presented by murine splenic cells. Further EMMPRIN antibodies against human EMMPRIN were unable to act against murine splenocytes. Murine anti human EMMPRIN did not block the T cell proliferation in this system i.e., Balb/c mediated T cell proliferation. Irradiated monocytic cell lines (THP-1 and U937) and mouse splenocytes (Balb/c) were used as accessory cells and were treated with 10 µM statins (pravastatin, fluvastatin and atorvastatin). Neutralising antibodies against EMMPRIN (MEM6/6) and (H2K) at a concentration of 0.1 µg/ml were used in this inhibitory assay. Prepulsing of neutralising antibodies on THP-1 and U937 resulted in inhibition of T cell proliferation whereas prepulsing of the same on mouse splenocytes did not result in inhibition of T cell proliferation neither in comparison to the control, nor in comparison to isotype control. The cellular specificity of inhibitory antibody (through APC or T cells) was effectively judged by this assay.

Costimulatory signals from THP-1 and U937 effectively reduced T cell proliferation but the inhibition was not drastic when compared with statins throughout the culture (Fig 4.15).

4.2.7 Inhibition of T cell proliferation by EMMPRIN antagonists

MEM6/6 (EMMPRIN) antibody inhibits CD3 mAb activation of human T cells enriched from peripheral blood. Epitope mapping of this mAb directed to a unique epitope located to the membrane proximal Ig-like domain of EMMPRIN (Chiampanichayakul, 2006). To examine the inhibitory effects of MEM6/6 on T cells neutralising antibodies were prepulsed on culture system and stimulated with anti-CD3 antibody at suboptimal concentration 0.1 µg/ml. Downregulation of T cell proliferation (Fig 4.16) by inhibitory MEM6/6 was achieved in range of 0.1-1 µg/ml. This was in line with earlier data and indicated that appropriate antigen:antibody ratio was necessary to mediate the inhibitory effect on T cell proliferation. This was also the case with AP-9 (antagonistic peptide) which works in a narrow concentration range of 25-50 µg/ml. Concentrations lower or higher to this range, increased proliferation. Fluvastatin inhibited T cell proliferation in a dose dependent manner (1-10 µM).

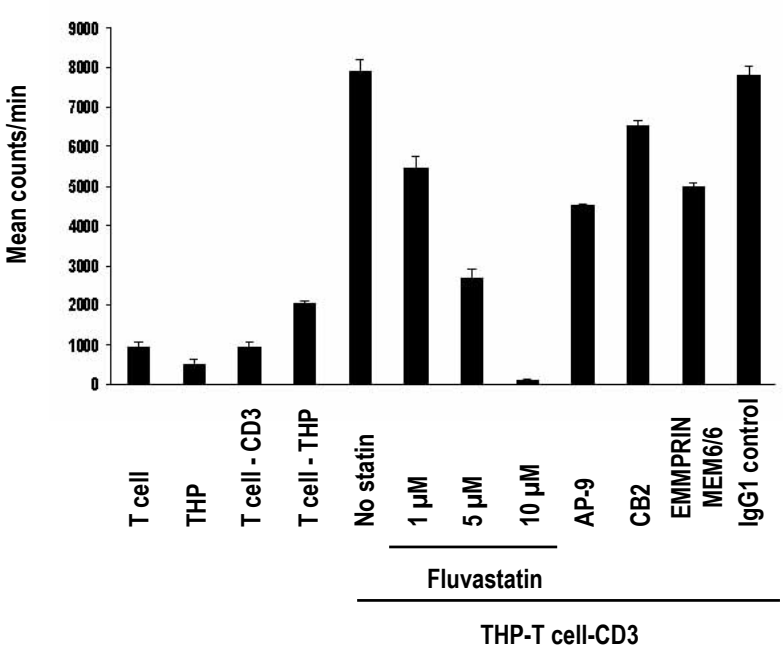


Figure 4.16: Inhibition of T cell proliferation by EMMPRIN antagonists. Accessory cell-dependent T cell proliferation was assessed. The primary stimulus was provided by anti-CD3 antibody (0.1 µg/ml), while THP-1 cells were used as secondary signal mediators. PBMCs were depleted of accessory cells by incubating them with 5 mM leucine ester for 45 min in complete RPMI medium. PBMCs were washed and coincubated with THP-1 cells and fluvastatin or AP-9 (100 µg/ml), or its control CB-2 (100 µg/ml), or EMMPRIN neutralising antibody MEM6/6 (1 µg/ml), was added as indicated. These cocultures were then stimulated with anti-CD3 for three days. (Methyl-^{3}H)-thymidine was added overnight and proliferation was estimated as counts per minute of thymidine (±SE). Data represents one mean of 5 independent experiments.

4.2.8 Induction of anergy by fluvastatin and EMMPRIN antagonists

Statins (Fig 4.10), and EMMPRIN antagonists (Fig 4.16) inhibited T cell proliferation. We hypothesized that T cells are rendered anergeic by statin treatment. Anergy is hypo-responsiveness to secondary stimuli. T cells were activated in a costimulation assay.

Primary stimulation was through CD3 antibody. After 3 days of incubation with the primary stimulation, cell proliferation was monitored by (methyl-^{3}H)-thymidineincorporation. Statins reduced proliferation of T cells and EMMPRIN antagonists mimicked this effect (Fig 4.17). After three days, cells were washed-off the primary stimulus, statins and EMMPRIN antagonists. Later, the secondary stimulus was provided by IL-2. Both statin treated cells and EMMPRIN antagonists induced anergy, and inhibited cell proliferation. Lower concentrations of statins were able to respond to secondary stimulus, 10 µM fluvastatin failed to respond to IL-2. This was due to downregulation of CD25 (receptor for IL-2) at higher concentration in contrast to upregulation at lower concentration. Isotype control responded positively to the secondary stimulation. Isolated control T cells responded to a lesser extent to IL-2 secondary stimuli, while both statin treated T cells and EMMPRIN neutralising antibody treated cells responded in a similar fashion to IL-2 restimulation (Fig 4.17).

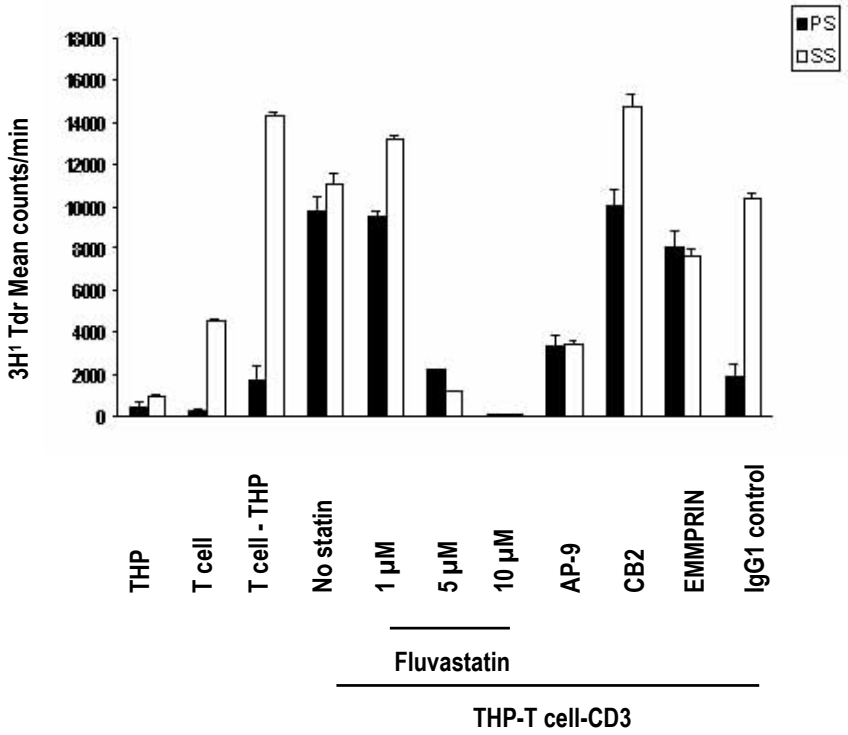


Figure 4.17: EMMPRIN antagonists mimick fluvastatin in inducing anergy. Accessory cell-dependent T cell proliferation was assessed. The primary stimulus (PS) was provided by anti-CD3 antibody (0.1 µg/ml), while THP-1 cells were used as secondary signal mediators. PBMCs were depleted of accessory cells by incubating them with 5 mM leucine ester for 45 min in complete RPMI medium. PBMCs were washed and coincubated with THP-1 cells and fluvastatin or AP-9 (100 µg/ml), or its control CB-2 (100 µg/ml), or EMMPRIN neutralising antibody MEM6/6 (1 µg/ml), was added as indicated. These cocultures were then stimulated with CD3 for three

days. These cells were divided into two fractions and to one(methyl-^{3}H)-thymidinewas added overnight and proliferation was estimated immediately. The other fraction of cells was washed and incubated with IL-2 (50 units) as a secondary stimulus (SS) for two days. (Methyl-^{3}H)-thymidinewas then added overnight to this fraction and proliferation was measured as counts per minute of thymidine (±SE). Data represents one mean of three independent experiments.

