Università degli Studi di Cagliari

DOTTORATO DI RICERCA

"Sviluppo e sperimentazione di farmaci antinfettivi"

Ciclo XXVIII

TITOLO TESI

Studio dell'attività antivirale e/o anti-infiammatoria di nuove molecole di sintesi e/o di farmaci noti

Settore scientifico disciplinare di afferenza

BIO/19 – Microbiologia Generale

Presentata da: Dott.ssa Elisa Carta

Coordinatore Dottorato Prof.ssa Alessandra Pani
Tutor Prof.ssa Alessandra Pani

Esame finale anno accademico 2014 – 2015

Università degli Studi di Cagliari

RESEARCH DOCTORATE IN

"Research and Development of Anti-infective Drugs"

Cycle XXVIII

THESIS TITLE

Studies on the antiviral and/or anti-inflammatory activity of novel synthetic inhibitors and/or of known drugs

Scientific Area

BIO/19 –General Microbiology

Candidate: Dott.Elisa Carta

Doctorate Coordinator Prof. Alessandra Pani
Supervisor Prof. Alessandra Pani

Academic year 2014 – 2015

Acknowledgements

Elisa Carta gratefully acknowledges Sardinia Regional Government for the financial support of her PhD scholarship (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2007-2013 - Axis IV Human Resources, Objective l.3, Line of Activity l.3.1.).

La presente tesi è stata prodotta durante la frequenza del corso di dottorato in Sviluppo e Sperimentazione dei Farmaci anti-infettivi dell'Università degli Studi di Cagliari, a.a. 2014/2015 - XXVIII ciclo, con il supporto di una borsa di studio finanziata con le risorse del P.O.R. SARDEGNA F.S.E. 2007-2013 - Obiettivo competitività regionale e occupazione, Asse IV Capitale umano, Linea di Attività l.3.1 "Finanziamento di corsi di dottorato finalizzati alla formazione di capitale umano altamente specializzato, in particolare per i settori dell'ICT, delle nanotecnologie e delle biotecnologie, dell'energia e dello sviluppo sostenibile, dell'agroalimentare e dei materiali tradizionali"

I would like to thanks everyone who have helped me in realizing this thesis:

My supervisor, Prof. Alessandra Pani, for her scientific and personal support during these three years.

Dott. Bruce Chesebro, for allowing me in his laboratories.

James Striebel, for his support and affection during my stay at the RML Laboratories.

Dr. Giuseppina Sanna, for her precious help and for encouraging me.

Dr. Sarah Vascellari, Dr. Elisabetta Pinna, Dr. Silvia Madeddu, Dr. Roberta Melis, Dr. Rossana Tuveri and Dr. Daniela Perra, for their support and friendship.

Abstract

This thesis collects the work I have done during the three-year of my PhD Course. During my first year I have started a path that has allowed me to acquire different techniques to set up and culture animal cell lines as well as to evaluate the cytotoxic and anti-picornavirus activities of a class of novel synthetic quinoxaline (**Part I**). Part of the second and all the third year was spent at the NIAID/NIH, Hamiltion MT, USA, in the laboratory of persistent viral diseases under the supervision of dr. Bruce Chesebro investigating the effect of statins for the control of neuroinflammation (**Part II**).

Part I - Antiviral activity of quinoxalines derivates as new leads against Picornavirus

Aim: New quinoxaline derivatives were synthesized and tested for cytotoxicity and antiviral activity against human coxsackie virus B5 [CVB-5] and polio virus type-1 [Sb-1], focusing on the mode of action of the more potent compound *Ethyl 4-[(2,3-dimethoxyquinoxalin-6-yl)methylthio]benzoate* (**7a**). **Methods:**Virus multiplication in Vero76 cells were monitored by plaque reduction assay. Time of addition assays were performed to determine the influence of the time of treatment on the antiviral activity. Initial steps of virus infection were dissected into adsorption and penetration steps by switching the incubation temperature from 4°C to 37°C. **Results:** Four compounds (**7a**, **7b**, **8a**, **8b**) showed a very potent antiviral activity (EC_{50}s range 0.06-3.8 μM) against CVB-5. Compound **7a** resulted the most active and selective derivative (SI >1000). Time of addition assays revealed that compound **7a** interfered with the penetration/uncoating stage of the virus cycle. **Conclusion:** Further experiments to confirm the mechanism of action of the compoundare ongoing.

Part II - In vitro studies on the anti-inflammatory activity of statins during neuroinflammation

Aim: Due to the known pleiotropic activity of statins, Four different types of statins were investigated for their effect on the production of pro-inflammatory cytokines (IL-6 and CXCL10) in mouse and rat cortical microglia and astrocytes primary cultures. **Methods:** Primary cell cultures were obtained from 2 days old mice and Sprague-Dawley rats. The purity of the cell cultures was verified by immunofluorescence staining. The production of pro-inflammatory cytokines was stimulated by the mitogen LPS. The amount of cytokines produced in the absence and in the presence of statins was determined by using an extracellular protein kit specific for the cytokines analized. An MTT assay was performed in order to verify the viability of the cell cultures and to exclude a possible stimulation by statins themselves. **Results:** No LPS/stimulation and no statin/ cytokine inhibition was obtained in rat cells, while in mice derived cells, there was a good LPS stimulation and three out of the four statins inhibited of the production of IL-6 by mouse microglia.

Conclusion: Statins seems to have a good anti-inflammatory activity on mouse cells, however, further studies should be performed in order to confirm these results. In rat cells, further studies should be performed in order to find better conditions of stimulation. *In vivo* testing of the effect of Atorvastatin and Simvastatin in mice affected by scrapie are ongoing.

TABLE OF CONTENTS

PART I - Antiviral activity of quinoxaline derivates as new leads against Picornavirus

1. INTRODUCTION 8

2. RESULTS 11

3. CONCLUSIONS 14

4. MATERIALS AND METHODS 15

4.1 Quinoxaline derivates synthesis 15

4.2 Cells and viruses 16

4.3 Cytotoxicity assay 16

4.4 Antiviral assay 17

4.5 Yield reduction assay 17

4.6 Time of drug addition 17

4.7 Virucidal activity assay 18

4.8 Adsorption assay 18

4.9 Virus penetration assay 18

PART II- Study of anti-inflammatory activity of statins during neuroinflammation in vitro

1. INTRODUCTION 19

1.1 Neuroinflammation 19

1.2 Glia Cells and neuroinflammation 22

1.2.1 Microglia 23

1.2.2 Astrocytes 28

1.3 Cytokines 35

1.3.1 Interleukin (IL)-6 39

1.3.1.1 IL-6 and neuroinflammation 41

1.3.2 CXCL10 45

1.4 Prion diseases 47

1.5 Statins 52

1.5.1 What are statins and what is their function? 52

1.5.2 Anti-inflammatory properties of statins 55

1.5.3 Statins used in this work 57

2. AIM OF THE PROJECT 61

3. RESULTS AND DISCUSSION 62

3.1 Immunofluorescent staining 62

3.2 Inhibition of cytokine production with statins 65

3.2.1 Inhibition of the production of CXCL10 in mouse astroglia 66

3.2.2 Inhibition of the production of IL-6 in mouse astroglia 70

3.2.3 Inhibition of the production of IL-6 in mouse microglia 72

3.2.4 Inhibition of the production of IL-6 in rat astroglia 75

3.2.5 Inhibition of the production of IL-6 in rat microglia 77

3.3 MTT Assay 81

3.3.1 MTT Assay on the inhibition of the production of IL-6 in mouse astroglia 81

3.3.2 MTT Assay on the inhibition of the production of IL-6 in mouse microglia 82

3.3.3 MTT Assay on the inhibition of the production of IL-6 in rat astroglia 85

3.3.4 MMT Assay on the inhibition of the production of IL-6 in rat microglia 87

4. CONCLUSIONS 91

5. MATERIALS AND METHODS 93

5.1 Cell cultures 93

5.2 Immunofluorescent staining 94

5.3 Reagents 95

5.4 Induction of cytokine production 96

5.5 Determination of the inhibition of cytokine production 96

5.6 MTT Assay 98

PART I and II References (alphabetical order) 99

PART I

Antiviral activity of quinoxalines derivates as new leads against Picornavirus

1. INTRODUCTION

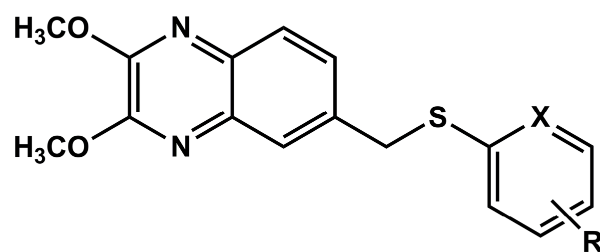
One of the major cause of morbidity and mortality in elderly people and young children are viral infections. Among them there are infections caused by human enteroviruses (HEVs), which are pathogens circulating commonly in the environment, with a seasonal peak during early fall. The infection is normally asymptomatic or mild, but occasionally the virus spreads to secondary organs leading to more severe diseases such as aseptic meningitis or myocarditis (Kandolf et al., 1999; Rotbart, 1995). Enteroviruses, which include Coxsackie A and B, polio, and ECHO viruses, cause systemic infection in man after ingestion and replication in the gastrointestinal tract (Woodruff, 1980). They belong to the *Picornaviridae* family, characterized positive single-stranded RNA genomes that are translated as monocistronic polyproteins to rapidly generate mature viral particles (Lim et al., 2013).

Coxsackieviruses are divided into group A and group B viruses based on early observations of their pathogenicity in mice, 23 serotypes of Coxsackie A viruses (CAV) and 6 serotypes of Coxsackie B viruses (CBV). Group A coxsackieviruses were noted to cause a flaccid paralysis, which was caused by generalized myositis, while group B coxsackieviruses were noted to cause a spastic paralysis due to focal muscle injury and degeneration of neuronal tissue; group B coxsackieviruses tend to infect the heart, pleura, pancreas, and liver, causing pleurodynia, myocarditis, pericarditis, and hepatitis. Coxsackie B viruses attach to target cells by receptors that are not shared with other members of the enteroviruses group. Thus all 6 Coxsackie B viruses compete for the same receptor, while polioviruses attach to a different

receptor on target cells. A large number of particles (60-100) are required for infection of a cell and once absorption, uncoating, and penetration occurs, replication of new infectious particles is rapid. Replication is confined to the cytoplasm and is directed by the single-stranded RNA genome that also serves as the messenger RNA for protein synthesis by the ribosomes (Fenner et al., 1974; Luria et al., 1978). Once the virus enters in the cells, macromolecular synthesis is rapidly suppressed; cell protein, RNA, and DNA synthesis are shut down after synthesis of viral-coded proteins. In particular, we worked on a Coxsackie B virus, Coxsackievirus B5(CVB-5) which is associated with several human diseases including encephalitis and myocarditis in immunocompromised children and CNS disease in older adults (Zhong et al., 2009).

Until now, there has been no enterovirus-specific vaccine or therapeutic reagent available for clinical use (); there are some inhibitors that has been use on picornaviruses but they have not effect on entetrovirus-induced myocarditis. So, in an attempt to discover new antiviral agents for these infections, during this part of my work we investigated the antiviral activity of quinoxaline derivates against Coxsackievirus B5.

Quinoxaline is also called as benzopyrazine. It is heterocyclic compound containing benzene ring and pyrazine ring (Patidar et al., 2011). Quinoxaline derivates are an important class of compound in which N replaces some carbon atoms in the ring of naphthalene. They are rare in natural state but their synthesis are easy to perform (Pereira et al., 2015). These compound seems to be interesting because they have been reported to show antibacterial, anticancer, antifungal, anti-inflammatory and antidepressant activities (Patidar et al., 2011).



2a-d, 3-6, 7-10a,b

2a) R = 4-OCH_3, X = CH
2b) R = 3,4-Cl, X = CH
2c) R = 3,4-CH_3, X = CH
2d) R = 2-$COOCH_3$, X = CH
3) R = 2-COOH, X = CH
4) R = 2-CO-Glu-Et, X = CH
5) R = 2-CO-Glu-H, X = CH
6) R = H, X = N

7a) R = 4-COOEt, X = CH
7b) R = 3-COOEt, X = N
8a) R = 4-COOH, X = CH
8b) R = 3-COOH, X = N
9a) R = 4-CO-Glu-Et, X = CH
9b) R = 3-CO-Glu-Et, X = N
10a) R = 4-CO-Glu-H, X = CH
10b) R = 3-CO-Glu-H, X = N

Fig. 1: Chemical structure of quinoxaline derivatives 2a-d, 3-6, 7-10a,b

2. RESULTS

Quinoxaline derivateswere tested in cell-based assays against two *Picornaviridae*, human enterovirus B [coxsackie virus B5, CVB-5] and human enterovirus C [polio virus type-1, Sb-1]. Results are reported in Table 1. Four compounds (7a, 7b, 8a, 8b) showed a very potent antiviral activity (EC_{50}s range of 0.06-3.8 μM) against CVB-5**.** Interestingly, **7a** showed a very selective activity, with a selectivity index (SI) >1000 and none cytotoxicity against all tested cell lines.

The potential virucidal activity of **7a** was investigated, incubating a CVB-5 solution containing $5x10^5$ PFU/ml for 1 hour at 37°C with two concentrations (4 and 20μM). Tested compounds failed to affect the CVB-5 infectivity, suggesting that the inhibition of CVB-5 replication observed in cell-based assays is not due to the infectivity inactivation of virions, but can be totally attributed to an interference of compounds with the viral life cycle.

To investigate the mechanism of **7a** on inhibition of CVB-5, we performed a time of drug addition experiment to determine at what stage of infection **7a** inhibits (Fig.2). Pretreatment with **7a** or addition of **7a** simultaneously to infection, followed by the removal of the sample after two hours, does not reduce the virus titre. The sample addition, simultaneously or at 2 hours post infection, significantly reduces the virus titre. The addition of sample at 4, 6, 8 or 12 hours instead doesn't reduce the virus titre, compared with the untreated control. These data suggest that **7a** inhibits CVB-5 infection by targeting an early event of the life cycle, probably during the attachment or the entry of virus.

Tab. 1 Cytotoxicity and antiviral activity of Quinoxalines derivatives against *Picornaviridae* (CBV-5, Sb-1) viruses.

Compds	Vero76	CVB-5	Sb-1
	[a]CC_{50}	[b]EC_{50}	[c]EC_{50}
2a	>100	>100	>100
2b	75	>75	>75
2c	>100	>100	>100
2d	>100	>100	>100
3	-	-	-
4	>100	>100	>100
5	-	-	-
6	>100	>100	>100
7a	>100	**0.09**	>100
7b	>100	**0.3**	>100
8a	65	**0.06**	>65
8b	90	**3.8**	>90
9a	-	-	-
9b	>100	56	>100
10a	-	-	-
10b	>100	>100	>100

[a]Compd concn (μM) required to reduce the viability of mock-infected Vero-76 cells by 50%, as determined by the MTT method;

[b]Compd concn (μM) required to reduce the plaque number of CVB-5 and Sb-1[(c)], by 50%, respectively, in Vero-76 cells.

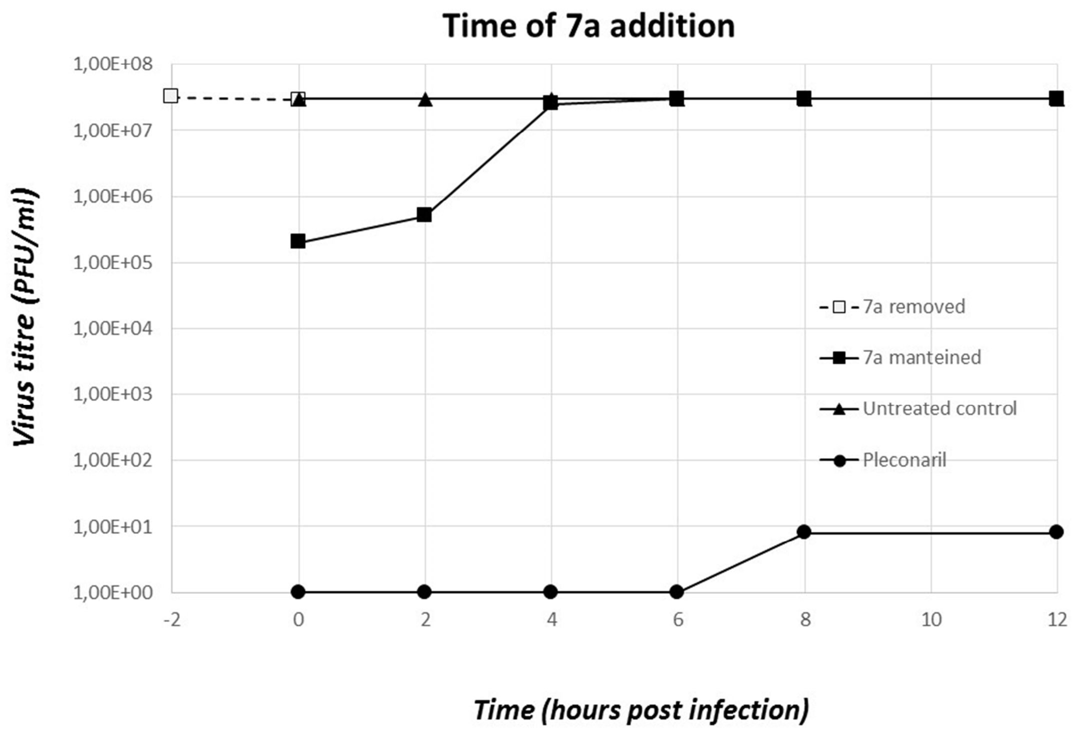


Fig. 2Time of drug addition experiment to determine at what stage 7a inhibits. The addition of sample simultaneously or at 2 hours post infection significantly reduces the virus titre.

3. CONCLUSIONS

With the aim of developing new therapeutic agents against Enterovirus, important human pathogens that cause both acute and chronic diseases in infants, young children and immunocompromised individuals, we tested quinoxaline derivates against Coxsackievirus B5(CVB-5) and Sb-1.

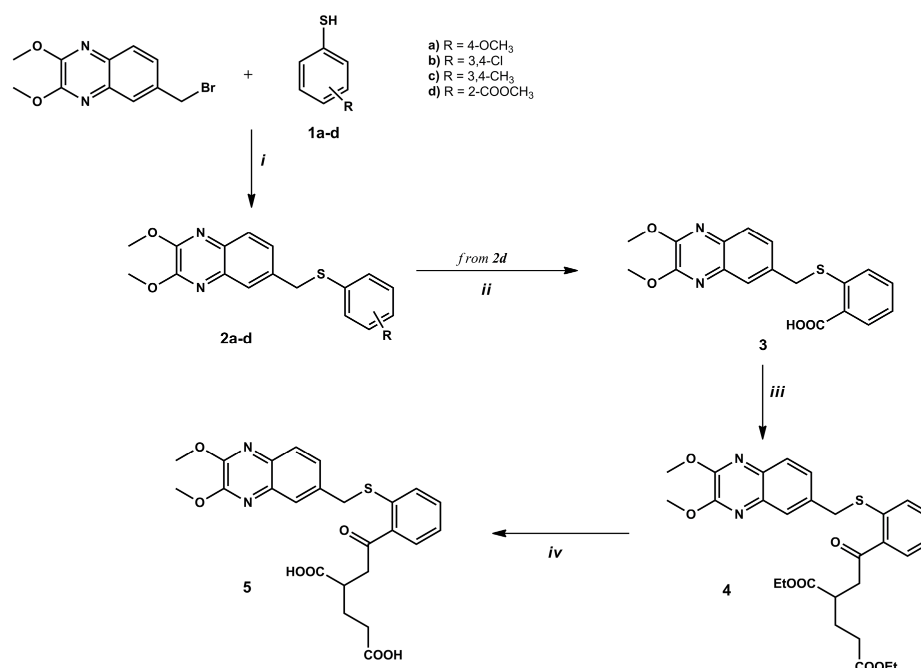
Unfortunately, none of compounds showed anti Sb-1 activity, but four of these derivatives resulted strongly active against CVB-5; in particular, **7a** and **8a** resulted endowed with potent and selective antiviral activity (EC_{50} = 0.09μM and 0.06 μM, respectively). Time of addition experiments suggest that **7a** inhibits CVB-5 infection by targeting an early event during the life cycle.

Coxsackieviruses type B is associated with several human diseases including encephalitis and myocarditis in immunocompromised children and CNS disease in older adults (Zhong et al., 2009) and unfortunately there has been no enterovirus-specific vaccine or therapeutic agents available for clinical use. Therefore, further studies are ongoing in order to better define the mechanism of action of **7a** and **8a**compounds. Overall, these findings may represent a good starting point for the development of novel and selective antiviral drugs for infections caused by Enterovirus, for which there is a continued need.

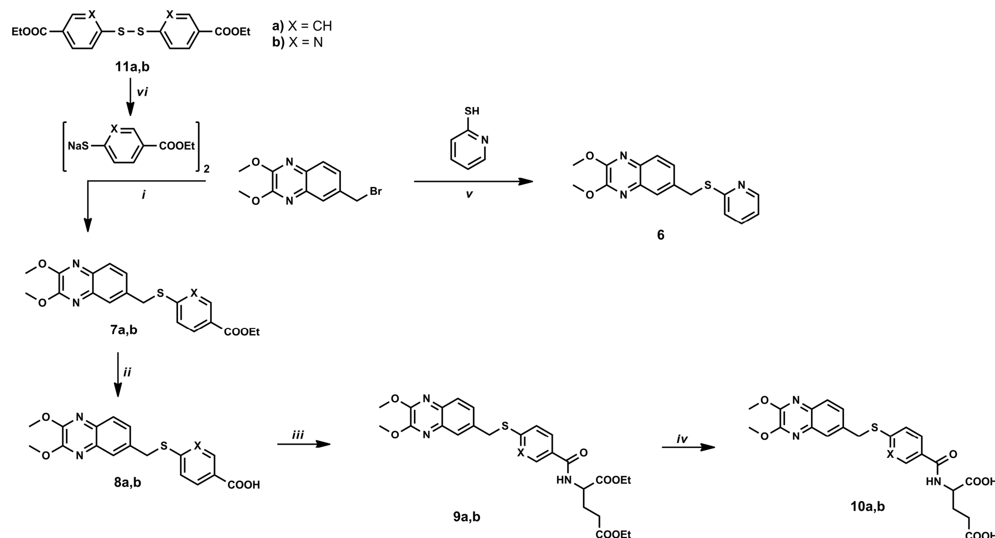
4. MATERIALS AND METHODS

4.1 Quinoxaline derivates synthesis

Quinoxaline derivatives **2a-d, 3-5** were prepared according to Scheme 1 while compounds **6, 7-10a,b** were prepared according to Scheme 2. The key intermediate 6-(bromomethyl)-2,3-dimethoxyquinoxaline was prepared according to the procedures already described in the literature. Its reaction with thiophenole derivatives (**1a-d**) was carried out in DMFa at 70 °C to give the expected compounds **2a-d**. From compound **2d** *via* basic hydrolysis the corresponding carboxylic acid **3** was obtained. This compound was amidified with diethyl-L-glutamate hydrochloride in DMFa, in presence of triethylamine (TEA) and diethyl dicarbonate (DEPC), to give derivative 4. The free acid **5** was finally obtained by alkaline hydrolysis.



Scheme 1: synthesis of 2,3-dimethoxy-6-((phenylthio)methyl)quinoxaline derivatives **2a-d**, **3-5**. Reaction condition: *i*) DMFa, $CsHCO_3$, 70 °C, 2h; *ii*) EtOH/H_2O, NaOH 1M, 70 °C, 7h; *iii*) L-diethylglutamate, DMFa, DEPC, TEA, r.t., 2h; *iv*) EtOH, NaOH 1M, r.t., 3h.



Scheme 2: synthesis of 2,3-dimethoxy-6-((phenylthio)methyl)quinoxaline derivatives **6, 7-10a** and 2,3-dimethoxy-6-((pyridin-2-ylthio)methyl)quinoxaline derivatives **7-10b**. Reaction condition: *i*) DMFa, r.t., 5 days; *ii*) EtOH/H_2O, NaOH 1M, 70 °C, 7h; *iii*) L-diethylglutamate, DMFa, DEPC, TEA, r.t., 2h; *iv*) EtOH, NaOH 1M, r.t., 3h.*v*) DMFa, $CsHCO_3$, 70 °C, 2h; *vi*) EtOH, $NaBH_4$, N_2, r.t., 30 min

4.2 Cells and viruses

Cell lines were purchased from American Type Culture Collection (ATCC). Cell line supporting the multiplication of the RNA virus was Monkey kidney (Vero 76) [ATCC CRL 1587 Cercopithecus Aethiops]. Viruses representative of ssRNA+ was Picornaviridae human enterovirus B [coxsackie type B5 (CVB-5), strain Ohio-1 (ATCC VR-29)] and Human Poliovirus type-1 Sabin (Sb-1) [strain Chat (ATCC VR-1562)].

4.3 Cytotoxity assay

Vero-76 cells were seeded in 24-well plates at an initial density of $4x10^5$ cells/mL, in Dulbecco’s Modified Eagle Medium (D-MEM) with L-glutamine and 25mg/L kanamycin, supplemented with 10% FBS. Cell culture was then incubated at 37 °C in a humidified, 5% CO_2 atmosphere, in the absence or presence of serial dilutions of test compounds. Cell

viability was determined after 48-96 hrs at 37 °C by the crystal violet staining method for Vero-76.