4.2.9 Fluvastatin entraps MCT1 inside activated PBMCs

Statin treated T cell inhibited EMMPRIN (Fig 4.13), paralleled by a down regulation of associated molecules CD98 and CD29 (Fig 4.14). We than anticipated that MCT1 transporter, which is chaperoned by EMMPRIN, could also be downregulated. MCT1 is highly expressed on the surface of activated T cells. Con A-activated PBMCs upregulated MCT1 on the surface of T cells. This upregulation was to cope up with the extensive glycolytic rate observed in activated T cells to sustain proliferation. T cells are more susceptible to MCT1 inhibition owing to dependence on aerobic glycolysis during lymphocyte activation to supply the high-energy requirement for cell growth and proliferation resulting in large increases of lactate production (Kirk, 2000). Fluvastatin inhibited MCT1 transporters in the cell, which was reversed by mevalonate and FPP. Isoprenylation inhibitors FTI and GGTI mimicked the effects of statins with FTI expressing a greater inhibition than GGTI. Rescue experiments with mevalonate and FPP rescued statin-mediated inhibition of MCT1 expression (Fig 4.18).

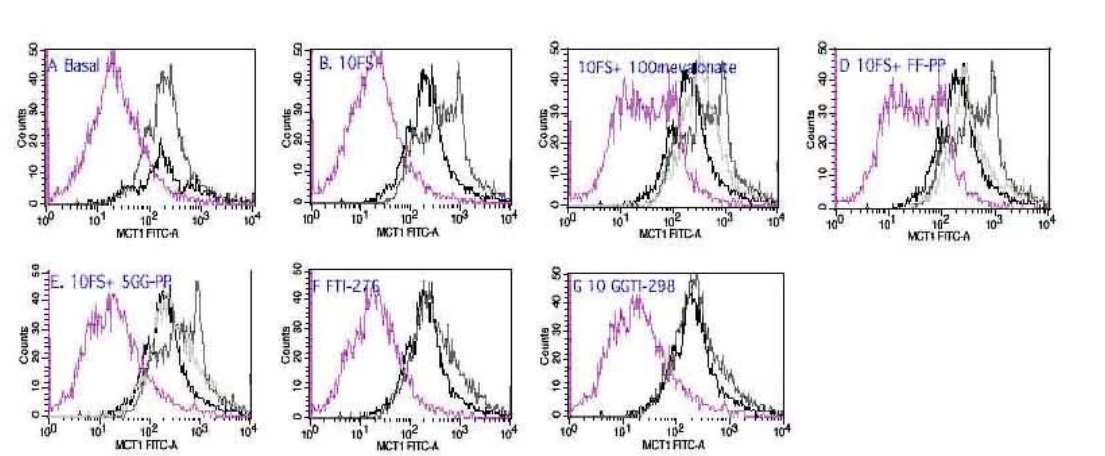


Figure 4.18: MCT1 inhibition upon fluvastatin treatment. 10^5 PBMCs were activated with 2 µg/ml Con A for three days. Intracellular MCT1 was quantified with MCT1 primary rabbit anti human antibody at a concentration of 1:100 in FACS buffer. After 30 min of incubation in dark, cells were washed in FACS buffer and incubated with secondary mouse anti rabbit FITC for another 30 min and acquired on FACS Scan in FL1 channel. Data represents one of five consecutive experiments. Data represents overlay of MCT1 mean fluorescent intensity expression of A) Unactivated cells with activated cells B) 10 µM fluvastatin with Con A , C) 10 µM fluvastatin with 10 µM fluvastatin coincubated with 100 µM mevalonate, D) 10 µM fluvastatin with 10 µM fluvastatin coincubated with the farnesylation rescue 5 µM FPP, E) 10 µM fluvastatin with 10 µM fluvastatin coincubated with its geranylated rescue 5 µM GGPP, F) Con A-activated cells with farneyslation inhibitor 10 µM FTI and G) Con A-activated cells with geranylation inhibitor 10 µM GGTI. (pink represents isotype, black represents con A activated and green represents drug treatment)

4.2.10 Fluvastatin induces intracellular acidification

Fluvastatin inhibited EMMPRIN (Fig 4.13) paralleled by a downregulation of MCT1 (Fig 4.18). We anticipated that inhibition of MCT1 might disturb lactic acid influx. In accordance to our hypothesis, we measured intracellular pH (pH_i) using the pH-sensitive fluorescent dye 2´,7´-bis(carboxyethyl)-5(6)-carboxy-fluorescein (BCECF) to detect rapid changes in intracellular pH_i accompanying proton-linked lactate transport. Statin-treated cells and control cells in tyrode buffer, pH 7.4 were loaded with 1 µM BCECF and incubated for 1 h. Cells were then subjected to FACS analysis. The change in pH_i was determined from a calibration curve using phosphate buffer of increasing pH (5.6- 8.0) containing nigercin to equilibrate internal and external pH. In accordance to our hypothesis, Con A-activated T cells showed increased pH after incubation with Con A. Fluvastatin treated cells dose dependently decreased intracellular pH (Fig 4.19). This was restored by mevalonate-coincubated cells. Thus, the intracellular acidification caused by fluvastatin was reversible in concordance to the MCT1 levels (Fig 4.18).

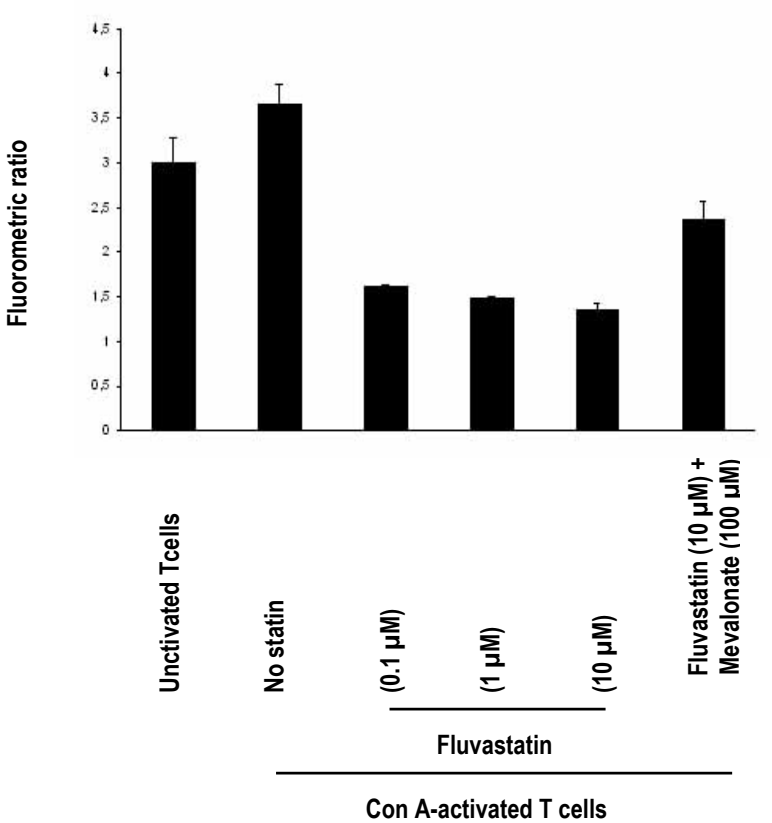


Figure 4.19: Fluvastatin induces intracellular acidification. 10^5 PBMCs were treated with fluvastatin and/or mevalonate and either activated or not with Con A (2 µg/ml) as indicated for three days. Later, cells were washed and incubated with BCECF for 1 h. Cells were then subjected to FACS analyses at FL1 and FL2 channels simultaneously. Data represents one representative of three independent experiments. .Data analysis was performed by flurometric ratio of BCECF emission in the FL1 and FL2 channels and by the ratio generated by cell quest software. Calibration of intracellular pH was made by increasing pH from 5.6 - 8.0 (phosphate buffers) equilibrated with nigercin.

4.2.11 Fluvastatin induces metabolic starvation

Entrapped MCT1 protein (Fig 4.18) resulted in intracellular acidification (Fig 4.19) and decreased lactic acid efflux. Additional impaired lactic acid influx from outside results in metabolic starvation due to associated impaired input of nutrients into the cell. We confirmed the same through inhibition of lactate transport into activated human T cells. It is well established that activated T cells rely on aerobic glycolysis for their energy metabolism, resulting in a large increase in lactate production from cells.

The CD28 costimulatory signalling pathway regulates enhanced expression of glucose transporters and glycolytic enzymes on T cell activation. As shown in (Fig 4.20), in-take of L-lactic acid by Con A-activated PBMCs at two different time points, 200, 800 sec resulted in drastic reduction of $pH_{i.}$ In comparison with control (activated PBMCs), 10 µM fluvastatin, treated cells displayed impaired transport of L-lactic acid into the cell. MCT1 impairment results in impaired lactic acid-shuttle into the cell thus disturbing the cellular homeostasis of the cell. Activated T cells need energy to sustain the extensive cell growth and proliferation. $CD45RO^+$ T cells (activated T cells) numbers were relatively decreased in comparison to $CD45RA^+$ (naive cells) on treatment with statins as the relative expression of MCT1 on naive cells was less when compared to activated $CD45RO^+$ T cells. Thus, fluvastatin selectively impaired activated cells sparing naive cells.