4.4 Antiviral assay

Compound's activity against CVB-5 was determined by plaque reduction assays in infected cell monolayers, as described earlier (Sanna et al., 2015). Briefly, Vero-76 monolayers were infected for 2 hours with 250 µL of proper virus dilutions; following removal of un-adsorbed virus, 500 µL of maintenance medium containing 0.75% methyl-cellulose, with or without serial dilutions of test compounds, were added. Cultures were incubated at 37°C for 3days and then fixed with PBS containing 50% ethanol and 0.8% crystal violet, washed and air-dried. Plaques were then counted. The extent of cell growth/viability and viral multiplication, at each sample concentration tested, were expressed as percentage of untreated controls. Concentrations resulting in 50% inhibition (CC_{50} or EC_{50}) were determined by linear regression analysis.

4.5 Yield reduction assay

Virus-infected cultures were incubatedwith antiviral compounds for a period sufficient to permit virus replication and thenassayed for the presence of new progeny virus by titration on separate monolayercultures.

4.6 Time of drug addition

In the time of drug addition experiments, Vero-76 cells were infected with CVB-5 at 0.3 MOI. **7a** at 1 µM was added at 2 h prior to infection, during the infection and at varying times post infection. The confluent monolayers of Vero-76 cells, seeded in 24-well tissue culture plates, were infected and incubated for 3 days as previously described. Monolayers were collected and the viral titre was determined by a plaque reduction assay. Medium

containing a ten-fold EC_{50} concentration of **7a**was: i) added at -2 to 0 (pretreatment) and then removed; ii) added at the time of infection (time 0) and removed or maintained; iii) added at 2, 4, 6, 8 and 12 hours post infection.

4.7 Virucidal activity assay

This assay was carried out to determine whether **7a** directly inactivated the CVB-5 virus. A CVB-5 suspension containing $5x10^5$ PFU/ml was incubated with or without different concentrations of compounds for 1 hour at 37°C. At the end of incubation, the residual infectivity was determined by plaque reduction assay in Vero cells.

4.8 Adsorption Assay

Vero cells grown in 24-well plates were infected with CVB-5, with a m.o.i. of 0.3, in the presence or absence of compounds. Plates were incubated for 60 min at 4°C. Medium containing unadsorbed virus was then removed, cells were washed twice with PBS and covered with MEM containing 1% methylcellulose. Plaques were counted after 24 hrs of incubation at 37°C.

4.9 Virus Penetration Assay

Vero cells were infected with CVB-5 at a MOI of 0.3 at 4°C for 1hr.Cells were washed by ice-cold PBS or medium and then shifted to 37°C in the presence or absence of compounds. The effect of compounds at different concentrations were tested. After 2 hrs of virus penetration, un-penetreted virus was washed and inactivated by acid glycine buffer (8g of NaCl, 0.38 g of KCl, 0.1 g of MgCl2 .6H2O, 0.1 g of CaCl2 2H2O, and 7.5 g of glycine/L, pH adjusted to 3 with HCl). Then methylcellulose was added and plate was incubated at 37°C for 24hrs.The amount of penetrated virus, which surviving the acid glycine treatment, was determined by plaque assay after 24 hrs.

PART II

Study of anti-inflammatory activity of statins during neuroinflammation in vitro

1. INTRODUCTION

1.1 *Neuroinflammation*

Neuroinflammation is becoming a very common problem. For a long time it was thought to be only a consequence of infectious diseases, but in the last few years it has become clear that neuroinflammation is part of the process that contributes to the degeneration of the central nervous system. While it is becoming clear that activation of microglia and astroglia and the attendant expression of proinflammatory cytokines and chemokines often are associated with disease-, trauma- and toxicant-induced damage to the CNS, it is by no means clear that a cause-and-effect relationship exists between the presence of a neuroinflammatory process and neural damage (O'Callaghan et al., 2008).

Neuroinflammation is an inflammatory process that hits the central nervous system and it is caused by infections, traumas, toxic metabolites or autoimmunity. When one of these stimuli hits the CNS, the first type of response is the activation of glia cells, like microglia and astroglia.

The inflammation is a process that is used as defense mechanism against pathogens or traumas. It includes distinct stages: first of all, there is the triggering event and the consequent molecular recognition by the cells of immune system (like microglia). These cells have different types of receptors on their cellular membrane that allow them to recognize different types of specific molecules on the surface of the pathogens. The

interaction between pathogen and receptor brings to the activation of a number of genes that encode cytokines and molecules involved in the phagocytosis. The most important role of cytokines is to stimulate other types of cells to produce chemical mediators of inflammation, which are responsible for the inflammatory response (and for this reason they are called proinflammatory cytokines), but they can have also other functions (Pontieri G.M., 2012).

The term "neuroinflammation" has come to denote chronic, CNS-specific, inflammation-like glial responses that do not reproduce the classic characteristics of inflammation in the periphery but that may engender neurodegenerative events including plaque formation, dystrophic neurite growth and excessive tau phosphorilation (Streit et al., 2004). Cytokines can be useful at first, because they help to fight the pathogen but at the same time, when their action is particularly intense or extended for a long time, what would be a help for the organism can start to damage it. The protracted production of cytokines, for example, becomes detrimental for the cells of the CNS. These kind of molecules are produced by the glia cells for the purpose of recruiting mononuclear phagocytes in the developing brain (Rezaie et al., 1999), in CNS inflammation and in neurodegenerative diseases (Rezaie et al., 2001). They are produced by glia cells but at the same time they go to activate them. After the activation, microglia start to proliferate and change their morphology, becoming rounded (ameboid) cells that are capable of moving faster throughout the CNS in order to find other pathogens; when microglia are activated they become phagocytes, to remove cellular debris of pathogens and CNS cells, and they start to increase the production of pro inflammatory cytokines. Also astrocytes activate during neuroinflammation but all the processes are not very clear; the activation of astrocytes is called "reactive gliosis" and we know that also these type of cells produces cytokines during this process.

Neuroinflammation is generally considered a chronic inflammation, in which the cumulative ill effects of immunological microglial and astrocytic activation contribute to and expand the

initial neurodestructive effects, thus maintaining and worsening the disease process through their action (Streit et al., 2004). This situation is generally called “gliosis” which includes both microglial and astrocytic hyperactivation. One of the consequence of the chronic neuroinflammation is in fact the microgliosis; this process involves hyperactivated microglia that start to proliferate and also exogenous perivascular cells originating in the bone marrow, that migrate into the CNS after an injury, to help the microglia. In a chronic state of inflammation, these cells are over-activated and produce a number of molecules that can be toxic for the neuronal tissue, like pro-inflammatory cytokines, prostaglandin E2 and oxidative stress factors.

As regards astrocytes, it is important to understand that with their endfeet they contibute to the formation of the Blood Brain Barrier (BBB), a barrier that separates the brain from the circulating blood, preventing the entrance in the CNS of toxic molecules and pathogens, under normal physiological conditions. Establishment, maintenance and repair of the endothelial barrier depend on pericytes and astrocytes and both are also linked to the disruption of the BBB (Argaw et al., 2012). When the astrocytes are activated in a condition of chronic inflammation, they undergo several changes which include the hypertrophy of cell bodies and glial processes, increased expression of proteins like GFAP, vimentin, nestin, tenascin-C and chondroitin sulfate proteoglycans (Cabezas et al., 2014; Alvarez et al., 2013). Moreover, they also start to produce a great amount of pro inflammatory cytokines that have a modulatory effect on microglia (Croisier and Graeber, 2006) and all these factors contibute to distrupte the BBB, which ceases to be a barrier and let toxic molecules, macrophages and lymphocytes spread into the CNS. Another brain damage after the activation of astrocytes is the formation of glial scars, which should be a mechanism to heal the brain but it could also bring detrimental effects. In fact, injury to the CNS induces tissue damage, which creates barriers to regeneration. One of the main barrier is the glial scar, which consists

predominantely of reactive astrocytes and proteoglycans. Axons can not regenerate beyond the glial scar, and they take on a dystrophic appearance of stalled growth (Silver and Miller, 2004). In the acute phase, glial scar formation is crucial for sealing the lesion site to remodel the tissue, and temporally and spatially controlling the local immune response. The glial barrier seals off the area of damage to prevent further microbial infections and spread of cellular damage, maintains extracellular ion and fluid balance, prevents an overwhelming inflammatory and growth factor responses, as well as free radical scavenging. Furthermore, the glial scar stimulates revascularization of blood capillaries to increase the nutritional, trophic, and metabolic support of the nervous tissue (Huang et al., 2014).

The glial scar produced after severe astrogliosis may separate necrotic tissue from healthy one, but also has the detrimental effect of impairing axonal regeneration through the expression of molecules like CSPGs, semaphorins and ephrin (Cabezas et al., 2014; Fitch and Silver 2008; Duffy et al., 2009).

It is easy to understand that neuroinflammation and brain injuries are connected and the first feeds the last and viceversa. It is also clear that neuroinflammation can be a possible target for the treatment of several brain injuries like neurodegenerative disease.

1.2 *Glia cells and neuroinflammation*

The central nervous system includes, in addition to neurons, the Glial cells - or Glia – which are quite different from nerve cells. For a long time, they were thought to be only supportive cells and that they had little influence on information processing; this opinion is now changed (Silverthorn 2010). Glia are more numerous than nerve cells in the brain, outnumbering them by a ratio of perhaps 3 to 1. Although glial cells also have complex

processes extending from their cell bodies, they are generally smaller than neurons, and they lack axons and dendrites (Purves et al., 2001). There are different types of glial cells, but the most important are microglia, astrocytes and oligodendrocytes. Microglia have a different origin, being cells belonging to the immune system; their role is to protect the SNC from pathogens, toxic molecules and other factors that can be dangerous for the brain. Astrocytes are the most abundant type of glial cells, being almost 50% of the brain cells. They have numerous projections that take contact with neurons and with blood vessels, in this way the astrocytes provide nutrition to nerve cells. Besides, they provide neurons with substrates for the ATP synthesis, contribute to maintaining the extracellular homeostasis by absorbing K^+ ions and water molecules, and they are also part of the Blood Brain Barrier thanks to their processes (Silverthorn 2010). Finally, oligodendrocytes are the cells that provide support and isolation to axon by producing myelin. For my thesis project I used two of these types of glial cells, Microglia and Astrocytes.

1.2.1 *Microglia*

Microglia were discovered and characterized for the first time between 1919 and 1921 by the Spanish neuroscientist Pio del Rio Hortega. In 1932 he wrote a chapter called "Microglia" for the book *Cytology and Cellular Pathology of the Nervous System*, in which he described microglia as cells that enter the brain during early development; that have amoeboid morphology and are of mesodermal origin; that they use vessels and white matter tracts as guiding structures for migration and enter all brain regions; that they transform into a branched, ramified morphological phenotype in the more mature brain; in the mature brain, they are found almost evenly dispersed throughout the central nervous system and display

little variation; each cell seems to occupy a defined territory; after a pathological event, these cells undergo a transformation; transformed cells acquire amoeboid morphology similar to the one observed early in development; these cells have the capacity to migrate, proliferate and phagocytose (Kettenmann et al., 2011). This is how the microglia could be described even now.

Microglia are the resident mononuclear phagocytes of the central nervous system, belonging to the glial system of non-neuronal cells that support and protect neuronal functions (Ginhoux at al., 2013).

Originally, microglia were thought to originate in the bone marrow; instead these cells take origin from embryonic hematopoietic precursors in the extra-embryonic yolk sac (YS) and then they migrate in the CNS prior to birth. It seems that the cells that derive from the bone marrow are recruited after an injury or in reaction to inflammation to help the microglia in the CNS. However it is not clear yet if these cells persist and become integrated or are a temporary addition to the endogenous population (Ginhoux et al., 2013). In humans, from 4.5 weeks of gestation, ameboid microglial cells were seen to enter the cerebral wall from the ventricular lumen and the leptomeninges (Rezaie et al., 2005; Monier et al., 2007). Once in the brain, they differentiate into “fetal macrophage population” even before the onset of monocyte production by the fetal liver (Naito et al., 1990).

Microglia can have different shapes depending on their location and on the role. In a healthy brain they are called “resting microglia”, though they are always active, in every moment; in this case they have a ramified morphology, a small soma with fine cellular processes (Kettenmann e al., 2011). Various studies using two-photon microscopy have shown that ramified microglia are not "resting", as it has long been thought, but instead they are very motile cells that constantly move their processes (Davalos et al., 2005; Nimmerjhan et al., 2005; Vinet et al., 2012). Under this condition they display a striking down-regulated

phenotype with low expression of CD45, Fc receptors and MHC class II (Perry and Teeling, 2013). Microglia constantly screen their microenvironment, making them the sentinels of the CNS. Their processes are very sensitive to any change of brain homeostasis; infection, trauma, ischemia, neurodegenerative diseases, or altered neuronal activity, that is any disturbance or loss of brain homeostasis indicating real or potential danger to the CNS can evoke rapid and profound changes in the microglial cell shape, gene expression and the functional behavior which summarily is defined as "microglial activation" (Kettenmann e al., 2011).

When they become active, they undergo many changes, for example alterations in their morphology, such as hypertrophy of the cell soma, increased branching, upregulation or de novo synthesis of cell surface or intracellular molecules, and proliferation (Perry and Teeling, 2013). They assume a conformation called "ameboid", in which the processes are shorter and they have a rounded shape. Local densities of microglia can also increase by proliferation, to provide more cells for the defense against invading germs and to organize for the protection and restoration of tissue homeostasis. Induction and rearrangement of surface molecules for cell-cell and cell-matrix interactions, changes in intracellular enzymes as well as release of multiple factors and compounds with pro-inflammatory and immunoregulatory effects are additional elements of the activation process. Microglia can unfold their phagocytotic activities to clear tissue debris, damaged cells, or microbes. Release of chemoattractive factors recruits and guides immune cell populations to the CNS, and presentation of antigens to T cells can subsequently aid the adaptive immunity in fight against viral or bacterial invasion. Gerhmann and colleagues, after inducing ischemia in rats, saw that microglial cells showed an increased expression of cellular markers such as the MUC 101- and the MUC 102 determinant and the CR3 complement receptor, recognized by the OX-42 antibody. These cellular markers are already found on resting microglial cells

and are known to be increased on activated microglial cells in other pathologies (Gerhmann et al., 1992).

In a 2002 work, Combrinck and colleagues injected bacterial lipopolysaccharide (LPS) into mice affected by prion disease to mimic a peripheral infection and they showed that prior priming of microglia by the degenerative process, followed by further activation through signaling from the periphery, resulted in increased brain IL-1β synthesis and the consequent acute sickness behavioural responses (Combrinck et al., 2002). In another interesting work, a group used cultured BV-2 mouse microglia activated with DA quinone, HIV TAT protein and LPS to understand the gene expression when microglia are stimulated and activated and they found that a total of 9882 annotated genes were scored as present in resting microglia; a panel of 116 common genes was increased in expression by all three activating conditions: these genes included cytokines IL-1α, IL-1β, and IL-6, a number of chemokine genes, Nos2, Gch1, Mmp13, as well as Tnf receptor 5. Increased production of cytokines and chemokines would drive inflammatory processes and could act in a feed-forward manner to enhance and perpetuate microglial activation. The increase in Gch1 would synthesize increased levels of tetrahydrobiopterin, an essential cofactor for Nos2, thereby allowing heightened or sustained production of nitric oxide (Thomas et al., 2005). So, among the genes that are upregulated, many of them encode cytokines and chemokines, which are also produced by microglia in resting conditions, but during the neuroinflammation the over production of these molecules can worsen the condition. Stimulation rapidly induces cyto/chemokine gene transcription and effective release while intracellular protein amounts remain mostly very low. Members of the IL-1 family may exist as preformed precursors, allowing instant conversion and release of mature cytokine upon stimulation (Fassbender et al., 2000, Hanisch, 2002). The most important cytokines produced by activated microglia are:

- **IL-1,** where IL-1α is the cell-associated form and IL-1β is the soluble form; its targets are

T- and B-cells, monocytes, and macrophages/microglia. Activated microglia can serve as major sources upon infection, ischemia, stroke, excitotoxicity, and mechanic injury (Hanisch, 2001a) and suppression of endogenous IL-1 has thus often neuroprotective effects.

- **IL-6,** which can be produced by a various cell types, including activated microglia. Like IL-1 and TNFα, IL-6 is considered a proinflammatory cytokine acting in the initiation and coordination of inflammatory responses and limiting the spread of infectious agents.
- **TNFα,** which is produced by neurons, astrocytes and microglia (on injury, activated microglia is an early and prominent source of TNFα) (Gabay et al., 1997). TNFα seems to have direct toxic effects on neuronal structures and myelin; apparently, low levels of TNFα have neuroprotective consequences, whereas high levels can cause destructive outcomes. (Hanisch, 2002).

Microglia can produce also GROα (KC), MIP-1α, MIP-1β, MIP-2, MCP-1, RANTES, IP-10, and IL-8 in response to experimental stimulation by bacterial agents, Aβ peptides, as well as cytokines, such as TNFα and IL-1. (Hanisch, 2002).

What is the purpose of cytokine production? They can activated other types of cells, like astrocytes and endothelial cells. Activation of astrocyte, for example, is needed for reestablishment of tissue integrity following injury; astrogliosis and glial scar formation are needed to close wounds, to wall off necrotic areas, and to stabilize regions of neural injury (Hanisch, 2002). Therefore, the cytokine release is an important process for the restoration of CNS functions, but in a condition of inflammation there is an overproduction of these molecules and this overproduction becomes detrimental for the brain itself. For example, activated microglia also produce nitric oxide and oxygen intermediate which are cytotoxic at high concentrations. Other microglial mediators can induce secondary reactions in endogenous or infiltrated cells, which are harmful for neuronal structures or interfere with their signaling. Microglia can stimulate lymphocytes by (re)presenting antigens in

conjunction with co-stimulatory signals and the release of cytokines. However, excessive recruitment of these microglial functions certainly has a profound impact on detrimental neuroinflammation and autoimmune defects (Hanisch, 2002).

1.2.2 *Astrocytes*

Astrocytes (*astron,* star) are ramified glial cells that seems to constitute 50% of the brain cells, distributed throughout the brain and the spinal cord. The first scientist who spoke about glia cells was Rudolf Virchow in 1858, who said that neuroglia was a “substance… which lies between the proper nervous parts, holds them together and gives the whole its form in a greater or lesser degree”.But it was with Camillo Golgi and his black staining reaction that it was possible to see the morphological details of these cells; Golgi described stellate neuroglial cells and found that some glial cells (which were to all probability the protoplasmic astrocytes) send the processes to the blood vessels, where they establish the endfeet structures; he developed a concept of nutritive role of glia and suggested that glial cells establish the metabolic link between blood vessels and the brain parenchyma. Santiago Ramón y Cajal developed the first specific stain for astrocytes, the gold chloride-sublimate technique, which, as we know today, labeled glial fibrillary acidic protein (GFAP). Using this technique Cajal confirmed the origin of astrocytes from radial glia, and also demonstrated that astrocytes can divide in the adult brain, thus laying the basis for much later discoveries of the stem properties of astroglia (Parpura and Verkhratsky, 2012). Finally, in 1891 Michael von Lenhossek gave them the name of “astrocyes”.

Astrocytes are divided in various groups, forming a network in which they can communicate through junctions. Astrocytes have many functions; for a long time they were thought to have only support function, but they connect to the neuron through synapses, for the

exchange of chemicals, they provide substrates to neurons for the ATP production and contribute to maintain homestasis by absorbing K^+ ions and water molecules. Finally, their processes surround blood vessels, forming the Blood Brain Barrier (Silverthorn 2010). Astrocytes are traditionally divided into *protoplasmic* and *fibrous* types based on their morphologic appearance and distribution in the central nervous system (CNS). In most instances, protoplasmic astrocytes predominate in gray matter and fibrous astrocytes are most common in white matter (Montgomery, 1994). Protoplasmic astrocytes are found throughout all gray matter and exhibit a morphology of several stem branches that give rise to many finely branching processes in a uniform globoid distribution; fibrous astrocytes exhibit a morphology of many long fiber-like processes (Sofroniew and Vinters, 2010). Glial cells (astrocytes and oligodendrocytes) are generated from radial glia cells in the ventricular zone and subventricular zone. During CNS development, neurogenesis precedes gliogenesis, with radial glial serving as both the scaffolding for migration and the neural stem cell (NSC) substrate for both cell types (Chaboud and Deneen, 2013); the peak of astrogliogenesis occurs late prenatal to early postnatal stages, that is, E18-P7 in various rodent CNS regions (Yang e al., 2013). Adult astrocytes, regardless of their anatomical location (gray or white matter), typically occupy a large and non-overlapping domain, which is composed of a large number of branches and fine processes (Bushong et al., 2002).

Although not all astrocytes express this specific marker, glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed in astrocytes, is typically used to distinguish and identify astrocytes within the central nervous system (Oberheim et al., 2012). Expression of glial fibrillary acid protein (GFAP) has become a prototypical marker for immunohistochemical identification of astrocytes (Sofroniew et al., 2010). GFAP was first isolated as a protein highly concentrated in old demyelinated plaques from multiple sclerosis patients and was then found to be associated immunohistochemically with reactive

astrocytes in such plaques and in other pathological contexts (Eng et al., 2000). However, GFAP is not an absolute marker of all non-reactive astrocytes and is often not immunohistochemically detectable in astrocytes in healthy CNS tissue or remote from CNS lesions. Although GFAP is immunohistochemically detectable in many astrocytes throughout the healthy CNS, it is clear from double staining with multiple markers (including transgenic reporter proteins) that many mature astrocytes in healthy CNS tissue do not express detectable levels of GFAP and that GFAP expression by astrocytes exhibits both regional and local variability that is dynamically regulated by a large number of inter- and intra-cellular signaling molecules (Sofroniew, 2009; Sofroniew et al., 2010).

Astrocytes, by virtue of numbers and relationships with other structural and cellular components, play a diverse and vital part in development and normal functioning of the CNS:

- *Neuronal migration*: It seems that neurons migrate along the processes of astrocytes during their development. Immunocytochemical studies have shown that the processes of radial glia contain vimentin and glial fibrillary acidic protein; these cells are believed to be immature astrocytes. Dur- ing further maturation of the CNS, these cells lose vimentin and differentiate into astrocytes after neuronal migration is complete (Montgomery, 1994).
- *CNS development*: Astrocytes are important for the development of CNS, for example molecular boundaries formed by astrocytes take part in guiding the migration of developing axons and certain neuroblasts (Powell and Geller, 1999). Substantive evidence is accumulating that astrocytes are essential for the formation and function of developing synapses by releasing molecular signals such as thrombospondin (Barres 2008, Christopherson et al., 2005, Ullian et al., 2001).
- *Extracellular matrix proteins and adhesion molecules* : Astrocytes are the major source of extracellular matrix proteins and adhesion molecules in the CNS. These molecules are

important in the development and maintenance of structural relationships (Montgomery, 1994).

• *Regulation of blood flow*: Recent findings show that astrocytes produce and release various molecular mediators, such prostaglandins (PGE), nitric oxide (NO), and arachidonic acid (AA), that can increase or decrease CNS blood vessel diameter and blood flow in a coordinated manner (Gordon et al., 2007; Iadecola and Nedergaard, 2007; Sofroniew and Vinters, 2010). Moreover, thanks to their processes which are in contact with blood vessels and synapses, they can titrate blood flow in relation to levels of synaptic activity (Sofroniew and Vinters, 2010).

• *Angiogenesis*: Angiogenesis is the physiological process through which new blood vessels form from pre-existing vessels. Steps involved in angiogenesis include endothelial activation, basement membrane dissolution, replication and migration of endothelial cells to form cords and tubes, and maturation with reconstitution of the basement membrane. Astrocytes apparently are involved in at least some stages of angiogenesis and this may be important in repair of the CNS (Montgomery, 1994).

• *Brain homeostasis*: astrocytes are important for the maintenance of the fluid, ions, pH and transmitter homeostasis of the synaptic interstitial fluid. Astrocytes processes are rich in aquaporin 4, which is a water channel; this channel play a critical role in regulating fluid homeostasis in the CNS (Sofroniew and Vinters, 2010).

• *Constitution of Blood Brain Barrier (BBB)*: The Blood Brain Barrier is a highly selective barrier that separates the circulating blood from the brain and allows to control the molecules that enter the CNS, leaving out potential toxic molecules or pathogens. The barrier also plays an important role in the homeostatic regulation of the brain microenvironment necessary for the healthy function of the CNS (Abbott, 2002). The BBB is formed by endothelial cells that form tight junctions and are surrounded by a basal lamina,

pericytes and astrocyte end-feet. With their end-feet, astrocytes are believed responsible for the formation of the tight junctions between endothelial cells (Montgomery, 1994). In a study of 1997, a group showed that cultured astrocytes implanted into areas with normally leaky vessels were able to induce tightening of the endothelium, indicating that astrocytes were a major source of the inductive influence from neural tissue (Janzer and Raff, 1997). Other studies suggest a role for astrocytes in regulating BBB properties of cerebral endothelial in vivo in adult mice through specific BMP signaling mechanisms on astrocyte endfeet that when disrupted cause BBB leaks (Araya et al., 2008; Sofroniew and Vinters, 2010). It is clear, thus, that the role of astrocytes in the constitution of the BBB and their activation, in particular, is fundamental in the process of neuroinflammation.

Like microglia, also astrocytes are important during an insult to the CNS, since they start a response which serves to limit the areas of damage and for the post-insult remodeling and recovery of neural function; this response is called "reactive astrogliosis". Astrocytes respond to all forms of CNS insults such as infection, trauma, ischemia and neurodegenerative disease by a process commonly referred to as reactive astrogliosis, which involves changes in their molecular expression and morphology, and in severe cases, scar formation (Sofroniew, 2009). What happenes when astrocytes are stimulated and activated? They undergo through a process of hypertrophy and proliferation, in which there is a dramatic increase in glial fibrils throughout the cytoplasm and the increased width, length, number, and complexity of the processes. As a result, the cell bodies and processes enlarge, become increasingly eosinophilic, and may even be visible with hematoxylin and eosin (Montgomery, 1994). The changes undergone during reactive astrogliosis have the potential to alter astrocyte activities both through gain and loss of functions that can impact both beneficially and detrimentally on surrounding neural and non-neural cells (Sofroniew, 2009). Astrocytes have the potential under different conditions of stimulation to produce

intercellular effector molecules of many different categories, or to alter their expression of molecules involved in all aspects of cellular activity including cell structure, energy metabolism, intracellular signaling and membrane transporters and pumps (Sofroniew, 2009).

Primarily, there is the up regulation of expression of GFAP and the body hypertrophy but not astrocyte proliferation, however, the up regulation of GFAP expression in astrocytes that do not express detectable levels of GFAP in healthy tissue can lead to the staining of more cells, sometimes giving the false impression of proliferation (Sofroniew and Vinters, 2010). If the triggering mechanism is able to resolve, then mild or moderate reactive astrogliosis exhibits the potential for resolution in which the astrocytes return to an appearance similar to that in healthy tissue (Sofroniew, 2009). But if the pathogen, or whatever caused the reaction of astroglia, does not resolve, the astrogliosis keeps getting worse. In this case, besides the hypertrophy of cell body and the up regulation of GFAP, astrocytes start to proliferate; finally there is the formation of the glial scar, which is a barrier formed by reactive astrocytes, microglia, endothelial cells and basal membrane and act as neuroprotective barrier to inflammatory cells and infectious agents, and that they form in particular along borders to severe tissue damage, necrosis, infection or autoimmune-triggered inflammatory infiltration (Sofroniew and Vinters, 2010). In the acute phase, glial scar formation is crucial for sealing the lesion site to remodel the tissue, and temporally and spatially controlling the local immune response. The glial barrier seals off the area of damage to prevent further microbial infections and spread of cellular damage, maintain extracellular ion and fluid balance, prevent an overwhelming inflammatory and growth factor responses, as well as free radical scavenging (Rolls et al., 2009). Some studies have focused on ablation of proliferating scar-forming astrocytes, where it was used a model based on the transgenically targeted expression of herpes simplex virus thymidine kinase

(HSV-TK) specifically to astrocytes, which renders dividing astrocytes vulnerable to the antiviral drug ganciclovir; they found that loss or dysfunction of reactive astrocytes leads to a prolonged increase in leukocyte infiltration, failure of BBB repair with vasogenic edema, neuronal degeneration, and increased outgrowth of nerve fibers in injured CNS parenchyma (Bush et al., 1999). The glial scar also acts as an impediment to axon regeneration and thus prevents the recovery of CNS function in the chronic phase, because the reactive astrocytes secrete several growth-inhibitory molecules that chemically prevent axonal extensions (Huang et al., 2014). There are many triggers of reactive astrocytes and glial scar, like a number of cytokines, nitric oxide, neurotransmitters, that can be produced by many brain cells like neurons, microglia, oligodendrocyte lineage cells, pericytes, endothelia, and other astrocytes, in response to all forms of CNS insults (Sofroniew et Vinters, 2010). Molecular triggers that lead to proliferation of reactive astrocytes in vivo include EGF, FGF, endothelin 1, and ATP (Gadea et al., 2008; Levinson et al., 2000; Neary and Zimmermann, 2009).

During astrogliosis, activated astrocytes produce a number of different molecules with either pro- or anti-inflammatory potential, like cytokines (Eddleston and Mucke, 1993; John et al., 2003) and they can exert both pro- and anti-inflammatory effects on microglia. How is it possible to have both effects? For example, astrocytes can exert different activities at different times after insults, or in different geographical locations in relation to lesions. So, reactive astrocytes may exert pro-inflammatory roles at early times after insults and in the center or immediate vicinity of lesions, but exert anti-inflammatory roles at later times and at the borders between lesions and healthy tissue (Sofroniew and Vinters, 2010).

Among the many functions of astrocytes there is the constitution of the blood brain barrier; when astrocytes undergo astrogliosis, the blood brain barrier inevitably suffers damage. Several processes may affect the integrity of the BBB, including an increase in ROS production, elevated levels of proinflammatory cytokines, and other toxic substances

(Cabezas et al., 2014) and many of these molecules are produced during neuroinflammation by microglia and astrocytes. All these triggers bring to the disruption of the BBB and its breakdown leads to edema, metabolic imbalance, excitotoxicity, and ingress of factors that potentiate inflammation and inhibit repair and facilitates infiltration of T and B lymphocytes, macrophages, and neutrophils (Argaw et al., 2012).

1.3 *Cytokines*

Cytokines are a class of small proteins that act as signaling molecules at picomolar or nanomolar concentrations to regulate inflammation and modulate cellular activities such as growth, survival, and differentiation (Vilcek, 2003). Cytokine is a general name; other names include lymphokine (cytokines made by lymphocytes and attract other immune cells like macrophages and othe lymphocytes to an infected site), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by one leukocyte and acting on other leukocytes). Cytokines may act on the cells that secrete them (autocrine action), on nearby cells (paracrine action), or in some instances on distant cells (endocrine action)(Zhang and An, 2007). The binding of a cytokine or chemokine ligand to its cognate receptor results in the activation of the receptor, which in turn triggers a cascade of signaling events that regulate various cellular functions such as cell adhesion, phagocytosis, cytokine secretion, cell activation, cell proliferation, cell survival and cell death, apoptosis, angiogenesis, and proliferation (Devi, 2005). In the nervous system, cytokines and chemokines function as neuromodulators and regulate neurodevelopment, neuroinflammation, and synaptictransmission (Ramesh et al., 2013). .Cytokines and chemokines are crucial to the brain's immune function serving to maintain immune surveillance, facilitate leukocyte traffic, and recruit other inflammatory

factors (Takeshita and Ransohoff, 2012).

Among their functions, they activate inflammation; upon stimulation by pathogens or toxic molecules, immune cells as well as cells of the nervous system such as microglia (the resident macrophages of the brain), astrocytes, oligodendrocytes, the myelinating cells of the CNS, and Schwann cells in the peripheral nervous system (PNS), endothelial cells of the brain microvasculature, and even neurons can release cytokines and chemokines as well as respond to them by way of cytokine and chemokine receptors (Ramesh et al., 2013).

Cytokines can be both pro-inflammatory, which promote inflammation, and anti-inflammatory which suppress the activity of pro-inflammatory cytokines; some pro-inflammatory molecules are:

IL-8, which activates neutrophils to degranulate and cause tissue damage; IL-1 and TNF, which are inducers of endothelial adhesion molecules, essential for the adhesion of leukocytes to the endothelial surface prior to emigration into the tissues (Dinarello, 2000); IFN-α, produced by leucocytes and involved in innate immune response against viral infection; IFN-γ, generated by both innate and acquired immune cells, particularly T cells and NK cells and which can promote Th1 polarization, facilitate specific cytotoxicity by increasing the expression of MHC class-I and -II molecules, and boost antigen processing and immunoglobulin switching (Su et al., 2012); IL-23, which is heterodimeric cytokine composed of a p19 subunit and a p40 unit shared with IL-12 and it is produced by antigen presenting cells. IL-23 affects IFN-γ production, stimulates Th1-cell responses, activates memory T cells, and enhances inflammation by stimulating the production of proinflammatory cytokines. IL-23 is also essential to promote the expansion and survival of IL-17- producing cells (Riol-Blanco et al., 2010); IL-17, which has multiple functions in the regulation of tissue inflammation. There are 6 different IL-17, named IL-17A to IL17F (Su et al., 2012). IL-17 has a key role in tissue neutrophil recruitment and helps the clearance of

pathogens such as extracellular bacteria. IL-17 is produced by several cell types including activated T-cell subsets (CD4+, CD8+, CD4− CD8−, γδT cells), natural killer cells, and neutrophils (Dong, 2006).

Among anti-inflammatory cytokines there are: IL-1ra, produced by monocytes and macrophages, is a 152-amino-acid protein that functions as a specific inhibitor of the two other functional members of the IL-1 family, IL-1α and IL-1β (Dinarello, 1996; Dinarello 1998). IL-1ra, IL-1α and IL-1β are very similar, IL-1ra shares approximately 26% amino acid sequence homology with IL-1b and 19% homology with IL-1a and the genes that encode these proteins are very close, on the long arm of chromosome 2 (Dinarello, 1996; Opal 1996). IL-1ra blocks the action of IL-1a and IL-1b functional ligands by competitive inhibition at the IL-1 receptor level. IL-1ra binds with equal or greater affinity than does IL-1a and IL-1b to the type 1 (80 kd) membrane-bound IL-1 receptor. IL-1ra does not bind with high affinity to the type II (68 kd) IL-1 receptor (Dinarello, 1997; Simms et al., 1993). By occuping the receptor, IL-1ra prevent cellular activation by IL-1α or IL-1β. The anti-inflammatory cytokines IL-4, IL-10, and IL-13 inhibit the synthesis of IL-1β, yet they stimulate the synthesis of IL-1ra.1 (Schreuder et al., 1997).

IL-4, is a glycoprotein produced by mature Th2 cells and cells from the mast cell or basophil lineage and it is able to influence Th cell differentiation. IL-4 is an anti-inflammatory cytokines because it goes to block or suppress the monocyte-derived cytokines, including IL-1, TNF-a, IL-6, IL-8, and macrophage inflammatory protein (MIP)-1α (Opal and DePalo, 2000; Brown and Hural, 1997; Paul, 1991). This cytokine also suppresses macrophage cytotoxic activity, parasite killing, and macrophage-derived nitric oxide production (Vannier et al., 1992), and stimulates the synthesis of the cytokine inhibitor IL-1ra (Hart et al., 1989).

IL-10, which is the most important anti-inflammatory cytokine found within the human immune response. It is a potent inhibitor of Th1 cytokines, including both IL-2 and IFN-γ

(Opal and DePalo, 2000). IL-10 is primarily synthesized by CD41 Th2 cells, monocytes, and B cells and inhibits monocyte/macrophage-derived TNF-α, IL-1, IL-6, IL-8, IL-12, granulocyte colony-stimulating factor, MIP-1α, and MIP-2α (Clark et al., 1998; Marchant et al., 1994). ,IL-10 promotes the development of a type 2 cytokine pattern by inhibiting the IFN-γ production of T lymphocytes (Romagnani, 1995), directly inhibited the proliferation of CD4+ T cells and production of cytokines such as IL-2, IFNγ, IL-4, IL-5, and TNF-α (Joss et al., 2000), consequently impairing cellular immune responses, and regulates Th1/Th2imbalance. IL-10 also reduces the secretion of IL-23 by macrophages, which is essential for the existence of Th17 cells (Llorente et al., 1995).

IL-11 was initially described as a hematopoietic growth factor with particular activity in the stimulation of thrombopoiesis (Opal and DePalo, 2000); this anti-inflammatory cytokine has been shown to attenuate IL-1 and TNF synthesis from macrophages by up-regulating inhibitory NF-kB (inhibitory NF-kB) synthesis in monocyte/macrophage cell lines. Inhibitory NF-kB prevents NF-kB from translocating to the nucleus where NF-kB functions as a transcriptional activator for the proinflammatory cytokines (Trepicchio et al, 1997). IL-11 has also been shown to inhibit the synthesis of IFN-g and IL-2 by CD41 T cells.

IL-13, is secreted by activated T lymphocytes; IL-13 and IL-4 share a common cellular receptor, and this accounts for many of the similarities between these two anti-inflammatory cytokines (Callard et al., 1996). The principal functional difference between IL-4 and IL-13 lies in their effects on T cells. IL-4 is a dominant mediator of Th2 cell differentiation (Zurawski et al., 1994), whereas IL-13 has minimal effects on T-cell function. IL-13 can down-regulate the production of TNF, IL-1, IL-8, and MIP-1α by monocytes (Zurawski et al., 1994; de Waal Malefyt et al., 1993) and has profound effects on expression of surface molecules on both monocytes and macrophages (de Waal Malefyt et al., 1993).

TGF-β is an important regulator of cell proliferation, differentiation, and formulation of the

extracellular matrix (Litterio and Roberts, 1997). Like many cytokines, TGF-b has both pro- and anti-inflammatory effects. It functions as a biological switch, antagonizing or modifying the action of other cytokines or growth factors (Opal and DePalo, 2000). In an inflammatory state, TGF-β is produced by macrophages upon their phagocytosis of apoptotic cells and exerts an anti-inflammatory e□ect, reflecting a negative regulation of inflammation processes (Fadok et al., 1998). It can convert an active site of inflammation into one dominated by resolution and repair (Litterio and Roberts, 1997). An isoform of TGF-β, TGF-β1, is capable of suppressing the proliferation and differentiation of T cells and B cells and limits IL-2, IFN-g, and TNF production (Opal and DePalo, 2000).

With the potential exception of interleukin (IL)-1 receptor antagonist (IL-1ra), all anti-inflammatory cytokines have at least some pro-inflammatory properties as well (Opal and DePalo, 2000). For example, although the cytokine IL-6 can be also anti-inflammatory, in this work I will treat the argument considering its pro-inflammatory properties during neuroinflammation.

1.3.1 *Interleukin (IL)-6*

Interleukin-6 (IL-6) is one of the two cytokines that I studied and analyzed during this part of my PhD work. IL-6 is part of the family of cytokines that includes IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary inhibitory factor (CNTF), cardiotropin-1 (CT-1), cardiotrophin-like related cytokine and stimulating neurotrophin-1/B-cell stimulating factor 3 (NNT-1), neuropoietin (NPN), IL-27, and IL-31; with the exception of IL-31, all IL-6 type cytokines share the membrane glycoprotein gp130 as a common receptor and signal transducer subunit (Heinrich et al., 2003; Scheller et al., 2006). It was

originally discovered as a factor produced by lymphocytes that stimulated the final maturation of B cells to antibody-producing cells (Hirano et al., 1986). As I said, many cytokines have both pro- and anti-inflammatory properties and IL-6 is one of them; it was originally considered to be a pro-inflammatory cytokine, but several discoveries prompted a revision of its characteristics and indicated that IL-6 also has anti-inflammatory properties (Sporeen er al., 2011). For example, IL-6 inhibits neutrophil accumulation after LPS injectionand antagonizes the action of IL-1β and TNF-α via induction of the soluble IL-1 receptor antagonist and the soluble TNF-α receptor (Tilg et al., 1994; Ulich et al., 1991). On the other hand, IL-6 is considered a proinflammatory cytokine acting in the initiation and coordination of inflammatory responses and limiting the spread of infectious agents; it helps to initiate and regulate acute-phase responses, a complex of adjustments in metabolic and executive organ functions (liver, immune cells) and circulating serum components that assist in host defense. CNS activities relate to fever induction, sleep, increased pain perception, reduced food intake, and neuroendocrine mobilization of energy stores (Hanisch, 2002). The tendency for one over the other net effect is likely determined by the simultaneous presence of other factors (like cytokines). IL-6 seems to be one of the most important cytokines in the CNS: a vast range of stimuli, varying from neurotransmitters over depolarization to other inflammatory cytokines, is able to induce IL-6 production in the brain (Sporeen et al., 2011).

IL-6 belongs to a cytokine family of which all the members share a common signal transducer named gp130 (glycoprotein 130 or CD130). All the family members possess a similar tertiary structure, composed of 4 α-helices with an up-up-down-down topology. Of these members, several have a role in the CNS. The IL-6 family members bind to their respective membrane receptors and this binding leads to the dimerization of the signal-transducer protein gp130 (Sporeen., 2011).

The receptor complex mediating the biological activities of IL-6 consists of the IL-6 binding

type I transmembrane glycoprotein termed IL-6R (or CD126 or gp80) and the type I transmembrane signal transducer protein gp130. IL-6 first bind to the membrane-bound non-signaling α-receptor IL-6R. Then, this complex binds to two molecules of gp130 and leads to IL-6-signal transduction, which includes activation of JAK/STAT, ERK, and PI3K signal transduction pathways (Scheller et al., 2011). Once recruited, the STATs (especially STAT3) are phosphorilated by JAKs and this induces dimerization of STATs and their subsequent translocation to the nucleus where they modulate gene transcription Sporeen et al., 2011).

Some cytokines have soluble receptors, and they are important for the regulation of cytokine signaling (Scheller et al., 2011); IL-6 also has a soluble receptor, s IL-6r; this was discovered in urine and in plasma and is generated via either alternative splicing of IL-6R mRNA (Lust et al., 1992), or shedding of the membrane receptor (Mullberg et al., 1993). This soluble receptor amplifies IL-6-mediated signaling by activation of cell types that express the signal transducer protein gp130 but lack mbIL-6R expression (Scheller et al., 2011). Although gp130 is an ubiquitous protein, IL-6R expression is much more restricted. The generation of s IL-6R is a mechanism to conferring IL-6 responsiveness to cells lacking IL-6R (Spooren et al., 2011).

1.3.1.1 *IL-6 and neuroinflammation*

IL-6 is really important during neuroinflammation. Soon after its discovery, it was demonstrated that some astrocytoma and glioma lines expressed IL-6 when stimulated with IL-1β, which prompted speculation that IL-6 could have a role in the CNS (Yasukawa et al., 1987; Erta et al., 2012). Later, it was discovered that both glial and neuronal cells expressed IL-6 and IL-6R to various degrees throughout the brain (Cornfield and Sills, 1991; Vallieres

and Rivest, 1997). Besides neurons and glial cells, endothelial cells produce great amounts of IL-6, which can act on surrounding cells but also in an autocrine manner regulating a number of adhesion proteins but also IL-6 synthesis, particularly in the presence of sIL-6R (Jirik et al., 1989; Reyes et al., 1999).

It is clear that IL-6 is an important cytokine in the CNS because many cytokines and inflammatory factors as well as neurotranmitters and neuropeptides have been shown to affect is regulation (Erta et al., 2012). For example, when microglia and astroglia are infected by a virus, they both secrete IL-6 (Frei et al., 1989; Righi et al., 1989); IL-1β and TNFα induced IL-6 in cultured cortical neurons (Ringheim et al., 1995); the AMPc-PKA pathway may also induce astrocytic IL-6 (Norris et al., 1994; Cadman et al., 1994). IL-1β is a potent stimulator of IL-6 production in astrocytes (Lee et al., 1993); IFN-γ induces IL-6 (and NO) in the murine microglial cell line 6-3 (Hashioka et al., 2007), this cytokine does not induce IL-6 in astrocytes unless it is coincubated with IL-1β (Benveniste et al., 1990). IL-17 functioned in a synergistic manner with IL-6 (+ sIL-6R) to induce IL-6 expression in astrocytes (Ma et al., 2010). These are some examples that explain the central role of the IL-6 production in the CNS. There are dozens of other different pathways and stimuli that leads to its secretion.

IL-6 is produced also in physiological conditions. For instance, they have an influence on neurons; in normal conditions IL-6 KO mice show a 60% reduction of the compound action potential of the sensory branch of the sciatic nerve and a dramatic decrease of temperature sensitivity in the frontpaw withdrawal time in the hot-plate assay; and IL-6 is also important for neuronal recovery after injury (Zhong et al., 1999). IL-6 also has a role in adult neurogenesis (Bauer et al., 2007; Deverman and Patterson, 2009), the process of creating new neurons and glial cells from neural stem cells (NSCs) (Erta et al., 2012).

IL-6 has effect also on glia and endothelial cells: it seems that IL-6 could have proliferative

effects on astrocytes but in synergy with other factor (Levinson et al., 2000) while microglia in culture consistently proliferate when stimulated with IL-6 (Streit et al., 2000). Regarding oligodendrocytes, in addition to promoting oligondendrogenesis, IL-6 promotes survival and myelin production of oligodendrocytes (Valerio et al., 2002; Zhang et al., 2006; Pizzi et al., 2004; Zhang et al., 2007).

Besides, IL-6 stimulates the pituitary-adrenal axis (Navarra et al., 1992; Mastorakos e al., 1993), induces fever (Schöbitz e al., 1995; Klir et al., 1993; Chai et al., 1996) and it is very important in the control of body temperature following recovery from stroke (Herrmann et al., 2003).

CNS IL-6 is upregulated whenever neuroinflammation is expected, such as following CNS infection or injury or in a number of CNS diseases (Erta et al., 2012). Studies demonstrated that IL-6 was expressed and produced in CNS during viral meningitis, in encephalitis mouse models, and in CSF of patients with acute viral infections (Frei et al., 1988) and IL-6 was also found to be upregulated in mouse experimental cerebral malaria (ECM) (Grau et al., 1990). IL-6 was highly found in CSF of patients with systemic lupus erithematosus (SLE) with CNS involvement (Hirohata and Miyamoto, 1990) or in those in advanced stages of patients with human immunodeficiency virus (Pagliusi et al., 1990) infections (Laurenzi et al., 1990). Two studies, where transcriptomic analysis of IL-6KO mice versus WT mice and that of GFAP-IL-6 mice were used in a model of brain cortex cryoinjury, revealed that IL-6 modulates the expression of many genes involved in inflammation, apoptosis and oxidative stress among others (Poulsen et al., 2005; Quintana et al., 2008).

IL-6 production is also altered in neurodegenerative disease, where there always is inflammation. So, its expression is elevated in brain areas surrounding amyloid plaques and in cerebrospinal fluid in the brains of Alzheimer's disease (AD) patients (Bauer et al., 1991; Hampel et al., 2005). IL-6 also enhances neuronal damage induced by beta-amyloid peptide

in cultured rat cortical neurons (Qiu and Gruol, 2003). It has also been demonstrated the presence of IL-6 in acute and chronic active plaques of multiple sclerosis (MS) patients, mainly associated with astrocytes rather than macrophages or mononuclear infiltrating cells (Maimone et al., 1997). IL-6 seems to have a role also in Parkinson's disease (PD), because its level in the CSF of PD patients are elevated (Mogi et al., 1996). As regard another neurodegenerative disease, Huntington's disease (HD), IL-6 expression was found to be dramatically elevated in the striatum of HD patients (Björkqvist et al., 2008).

IL-6 is important for processes like astrogliosis and microgliosis. As regard the activation of astrocytes, it has been demonstrated that direct infusion of IL-6 in rat brain increases GFAP expression and leads to astrogliosis (Balasingam et al., 1994; Woiciechowsky et al., 2004); indirect delivery of IL-6 in the striatum also leads to astrogliosis (Tilgner et al., 2001). Furthermore, IL-6 $^{-/-}$mice have impaired neuroglial activation and reduction of astrogliosis after injury (Klein et al., 1997). IL-6 activates astrocytes through the JAK-2/STAT-3 pathway (Damiani and O'Callaghan, 2007; Sriram et al., 2004). The cytokine and this pathway seems to be implicated in driving astrogliogenesis (Bonni et al., 1997; He et al., 2005).

IL-6 affects astrocytes in multiple ways, influencing their proliferation, the secretion of inflammatory mediators and growth factors, astrocytic chemotaxis and astrogliogenesis (Sporeen et al., 2011). This cytokine induces a micro-RNA for the cyclin-dependent kinase inhibitor 2A (CDKN2A) in astrocytes and this allows down-regulation of the cell growth repressor CDKN2A and in this way the astrocytes can proliferate (Pogue et al., 2010). Moreover, astrocytes are induced by IL-6 to produce several other cytokines, chemokines, prostaglandins and acute phase proteins (Barnum et al., 1996; Bolin et al., 2005; Chikuma et al.,2009; Kordula et al 1998; Quintana et al., 2008; Sporeen et al., 2011). The stimuli that induce astrocytes to produce IL-6 are different: neurotransmitters (e.g. norepineprhine),

neuropeptides (e.g.vasoactive intestinal peptide, substance P), inflammatory cytokines (e.g. TNF-α, IL-1β and IL-6 itself) and viral and bacterial pathogens (e.g.LPS) (Sporeen et al., 2011).

IL-6 influences also the microglia and the process of microgliosis. In numerous studies, it has been seen the correlation between the overexpression of IL-6 and the enhanced microgliosis with astrogliosis, in vivo (Brunello et al., 2000; Chiang et al., 1994; Di Santo et al., 1996; Fattori et al., 1995); besides, it is possible to block the release of LPS-induced microgliosis by intracerebral injection of an anti-IL-6 antibody (Pang et al., 2006). IL-6 stimulated also microglia proliferation in vitro (Streit et al., 2000).

Finally, IL-6 seems to have an important role in controlling the integrity of the BBB, but not as a proinflammatory cytokine. In this case, IL-6 acts as an antiinflammatory cytokine, by helping to keep the integrity of the BBB during neuroinflammation (Milner and Campbell, 2006).

1.3.2 *CXCL10*

I also analyzed another cytokine, the CXC motif chemokine 10 (CXCL10 or IP-10). CXCL10 is a cytokine belonging to the CXC chemokine family. The chemokine family contains 48 structurally homologous chemotactic cytokines that induce leukocyte migration and activation by binding to seven transmembrane-spanning receptors shown to be coupled to pertussis toxin (PTX) sensitive Gai proteins (Klein, 2004). They are small secreted molecules that exhibit very specific cysteine motifs in their amino acid sequence. Most chemokines have four characteristic cysteines, and depending on the motif displayed by the first two cysteines, they have been classified into CXC or alpha, CC or beta, C or gamma,

and CX3C or delta chemokine classes (Rossi and Zlotnik, 2000). These structural motifs bear functional differences in terms of chemoattraction for various leukocyte cell types and so, CXC chemokines may attract neutrophils or lymphocytes (Klein, 2004).