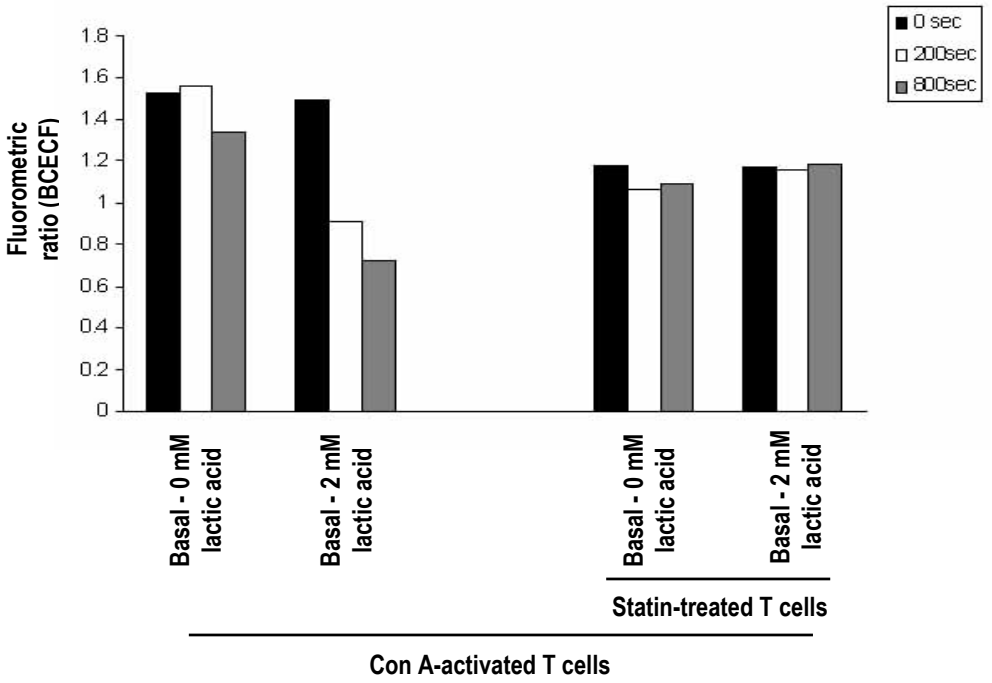


Figure 4.20: Fluvastatin induces metabolic starvation by impaired lactic acid shuttle10^5 PBMCs were treated with fluvastatin either activated or not with Con A (2 µg/ml) as indicated for three days. Later, cells were washed and incubated with BCECF for 1 h. Cells were then subjected to FACS analyses and lactic acid was added after 200 sec and absorbance in FL1 and FL2 channels was quantified throughout simultaneously for about

800 sec. Fluorometric ratio was calculated as the ratio of mean fluorescence intensity in FL1and FL2 channels . Data represents one representative of three independent experiments.

4.2.12 Fluvastatin impairs lactic acid shuttle in activated T cells

Impaired MCT1 translocation (Fig 4.18) to the cell surface led to intracellular acidification (Fig 4.19). This impaired lactic acid influx i.e., transport of lactic acid into the cell (Fig 4.20). The efflux of lactic acid into the cell exterior was then estimated using enzymatic lactic acid kit (Trinity biotech). In concordance with the decreased cellular proliferation (Fig 4.10), downregulated EMMPRIN (Fig 4.13) and entrapped MCT1 expression (Fig 4.18), all resulted in decreased lactic acid secretion into the cell exterior. We observed a decreased lactic acid level in the cell supernatant upon fluvastatin treatment (Fig 4.20).

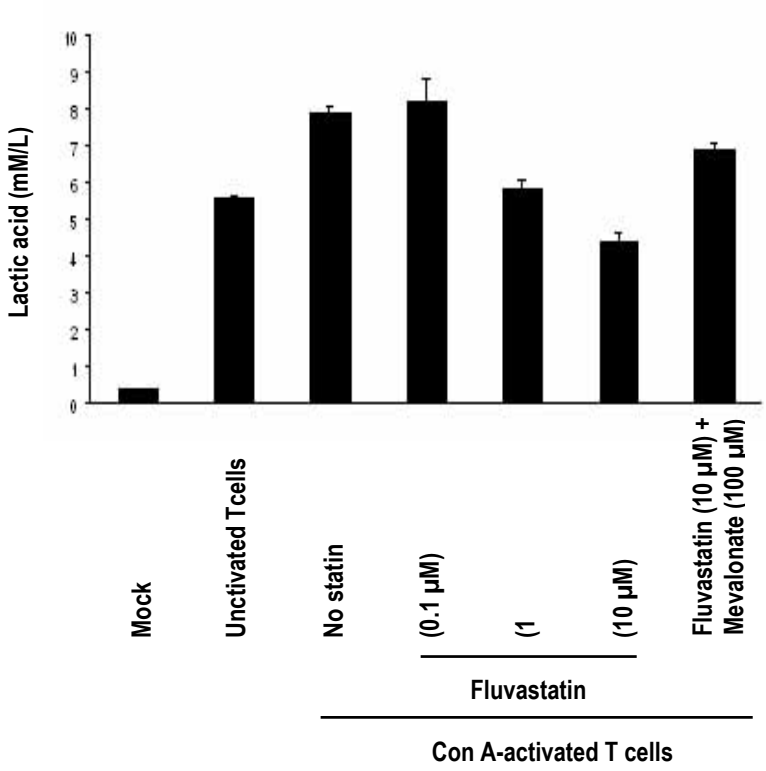


Figure 4.21: Fluvastatin impairs lactic acid shuttle. 10^5 PBMCs were treated with fluvastatin and/or mevalonate and either activated or not with Con A (2 µg/ml) as indicated for three days. Cell supernatant was collected and lactic acid was measured by enzymatic assay. Data represents one mean of three independent experiments.

Fluvastatin reduced lactic acid secretion into the external environment due to decreased MCT1 expression. Mevalonate rescued the lactic acid level to control Con A, thus showing that it was mevalonate-reversible and occurred through mevalonate dependent mechanisms. Thus, fluvastatin in total decreased expression of EMMPRIN (Fig 4.13), a master regulator/activation hub of the activated cell's metabolic health. MCT1 translocation was impaired to the cell surface (4.18), thus the lactic acid synthesised because of extensive proliferation to mitogenic

stimuli was entrapped decreasing the intracellular pH of the cell. Intake of nutrients and other monocarboxylates was also impaired thus leading to metabolic starvation of the cell (Fig 4.21). Therefore, statins in total can de-activate T cells in either a costimulatory-dependent or a costimulatory-independent setup.

4.3 PREVENTION OF NEURODEGENERATION BY DE-ACTIVATED T CELLS

4.3.1 T cell-mediated cytotoxicity is neuron specific

We demonstrated that T cells could kill neuronal cell lines, SHSY5Y (human neuronal cell line) when cocultured with polyclonally activated T cells. After 24 h of coincubation, neuronal injury or toxicity was measured by MTT assay. We found that unactivated T cells did not cause neuronal death in contrast to activated T cells, which consistently resulted in a rapid loss of neurons (Fig 4.22). In contrast to activated T cells, unactivated T cells did not reduce neuronal cell numbers at all time points examined. Further, the T cell cytotoxicity was specific to neurons. We then investigated whether activated T cells caused equal cytotoxicity to all members of the CNS family. No death of the astrocytic cell line (LN-18) was evident morphologically or by MTT assay. In contrast, neurons were highly susceptible to T cell-mediated cytotoxicity as long as they were activated indicating that the T cell cytotoxicity is specific to neurons.

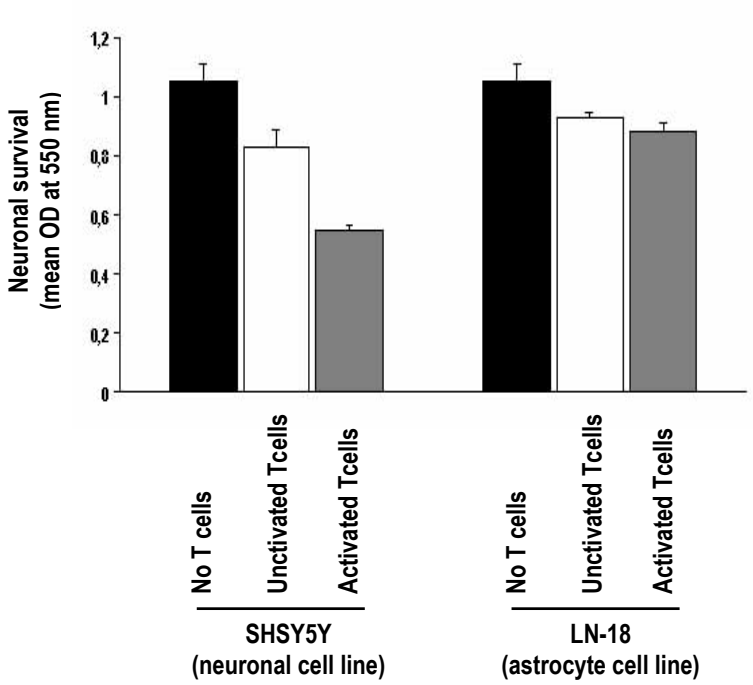


Figure 4.22: Neuronal specific cell death. 50,000 PBMCs were either unactivated or activated with Con A (2 µg/ml) for three days. Later these cells were washed and cocultured with 10,000 neuronal cells (SHSY5Y) or astrocytes (LN-18). After 24 h, the non-adherent T cells were washed-off and the neuronal or astrocyte cell

survival was assessed by MTT assay. Data represents mean of four individual wells and one of five independent experiments.

4.3.2 T cell cytotoxicity is MHC-independent

Neuronal toxicity was due to Con A-activated T cells and was neuron specific (Fig 4.22). The next question was to rule out apoptosis due to MHC mismatch. Neutralising antibodies against MHC-I and -II were used at a concentration of 5 µg/ml. PBMCs were activated by Con A at a concentration of 2 µg/ml. After 3 days of incubation, T cells were washed and incubated with SHSY5Y neurons treated with functional blocking MHC-I and MHC-II antibodies for 30 min.