CXCL10 is a IFN-γ-inducible chemokine; first, IFN-γ binds to the IFN-γ heterodimeric receptor that activates the JAK/STAT pathway, which leads to STAT1 activation, then STAT1 translocate to the nucleus and binds to the promoter region of immediate-early-IFN-γ-inducible genes, leading to the transcription of CXCL10 (Bach et al., 1997). This chemokine is produced by monocytes and activated astrocytes (in particular after stimulation with viruses), endothelial cells and fibroblasts; also microglia produce CXCL10, after viral stimulation or during neurodegenerative diseases like Alzheimer's disease and multiple sclerosis (Xia et al., 2000). Its production is induced by IFN-γ, but CXCL10 has been observed to enhance IFN-γ release both in vitro and in vivo. Besides, it is involved in the development of antigen-driven T-cell responses, suggesting that this chemokine plays an important role in the overall evolution of Th1 peripheral immune responses (Gangur et al., 1998; Hancock et al., 2001; Dufour et al., 2002).

CXCL10 is also involved in neuronal apoptosis pathways via activation of CXCR3 by neurons. Previous studies demonstrated that treatment of CXCR3-expressing neurons with CXCL10 results in a caspase-3 dependent apoptotic cell death (Sui et al., 2006) and so, the chemokine required for the recruitment of effector immune cells by virally infected neurons for their survival might also promote their death (Durrant et al., 2014).

CXCL10 has certainly a role in neuroinflammation, since it has been found in great amount in the SNC during infections and inflammations due to neurodegenerative diseases like Alzheimer's disease, multiple sclerosis (Xia et al., 2000) and prion disease (Tribouillard-Tanvier et al, 2009). Like cytokines, also chemokines can have a protective role within the CNS through their ability to orchestrate leukocyte entry and interactions at the endothelial

barriers of the CNS and initiate CNS repair of damaged tissue within the CNS, but at the same time, they can also enhance and promote the neuroinflammation and its detrimental effects. The pro or anti-inflammatory property of this chemokine seems to depend on the disease and on the contest; so, in many models of viral encephalitis induced by MHV, WND and HSV, CXCL10 has a beneficial role, helping to eliminate cells infected with viruses, in contrst, CXCL10-driven inflammation has detrimental effects in encephalitis induced by LCMV, malaria and trypanosomes (Michlmayr and McKimmie, 2014).

In a study of Sui and colleagues, they used the SHIV/rhesus macaque model of HIV-E to investigate whether there was a link between CXCL10 overexpression and neuronal degeneration and they found that CXCL10 was overexpressed in neurons in the brains of individuals with HIV-E and that exogenous CXCL10 led to activation of caspase-3 and neuronal apoptosis (Sui et al., 2004).

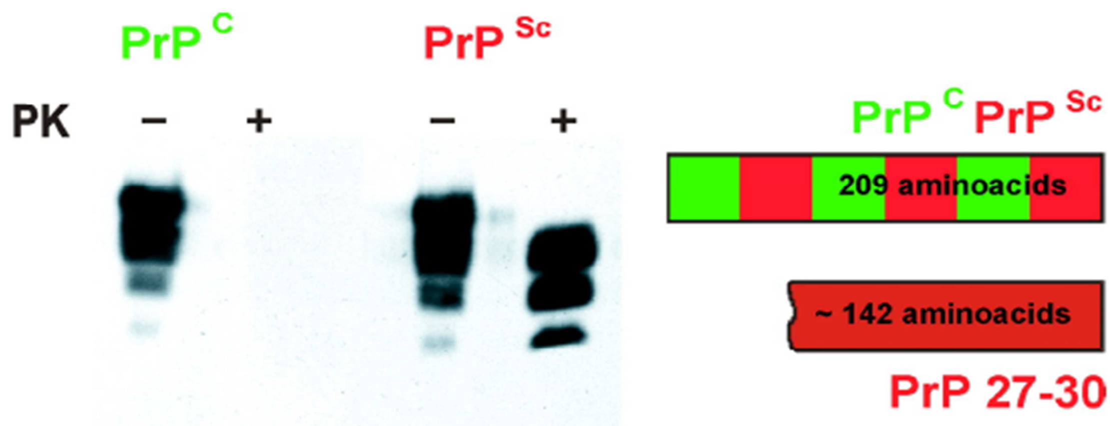
There are many works on the neuroprotective role of CXCL10, but at the same time, there are emerging studies about its detrimental effect, in particular on neurons. During neuroinflammation, it is very important for neuronal apoptosis, probably by calcium dysregulation (Sui et al., 2006).

1.4 *Prion diseases*

Among neurodegenerative diseases, there are prion diseases, which are disorders that affect both humans and animals (Prusiner 1991). They include scrapie (sheep and goats), bovine spongiform encephalopathy (cattle), chronic wasting disease (CWD in cervids) and Creutzfeldt-Jakob (CJD) in humans. They are caused by a conformational change of a host

protein, the PrP sen (protease-sensitive prion protein), also known as cellular PrP (PrP^{c}). This protein is a host-encoded membrane glycoprotein 208 amino acids long expressed in many tissues and cell type and it is called protease sensitive because it is sensitive to

Proteinase K resistance of PrP.



DetlevRiesner Br Med Bull 2003;66:21-33

©2003 by Oxford University Press

BRITISH MEDICAL BULLETIN

digestion with proteinase K (PK) and detergent soluble.

Fig.1Proteinase K resistance of PrP. A denaturing polyacrylamide gel electrophoresis with antibody staining is shown in left panel. 'Full-length' and N-terminal truncated PrP are presented in the right panel

In Fig.1the Western blot of an SDS-gel electrophoresis of PrPC and PrPSc, without and with proteinase K digestionis shown. The three bands of PrP disappear completely after digestion with PK, while, as regards PrP^{Sc}, the bands remain nearly undiminished in intensity although shifted to lower molecular weight (Riesner, 2003). PrPSc from different species or prion

strains may show different degrees of protease resistance (Colby and Prusiner, 2011). PrP is posttranslationally processed to remove a 22-amino-acid, amino-terminal signal peptide and a 23-amino-acid, carboxyterminal peptide, which directs addition of the GPI anchor that tethers the protein to the cell membrane. No post-translational modifications to the primary structure differentiate PrPC from PrPSc (Stahl et al. 1993).

The normal functions of PrP are not known although PrPnull mice have detectable deficits in both neurophysiological and memory functions (Aguzzi et al., 2008). The problem is when it changes its confomation becoming an abnormal protease-resistant disease-associated form (PrPres), also known as PrP scrapie (PrPSc) (Tribouillard-Tanvier et al., 2009). This form is PK resistent and this is in fact a way to distinguish and detect the presence of PrPres. This form of the protein is also insoluble and its accumulation in the brain is believed to be involved in the neurodegeneration which leads to the characteristic spongiform changes in the brains of infected animals (Brander et al., 2008). In the brains of some, but not all, animals and humans that have died of prion diseases, amyloid plaques are found which contain PrP, as determined by immunostaining and Edman protein sequencing studies (Roberts et al., 1988; Tagliavini et al., 1991). PrPsen and PrPres have the same primary structure, having the same aminoacidic sequence, but different secondary structure: PrPsen has more alpha-helices but during the formation of PrPres, PrPsen undergoes conformational changes that involve an increase of β-sheet secondary structure (Vorberg et al., 2001). Purified prions, either in the form of ‘full-length’ PrPSc or as PrP 27–30, are insoluble, even in mild detergents; in the brains of CJD or Kuru victims, PrP deposits can be detected as diffuse deposits, amyloidic fibres, condensed plaques, or florid plaques (Riesner, 2003).

In order to understand how the PrP^{C} form changes into PrP^{Sc}, many experiments have been carried out; for example, in an experiment of Morillas and collegues, the PrPC-like, α-helical state was established first as it is in the non-infected organism. Then the transition to

the PrPSc-like conformation was induced by slightly denaturing conditions. It was found that β-sheet formation is always connected with aggregation and that the most stable state, at least at acidic pH, is the β-sheet-rich aggregated state. Other studieson the in vitro conversion were carried out with natural PrP and at neutral pH (Riesner et al., 1996). Infectious PrP 27–30 was converted to an α-helical, oligomeric and non-infectious form by addition of 0.3% sodium-dodecylsulphate (SDS). These experiments were closest to natural conditions if the low concentrations of SDS were regarded as a membrane-like environment. It was also shown that the conversion occurs in steps, first fast formation of β-sheet structure concomitant with forming small oligomers, then larger oligomers in minutes to an hour, and finally large insoluble aggregates in hours to days (Riesner, 2003).

So, when the PrP^{Sc} takes contact with the PrP^{C}, it induces its conformational change, leading to the conversion of the normal protein on infective prion protein. Prion propagation requires conversion of PrPC to PrPSc, thought to occur by a templateassisted process in which PrPSc acts as a template onto which PrPC is refolded into the infectious conformation . PrPC may need to enter a partially unfolded, intermediate state to interact with PrPSc and undergo conversion; this intermediate state is referred to as PrP (Cohen et al. 1994). The conversion of PrPC to PrPSc may also require the assistance of one or more as-yet-unidentified cofactors, provisionally designated protein X. Presumably, protein X binds to PrPC and enables it to interact with PrPSc for conversion (Colby and Prusiner, 2011).

Prion diseases occur as sporadic, genetic, and transmissible disease in humans. Although infectious forms of prion disease are most well known to the general public, sporadic and heritable forms of the disease occur much more frequently in humans, with sporadic (s)CJD accounting for approximately 85% of cases (Colby and Prusiner, 2011). It seems that the cause of CJD is spontaneous misfolding of PrPC into PrPSc. Amyloid plaques are a nonobligatory feature of prion diseases. Approximately 10% of sCJD cases whereas 70% of

kuru cases show amyloid plaques; all vCJD cases show amyloid plaques surrounded by a halo of spongiform degeneration (Colby and prusiner, 2011).

A prominent feature of neurodegenerative diseases like prion diseases is the early onset of gliosis in the brains of infected hosts. The direct cause of this gliosis is unclear, but evidence of microglial and astroglial activation coincides with the detection of disease-associated protease-resistant prion protein (PrPres). As I mentioned before, gliosis is an event that occurs after microglia and astrocytes activation and it can be caused by the cytokines produced by glia cells during these events.

Na et al. detected phosphorylation of STAT1 and STAT3 in scrapie-infected mice, and it has been suggested that JAK2-STAT1 signaling may be important in facilitating astrogliosis during infection with scrapie strain ME7. This neuroinflammatory reaction appears to be a host response to PrPres accumulation and associated brain cell damage. However, this neuroinflammation might also act either to increase or to decrease prion disease pathogenesis, and if so, therapeutic intervention to increase or dampen this reaction might be beneficial (Tribouillard-Tanvier et al., 2012).

Unfortunately, no treatment is currently available to halt the progression of any of these illnesses. Studies of prions in mice have elucidated several aspects of neurodegeneration that may prove useful in developing effective therapeutics.

1.5 *Statins*

1.5.1 *What are statins and what is their function?*

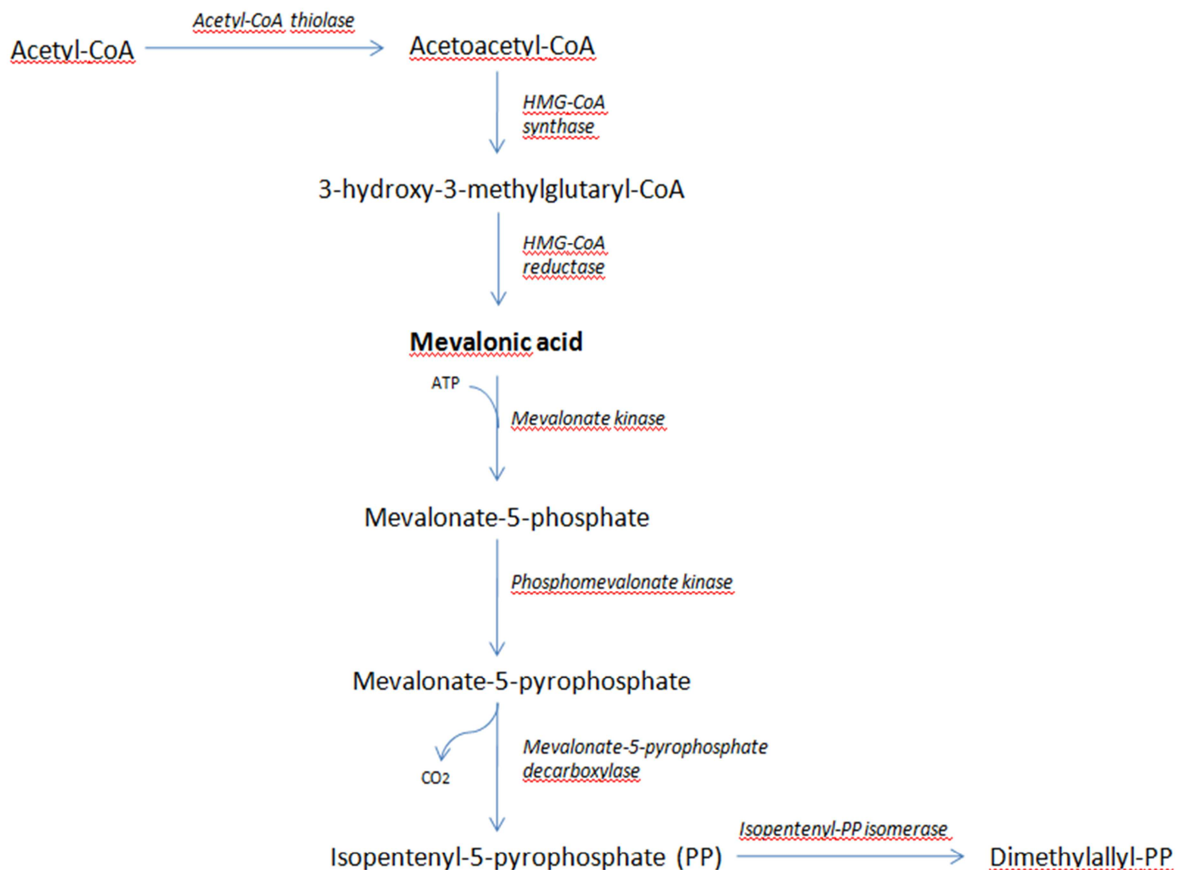


Fig. 2 Mevalonate pathway

Statins are a class of drugs whose main action is to lower cholesterol levels, so they are principally used as therapeutics for the treatment of cardiovascular diseases. Cholesterol in synthesized from acetyl-CoA, through the mevalonate pathway (**Fig.2**), which is a series of enzymatic reactions that leads to the production of polyisoprenoids (e.g. dolichol) and sterols (e.g. lanosterol, ergosterol, cholesterol) in fungi, plant cytoplasm, animals, most other eukaryotes, archaea and some eubacteria (Miziorko, 2011). Statins goes to block the

production of cholesterol by inhibiting the enzyme HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase), which is responsible for the reduction of HMG-CoA into Mevalonate: HMG-CoA reductase catalyses the rate-limiting step of cholesterol biosynthesis, a four electron reductive deacylation of HMG-CoA to CoA and mevalonate (Liao and Laufs, 2005). Mevalonic acid, or Mevalonate, is the precursor of isoprenoids, a class of compounds involved in diverse cellular functions such as sterol synthesis and growth control. Within cells, the concentration of mevalonate is tightly controlled through the HMG-CoA (Istvan et al., 2000). The statins do more than just compete with the normal substrate in the enzymes active site. They alter the conformation of the enzyme when they bind to its active site. This prevents HMG-CoA reductase from attaining a functional structure (Stancu and Sima, 2001). By inhibiting the HMG-CoA reductase, all the pathway is inhibited and there is no cholesterol synthesis.

The statin family is composed of eight unique compounds that are naturally derived or chemically synthesized (Wong et al., 2002). The common structural characteristic of all statins is a side chain that exists either in a closed ring (inactive, lactone) or an open ring (active, acid) form (Istvan et al., 2000; Istvan and Deinsenhofer, 2001). The open ring conformation of this drug blocks catalytically active HMG-CoA reductase by functioning as a molecular mimic of a reaction intermediate formed within the active site of this enzyme (Istvan and Deinsenhofer, 2001). The inhibition of this enzyme leads to a decrease in intracellular hepatic cholesterol levels which then induces expression of cell surface low-density lipoprotein receptors, enabling cholesterol to be removed from the circulation and replenish intracellular cholesterol stores (Ucar et al., 2000).

Although their action against hypercholesterolemia is the most important reason for their use as therapy, they seems to have other interesting effects. For example they has been tested as anti-cancer drugs: deregulated or elevated activity of HMGCoA reductase has been shown

in a range of different tumors and MVA pathway holds an important regulatory role in cellular proliferation and transformation, so statins has been used as antiproliferative to arrest cancer cells growth and to induce apoptosis in tumor-derived cells (Wong et al., 2002). They have been also used to reduce oxidative stress in patients with atherosclerotic cerebrovascular disease (statins had antioxidant effects against lipid peroxidation but not protein oxidation or DNA damage) (Moon et al., 2014).

Statins have been used also to reduce the accumulation of α-synuclein (whose aggregation in the brain is a typical sign of diseases like dementia with Lewy bodies and Parkinson's disease) and ameliorate the associated neuronal deficits, in the treatment of Parkinson's disease (Bar-On et al., 2008). Another neurodegenerative disease, Alzheimer's disease, has been treated with statins (Fassbender et al., 2001), since cellular cholesterol levels affect neuronal Aβ production in vitro and this protein is the main component of the amyloid plaques which accumulate in the brains of patients with Alzheimer's disease. Statins have been also used to treat mice infected by prion proteins: for example, in 2008, Haviv and colleagues used prion disease as a model for progressive neurodegeneretion in order to test the effect of Simvastatin on prion disease progression, PrP^{Sc} levels and neurotoxic effects in mice infected with scrapie. They decided to use a statin, though, not for the pleiotropic effects of these drugs but to see if lowering the cholesterol level in the brain would have had some kind of effect of the interaction of PrP^{Sc} and PrP^{C} ; they found that Simvastatin delayed disease progression and increased the survival of mice, but the beneficial effect of this drug was not correlated wih cholesterol level (Haviv et al., 2008). The same results were seen in two other works (Mok et al., 2006; Kempster et al., 2007). In another 2008 work, an italian group used Pravastatin to treat scrapie-infected mice and they found that high-dose and long-term oral Pravastatin treatment prolonged survival times of infected mice. They also reported that, although they were using an hydrophilic statin, this charactestic did not affect

the results (Vetrugno et al., 2008).

Finally, statins have been used to treat the neuroinflammation caused by cerebral malaria (CM), effect due to their pleiotropic activity. In this study, they propose that drugs with pleiotropic effects on inflammation and metabolism could be ideal candidates to target neuroinflammation and decrease cognitive impairment after severe infectious syndromes and that statins may be such agents because they can modify two processes leading to brain injury: neuroinflammation and activation of proinflammatory microglia (Reis et al., 2012).

1.5.2 *Anti-inflammatory properties of statins*

There are many studies in which statins are thought to be good anti-inflammatory drugs, because of their pleiotropic effect and many groups are using them to inhibit inflammation due to infections.

How statins may act as anti-inflammatory drugs? They can reduce systemic inflammation by lowering the proinflammatory tendencies of macrophages and neutrophils, by limiting endothelial cell activation, and by enhancing T-helper cell (Th)-1 function, all of this by influencing intracellular signaling (Terblanche et al., 2007). As I explained earlier, mevalonate is the precursor of many classes of sterol and non-sterol end-product, and among the non-sterol products there are numerous isoprenoid molecules, including farnesyl pyrophosphate and geranylgeranyl pyrophosphate. They serve for the isoprenylation of proteins and it allows the subcellular localisation and intracellular tra□cking of membrane-associated proteins such as heterometric G-proteins, haem-a, nuclear lamins, and small GTP-binding proteins (Takemoto and Liao, 2001). These proteins are important for intracellular inflammatory signaling because they modulate the response of various protein

kinases; so, when statins goes to inhibit the HMG-CoA reductase and consequently the production of farnesyl pyrophosphate and geranylgeranyl pyrophosphate, protein isoprenylation is slowed and this goes to influence the inflammatory response. For example, protein kinases activate the transcription factor nuclear factor kappa B (NKfB), which can be induced by bacteria and viruses and leads to the production of many cytokines. Statins can inhibit NkfB-associated signaling and consequently the production of inflammatory mediators (Terblanche et al., 2007). Also the mitogen-activated protein kinase (MAPK) family is part of the transcriptional regulation: these proteins are activated by proinflammatory cytokines like IL-1, TNF-α and LPS, and once activated they promote the production of more proinflammatory cytokines by monocytes and macrophages (Cuschieri et al., 2005; Lahti et al., 2003). So, also the inhibition of MAPK signaling brings to the reduction of proinflammatory mediators.

An important study that addressed the anti-inflammatory effect of statins was the PRINCE (pravastatin inflammation/CRP evaluation) trial. This trial demonstrated that oral administration of pravastatin for 24 weeks significantly reduced serum CRP levels in subjects with and without CVD, independently of any changes in LDL cholesterol (Albert et al., 2001). This study confirmed the anti-inflammatory effects of pravastatin in the primary and secondary prevention setting.

Usually, during inflammation, cytokines are not the only inflammatory molecules produced; many kind of cells produce also reactive oxygen species, leading to oxidative stress, which goes to worse the inflammatory situation. Different statins have been used successfully against these molecule, for example Hernandez-Perera and his group used two statins, Atorvastatin and Simvastatin to modulate the expression of the vasoactive factors ET-1 and NO and they showed that statins significantly reduce the synthesis of ET-1 and the expression of its precursor, the pre–proET-1 mRNA, in a process specifically related to the

inhibition of HMG-CoA reductase; they also observed that statins prevent the downregulation of eNOS mRNA and protein levels associated to oxLDL (Hernandez-Perez et al., 1998). Another interesting work that inspired my thesis was that of Pahan and colleagues, who used Lovastatin to inhibit the production of nitric oxide and cytokines in rat primary glia cells and macrophages (Pahan et al., 1997).

Finally, statins inhibit interferon-γ-stimulated CIITA promoter IV expression in numerous human cells, including endothelial cells and monocytes/macrophages (Jacobson et al., 2005) and this inhibition is highly specific for the inducible form of MHC-II expression, and does not a□ect MHC class I; so, statins can also modulate the adaptive immune system through the suppression of MHC-II expression by monocytes/macrophages (Terblanche et al., 2007).

1.5.3 *Statins used in this work*

Statins were originally discovered as secondary metabolites of fungi, the first statin discovered and used was mevastatin, which was isolated from *Penicillium citrinium* in 1976 (Endo et al, 1976). When it was used on rats, it inhibited the cholesterol production but it had also toxicity effects on hepatocytes, so it was abandoned. Three years later, in 1979, another statin, Lovastatin, was isolated from *Aspergillus terreus*, resulting in a more active and less toxic compound than mevastatin. Now we have a great number of statins, both natural and chemically modified.

Statins have different tissue permeability and because of that, they are classified into hydrophilic and lipophilic groups. Lipophilic statins are considered more likely to enter endothelial cells by passive diffusion than hydrophilic statins which are primarily targeted to

the liver (Liao and Laufs, 2005). Moreover, not all statins are in an active form and consequently ready to use; most of them are already in the active form (with the open ring), while lovastatin and simvastatin need to be activated by hydrolisis which allows the opening of the ring. *In vivo* this process occurs in the liver, while *in vitro* it has be done with ethanol, NaOH, and heat.

In my experiments for this part of my thesis, I used four different statins: Atorvastatin, Simvastatin, Lovastatin and Pravastatin. Three of these statins belong to the group of "lipophilic" statins (Atorvastatin, Simvastatin and Lovastatin), while the other one is "hydrophilic" statin (Pravastatin).

Atorvastatin

Atorvastatin was synthesized in 1985 and it is a lipophilic compound. It was approved for use in the United States in 1996 and has become one of the most commonly prescribed drugs in America . Besides the cholesterol lowering effect, this statin was used also to reduce inflammation, for example it was assigned to 430 patients with acute coronary syndromes (ACS) (in a randomized, double-blind, placebo-controlled study) and it was seen an anti-inflammatory effect (Macin et al., 2005). Atorvastatin reduced the neuroinflammation in mice after injection of the amyloid-beta protein $A\beta_{1-40}$ (Piermartiri et al., 2010). Experimental data have indicated that the treatment with atorvastatin for 1 week significantly reduced neurological deficits and increased the survival and synaptogenesis in the hippocampus after traumatic brain injury (Lu et al, 2004), suggesting neuroprotective effects.

Simvastatin

Simvastatin is more recent, it was developed in 1992 from *Aspergillus terreus* and it is a lipophilic molecule. Also Simvastatin has been used as an anti-inflammatory drug, for

example for treating the inflammation occurring with rheumatoid arthritis (RA), an autoimmune disease in which there is production of a great amount of proi-inflammatory cytokines (Leung et al., 2003). They showed that simvastatin can suppress murine collagen-induced arthritis (CIA), either prophylactically, or if administered after clinically evident onset of disease. Pruefer and his group were the first to demonstrate a protective effect of simvastatin under conditions of acute inflammation induced by an exotoxin within the microcirculation; in particular they proved that simvastatin is able to attenuate enhanced leukocyte-endothelium interaction after S aureus -toxin administration (Pruefer et al., 2002). An example of the use of simvastatin on neuroinflammation is a work of 2006, in which Zeinstra and her collegues, on the theory that the development of autoimmune lesions in the central nervous system (multiple sclerosis) may be due to a failure of endogenous inhibitory control of MHC class II expression on astrocytes, allowing these cells to adapt an interferon (IFN)- γ-induced antigen presenting phenotype, and reporting that Statins have been shown to reduce MHC class II expression in cultured microglia (Youssef et al., 2002), used simvastatin on astrocytes cultures from newborn rats to inhibit up to 70% of IFN-γ-induced MHC class II expression (Zeinstra et al., 2006).