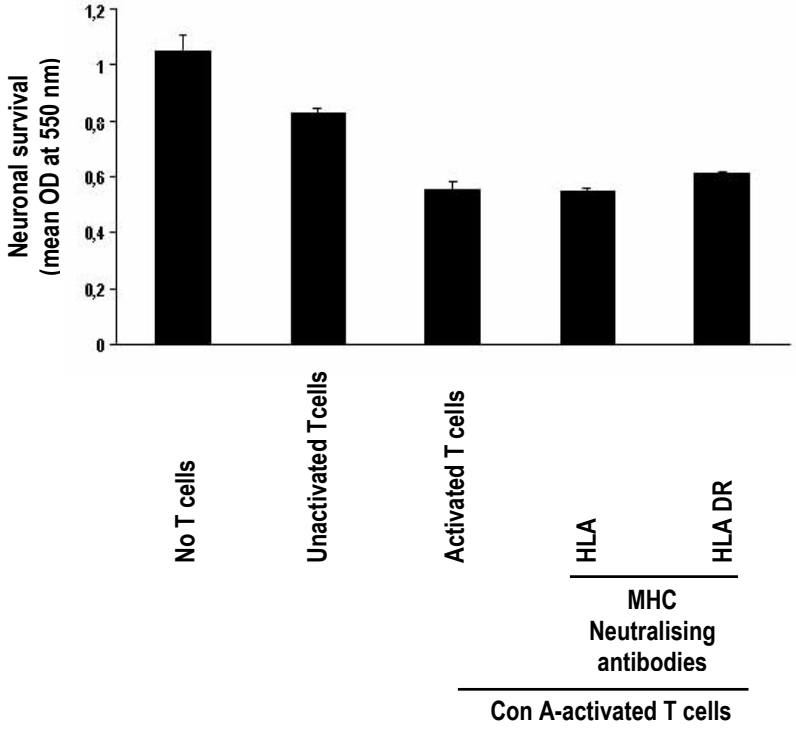


Figure 4.23: MHC-independent neuronal cell death. 50,000 PBMCs were either unactivated or activated with Con A (2 µg/ml) for three days and then incubated with neutralising antibodies HLADR and HLA ABC for 1 h. The cells were washed and cocultured with 10,000 neuronal cells (SHSY5Y). After 24 h, the non-adherent T cells were washed off and neuronal cell survival was assessed by MTT assay. Data represents mean of four individual wells and one of five independent experiments

Neuronal loss was neither prevented by this antibody nor was it attenuated by MHC–II function blocking antibody. Consistent with the lack of MHC-I involvement in killing neurons by T cells was the finding that human neurons in culture did not express MHC-I unlike their astrocyte counterparts (Fig 4.23). Taken together, activated T cells were independent of MHC-mediated mechanisms in their neuronal cytotoxicity.

4.3.3 Statins inhibit neuronal cytotoxicity

Neuronal toxicity was due to activated T cells (Fig 4.22). Statin treated T cells were antiproliferative (Fig 4.10) de-activated (Fig 4.20) and anergic (Fig 4.17). PBMCs were activated with Con A and coincubated with pravastatin, fluvastatin or atorvastatin. Statin-treated T cells were coincubated with mevalonate. The T cells were incubated with statins and activated with Con A for three days, and later washed to remove statins and Con A.

Statin-treated PBMCs rendered cells inactive and thereby protected neurons from cytotoxicity. All the statins tested rendered cytotoxic protection. Mevalonate completely restored cytotoxicity of activated T cells. This shows that statin induced neuroprotection was reversible by mevalonate as shown by the neuronal survival by MTT assay (Fig 4.24).

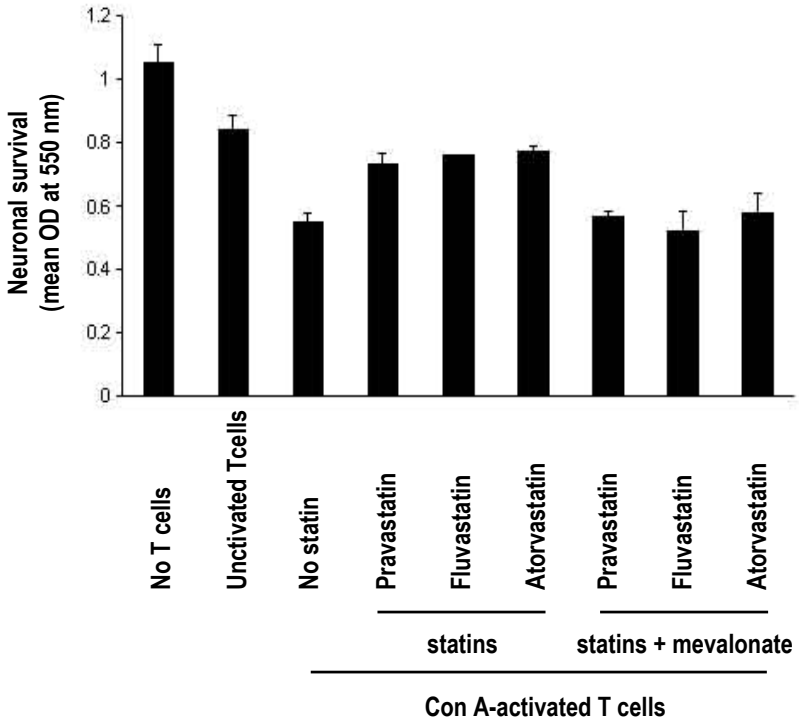


Figure 4.24: Neuroprotection by statins. 50,000 PBMCs were either unactivated or activated with Con A (2 µg/ml) and treated with statins (10 µM) and/or mevalonate (100 µM) for three days. PBMCs were then washed and cocultured with 10,000 neuronal cells (SHSY5Y). After 24 h, the non-adherent T cells were washed off and neuronal cell survival was assessed by MTT assay. Data represents mean of three independent experiments.

To further confirm the results derived from the MTT assay represents neuronal cell death clearily, we performed a flow cytometric assay in which neuronal cells were selectively incubated with a fluorescent dye DioC18. This dye integrates with the neuronal cells and does not leak to the neighbouring T cells. Neuronal toxicity with fluvastatin treated cells was dose dependent and was in concordance with the biphasic effects of statins on T cells (Fig 4.25).

The lower concentration of fluvastatin caused complete neuronal toxicity. The higher concentration of fluvastatin decreased neuronal toxicity to a substantial level.

Flow cytometric analysis of neuronal cell death was conducted to clarify the actual cell type that was killed in the coculture assay. Data analysis was performed by measuring the total number of dead cells [staining with propodium iodide (PI)] in the upper right quadrant, ($Dio^+ PI^+$). The lower right quadrant represented the living Dio-labelled SHSY5Y cells. Further fate of T cells in the coculture was also determined in this assay. Figure 4.25 shows that T cells were unaffected in this assay.

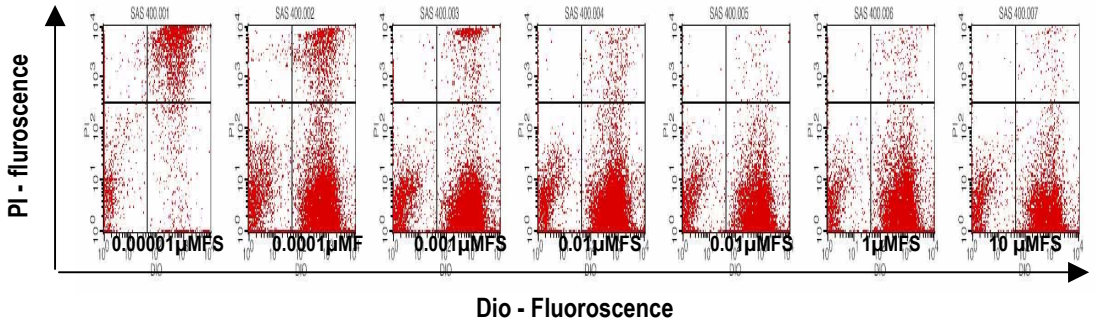


Figure 4.25: Biphasic effect of fluvastatin treated T cells on neuroprotection. 50,000 PBMCs were either unactivated or activated with Con A (2 µg/ml) and treated with statins as indicated for three days. PBMCs were then washed and cocultured with 10,000 DiO-labelled neuronal cells (SHSY5Y). After 24 h, the non-adherent T cells were washed off and neuronal cell survival was assessed by FACS analyses. Dead neuronal cells were represented by Dio-positive, propidium iodide (PI) positive upper right quadrant. Data represents mean of three independent experiments.

4.3.4 Neuronal protection is mimicked by EMMPRIN antagonists and neutralising cell adhesion antibodies

Activated T cells caused neuronal cytotoxicity (Fig 4.22), which was reduced by statins (Fig 4.24). We further proceeded to see if neuronal apoptosis, which was decreased upon treatment with statins, could be mimicked by PBMCs treated with EMMPRIN antagonists. PBMCs were treated with EMMPRIN neutralising antibodies (MEM6/6) at two different concentrations 0.1 µg/ml and 1 µg/ml. We reported previously (Fig 4.12) that EMMPRIN neutralising antibodies worked better at lower concentrations and higher concentrations were unable to cause inhibition. There was a direct correlation to the inhibitory effect of neutralising antibodies on the T cell proliferation with the neuronal apoptosis. Further neutralising antibodies against CD98, an activation and adhesion molecule, and LFA-1, an adhesion molecule, also resulted in neutralisation of T cell mediated cytotoxicity. These treatments protected neurons as evident by 70-80% viability, compared with cultures not treated with activated T cells (Fig 4.26). Thus, in summary EMMPRIN antagonists were able to protect

neurons from T cell mediated cytotoxicity similar to statin treated PBMCs thus incurring protection from cytotoxicity.