<u>*Lovastatin*</u>

As I already mentioned, Lovastatin is one of the first statins discovered, in 1979, from *Aspergillus terreus*; also Lovastatin has lipophilic characteristics. Lovastatin has been used to inhibit the production of nitric oxide and cytokines induced by LPS in rat primary glia cells and macrophages (Pahan et al., 1997). Moreover, in another study of 2014, a group investigated the effects of f lovastatin on mRNA expression of proinflammatory cytokines (interleukin-1β, tumor necrosis factor α, interleukin-6) and the antiinflammatory cytokine IL-10 in the hippocampus during epileptogenesis on rats, reporting that lovastatin decreases the inflammatory process in the hippocampus induced by long-lasting SE (Gouveia et al.,

2014), after they showed, in another work, that t lovastatin is able to decrease the neuronal death rate induced by long-lasting status epilepticus (Gouveia et al., 2011).

Pravastatin

Pravastatin is the only hydrophilic molecule that I used; it is a derivative of compactin, which was identified in he fungus *Penicillium citrinium* in the 1970s (like mevastatin). It was used in clinical trials in healthy volunteers, showing to be effective for lowering LDL cholesterol with no severe side effects. After an interruption due to problems with compactin (it seems that it was seriuosly toxic on animals), the trials with lovastatin were started again in 1982 with good results on very high-risk patients (Tobert, 2003).

Also pravastatin seems to have anti-inflammatory properties in addition to its principal function of cholesterol-lowering drug. For example, in a study of 2003, its anti-inflammatory properties were tested on 50 patients with type 2 diabetes; they finally found that pravastatin reduces levels of coagulation and inflammation markers in type 2 diabetic patients(Sommeijer et al., 2004). However, there are not many studies, in particular as regard a possible anti-inflammatory and neuro-protective property during neuroinflammation. For this reason, it could be interesting to try also this statin in my experiments.

2. AIM OF THE PROJECT

Since statins seems to have anti-inflammatory and neuroprotective activities besides their principal role as cholesterol-lowering drugs, during this part of my PhD I used four different statins (Atorvastatin, Simvastatin, Lovastatin and Pravastatin) to inhibit the production of pro-inflammatory cytokines (IL-6 and CXCL10) in vitro. In order to do this, I used cortical astrocyes and microglia primary cultures from newborn C57BL/10SnJ mice and Sprague-Dawley rats, which I stimulated with LPS to recreate a situation of neuroinflammation. Statins were used to inhibit the production of cytokines, stimulated by the activation with LPS.

Future projects at NIH-LPVD laboratories contemplate the use of statins to inhibit the neuroinflammation in vivo, on mice infected by prion diseases.

RESULTS AND DISCUSSION

3.1 Immunofluorescent staining

For my experiments, pure primary cell culture of astrocytes and microglia were needed; it is nearly impossible to have a 100% pure culture but usually, with the protocol used in this work to separate a mixed glia cell culture it is possible to obtain a culture with > 90% of purity.

The aim of the fluorescent staining was to determine the purity of the cell culture. This technique was used every time different conditions were introduced, such as different type of cells or separation method or different animal, to verify if the procedure used was successful. **Fig.1** reports an example of the staining for mice and rats cells: the **fig.1a** represents a well of a chamber slide in which astrocytes from mice brains were seeded (after the separation procedure) and stained with the antibody α-GFAP (in red), which goes to bind to the GFAP on astrocytes. This allows to put in evidence the astrocytes in the wells and also their morphology. The cells were stained also with DAPI, which is a blue fluorescent stain that binds to the DNA, thus allowing to put in evidence the nuclei and to distinguish the cells from any debris. As can be seen from the picture, the most part of the nuclei match to the cells highlighted by the antibody GFAP; furthermore, the cells have typical astrocyte morphology and the color of the dye appear to be very bright; this indicates that most of the cells in the picture are effectively astrocytes and so, the culture was almost completely pure. There are some cells which are not surrounded by the red dye: they are probably microglia. It s normal to have some other type cells, mostly microglia, also after the separation since it is very difficult to separate completely these two kind of glia cells. In **fig.1b** there is the picture of well of a chamber slide in which microglia from mice brains were seeded and stained with the antibody α-IBA-1 specific for microglia (in green) and DAPI (in blue). Also

in this case, the purity of the culture was very high, as can be deduced from the brightness of green dye which put in evidence microglia and from the high matching between nuclei stained with DAPI and green cells. Most of the cells are microglia. In both cases there are not many cells, but it is normal since the cells are incubated in chamber slides for almost three days and usually mice cells grow very slowly in vitro, especially microglia. **Figures** from **1g** to **1h**, report pictures of rat glia cells; this time there are single pictures of cells stained with an antibody and then a picture where the first two are merged, to see the matches between the two dyes. **Figures** from **1c** to **1e** represent cells from rat brains; in particular they are pictures of the same well in which astrocytes from rat brains were seeded: in **1c** it is possible to observe cells stained with the antibody α-GFAP while in **1d** there are the same cells stained with DAPI; in **fig.1e** there are the two pictures merged. First af all, in this case it is possible to observe that there are more cells than that in mice astrocyte culture and this is because rat cells grow very easily and faster than mice cells in vitro. Also in this case the cells in red are almost completely matching to the nuclei in blue, meaning that most of them are astrocytes and also in this case the separation procedure was successful, even though it was used a different time for shaking for rat cells, almost 18 hours vs 4 hours used for mice cells. This prolonged time period could bring to the detachment of astrocytes from the surface of the flasks, but it did not ever occurred.

Finally, **figures** from **1f** to **1h** represent the same well in which rat microglia were seeded; in **1f** it is possible to observe cells stained with the antibody α-IBA-1 while in **1g** there are the same cells stained with DAPI; in **fig. 1h** there are the two pictures merged. As for mouse microglia, even though rat microglia grow in a greater number, they are however less than astrocytes, mainly because of their slow growth. As can be seen in **fig. 1h**, they are mainly microglia.

In this work, it is reported only an example for each case, but this staining procedure was

repeated every time different conditions were used, always obtaining similar results. Therefore, the cultures used in the experiments hada very few contamination by other type of cells, which would not constitute a problem for the results of the stimulations.

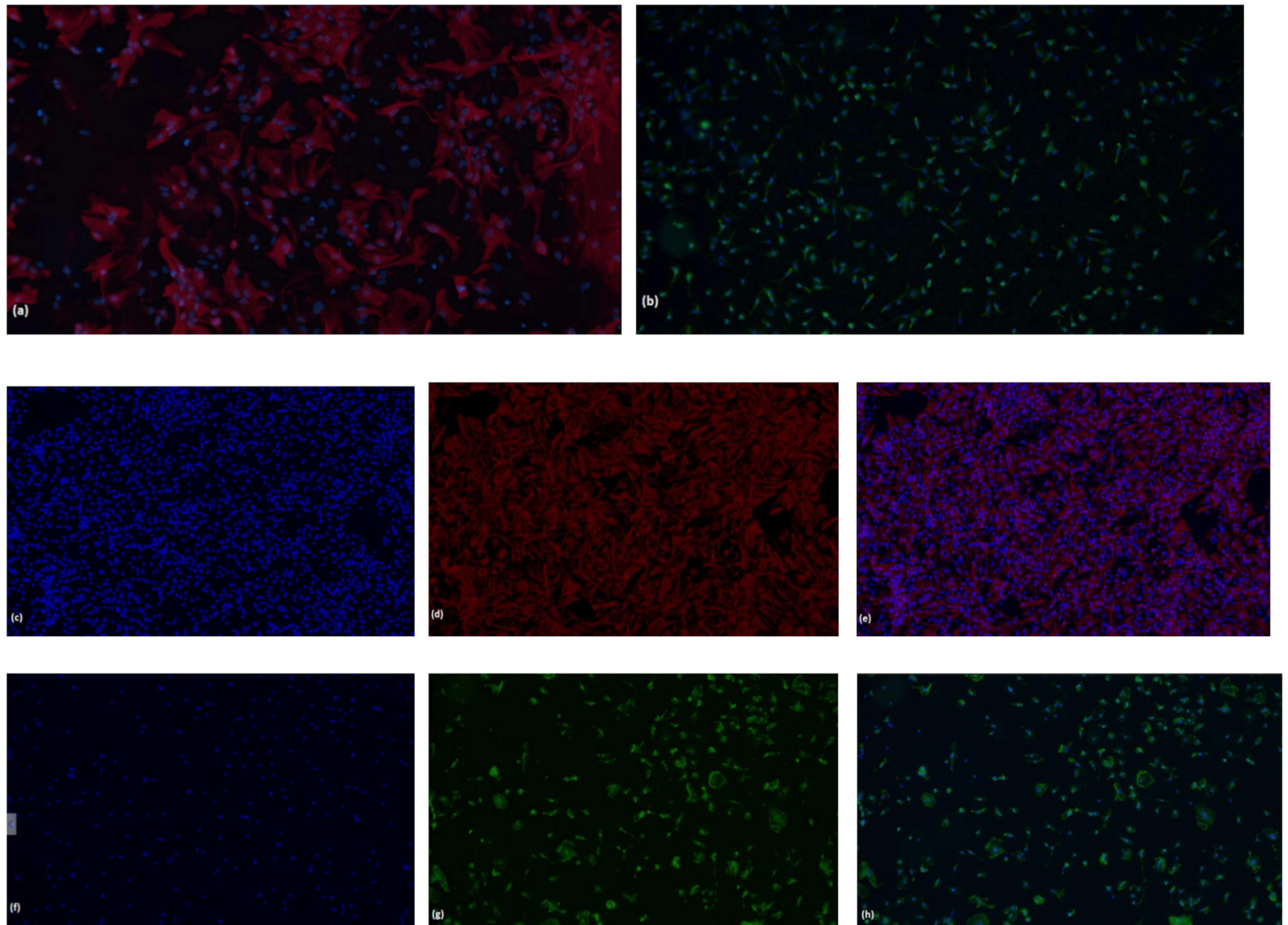


Fig.1 (a) Chamber slide containing mouse astrocytes cell culture, in which the cells have been stained with α-GFAP and DAPI; (b) Chamber slide containing mouse microglia cell culture, in which the cells have been stained with α-IBA-1 and DAPI; (c) Chamber slide containing rat astrocytes cell culture, cells stained with DAPI; (d) same chamber, in which the cells have been stained with α-GFAP; (e) merged images, α-GFAP+DAPI; (f) Chamber slide containing rat microglia cell culture, cells stained with DAPI; (g) same chamber, in which the cells have been stained with IBA-1; (h) merged images, α-IBA-1+DAPI.

3.2 Inhibition of cytokines production with statins

To see if statins could be helpful in the inhibition of neuroinflammation, it was stimulated the activation and production of cytokines in glia cells from newborn C57BL/10SnJ mice and Sprague-Dawley rats. After obtaining cortical astrocytes and microglia cell culture, these cells were stimulated with lipopolysaccharide (LPS); in order to decide the concentration for the best stimulation similar experiments described in literature were considered, in which the same cells and the same stimulant were used and it was also performed a LPS curve to see which concentration would have the best stimulation and a higher production of cytokines; this LPS curve was performedusing a different kind of cell every time, using 10 different concentrations of LPS, with a dilution of 1:10 (data not shown). Based on these experiments and on literature, for the first experiments on mouse cells 1 ng/ml of LPS were used; after some experiments, though,1 µg/ml of LPS were prefered because of the poor stimulation.

With LPS stimulation, the cells were activated and they produced cytokines, thanit was tried the inhibition with the use of 4 statins, at different concentrations and dilutions. After 20 hours, the supernatant was collected and on this, the Extracellular protein kit (BIOPLEX) was used to determine the presence and the eventual inhibition of two pro-inflammatory cytokines, CXCL10 and IL-6.

In the following paragraphs the production and inhibition of these two cytokines in different types of cells will be analyzed.

3.2.1 Inhibition of the production of CXCL10 in mouse astroglia

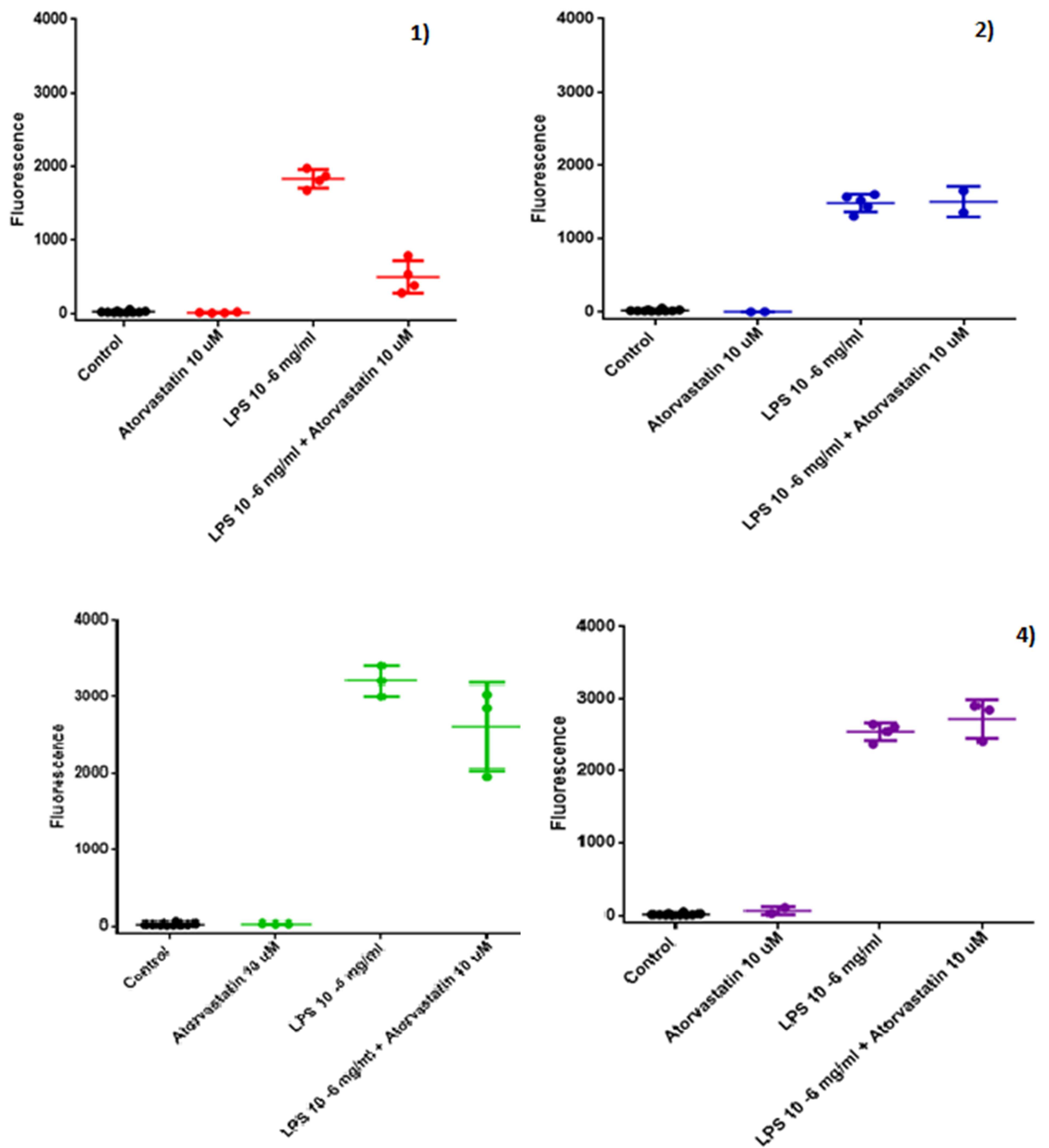


Fig.2. Graphs of 4 experiments on stimulation of mouse astroglia with 1 ng/ml of LPS and inhibition of CXCL10 production with Atorvastatin 10 µM.

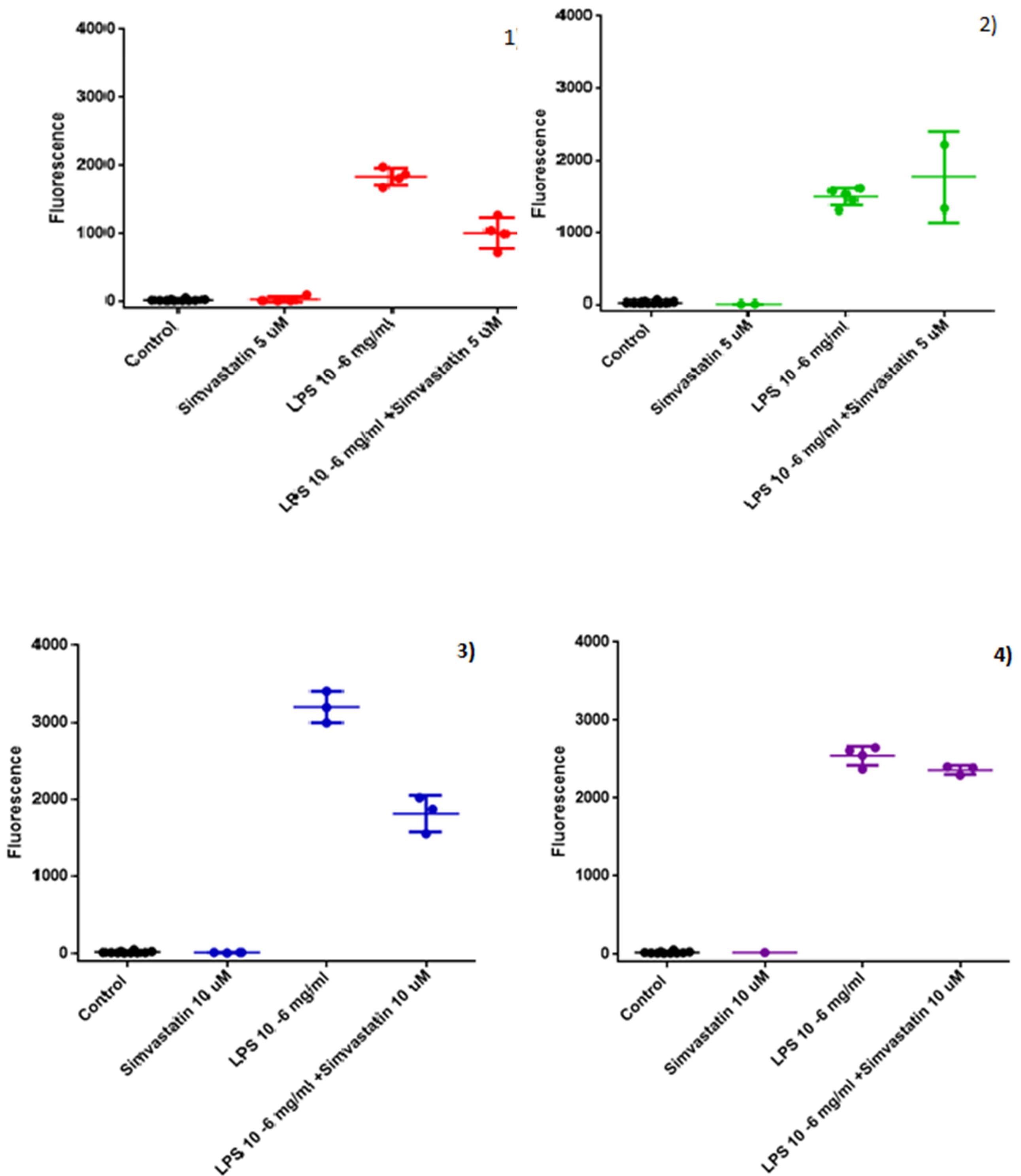


Fig.3. Graphs of 4 experiments on stimulation of mouse astroglia with 1 ng/ml of LPS and inhibition of CXCL10 production with Simvastatin 5 µM (a-b) and 10 µM (c-d).

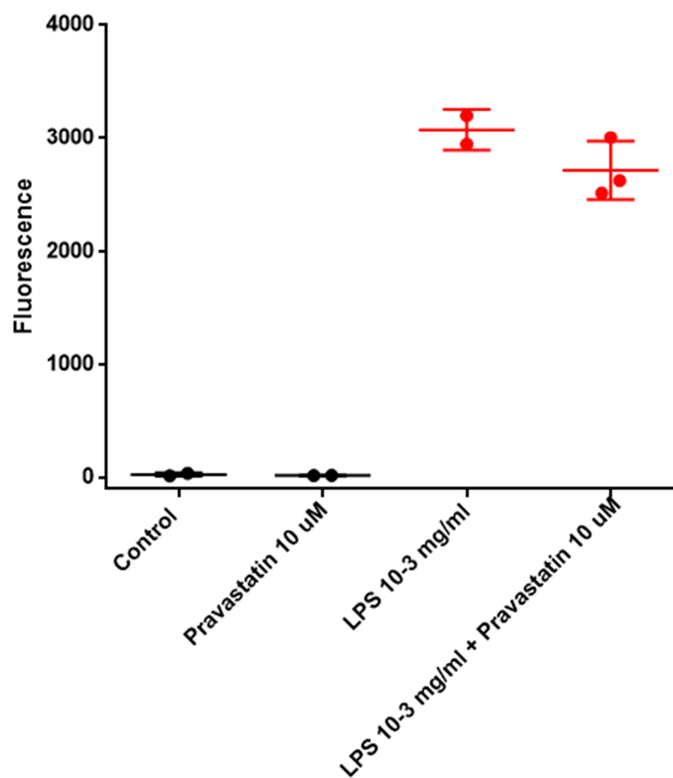


Fig.4. Graph on stimulation of mouse astroglia with 1 µg/ml of LPS and inhibition of CXCL10 production with Pravastatin 10 µM.

These graphs represent the first experiments in which it was analyzed the inhibition of the production of the pro-inflammatory chemokine CXCL10 by activated mouse cortical astrocytes. On these type of cells Atorvastatin and Simvastatin were tested four times and Pravastatin one time. As it has been mentioned before, it was used 1 ng/ml of LPS to stimulate the cells. In each experiment different concentrations of statins were used, in order to verify if there could be a dose-dependent response. thus, in the graphs it is reported only the highest concentration used 10 or 5 µM; in each graph there is the control, which is constituted by cells and medium as negative control. In addition, statin alone was used as a

control, to see if statins could activate cells and to see any possible toxicity on cells with the MTT assay. As regard the experiments with Atorvastatin, using 1 ng/ml of LPS it has been always obtained a good stimulation, especially in the third and last experiment (Fig.2.3 and 2.4) but there was a significant reduction of CXCL10 production only in the first (Fig.2.1) and third (Fig.2.3) experiment.

In the experiments with Simvastatin (Fig.3) there is a very similar situation. Also in this case it was used 1 ng/ml of LPS with a good stimulation. In the last two experiments (Fig.3.3 and 3.4) 10 mM of Simvastatin instead of 5 µM but the inhibition seems to follow the same trend regardless of the concentration of the drug used.

Finally, Pravastatin was used in one experiment. There is not much literature about the anti-inflammatory property of Pravastatin, especially on possible neuroprotective effects so it was decided to try it on mouse astrocytes but this drug had no effects on CXCL10 production.

<u>*3.2.2 Inhibition of the production of IL-6 in mouse astroglia*</u>

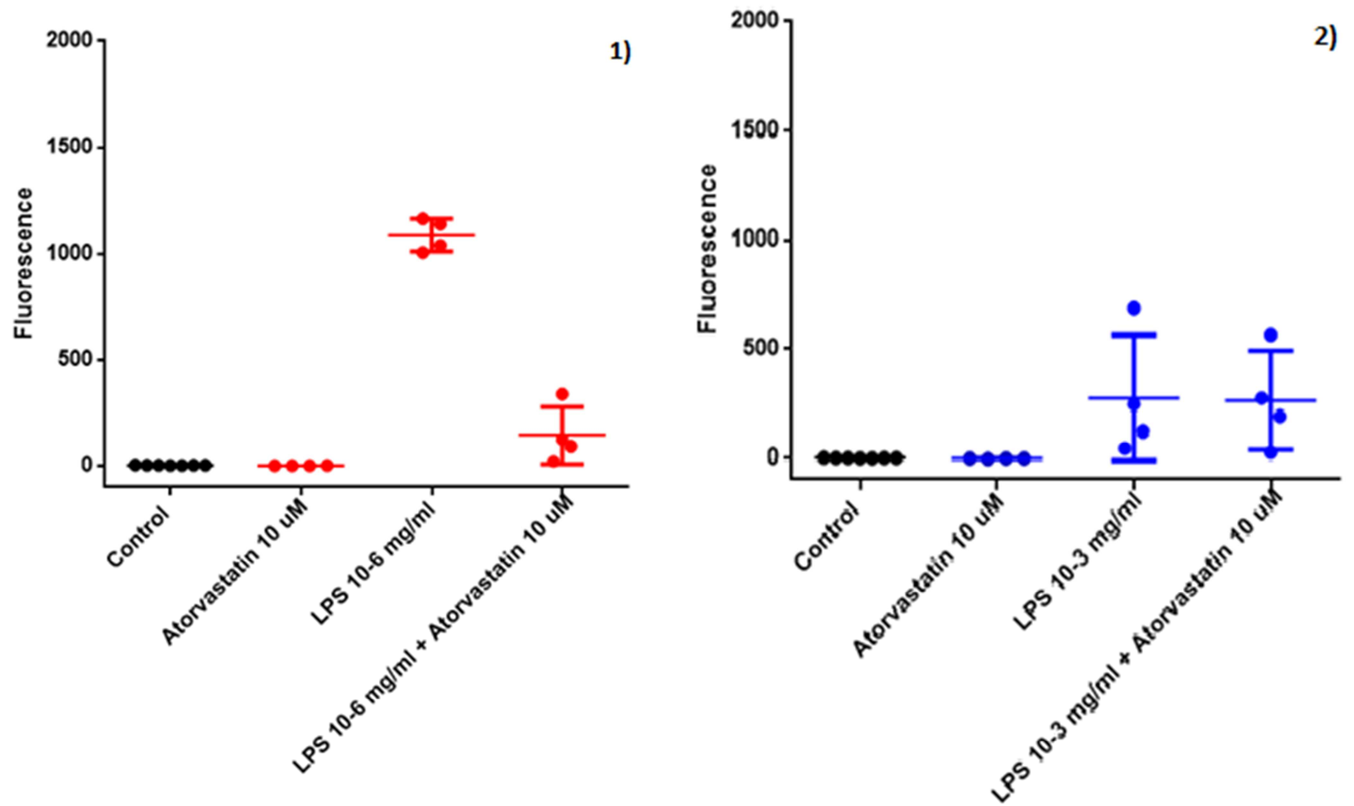


Fig.5. Graphs of 2 experiments on stimulation of mouse astroglia with 1 ng/ml and 1 µg/ml of LPS and inhibition of IL-6 production with Atorvastatin 10 µM.

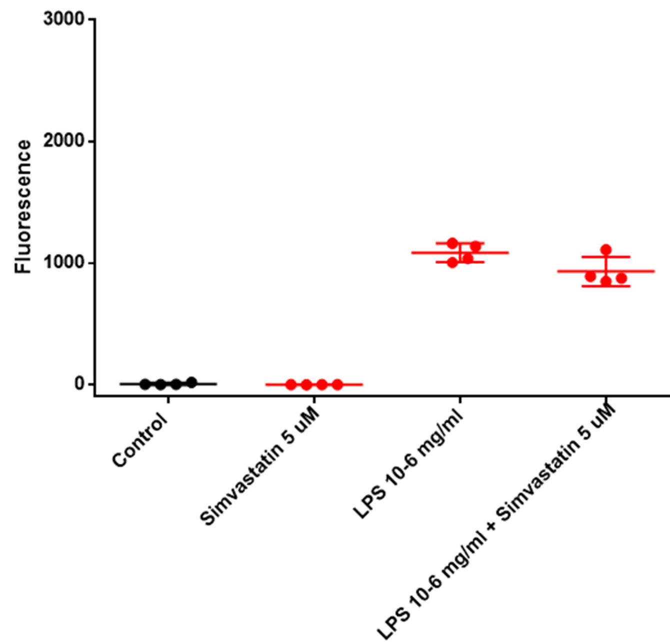


Fig.6. Graph on stimulation of mouse astroglia with 1 µg/ml of LPS and inhibition of IL-6 production with Simvastatin 5 µM.

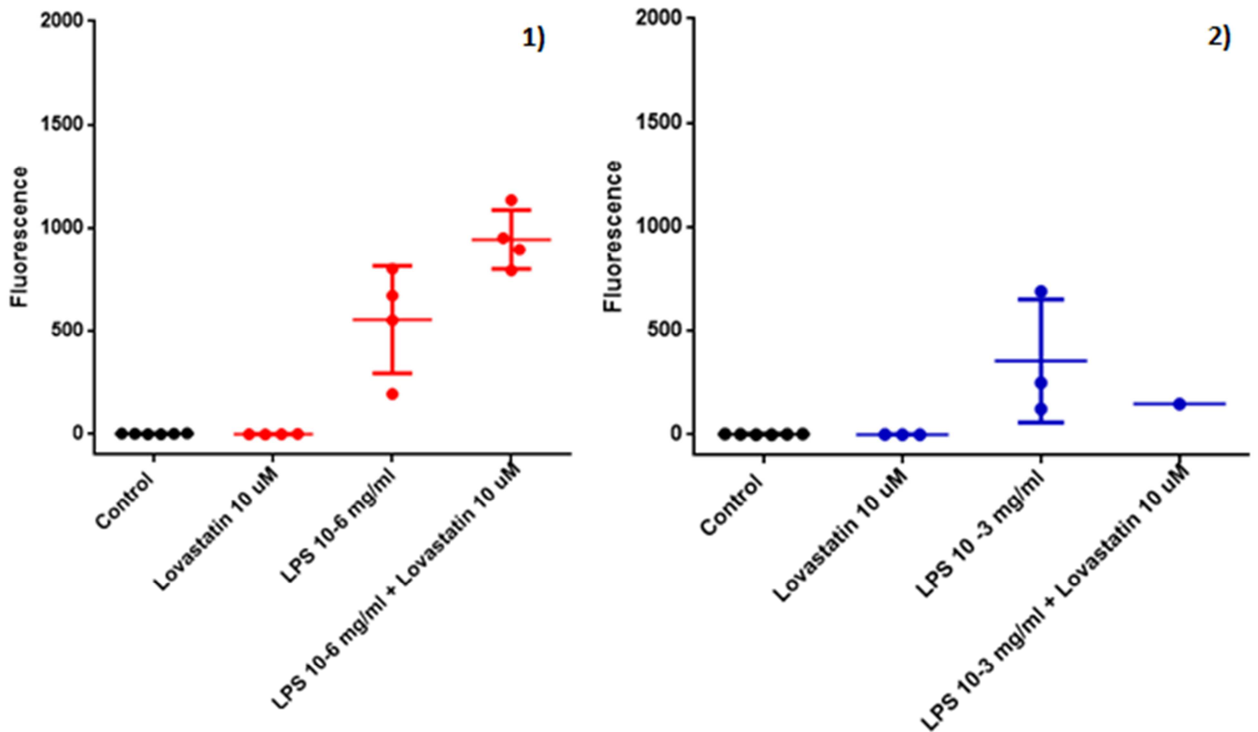


Fig.7. Graphs of 2 experiments on stimulation of mouse astroglia with 1 ng/ml of LPS and inhibition of IL-6 production with Lovastatin 10 µM.

Together with the experiments on CXCL10 the statins were tested also on the production of the other pro-inflammatory cytokines like IL-6. For this reason, also in this case the conditions can vary (concentrations of LPS or statins). As regard IL-6 production by mouse astrocytes, three statins were , Atorvastatin, Simvastatin and Lovastatin. Atorvastatin was used in two experiments: the drug was always used starting from 10 µM in both cases, while as regards LPS, in the first experiment (Fig.5.1) the cells were stimulated with 1 ng/ml of LPS, obtaining a good stimulation but not as good as in CXCL10 experiments, so it was decided to use more LPS (1 µg/ml) in the second experiment (Fig.5.2), but it was not successful; plus, it was obtained an inhibition of cytokine production only the first time. Also Lovastatin was used two times at the concentration of 10 µM (Fig.7.1-2), using two concentration of LPS, but it was never obtained any inhibition. Simvastatin was used only in one experiment (Fig.6) at the concentration of 5 µM but with no inhibition of IL-6.

3.2.3 Inhibition of the production of IL-6 in mouse microglia

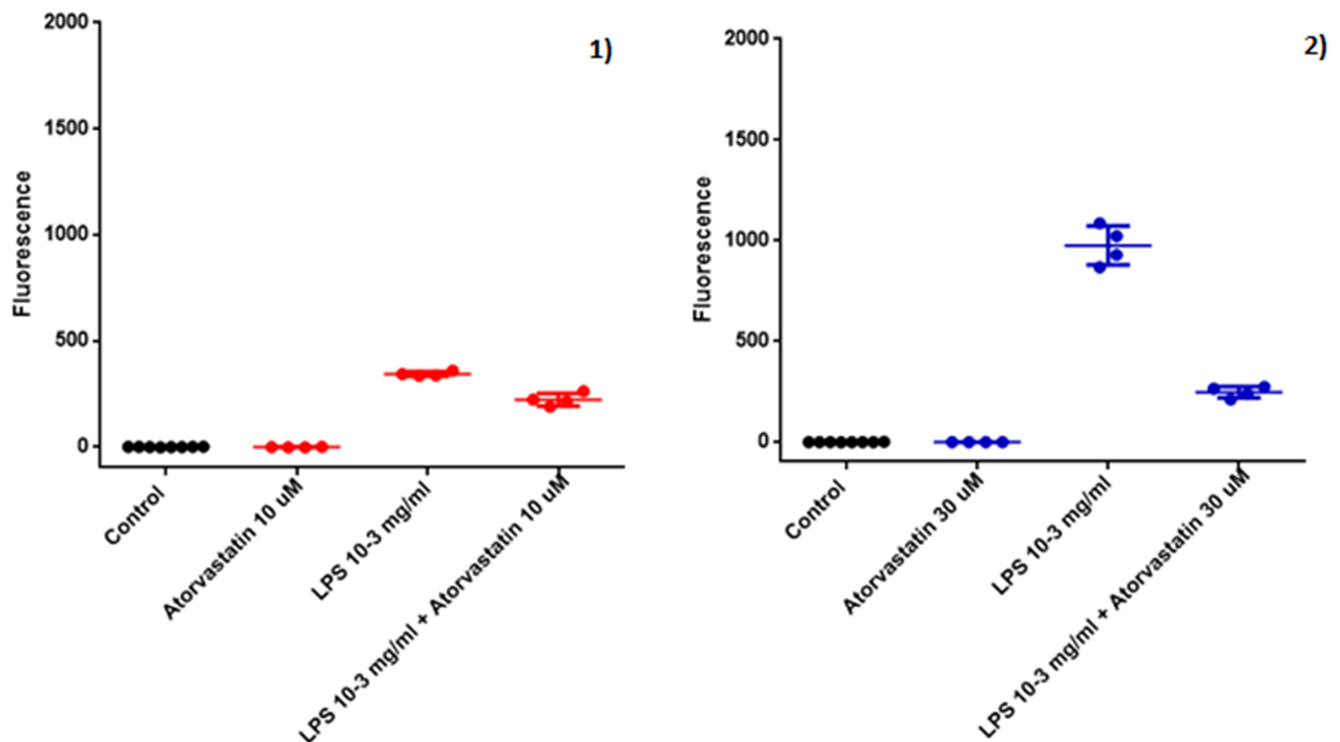


Fig.8. Graphs of 2 experiments on stimulation of mouse microglia with 1 µg/ml of LPS and inhibition of IL-6 production with Atorvastatin 10 (8.1) and 30 µM (8.2).

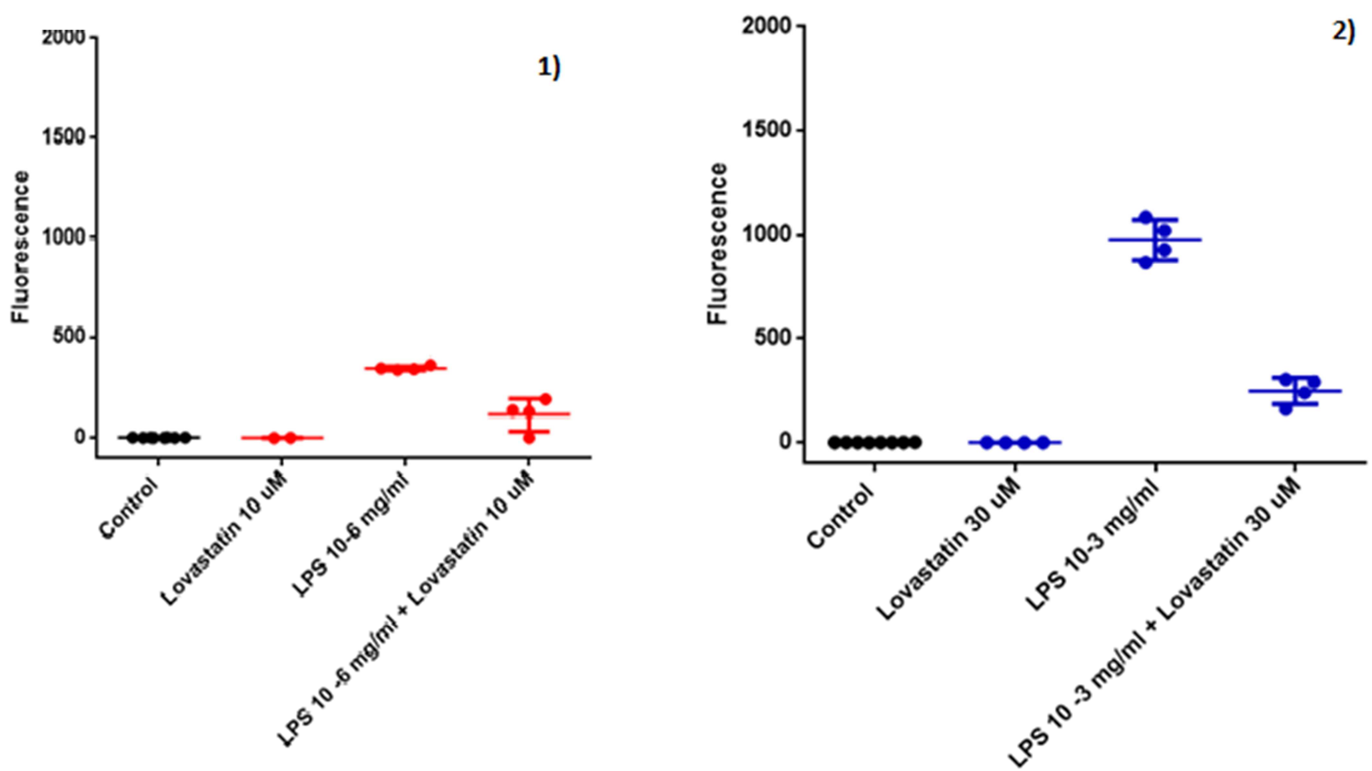


Fig.9. Graphs of 2 experiments on stimulation of mouse microglia with 1 ng/m1 and µg/ml of LPS and inhibition of IL-6 production with Lovastatin 10 (9.1) and 30 µM (9.2).

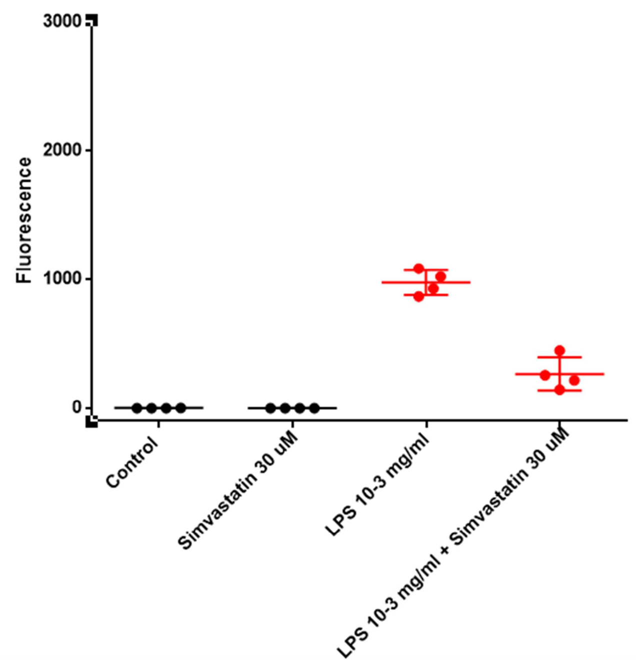


Fig.10. Graph on stimulation of mouse microglia with 1µg/ml of LPS and inhibition of IL-6 production with 30 µM of Simvastatin.

In this work also microglia were used in order to verify the production of IL-6 since these cells are the major producers of this cytokine and since they are the cells that are primarily activated during neuroinflammation. On this type of cells Atorvastatin, Lovastatin and Simvastatin were used. As regards Atorvastatin, it has been used for the inhibition in two experiments, after a stimulation of microglia with 1 µg/ml of LPS; in the first experiment (Fig.8.1) it was used 10 µM of statin but there was no inhibition, probably because there was not stimulation too. In the second experiment (Fig.8.2) it was increased the concentration of Atorvastatin (30 µM), having a successful stimulation and inhibition of cytokine production. I wss also used Lovastatin two times: in the first experiment (Fig.9.1) it was used less LPS (1 ng/ml) and 10 µM having no stimulation and inhibition, while the second time (Fig.9.2) they were increased both, resulting in a good stimulation and inhibition of IL-6. Finally Simvastatin was tried using directly the highest concentration of LPS and statin and there was also in this case a good stimulation and a successful inhibition of IL-6 (Fig.10).

3.2.4 Inhibition of the production of IL-6 in rat astroglia

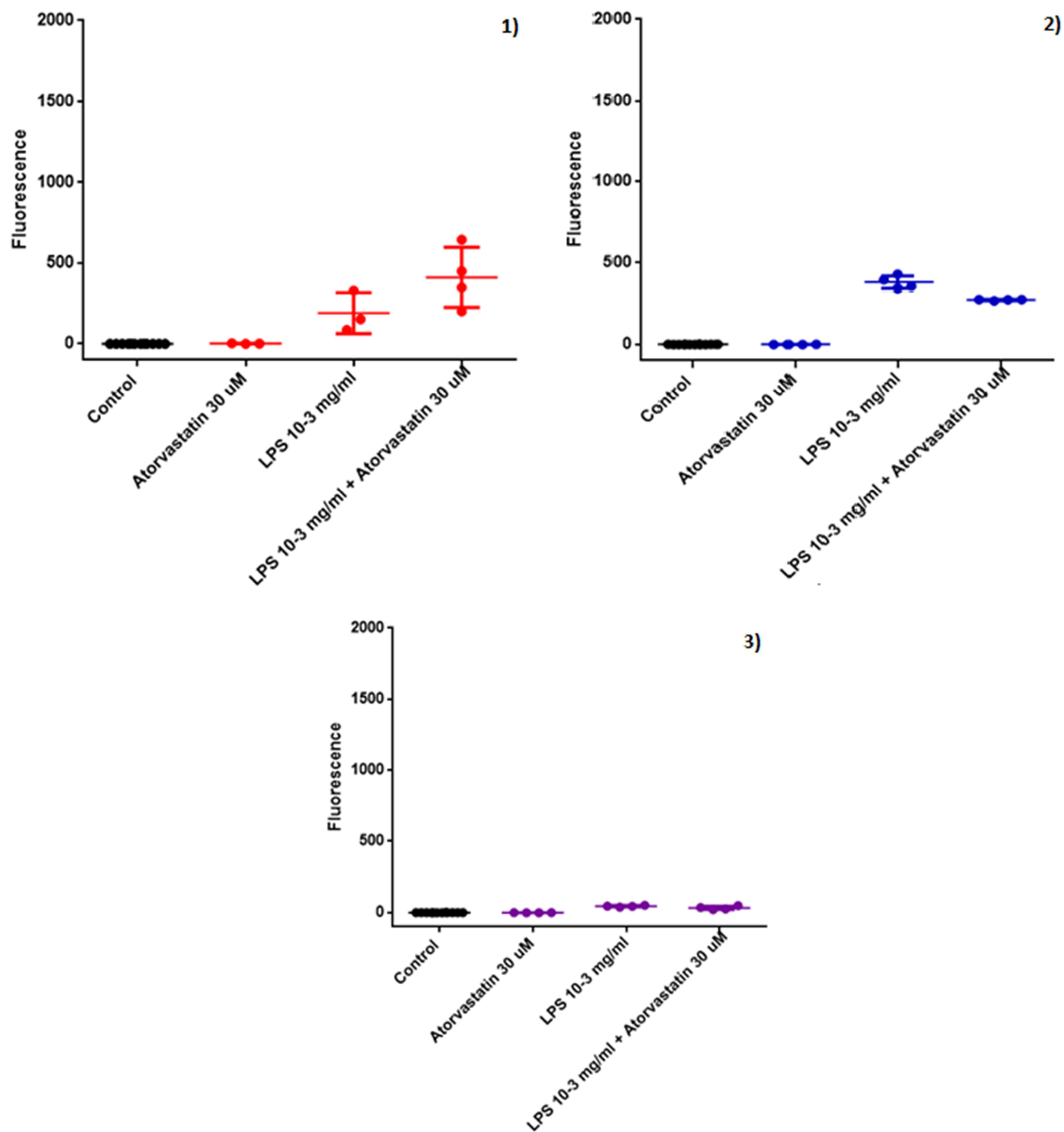


Fig.11. Graphs on stimulation of rat astrocytes with 1 µg/ml of LPS and inhibition of IL-6 production with 30 µM of Atorvastatin.

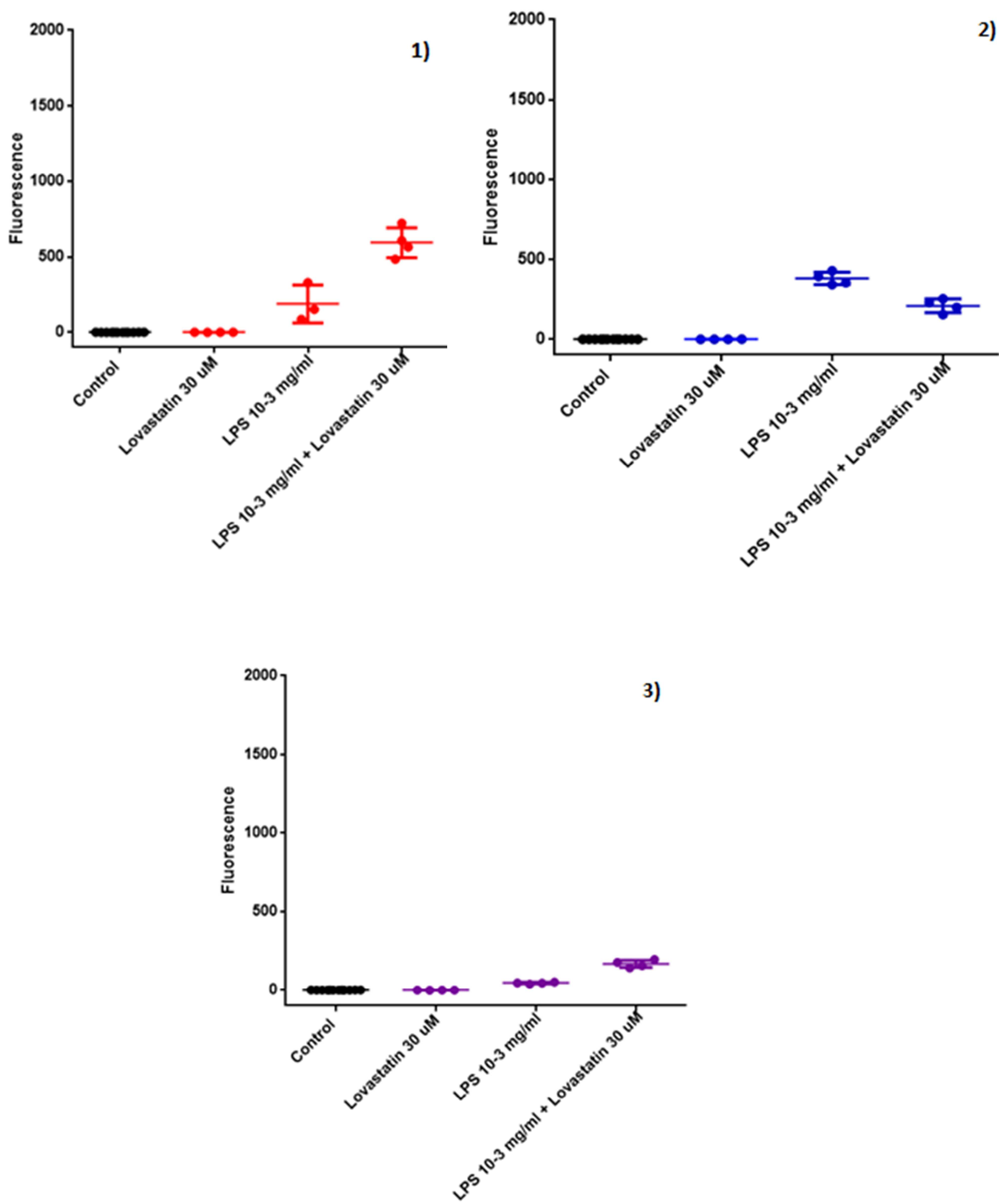


Fig.12. Graphs on stimulation of rat astrocytes with 1µg/ml of LPS and inhibition of IL-6 production with 30 µM of Lovastatin.

At a certain pointalso rat glia cells have been used, since the results with mouse cells were not fully satisfying yet and there was the possibility that statins would work better with rats, since most of the works in literature are on experiments with rat cells. We started with rat cortical astroglia, using two statins, Atorvastatin and Lovastatin. For the stimulation it was used 1 µg/ml of LPS and for the inhibition it was used 30 uM for both statins. In both cases three experiments were performed. Unfortunately, rats astroglia never reached a good stimulation and they never produced a sufficient amount of IL-6, so it is very difficult to understand if statins could inhibit its production; in fact, with Atorvastatin (Fig.11) There was never any inhibition, while, in Lovastatin case, it could be some inhibition in the second experiment (Fig.12.2) but the third experiment (Fig.12.3) did not confirm the result. In the first experiments of both statins (Figs. 12-13(1-4)), in the samples with LPS and statins there is a higher stimulation than that in the samples with LPS alone.