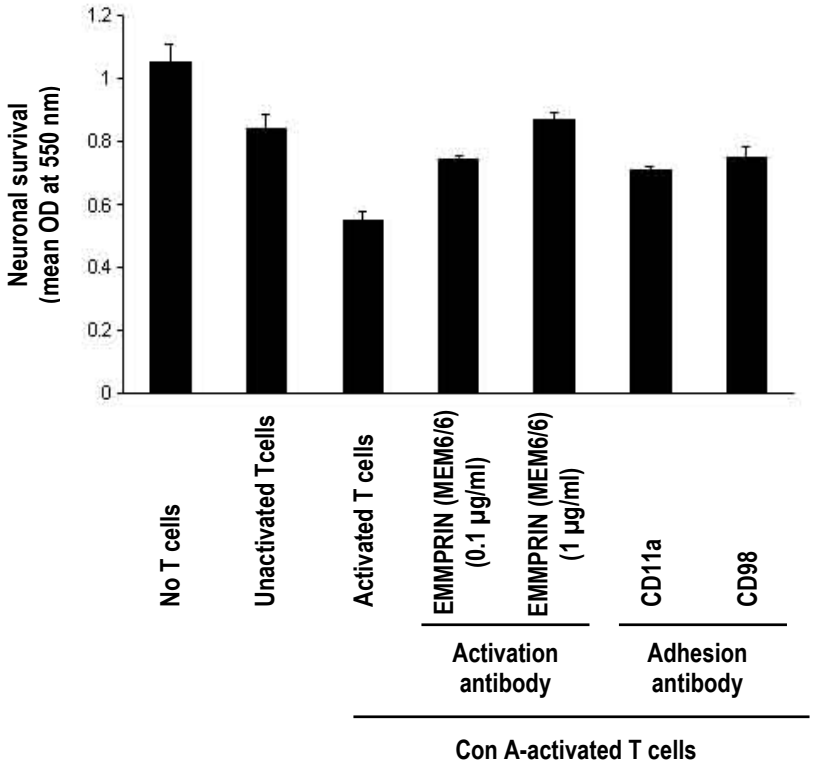


Figure 4.26: EMMPRIN and LFA-1 neutralising antibodies mimick neuroprotection. 50,000 PBMCs were either unactivated or activated with Con A (2 µg/ml) and treated with either neutralising activation or adhesion antibodies as indicated for three days. PBMCs were then washed and cocultured with 10,000 neuronal cells (SHSY5Y). After 24 h, the non-adherent T cells were washed off and neuronal cell survival was assessed by MTT assay. Data represents mean of four individual wells of a single experiment out of five independent experiments.

5 DISCUSSION

Immunosuppressive effects of statins on monocytes and T cells are intriguing (Mach, 2002). Independent of their effects on inhibition of cholesterol, statins exhibit pleiotropic effects that were mediated through inhibition of isoprenylation and other pathways downstream of mevalonate (Neuhaus et al., 2005a). Modulation of EMMPRIN structure and its functions by statins are discussed in the below sections:

5.1 STATIN EFFECTS ON MONOCYTES

Differentiation of THP-1 cells resulted in an adherent phenotype (Fig 4.1). Treatment with statins reduced adherence to the surface and altered the morphology of THP-1 cells indicating that statins interfere with the cellular differentiation mechanisms. The morphology of statin-treated cells was similar to that of THP-1 cells treated with AP-9, a specific antagonistic peptide to EMMPRIN, indicating that cellular differentiation is governed by EMMPRIN itself or its associated molecules. EMMPRIN interacts with adhesion markers such as CD29 (β1-integrin) or CD98 (a pleiotropic molecule participating in integrin-mediated cell adhesion, cell differentiation and apoptosis) In addition, CD98 has been reported to associate with L-type amino acid transporters (LATs) that shuttle amino acids into the cell. Furthermore, EMMPRIN is intimately associated with proton-linked monocarboxylate transporters (MCTs) which regulate the inflow of lactate into the cell. EMMPRIN also serves as a signalling receptor for extracellular cyclophilins, i.e., molecules that regulate protein trafficking in cells and, in addition, possess chemotactic properties for immune cells. Hence, EMMPRIN and its molecular partners play key roles in (inter-)cellular adhesion as well as metabolism pathways, making EMMPRIN an attractive target for anti-inflammatory or anti-neoplastic therapeutic strategies (Zucker et al., 2001).

The major finding of the present study was that statins inhibited the expression of EMMPRIN in PMA-differentiated THP-1 cells on the cell surface (Fig 4.2) and affected its structure (glycosylation) (Fig 4.6) and function (MMP activation) (Fig 4.5) through inhibition of isoprenylation and glycosylation. Statins interfere with both isoprenylation and N-linked glycosylation of EMMPRIN resulting in the accumulation of LG-EMMPRIN (Fig 4.7). In parallel, the translocation of EMMPRIN to the cell surface is impaired (Fig 4.8) resulting in inhibition of MMP-2 and -9 activation (Fig 4.5).

Treatment with statins resulted in downregulation of EMMPRIN expression, which was rescued by co-incubation with mevalonate, by which we can infer that inhibition is due to a

mechanism downstream of mevalonate. Cell permeabilisation studies conducted with statin-treated THP-1 cells revealed that the inhibition of the expression of EMMPRIN was more profound on the cell surface compared to the intracellular compartment. Hence, we hypothesise that the expression of EMMPRIN is regulated by post-translational mechanisms, i.e., isoprenylation, N-glycosylation or entrapment of immature pro-forms of EMMPRIN inside the cell rather than by mechanisms on the genomic level.

To dissect the pathway through which statins modulate their influence on EMMPRIN, we used specific inhibitors of the mevalonate pathway. Inhibitors of isoprenylation, i.e. FTI as a farnesylation inhibitor and GGTI as a geranylgeranylation inhibitor partially inhibited the surface expression of total EMMPRIN and MMP activation, indicating that isoprenylation plays only a minor role in these pathways. However, the same isoprenylation inhibitors did induce the expression of lower glycosylated forms of EMMPRIN to some extent. Thus, it can be speculated that this discrepancy is due to a potential effect of statins on caveolin-1 itself (mediated by isoprenylation), as upregulation of caveolin-1 also promotes lower glycosylated forms and subsequently decreases EMMPRIN self-association on the cell surface. Tunicamycin, a specific inhibitor of N-glycosylation of newly synthesised proteins (Tang et al., 2004a), induced downregulation of MMP activation. Furthermore, it drastically prevented *de novo* N-glycosylation of EMMPRIN, affecting both lower and higher glycosylated forms, and resulted a non-glycosylated core protein.

The extracellular domain of EMMPRIN has three potentially N-linked glycosylated sites that could be potentially inhibited by statin treatment. Since inhibition of mevalonic acid synthesis leads to a decreased rate of N-linked glycosylation, a possibility is that mevalonic acid might be rate-limiting factor for the function of EMMPRIN. For example, mevalonic acid-regulated N-glycosylation might be required for the translocation of EMMPRIN from the intracellular compartment to the cell membrane. If this was the case, suppression of HMG-CoA reductase activity would reduce the number of EMMPRIN molecules at the surface (Fig 4.8). This would then lead to decreased interactions with other EMMPRIN-associated molecules, which in turn leads to decreased MMP induction (Fig 4.5).

We provide evidence (Fig 4.2) that stains downregulated cell surface expression of EMMPRIN, which was rescued by mevalonate. Similarly, tunicamycin, which inhibits N-linked glycosylation of newly formed proteins, also inhibited the cell surface expression of EMMPRIN and modulated its glycosylation status (Fig 4.2 and Fig 4.7). As inhibition of the mevalonate pathway by statins, leads to reduced production of dolichol phosphate, which plays an

important role in N-linked glycosylation, it can be speculated that statin-mediated downregulation of dolichol phosphate along with other isoprenylated products lead to the intracellular entrapment of EMMPRIN by affecting its N-linked glycosylation. N-linked glycosylation has been reported to be a prevalent post-translational mechanism of all proteins that are to be transported to the cell membrane (Jia et al., 2006a). The core structure of the oligosaccharide complex is commonly composed of two N-acetyl-GlcN molecules, nine mannose and three glucose residues. Dolichol-phosphate acts as membrane-bound carrier of this complex inside the endoplasmic reticulum and makes it available to an oligosachharyl transferase, which couples it to aspargine residues of *de novo* synthesised proteins. Only sites containing Asn-X-Thr/Ser sequences (X is an amino acid with the exception of proline) are recognised by the transferase (Li et al., 2003). Since N-linked glycosylation occurs post-translationally, only newly synthesised proteins are modified in this manner. In this study we demonstrated that N-linked glycosylation of EMMPRIN was efficiently blocked upon inhibition of HMG-CoA reductase, and that the amount of de novo synthesized EMMPRIN at the cell membrane correlated to the MMP secretion (Fig 4.5) and activation. This strongly suggests that mevalonate controlled translocation of newly synthesized receptors and glycosylated receptors play an important role in secretion of MMP-2 and -9.