3.2.5 Inhibition of the production of IL-6 in rat microglia

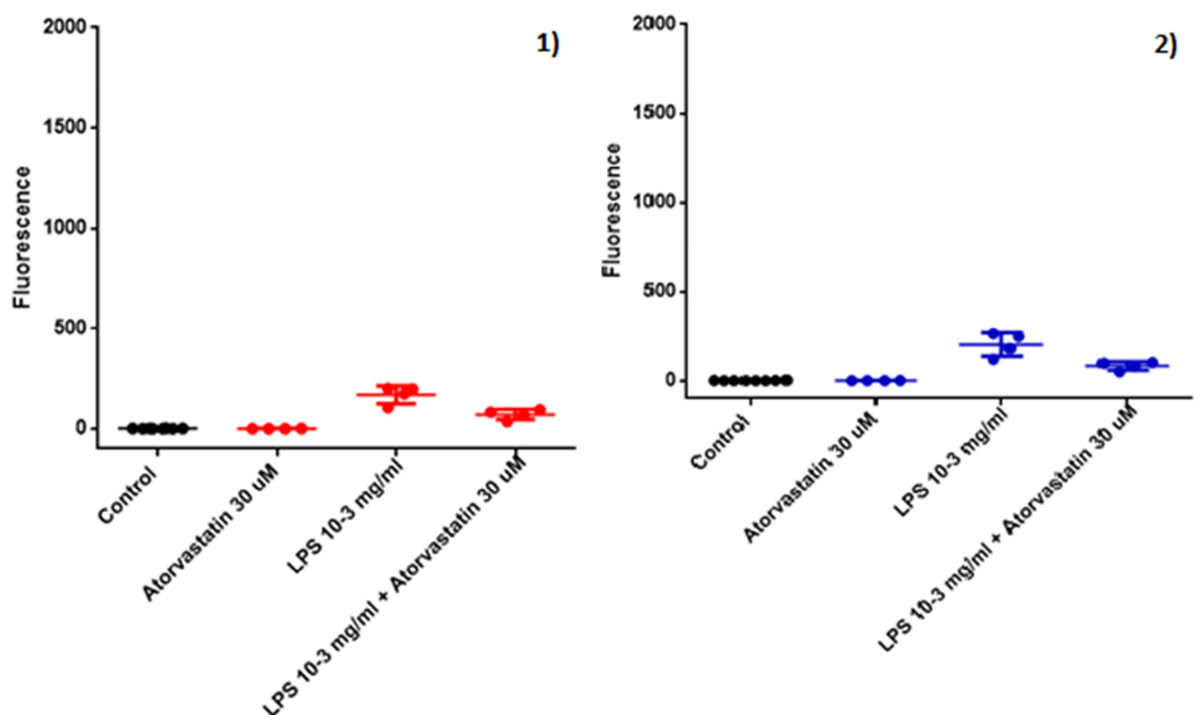


Fig.13. Graphs on stimulation of rat microglia with 1µg/ml of LPS and inhibition of IL-6 production with 30 µM of Atorvastatin.

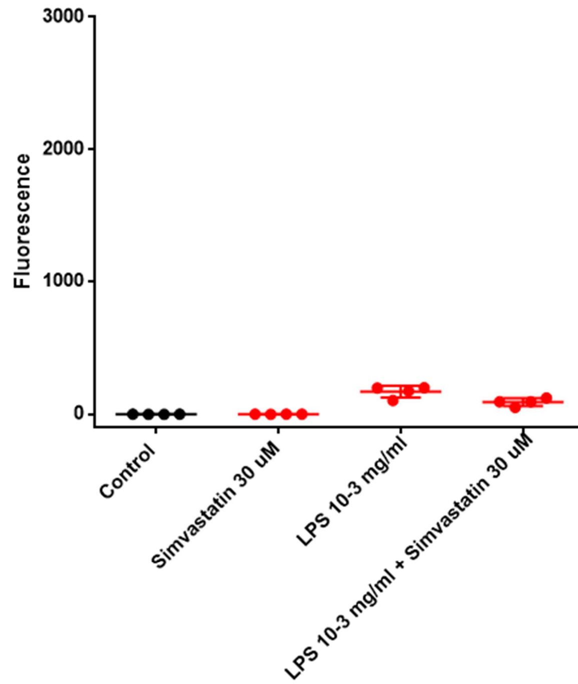


Fig.14. Graph on stimulation of rat microglia with 1µg/ml of LPS and inhibition of IL-6 production with 30 µM of Simvastatin.

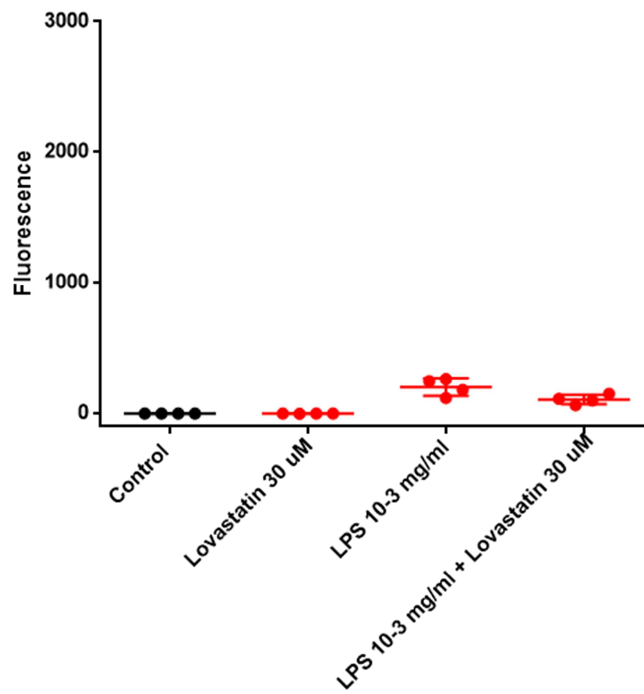


Fig.15. Graph on stimulation of rat microglia with 1µg/ml of LPS and inhibition of IL-6 production with 30 µM of Lovastatin.

On rat microglia experiments in total were performed, of which two experiments were on Atorvastatin (Fig.13.1-13.2), one on Simvastatin (Fig.14) and one on Lovastatin (Fig.15). In every experiment the same conditions and concentrations for stimulation and inhibition (1µg/ml of LPS and 30 uM of statin) were used. Unfortunately, rat microglia have never been stimulated and for this reason the inhibition was impossible to obtain.

CXCL10 fluorescence (mouse microglia)				
Pretreatment	**Average+SD LPS**	**Average+SD ATV+LPS**	**Average+SD SIM+LPS**	**Average+SD PVS+LPS**
8 hours	1833.5 ±130	497.25 ±222	1006.25 ±227	-
8 hours	1488 ±121	1505 ±209	1770.5 ±634	-
8 hours	3200.5 ±204	2605 ±570	1816 ± 238	-
8 hours	2540.4 ±122	2716.8 ± 268	2358.5 ±58	-
8 hours	2151.5 ±133	-	-	1759.8 ± 274

Tab.1 Summary table of the experiments on the production of CXCL10 with mouse astroglia.

IL-6 fluorescence (mouse astroglia)				
Pretreatment	**Average+SD LPS**	**Average+SD ATV+LPS**	**Average+SD SIM+LPS**	**Average+SD LOV+LPS**
8 Hours	1087.12 ± 77.1	143.37 ±137	930.5 ±122	-
8 Hours	555.3 ±261	-	-	944.17 ±143
8 Hours	277.25 ±287.4	265.5 ±226.3	-	54.16 ±85.6

Tab.2 Summary table of the experiments on the production of IL-6 with mouse astroglia.

IL-6 fluorescence (mouse microglia)				
Pretreatment	**Average+SD LPS**	**Average+SD ATV+LPS**	**Average+SD SIM+LPS**	**Average+SD LOV+LPS**
8 Hours	343.9 ±11	(ATV 10 uM) 223 ±30	-	(LOV 10 uM) 158 ±36
8 Hours	976.55 ± 96	247.3 ±27.7	268.1 ±129	250 ±64

Tab.3 Summary table of the experiments on the production of IL-6 with mouse microglia.

IL-6 fluorescence (rat astroglia)				
Pretreatment	**Average+SD LPS**	**Average+SD ATV+LPS**	**Average+SD SIM+LPS**	**Average+SD LOV+LPS**
8 Hours	189 ±126.7	411.3 ±186.7	-	595.7 ±99.4
8 Hours	381.5 ±39	275.6 ±4.1	-	209 ±42.4
8 Hours	45.1 ±5.5	33.25 ±12.3	-	167.1 ±23.6

Tab.4 Summary table of the experiments on the production of IL-6 with rat astroglia.

IL-6 fluorescence (rat microglia)				
Pretreatment	**Average+SD LPS**	**Average+SD ATV+LPS**	**Average+SD SIM+LPS**	**Average+SD LOV+LPS**
8 Hours	169.1 ±44.5	69.6 ±25.3	90.6 ±29.5	-
8 Hours	114.4 ±41.2	97.4 ±21.7	-	62.7 ±36.1

Tab.5 Summary table of the experiments on the production of IL-6 with rat microglia.

3.3 MTT Assay

After removing the supernatant, the cells in the plate were used to performe an MTT Assay. This assay exploits the capacity of mitochondrial dehydrogenase to reduce the MTT to an insoluble purple formazan which is then solubilized and whose quantity is determined spectrophotometrically. Since only living, active cells can do this reduction, the MTT Assay is generally used to determine the cell viability. This assay was used to verify any possible cytotoxic activity of statins. I started to use this assay late, so unfortunately it was not performed on each experiment. For the procedure, see the chapter "*Materials and methods*".

3.3.1 MTT Assay on Inhibition of the production of IL-6 in mouse astroglia

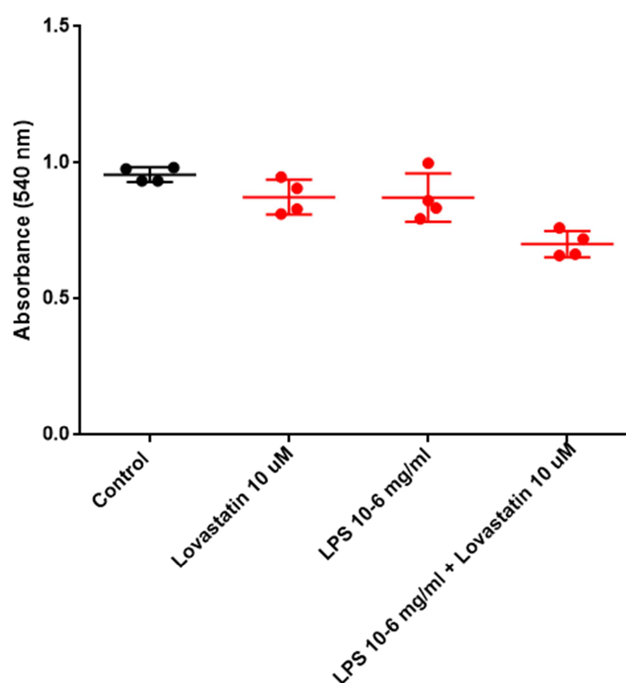


Fig.16. Graph of MTT assay on stimulation of mouse astrocytes with 1 ng/ml of LPS and inhibition with 10 µM of Lovastatin.

The cells were tested for their viability late, so for mouse astroglia it can be shown only the results of a MTT Assay on one of the last experiment, in which it was used Lovastatin (for the inhibition graph, see Fig.16). Both the wells in which the cells were treated with the statin or he LPS show absorance very similar to that of the control, meaning that they were not toxic in any way. There seems to be lower viability in the wells were I performed the inhibition with LPS and statin though, as if they are exerting some kind of synergism and they result toxic together for the cells.

3.3.2 MTT Assay on Inhibition of the production of IL-6 in mouse microglia

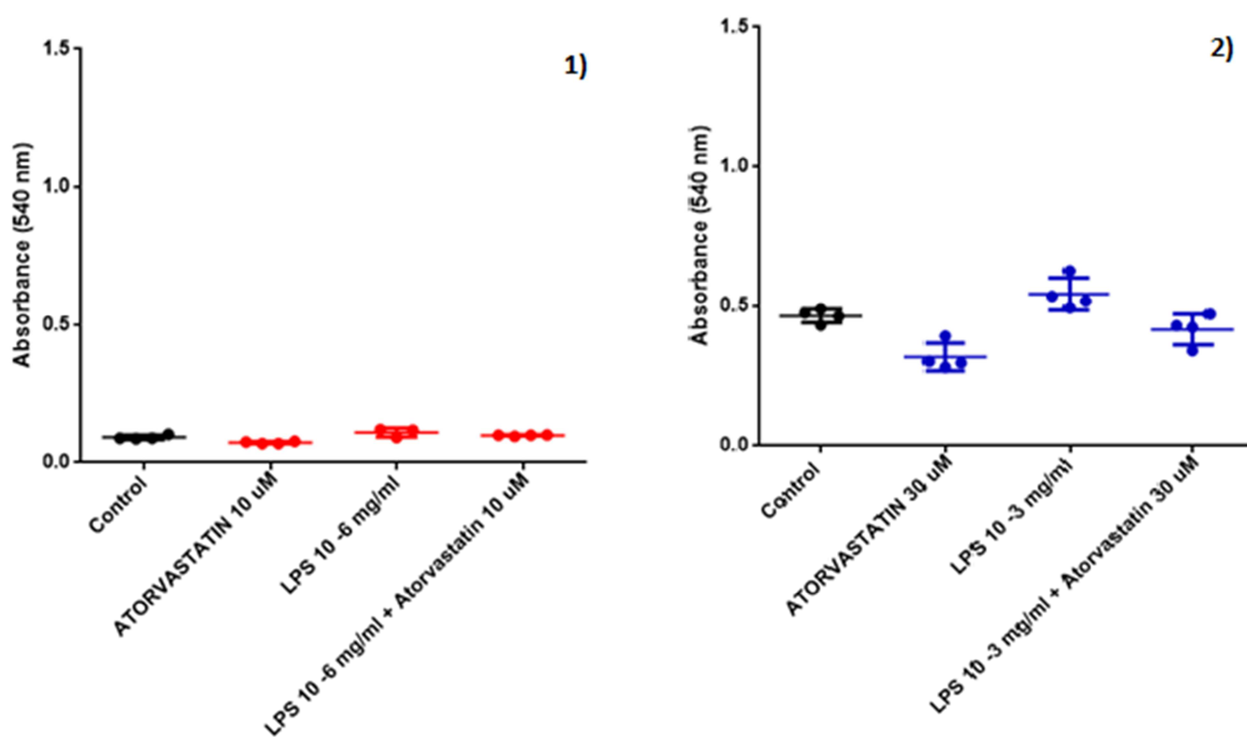


Fig.17. Graph of MTT assay on stimulation of mouse microglia with 1 ng/ml (17.1) or 1 µg/ml (17.2) of LPS and inhibition with 10 µM (17.1) or 30 µM (17.2) of Atorvastatin.

Fig.17. Graph of MTT assay on stimulation of mouse microglia with 1 ng/ml (17.1) or 1 µg/ml (17.2) of LPS and inhibition with 10 µM (17.1) or 30 µM (17.2) of Atorvastatin.

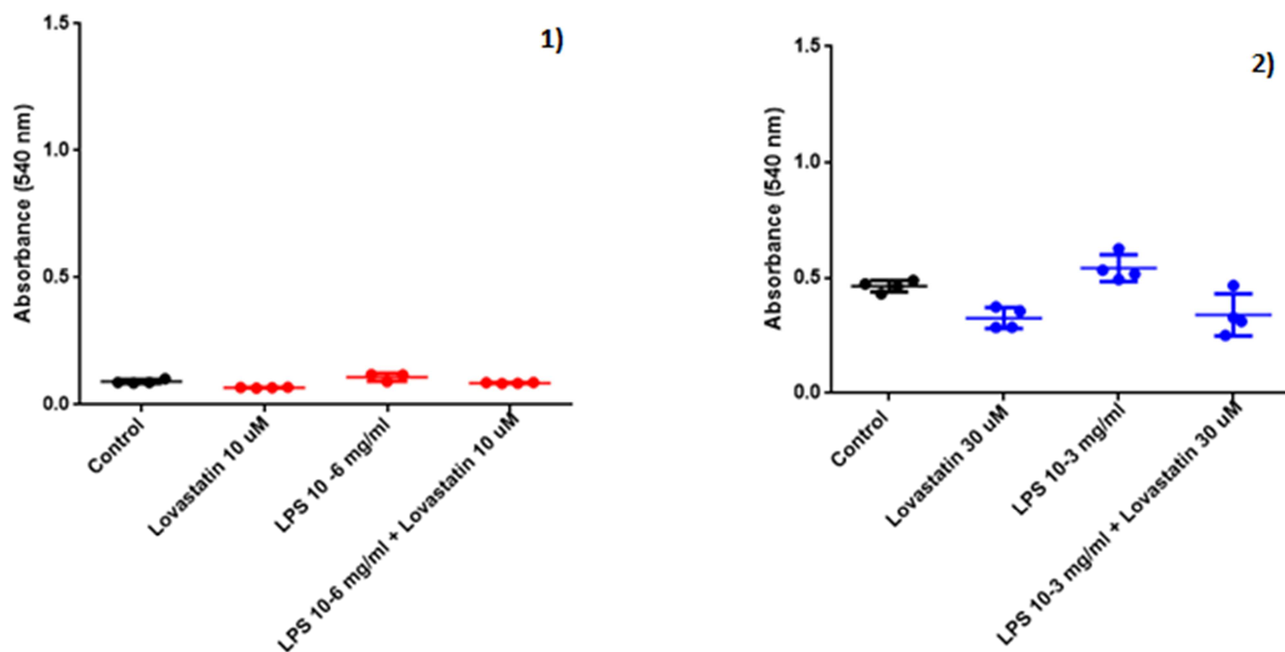


Fig.18. Graph of MTT assay on stimulation of mouse microglia with 1 ng/ml (18.1) or 1 µg/ml (18.2) of LPS and inhibition with 10 µM (18.1) or 30 µM (18.2) of Lovastatin.

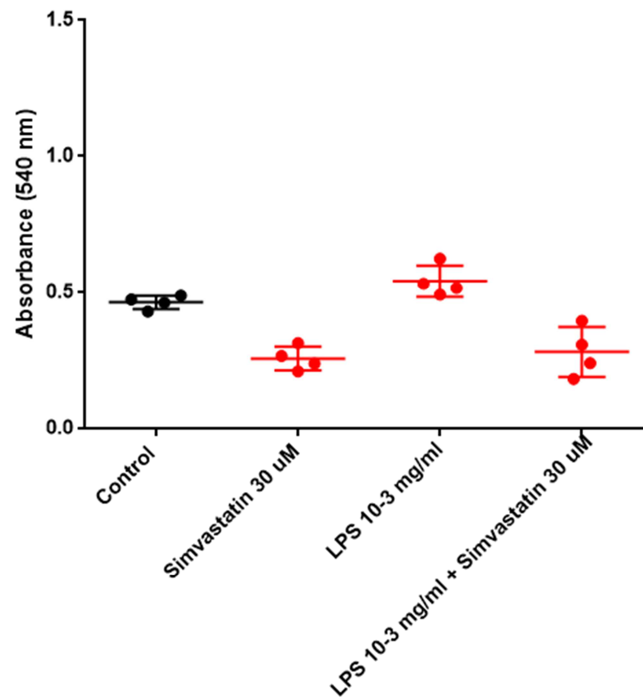


Fig.19. Graph of MTT assay on stimulation of mouse microglia with 1 µg/ml of LPS and inhibition with 30 µM of Simvastatin.

In this case the MTT assay was perfomed on every experiment with mouse microglia. As regards Atorvastatin, in the first experiment of inhibition (Fig.17.1), the cells were not stimulated very well by 1 ng/ml of LPS and there was not inhibitionalso, so in the second experiment (Fig.17.2) I was used more LPS, having a good stimulation and inhibition. The graphs of MTT assay show that it is possible that in the first experiment there was not any stimulation because the cells were not grown or it can be that most of them were already dead. In fact, in the second experiment were there was stimulation and inhibition, the reason could be that there was a good viability. The situation is very similar for Lovastatin. In fact, also in this case, in the first experiment (Fig.18.1) the lack of stimulation and inhibition could be due to the great amount of mortality of the microglia, while in the second experiment (Fig.18.2) they grew and they were stimulated by LPS. In this case, though, the MTT assay suggest that the inhibition of IL-6 production seen in Fig 9.2, could be due to a

possible cytotoxity of the statin, because in the wells were the cells were treated with 30 mM of Lovastatin (Fig.9.2), the viability is lower than that of the control and it is very similar to that of the wells in which the cells were stimulated with LPS and inhibited with Lovastatin. Finally, on cells treated with Simvastatin it was performed only an MTT assay (Fig.19) and, as for Lovastatin, also Simvastatin seems to be toxic for mouse microglia.

3.3.3 MTT Assay on Inhibition of the production of IL-6 in rat astrocytes

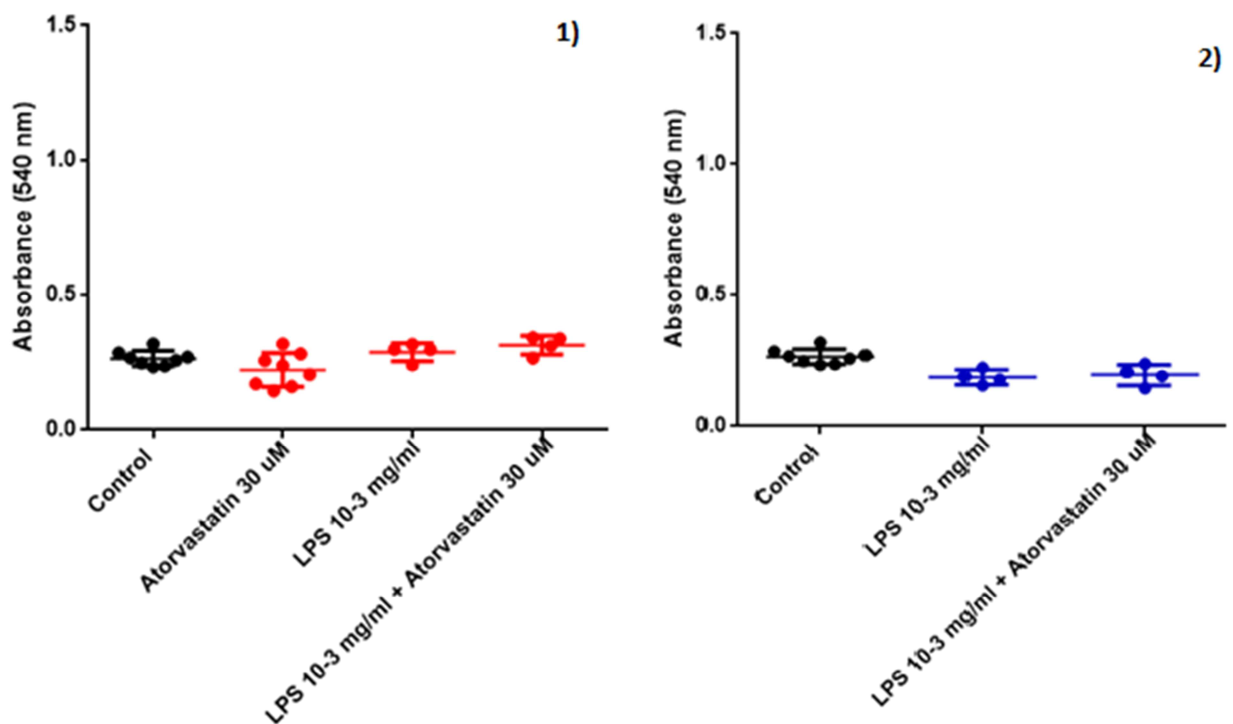


Fig.20. Graph of MTT assay on stimulation of rat astrocytes with 1 µg/ml of LPS and inhibition with 30 µM of Atorvastatin.

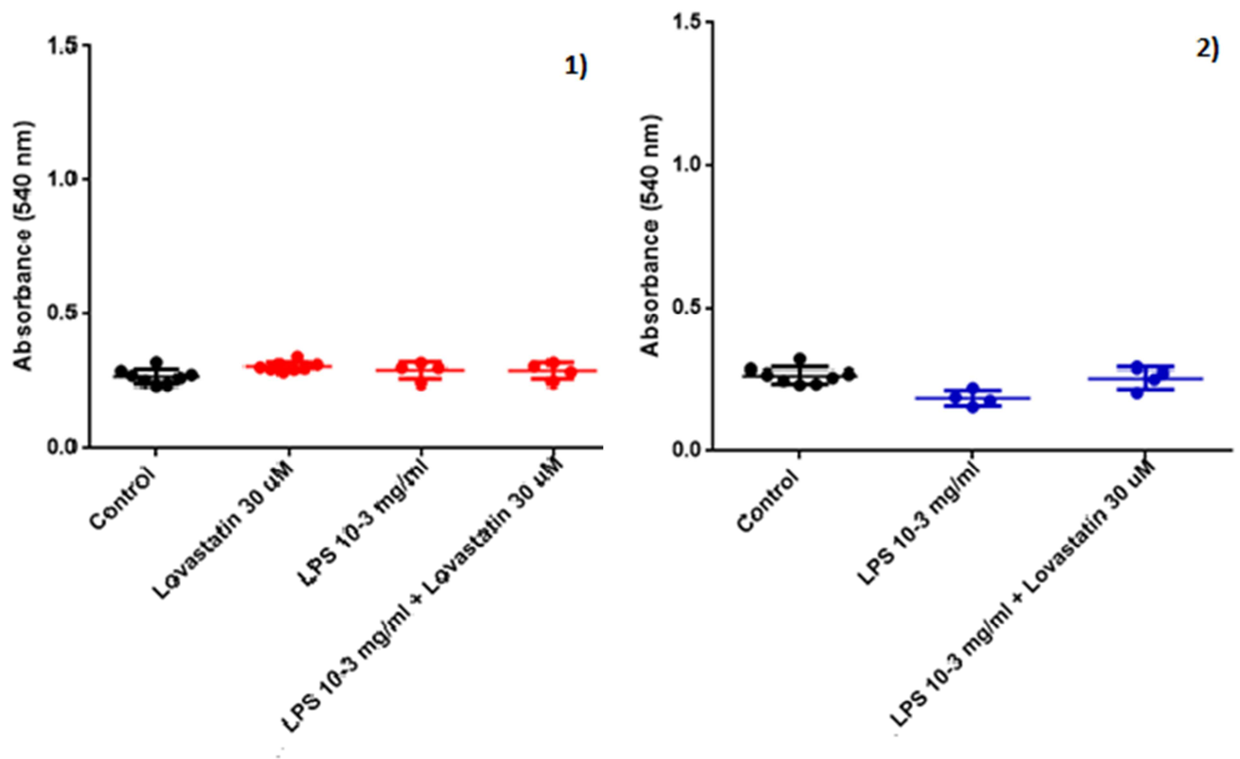


Fig.21. Graph of MTT assay on stimulation of rat astrocytes with 1 µg/ml of LPS and inhibition with 30 µM of Lovastatin.