It has been already shown that caveolin has a specific affinity towards lower glycosylated form of EMMPRIN and further upregulation of caveolin has a negative effect on the formation of oligomeric EMMPRIN tilting the dynamics towards LG -EMMPRIN (Tang and Hemler, 2004). However, statins have been reported to decrease caevolin-1 levels, in which case higher forms of EMMPRIN should be predominant. However, as statins promoted the formation of lower forms of EMMPRIN, in comparison to its higher forms, it can be speculated that statin-mediated induction of LG-EMMPRIN is caveolin-1-independent, and is more likely due to dolichol phosphate depletion.

Activation of MMP-2 and -9 was upregulated in PMA-differentiated THP-1 cells, which again was predominantly inhibited by fluvastatin. Thus, we confirm previously published data that EMMPRIN regulates MMP secretion and activation (Li et al., 2006a) and postulate that statins reduce MMP-2 and -9 by inhibition of EMMPRIN expression.

MMP induction is regulated by high glycosylated EMMPRIN, where, EMMPRIN acts as a molecular switch depending on its glycosylation status (Tang and Hemler, 2004). We observed a statin-induced shift from higher glycosylation form towards a lower glycosylated form of EMMPRIN (Fig 4.7) paralleled by an impeded intracellular translocation of EMMPRIN to

the cell surface (Fig 4.8). From these results, we hypothesise that both isoprenylation and N-glycosylation contribute to the expression and function of EMMPRIN and that both pathways that are mevalonic acid-dependent are impeded by statins.

Most other transmembranous proteins are less sensitive to changes in their glycosylation status. The requirement of higher glycosylation for proper functioning of the MMP stimulating activity of EMMPRIN is indeed unusual but not unique. Like EMMPRIN, insulin-like growth factor (IGF)-1, a membrane-targeted molecule also requires N-glycosylation for promoting its effects (Siddals et al., 2004); incomplete glycosylation due to lovastatin caused a proreceptor retention within the endoplasmic reticulum. Consistent with our findings in EMMPRIN, two other groups observed a downregulation of IGF-1 by statins and demonstrated a synergistic inhibitory effect via the isoprenylation and N-glycosylation pathways (Siddals et al., 2004; Miller et al., 2005).

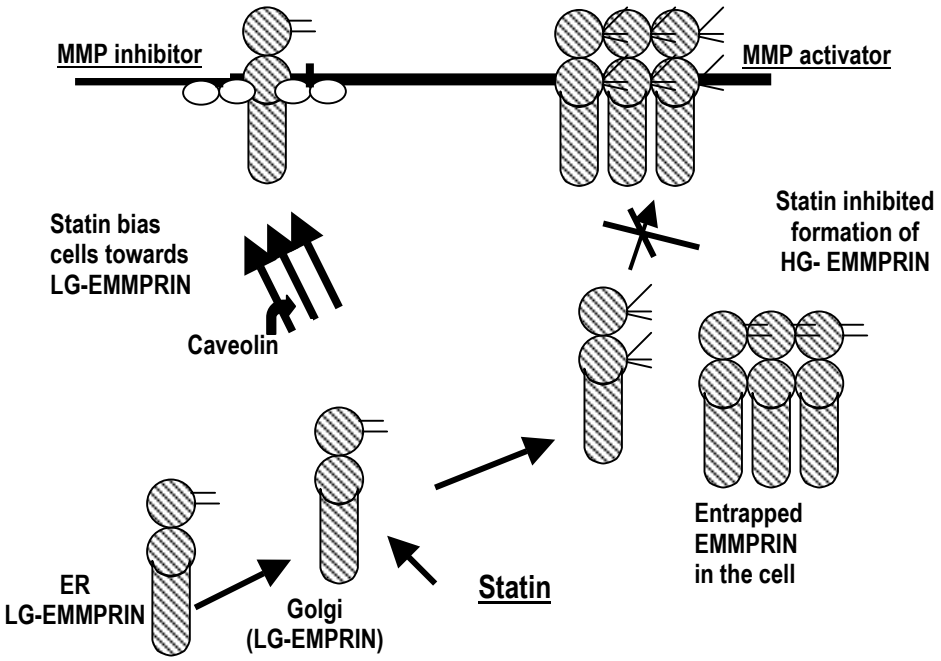


Figure 5.1: Hypothetical model of statin-mediated EMMPRIN modulation. Statins inhibit isoprenylation and N-linked glycosylation in the cell resulting in entrapment of EMMPRIN in the cell. Further EMMPRIN is deglycosylated due to inhibition of dolichol, resulting in formation of LG-EMMPRIN. This form of EMMPRIN is unable to form homoaggregates thus resulting in a bias towards the LG-EMMPRIN, which antagonises MMP secretion and activation [modified from (Tang et al., 2004a)].

The link between statin and EMMPRIN raises the possibility that an impaired control of mevalonate synthesis may contribute to uncontrolled growth of certain malignant cells. It has been reported that level of dolichol-phosphate and N-linked glycosylation is increased in malignant cells and tissues. The increase in the dolichol-phosphate synthesis might at least partially be a result of the uncontrolled regulation of HMG-CoA reductase. An increase in

N-linked glycosylation, due to an upregulated dolichol-phosphate biosynthesis may contribute to an overexpression of growth factor receptors or other functional molecules at tumour cell membrane. Thus, statins might be potential therapeutic targets in the treatment of malignant cells/tumour cells (Siddals et al., 2004).

5.2 STATIN EFFECTS ON T CELLS VIA MONOCYTES

Macrophages play a major role in mitogen induced human lymphocyte activation (Stonehouse et al., 1999). Characterisation of the role of non-lymphocytic accessory cells in immune responses requires the ability to separate candidate accessory cells from their responsive lymphocytes. Inhibitors of lysosomal enzyme activity (NH_4Cl) effectively protect macrophages from Leucine methyl ester-induced lysosomal disruption and subsequent cell death (Chiampanichayakul et al., 2006). This suggests that ester induced macrophage toxicity and their subsequent removal can be used to further test the costimulatory secondary signals. Leucine ester mediated cytotoxicity has been shown to be highly selective in depleting PBMCs virtually of all macrophages while leaving behind functionally unchanged B and T cell populations required for a mitogen-induced lymphocyte responses (Woodhead et al., 2000). In summary, studies (Fig 4.9) define the mechanism and conditions whereby the lysosomotropic agent Leu-OMe can be used to deplete human PBMC of macrophages. Further use of a xenogenic system (Fig 4.15) delineates the cell type needed during an immune reaction.

In the costimulation assay employed, T cells were stimulated with primary soluble anti-CD3 antibody rather than anti-CD3 antibody bound to the plastic surface. Costimulatory signal pathways that have been explored extensively were profoundly the B7 family on APC with CD28 on T cells (Stonehouse et al., 1999). But the relative expression of these costimulatory molecules on the cell lines (U937 and THP-1) was scanty for the cells to signal via CD28-B7 pathway. However, T cell proliferation does exist in U937 and THP-1 costimulated T cells which raise the possibility of alternative costimulatory pathways involved in these cell types.

Table 7: Relative expression of costimulatory markers on accessory cell lines

Cell lines	Median value of fluorescence intensity								
Cell lines	CD80	CD86	CD14	HLADR	CD11c	CD33	CD13	EMMPRIN	CD98
THP-1	-	-	-	+ + +	-	+ +	-	+ +	+
U937	-	-	-	-	-	+ +	-	+	+

Table 7 illustrates relative expression of markers based on median fluorescence intensity (- = <50; + = 50- 300; ++ = 300-1000) as measured on a log scale using the Lysis II software (Stonehouse et al., 1999).

Thus, this costimulation assay uses novel costimulatory molecules have not been well characterised to date. Two novel molecules that were recently identified in mediating costimulation of T cells are CD98 and EMMPRIN. Since EMMPRIN is expressed on both APC and T cells, we were interested in the cellular specificity of EMMPRIN inhibition. Irradiated murine splenocytes (xenogenic system) and THP-1 cells were used as accessory cells. PBMC were treated with leucine methyl ester and coculture experiments were performed in the presence of EMMPRIN neutralising antibodies. These experiments confirmed the role of EMMPRIN in T cell costimulation and directed that accessory cells were of major importance and that downregulated EMMPRIN on accessory cells has greater impact on inhibition of T cell proliferation. These studies (Fig 4.16) show that EMMPRIN mediates T cell responses through the accessory cells, both in a T cell costimulatory assay and in a xenogenic system (Fig 4.15). This is in agreement with the published results that EMMPRIN knockout mice-derived lymphocytes produced a greater proliferative responsive in mixed lymphocyte reaction (MLR), than those of wild littermates (Khunkeawla et al., 2000).

EMMPRIN has been previously reported to function as a costimulatory agent. As statins dose dependently inhibited EMMPRIN, we could establish a direct role of the statin anti-proliferative nature through inhibition of EMMPRIN. Statins dose dependently inhibited T cell proliferation in the costimulatory assay (Fig 4.10). Further we observed a biphasic effects of statins on T cell proliferation, i.e., lower concentrations of statins promoted T cell proliferation, while higher concentrations inhibited. Probably there could be number of factors playing in cohort upregulating the T cell activation and proliferation some of the factors could be escape phenomenon where the cell tries to gear its metabolic machinery to escape from the statin mediated inhibition. Further Weis *et al* reported biphasic effects of statins on angiogenesis *in vitro* and *in vivo.*

In this culture system, isoprenylation intermediates of the mevalonate pathway were responsible for T cell proliferation to some extent and GGTI partially mimicked the effects of statin-mediated inhibition (Fig 4.12). We further investigated the effect of isoprenylation on the anti-proliferative nature of statins by rescuing individual components of the mevalonate pathway. Addition of mevalonate completely rescued statin-mediated T cell proliferation, whereas addition of GGPP and FPP did not rescue proliferation to a substantial level. This is consistent with the notion that mechanisms other than inhibition of prenylation are involved in the statin-mediated functions. This also suggests that statins act through a synergy of various pathways to elicit a complete arrest of proliferation.

In vitro treatment with statins inhibited T cell proliferation and was paralleled by inhibition of EMMPRIN. EMMPRIN antagonists AP-9 and neutralising anti-EMMPRIN antibody MEM6/6 inhibited T cell proliferation in mixed lymphocyte reaction in a narrow concentration range (Fig 4.15) [AP-9 (25-75 µg/ml) and EMMPRIN MEM6/6 (0.1 µg - 1 µg/ml)]. This is in line with the existing notion that EMMPRIN may require dimerisation for initiation of a signalling function (Koch et al., 1999). Functional inhibition was observed neither at higher nor at lower concentration of EMMPRIN antagonists. In conclusion, we provided evidence that EMMPRIN antagonists inhibit T cell proliferation in the T cell costimulatory assay (Fig 4.15 & 4.16).

Induction of T cell tolerance by immunosuppressive or immunomodulatory agents has recently emerged as an important approach for treatment of inflammatory autoimmune diseases such as MS. Anergy constitutes one mechanism of imposing tolerance by rendering autoreactive cells functionally inactive. In the present study, we report an induction of T cell anergy to the secondary stimulus IL-2, followed by *in vitro* coculture of T cells and monocytic cell lines, upon application of fluvastatin and EMMPRIN antagonists, i.e., AP-9 or EMMPRIN neutralising antibody (Fig 4.17). All the inhibitory treatments resulted in downregulation of CD25 (Interleukin-2 receptor). Thus the secondary stimulation with IL-2 was unable to stimulate secondary response in these populations of cells. These *in vitro* findings shed light on the novel putative mechanisms of statins action. All the immunomodulatory properties of statins were attributed to inhibition of isoprenylation (Ras GTP-binding proteins prenylation). The underlying mechanism of most of these functions was unexplored. We reported here that fluvastatin - an immunomodulatory agent currently under investigation for treatment of autoimmune disorders - induces anergy in human T cells as a consequence of decreased EMMPRIN. Similar phenomena were observed when EMMPRIN antagonists were employed in the assay.

5.2.1 Statin effect on T cells in mixed lymphocyte reaction (MLR)

EMMPRIN is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression (Sepponen et al., 2006). EMMPRIN family members have a centrally positioned glutamic acid residue. A charged amino acid deep within the transmembrane region is a common feature of proteins with multiple transmembrane domains (Manoharan et al., 2006). It has been previously reported that EMMPRIN and two members of the proton-coupled monocarboxylate transporter family, MCT1 and MCT4 are co-associated. Evidence of a close physical association between EMMPRIN and specific monocarboxylate transporters was also provided by their colocalisation at the cell surface (Xu and Hemler, 2005). In highly glycolytic cells such as white muscle cells, activated lymphocytes, an excess of lactic acid is produced, which must be exported from the cell to a avoid a drop in intracellular pH. In these cells the predominant monocarboxylate transporter is MCT4. Conversely, cells with a high oxidative capacity, such as heart and red skeletal muscles, which are rich in mitochondria, may utilise lactate as an energy source. In these cells there is a net influx of lactic acid and MCT1 predominates.

In our study, both EMMPRIN and MCT1 were increased in response to mitogenic stimulation (Fig 4.13 & 4.15). Studies with T cells labeled with fluorescent dye CFSE identified an extremely rapid phase of T cell division in which T cell blasts undergo upto to 5 divisions in 24 h. In the present study, fluvastatin rapidly downregulated EMMPRIN expression on proliferating cells (Fig 4.13 b). Fluvastatin dose dependently decreased EMMPRIN expression and mevalonate rescued it. This effect was mimicked by GGTI and rescued by GGPP. Further CD98 and CD29 were also simultaneously downregulated on the cell surface (Fig 4.14).

In conjunction to EMMPRIN, MCT1 was entrapped inside the cell which led us to monitor the intracellular pH (Fig 4.19) and extracellular lactic acid levels (Fig 4.20). Monocarboxylate transport in these cells was measured by flurometric ratio with the pH sensitive dye BCECF. The addition of lactic acid caused no shift in pH_i in fluvastatin treated cells whereas control Con A cells showed a drastic reduction in pH_i (Fig 4.21). Further fluvastatin treated cells coincubated with mevalonate, resulted in an increase of pH_i owing to MCT1 surface translocation, resulting in neutralisation of intracellular acidification

We then investigated the effect of MCT1 inhibition on extracellular/secreted lactate levels. Fluvastatin dose dependently decreased lactic acid release into the supertnant which was reversed by mevalonate (Fig 4.21). Changes in lactate levels correlated with EMMPRIN expression and T cell proliferation (Fig 4.20). These results provide a direct link between

lactate efflux via MCT1 and inhibition of proliferation via EMMPRIN. The increase in glycolytic rate observed in activated T cells is essential both for providing adenosine triphosphate (ATP) for ATP-consuming processes and for supplying metabolic intermediates that are channeled through the pentose-phosphate pathway to feed *de novo* nucleotide biosynthesis (Murray et al., 2005).

Our results suggest that statins inhibit T cell proliferation by blocking MCT1 translocation to the plasma membrane (Fig 4.18) through inhibition of EMMPRIN (Fig 4.13), such that the rapid phase of cell division which is triggered by mitogen or CD3 stimulation cannot be further sustained. T cells might be more sensitive than other cells, owing to their dependence on aerobic glycolysis during lymphocyte activation.

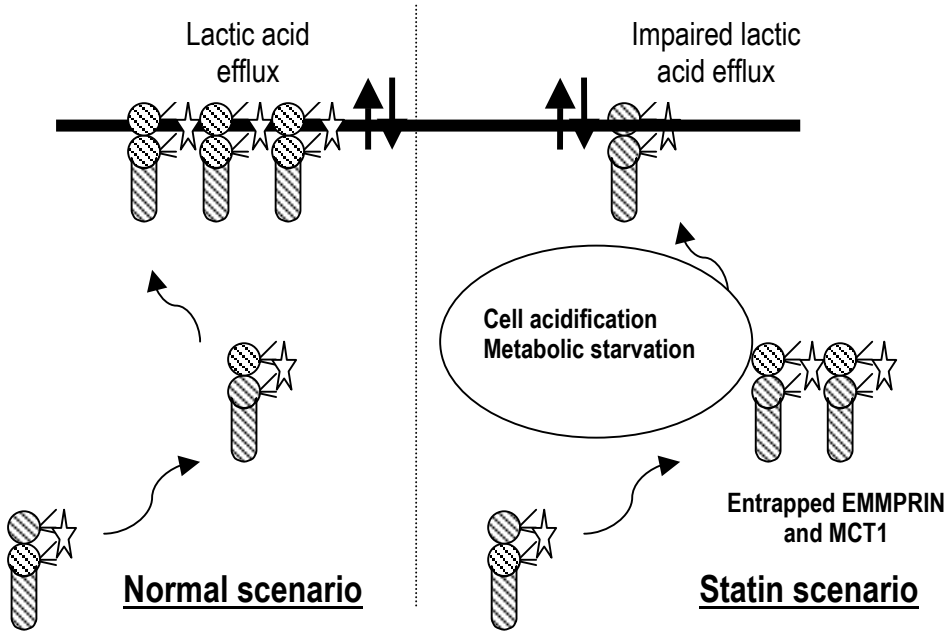


Figure 5.2: Hypothetical model of statin inactivation by MCT1 entrapment. Statins inhibit EMMPRIN and block MCT1 translocation to the cell surface. Entrapment of MCT1 in the cell blocks lactic acid efflux, thus decreasing intracellular pH leading to intracellular acidification. Due to improper MCT1 translocation on cell surface, nutrients and other monocarboxylate acids are unable to influx into the cell resulting in metabolic starvation (modified from Tang et al., 2004a).

In summary, figure 5.2 illustrates that statins inhibit T cell proliferation and that these effects are mimicked by EMMPRIN antagonists. The background mechanism that causes inhibition of proliferation can be attributed to MCT1 blockade. This results in disruption of lactate shuttle bidirectionally. Thus intracellular acidification occurs due to entrapement of lactic acid inside the cell and metabolic starvation occurs due to impairement of nutrient shuttle into activated cells. Statins might be a therapeutic option where autoactive immune cells were to be selectively killed sparing naïve cells without disturbing the immune homeostasis.

5.2.2 Statin effects on neurons via T cells

In a disease scenario like MS, neuronal apoptosis is a prime feature where neurons and axons are lost in large numbers (Bosel and Endres, 2006). This degenerative process is of importance in determining the clinical outcome. In MS, loss of neurons and axons correlates with the RR-MS to SP-MS. in transition from RR-MS to SP-MS. The direct cause of neuronal and axonal injury has not been elucidated and occurs in parallel to accumulation of several inflammatory cell types. Despite the presence of inflammatory cells in the lesion sites, a direct correlation of inflammatory cells and lesions were not yet confirmed. We studied the effect of de-activated T cells on neuronal injury (Fig 4.22). Our present study describes that activated T cells are potent neurotoxic agents and that the degree of activation of these cells correspond to the degree of neurodegeneration. In this scenario we also confirmed that neuronal cytotoxicity is contact dependent and MHC-I independent (Fig 4.23). Further this neuronal apoptosis is specific to neurons and is not wide spread to astrocytes. We had already reported statin-mediated T cell de-activation and these drugs exhibit a biphasic effects with upregulation of T cell proliferation at lower concentrations and complete immunosuppression at higher concentrations (Fig 4.10). We found an exact correlation to this scenario in terms of neuronal injury (Fig 4.25). Further the T cells used here were a mixed population of other cell types; we did not delineate the specific cell type responsible for neuronal injury and further did not probe into the mode of cell death on interaction with T cells.

When autoreactive T cells are transferred into susceptible animals, they migrate into the CNS where they face death due to upregulation of Fas-L by neurons (Elovaara et al., 1999). In a normal scenario the CNS has a high potential to eliminate T cell-dependent inflammation; in a MS scenario activated T cells infiltrate the CNS environment in significant numbers. In our studies, it was found by flow cytometric analyses that T cells were not subjected to cell death (Fig 4.25). The effector molecules that account for the T cell cytotoxicity may be either granzymes and perforins or Fas ligands, which were both reported to be downregulated by statins (Blanco-Colio et al., 2003).

In our *in vitro* model of neurodegeneration neutralising Abs against EMMPRIN and LFA-1 (cell adhesion molecule) inhibited cell death (Fig 4.26). This result was in accordance with the fact that statins and de-activation antibodies work in similar fashion. Both of them de-activate T cells which conceivably are unable to cause neuronal cytotoxicity. T cells are known to patrol the CNS under normal conditions; the reason why T cells do not kill the neurons in the normal state might be due to their relatively low cell numbers and might also

depend on the activation status of these cells. In summary, this study revealed that neurons are selectively vulnerable to T cell cytotoxicity, and that this might be responsible for neurodegeneration observed in inflammatory CNS diseases. Our studies suggest that while the initiation of MS may be attributed to the T cells that are antigen-specific, the subsequent arrival of activated T cells of unrelated specificities might damage CNS in a contact-dependent non-antigen restricted manner.

Further in EAE, Th1 cells are responsible for disease induction while Th2 cells can be protective (Hendrix and Nitsch, 2007). Statins are reported to bias cells towards Th2 environment. The neuroprotective nature of statins may be attributed to the following reasons: statins mediate direct binding to LFA-1 which is a ligand for ICAM-1, thus acting as antagonist of the ICAM-1/LFA-1 interaction, with the evidence which suggests that statins exert anti-proliferative effect on leukocyte expansion and result in a Th2 biased immune response. Taken together these data clearly suggest that statins limit CNS leukocyte accumulation through a number of mechanisms.

In conclusion statins modulate EMMPRIN structurally and functionally by depleting isoprenylation and dolichol. Thus statins are able to

- Inhibit MMP secretion and activation through cellular EMMPRIN bias towards lower glycosylated form which is an antagonist of MMP activation.
- Inhibit T cell activation and proliferation by rendering T cells anergic in a T cell-monocyte cocultures.
- Inhibit T cell activation through downregulation of monocytic EMMPRIN.
- Disruption of EMMPRIN activation complex ((CD98 –LAT1) - (CD29) - CD147-MCT1) on the cell surface.
- Inhibit translocation of MCT1 to cell surface leading to intracellular acidification due to accumulation of lactic acid.
- Cause metabolic starvation due to impaired surface MCT1.
- De-activated T cells are unable to cause neurotoxcity and this effect is mimicked by EMMPRIN antagonists.

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8 DECLARATION

EIDESSTATTLICHE ERKLÄRUNG

Hiermit versichere ich, dass die vorliegende Arbeit von mir selbst verfasst wurde und dass ich keine anderen als die von mir angegebenen Hilfsmittel verwendet habe. Alle Stellen, die aus anderen Werken im Wortlaut oder dem Sinn entsprechend übernommen wurden, habe ich mit Quellenangaben kenntlich gemacht.

Duesseldorf, 20. May 2007 ----------

(Venkata Sasidhar Manda)

9 CURRICULUM VITAE

Personal details

Name	:	Manda Venkata Sasidhar
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Date of Birth	:	18-08-1975
Address	:	Universität straße 1, Appartment 20315, 40225, Düsseldorf Germany Lab phone – +492118118678, Personal home – +492112058960, email – mvsasi1@gmail.com

Educational qualifications

02/2004 to date	:	PhD - Department of Neurology, Heinrich Heine University, Duesseldorf, Germany
1997 – 1999	:	Masters of Science - Biotechnology, Andhra University, Visakhapatnam, India (First division with distinction)
1993 – 1996	:	Bachelors of Science - Biochemistry, Botany and Chemistry, Andhra University, Visakhapatnam, India (First division)
1990 – 1992	:	Intermediate Education - Ushodhaya College, Visakhapatnam, India (First division)
1989 – 1990	:	Indian Certificate of Secondary Education - Visakhapatnam, India (First division)

Professional qualifications

2003	:	Specialised course in Biotechnology and Intellectual property - World Intellectual property Organization, Geneva, Switzerland.

1997 : Certificate course in computer applications - Institute of Computer Sciences, Visakhapatnam, India.

Research experience

9/2003 – 1/2004 : Research Associate (Biotechnology) - GVK Bio, Hyderabad, India

Summary of work details

Technical due diligence studies for method development, validation and quality control of diagnostic kits.

11/2001 – 8/2003 : Research Associate - Gland Pharma Ltd, Hyderabad, India

Summary of work details

Participation in Technology transfer - establishment of quality control parameters for testing recombinant bio-generics, (Granulocyte macrophage colony stimulating factor, GM-CSF).

Participation in training for testing bulk rhGM-CSF - Genemedix (United Kingdom) - in terms of bio-identity, bulk protein estimation, viral safety, potency, bio-activity, purity and functional activity of the drug followed by formulation of the drug and testing the drug in terms of bioactivity to United States Pharmacopeia (USP) standards.

Preparation of regulatory dossiers for bio-safety and regulatory clearance for the Department of Biotechnology, India, basing on data generated for the drug in China.

1/2000 – 10/2001 : Executive (Quality assurance) - VINS Bio-products, Hyderabad, India.

Summary of work details

Testing of in-process samples (potency and quality of venom), finished products (purified snake venom antiserum in terms of strength, dose and potency. QA tests including stability studies, flocculation studies, *in vivo* neutralisation and potency of antivenom in mice and endotoxin levels in rabbits and guinea pigs.

Research and Development work included “Extraction of hyaluronic acid from streptococcus zooepidemicus” - microbiological and biochemical screening of streptococcus and establishment of pilot scale fermentation parameters for culture and downstream processing of bacterial cell walls for extracting hyaluronic acid and further testing of hyaloronic acid.

Familiar techniques

Biochemical and Molecular biology

- Qualitative and quantative analysis of proteins (glycoproteins), Electrophoretic techniques involving SDS PAGE, Western blots, immunoprecipitation and surface biotinylation.
- Zymography and reverse zymography – casein, gelatine and collagen.
- Elisa – cytokines, lactic acid, soluble proteins and nitric oxide. B and T cell ELISPOT.

Flow cytometry

- Multiparameter flow cytometry – experience with FACS Calibur and FACS Cantor (8 color) for monocyte, T and B cell activation and differentiation.
- Cell tracking dyes CFSE, DioC18 for assessment of proliferation.
- Assessment of lymphocyte-mediated cytotoxicity and neuronal apoptosis by caspase substrates and mitochondrial potential.
- Phenotypic and functional analysis of T cell subsets (regulatory, memory T and B cells), intracellular cytokine staining.
- Functional analysis of membrane potential, intracellular calcium and intracellular pH.
- Functional analysis of lipid raft proteins in live cells by flow cytometry.

Microbiology and cell culture

- Microbiological and biochemical screening of bacteria
- Isolation and maintenance of primary and secondary T cell cultures (both short term and long term)
- Co-culture assays - T, B and monocytic cells with neuronal and epithelial cells .
- Quantative and qualitative microscopy - fluorescence and phase microscopy

Posters and Conferences

10/2006 : "Statins Inhibit Neuronal Cell Injury Promoted by Activated T Lymphocytes" - International Society of Neuroimmunology, Nagoya, Japan.

03/2006 : "Mechanisms of Statins in Multiple Sclerosis - Modulation of T cell Activation through Signals generated from EMMPRIN on Antigen Presenting Cells'' - American Academy of Neurology, San Diego, CA.

10/2004 : "De-activation of T cell activation by HMG-CoA reductase inhibitors'' - International Society of Neuroimmunology, Venice, Italy.

Publications

Neuhaus O, Manda VS, Scarpatetti , Hofler G, Hartung HP, Archelos JJ. New diagnostic options in tuberculous meningitis? **Int J Tuberc Lung Dis. 2006 Aug; 10(8):944.**

V. Sasidhar Manda, S. Manda Krishnaveni, Oliver Eickelberg, Hans-Peter Hartung, and Oliver Neuhaus. Down regulation of Monocytic Activation through Modulation of EMMPRIN by 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors (submitted to JBC)

V. Sasidhar Manda, Hans-Peter Hartung, and Oliver Neuhaus. Down regulation of Monocytic homo-aggregation and differentiation through Modulation of EMMPRIN by 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors (in preparation)

V. Sasidhar Manda, Hans-Peter Hartung, and Oliver Neuhaus.
Statin dependent immunosupression is MCT1 mediated by downregulation of EMMPRIN (in preparation)

V. Sasidhar Manda, Hans-Peter Hartung, and Oliver Neuhaus. Novel Co-stimulatory Signals EMMPRIN and its associated proteins CD98 and CD29 modulate T cell activation in monocyte - T cell co-culture (in preparation)

References

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