On rat astrocytes three inhibition experiments were perfomed using atorvastatin, but the MTT assay was perfomed only on two of them. In the experiments in which this statin was used, there were never any stimulation by LPS and consequently any inhibition by this statin. If we consider the graphs of MTT assay (Fig.20.1-20.2), this situation could be explained with a low viability or growth of rat astrocytes (though, when observed at the microscope prior to the experiments, the cells formed a monolayer on the bottom of the plate). This situation occurred also in the case of Lovastatin (Fig. 21.1-21.2).

3.3.4 MTT Assay on Inhibition of the production of IL-6 in rat microglia

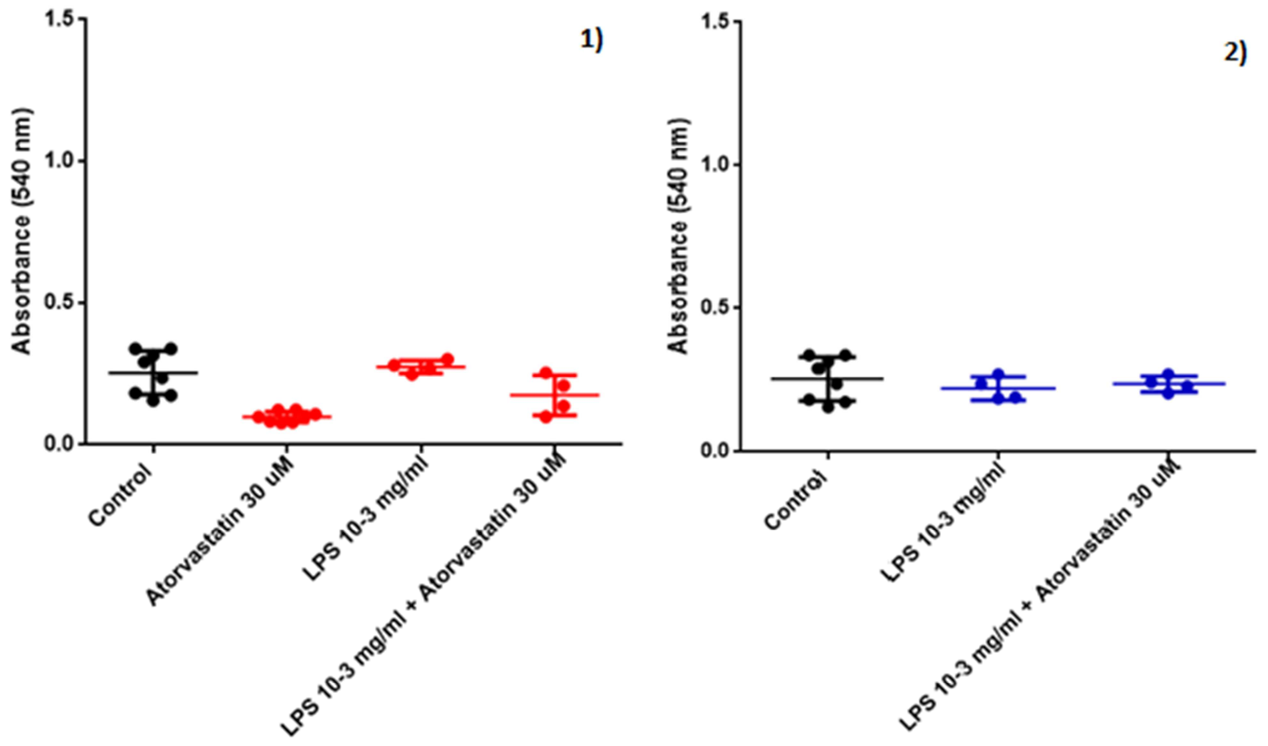


Fig.22. Graph of MTT assay on stimulation of rat microglia with 1 µg/ml of LPS and inhibition with 30 µM of Atorvastatin.

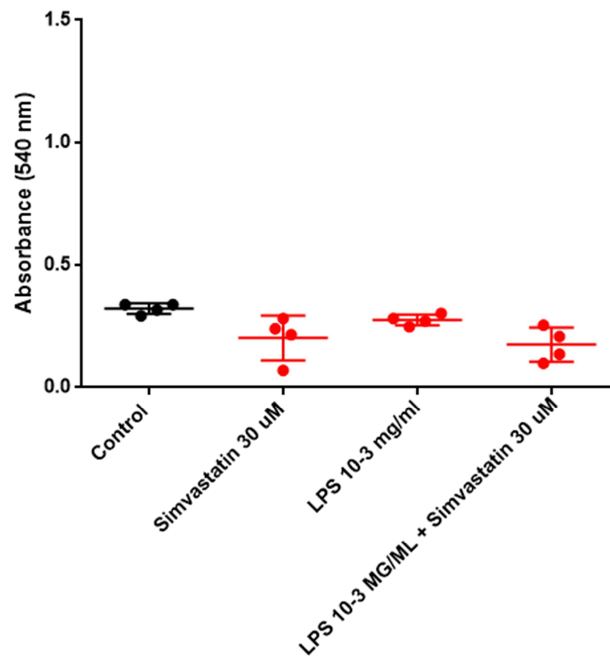


Fig.23. Graph of MTT assay on stimulation of rat microglia with 1 µg/ml of LPS and inhibition with 30 µM of Simvastatin.

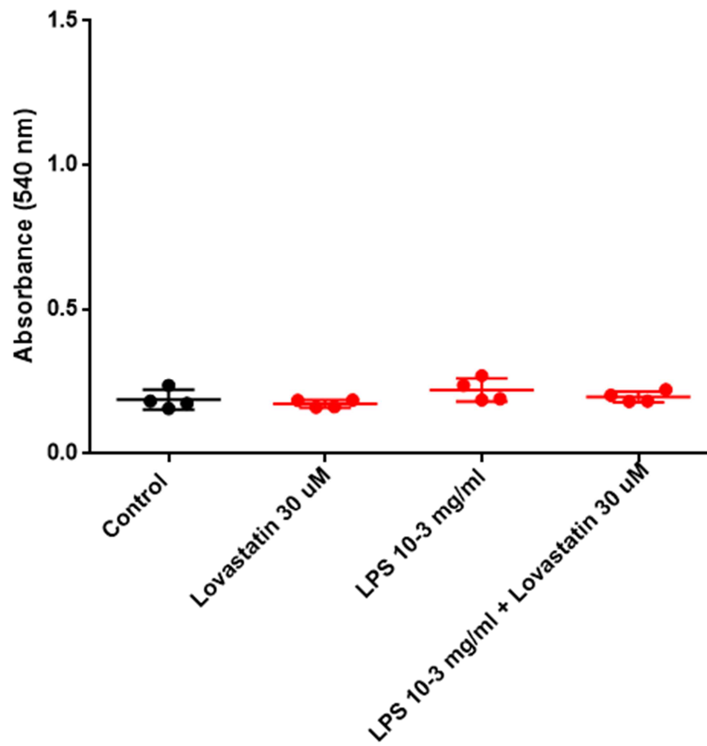


Fig.24. Graph of MTT assay on stimulation of rat microglia with 1 µg/ml of LPS and inhibition with 30 µM of Lovastatin.

The last type of cells used were rat microglia. As regards the experiments of stimulation and inhibition of IL-6, good results were never obtained on these cells, with any statin; there was never stimulation and consequently inhibition of the cytokine. Looking at the MTT graphs, it is clear that statins or LPS do not have any toxic activity but, as for rat astrocytes, there was a low number of cells and maybe this fact could be the reason why the experiments on these cells never worked. Another reason could be that the LPS concentration was not sufficient for a good stimulation.

MTT Assay (mouse astroglia)				
Pretreatment	**Average+SD O.D. Control**	**Average+SD O.D. ATV**	**Average+SD O.D. SIM**	**Average+SD O.D. LOV**
8 Hours	0.96 ± 0.03	-	-	(LOV 10 uM) 0.87 ± 0.06

Tab.6 Summary table of the experiments of cell viability on mouse astroglia

MTT Assay (mouse microglia)				
Pretreatment	**Average+SD O.D. Control**	**Average+SD O.D. ATV**	**Average+SD O.D. SIM**	**Average+SD O.D. LOV**
8 Hours	0.29 ±0.04	(ATV 10 uM) 0.19 ±0.03	-	(LOV 10 uM) 0.16 ±0.02
8 Hours	0.46 ±0.02	0.32 ±0.05	0.3 ±0.04	0.33 ± 0.05

Tab.7 Summary table of the experiments of cell viability on mouse microglia

MTT Assay (rat astroglia)				
Pretreatment	**Average+SD O.D. Control**	**Average+SD O.D. ATV**	**Average+SD O.D. SIM**	**Average+SD O.D. LOV**
8 Hours	0.25 ±0.025	0.275 ±0.03	0.34 ±0.006	0.3 ±0.007
8 Hours	0.27 ±0.03	0.17 ±0.02	-	0.3 ±0.03

Tab.8 Summary table of the experiments of cell viability on rat astroglia

MTT Assay (rat microglia)				
Pretreatment	**Average+SD O.D. Control**	**Average+SD O.D. ATV**	**Average+SD O.D. SIM**	**Average+SD O.D. LOV**
8 Hours	0.32 ±0.02	0.08 ±0.008	0.24 ±0.03	-
8 Hours	0.21 ±0.04	0.24 ±0.03	-	0.2 ±0.02

Tab.9 Summary table of the experiments of cell viability on rat microglia

4. CONCLUSIONS

There are many studies, especially in recent years, about neuroinflammation and its role in infectious and neurodegenerative disease, because it has become clear that neuroinflammation is part of the process that brings to the degeneration of the central nervous system. Here, the cells that have a central role in this process are glia cells, especially astrocytes and microglia. In fact, when a trauma or a pathogen hits the brains, these cells are activated, generating a number of processes including the production and secretion of pro-inflammatory cytokines.

The most important role of cytokines is to stimulate other types of cells to produce chemical mediators of inflammation, which are responsible for the inflammatory response (Pontieri G.M., 2012). There are a great number of cytokines with different roles; most of them have both pro and anti-inflammatory activity but for this project only pro-inflammatory cytokines were studied. In particular, I focused on two cytokines, IL-6 and CXL10.

Statins are a class of drugs whose main action is to lower cholesterol levels, so they are principally used as therapeutics for the treatment of cardiovascular diseases, but in the last few years, this type of drugs seems to have other properties, like anti-inflammatory properties, and for this reason, they have already been tested in a number of infectious and neurodegenerative diseases (Bar-On et al., 2008; Fassbender et al., 2001; Reis et al., 2012).

Thus, during this part of my PhD I used four different statins (Atorvastatin, Simvastatin, Lovastatin and Pravastatin) to inhibit the production of two pro-inflammatory cytokines, IL-6 and CXCL10, after stimulating cortical astrocyes and microglia primary cultures from newborn C57BL/10SnJ mice and Sprague-Dawley rats with LPS.

From the results of these experiments, I would assume that the more interesting statins are Atorvastatin, Simvastatin and Lovastatin, while Pravastatin was used only at the beginning

with no results. This confirm the fact that Pravastatin is not often used for this purpose because, Pravastatin has anti-inflammatory properties but there are not many studies about a neuro-protective effect during neuroinflammation. So, after using Pravastatin in an experiment to try to inhibit the production of CXCL10 with no result it has been decided to focus on the other three statins that generally show more activity on different diseases.

Under different conditions for stimulation and inhibition, as two different concentrations of LPS and different concentrations and dilutions of statins, the best results were obtained with 1 µg/ml of LPS and 30 µM of statins for mouse cells. As regards rat cells, when the LPS curve was performed on rat microglia and astrocytes, the concentration of LPS that would give the best production of IL-6 seemed to be 1 µg/ml as for mice; moreover, in literature this seems to be the most used concentration. For example, in a work of 2014, Villa and her group stimulated primary microglia mouse cells with 1 µg/ml of LPS, having a great stimulation (Villa et al., 2014). Unfortunately,in my hands there was never any stimulation of either microglia and astrocytes, with this amount of LPS. From the viability assays (MTT), and from direct observation under the microscope prior to the experiments, the cells were not affected in terms of numbers and morphology, but it is possible that this type of cells need higher concentration of LPS to be stimulated. So, although statins have been used on rat cells before, I was not able to determine the optimal conditions to test statins.

Better results were obtained with mouse cells, especially with microgliain which, when the appropriate conditions of stimulation and treatment were determined, a moderate inhibition of IL-6production was observed. Unfortunately, I did not have enough time left in the US to confirm this hypothesis. Based on the results of my preliminary experiments in the mouse model, indicating that statins inhibit the inflammation by acting on glia cells, *in vivo* testing of the effect of Atorvastatin and Simvastatin in mice affected by scrapie are ongoing.

5. MATERIALS AND METHODS

5.1 Cell cultures

Astroglia and microglia primary cells from 2 days old mice and Sprague-Dawley rats were used in these experiments. The use of 1- to 2-d-old pups ensures the absence of viable neurons in the cell suspension obtained from dissociating the cerebral cortex. The procedures used for these two types of cells and animals were very similar. It is not necessary to work in sterile conditions but it must be always used 70% ethanol to clean all the materials like surgical scissors, smooth fine forceps and flat tip forceps.

The animals were sacrificed by rapid decapitation using the scissors. After removing the head, perform a midline incision, posterior to anterior, along the scalp to reveal the skull, then cut the cranium carefully from the neck to the nose. Using the flat tip forceps, the skull was opened and the brain was removed , then it was placed in a Petri dish with PBS and ice. For the experiments cortical cells were needed, so, first the olfactory bulbs and the cerebellum were removed using dissecting forceps, then the two hemispheres were divided and a second set of forceps was used to the created grove and peel away the plate-like structure of the cortex from the brain. Finally the meninges were removed, in order to avoid contamination by meningeal astrocytes and fibroblasts. The cortex hemispheres were transferred in a 15 ml tube containing 4 ml of DMEM with no FBS in it. For rats, I put one brain in each tube, while for mice I put 3 or 4 brains in each tube.

1 ml of 0.05% trypsin was added in each tube, the cells were then triturated by pipetting and the tubes were placed in the incubator at 37ºC for 10 minutes. Then the brains were centrifuged for 5 minutes at 800 rpm and re-suspended in 5 ml of fresh medium (DMEM with 10% FBS, 1% Pen/Strep, 1% L-Glutamate). This suspension was finally put in flasks (for rats I used T75 flasks containing 5 ml of suspension and 15 ml of DMEM, for mice I used T25 flasks containing 5 ml of suspension and 5 ml of DMEM). The flasks were

incubated at 37ºC, 5% CO_2, for 10-13 days (generally, 10 days for rats and 13 days for mice cells).

After this time period, generally the cells were confluent but they were a mixed cortical cell culture; so, in order to obtain astrocytes and microglia pure cell cultures, the cells needed to be separated. The separation procedure is based on the fact that, when shaken, astrocytes tend to stay adherent to the surface of the flask, while microglia detach from the flask and go in the suspension. So, the flasks with mixed glia cells were put in a shaker at 37ºC, 500 rpm, for 4-18 hours (4 h for mice cells, 18 hours for rats cells); then, microglia in the suspension were taken, centrifugated for 5 minutes, re suspended in fresh DMEM and seeded in 96 well plates at the density of 50000 cells/well. As regard astrocytes, they were detached from the flask using 1 ml of trypsin, then re-suspended in fresh DMEM and seeded in 96 well plates at the density of 50000 cells/well. The plates were put in an incubator at 37ºC, 5% CO_2 for 3 days.

I also seeded 400 µl of cell suspension in a chambered slide in order to perfom a fluorescent staining procedure.

5.2 Immunofluorescent staining

In order to stain the cells cultures contained in the chambered slides I used the following protocol:

- Remove the medium and wash the cells 3 times with PBS, in order to remove any trace of medium and dead cells;
- Wash the cells 3 times with PBS;
- Fix the cells with 3.7% formaldehyde for 15 minutes;
- Wash the cells 3 times with PBS;
- Permeabilize the cells with0,1% Triton X-100 for 10 minutes;

- Wash the cells 3 times with PBS;
- Block with 3% BSA (bovine serum albumin) for 30 minutes;
- Wash the cells 3 times with PBS;
- Block with 0.2 M Glycine in PBS for 30 minutes;
- Wash the cells 3 times with PBS;
- Incubation with primary antibody for 1 hour (for astrocytes I used αGFAP 1:2500, for microglia I used αIBA-1 1:1000);
- After 1 hour of incubation, wash the cells 3 times with PBS;
- Incubation with secondary antibody for 1 hour (for astrocytes I used α-rabbit AlexaFluor-568 1:5000, for microglia I used α-rabbit AlexaFluor-488 1:5000);
- Wash the cells 3 times with PBS;
- After removing the chambers, put a drop of Prolong Gold Antifade with DAPI on cells, followed by a coverslip.;
- Observe with fluorescence microscope.

5.3 Reagents

Lipopolysaccharide (LPS), Atorvastatin (ATV), Simvastatin (SIM), Lovastatin (LOV) and Pravastatin (PVS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). It was used a LPS stock of 1 mg/ml prepared in PBS and stored at -80°C. The stock of Atorvastatin and Pravastatin were prepared in DMSO, at the concentration of 10 μM and stored at -20ºC; as regard Simvastatin and Lovastatin, they need to be activated prior to use in experiments; the activation allows the opening of the lactone ring: 8 mg of SIM were dissolved in 0.2 ml of 100% ethanol (19 μM), then 0.3 ml of 0.1 N NaOH were added. The solution was heated at 50ºC for 2 h and then neutralized with HCl to pH 7. Finally I brought to a final volume (1 ml) with distilled water, and stored at –20°C until use. Similar protocol was used for a 10 μM

stock of Lovastatin: 25 mg of the lactone form weredissolved in 500 µl of 100% ethanol, heated to 50°C, alkalinized by adding 250 µl of 0.6 M NaOH, and incubated at 50°C for 2 h. After incubation, the solution was neutralized with 0.4 M HCl at pH 7.5 and stored at -20ºC (Morimoto et al., 2006).

For immunofluorescence primary and secondary antibodies were used. For astrocytes I used α-GFAP as primary antibody (dilution 1:2500) and α-rabbit AlexaFluor-568 as secondary antiboby (dilution 1:5000); for microglia I used αIBA-1 as primary antibody (dilution 1:1000) and α-rabbit AlexaFluor-488 as secondary antibody (dilution 1:5000).

5.4 Induction of cytokines production

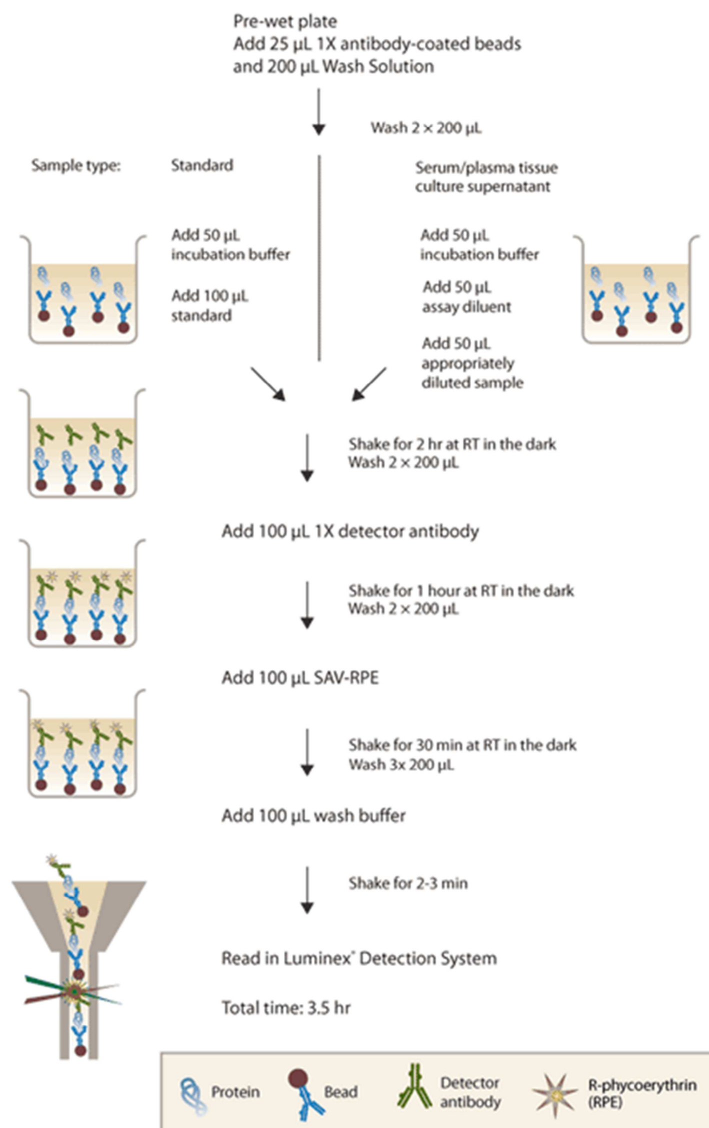
Prior to stimulation with LPS, the cells were pretreated with different concentrations of statins for 8 hours, preparing the dilutions and putting 50 µl of each dilution in the wells. Then, the plate were put in the incubator for 8 hours. After that, the cells were stimulated to produce cytokines with LPS (at the concentration of $1x10^{-6}$ or $1x10^{-3}$ mg/ml), 50 µl/well. In the wells containing the control for the LPS 50 µl of LPS and 50 µl of DMEM were seeded; in the wells where the cells were stimulated with LPS and treated with statins, 50 µl of LPS and 50 µl of the proper statin dilution were seeded. The wells containing the negative controls had 100 µl of DMEM. For the experiments it was always used DMEM without FBS. Then, the plates were incubated at 37ºC, 5% CO_2 for 24 hours. After the incubation period the supernatant was taken for the analysis of cytokines, while the cells were used for the MTT assay.

5.5 Determination of the inibition of cytokines production

To determine if the treatment with statins could inhibit the production of cytokines by mouse and rat cell cultures, the Exracellular protein kit specific for mouse IP-10 or IL-6 and

for rat IL-6 (Bioplex), purchased from Invitrogen (Carlsbad, CA, USA) for Luminex® Platform was used.

24 hours after stimulation with LPS and treatment with statins, I took the supernatant and I used the kit I mentioned above specific for the cytokine I wanted to analyzed, using the following protocol provided by the company:



The fluorescence of each sample was finally read using the Luminex® Detection System.

5.6 MTT Assay

In order to see if the treatment with statins could influence the cell viability, the MTT assay was performed on the cells, after removing the supernatant (which I used for the Bioplex Assay). In the first experiments the Vybrant® MTT Cell Proliferation Assay Kit purchased from ThermoFisher Scientific was used. This kit contained MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Component A), 10 vials, each containing 5 mg and SDS sodium dodecyl sulfate (Component B), 10 vials, each containing 1 mg; then it was used the MTT powder (ThermoFisher Scientific) which was dissolved in PBS (5mg/ml) and DMSO (dimethylsulfoxide) instead of the Component B to read the absorbance of the wells. Immediately after removing the supernatant, 100 µl of DMEM without phenol red (as this could interfere with the reading of the absorbance) were put in each well, plus 10 µl of MTT solution. The plates were incubated at 37ºC for 4 hours. During this incubation period the MTT is reduced to an insoluble purple formazan product by mitochondrial dehydrogenase; after the incubation the formazan in dissolved with the component B or the DMSO and the absorbance is finally quantified spectrophotometrically, at 540 nm if using component B or at 570 nm if using DMSO.

PART I and PART II

REFERENCES (alphabetical order)

Abbott N J - Astrocyte–endothelial interactions and blood–brain barrier permeability – 2002, J Anat 200, 629-638

Aguzzi A, Baumann F, Bremer J The prion's elusive reason for being. Annu Rev Neurosci 2008, 31:439–477.

Albert M A, Danielson E, Rifai N, Ridker P M - Effect of statin therapy on C-reactive protein levels: the pravastatin inflammation/CRP evaluation (PRINCE): a randomized trial and cohort study – 2001, JAMA 286(1), 64-70

Alvarez J L, Katayama T, Prat A - Glial influence on the blood brain barrier – Glia 61, 1939-1958

Araya R, Kudo M, Kawano M, Ishii K, Hashikawa T, Iwasato T, Itohara S, Terasaki T, Oohira A, Mishina Y, Yamada M - BMP signaling through BMPRIA in astrocytes is essential for proper cerebral angiogenesis and formation of the blood-brain barrier – 2008, Mol Cell Neurosci 38:417–430

Argaw A T, Asp L, Zhang J, Navrazhina K, Pham T, Mariani J N, Mahase S, Dutta D J, Seto J, Kramer E G, Ferrara N, Sofroniew M V, John G R – Astrocytes-derived VEGF-A drives blood brain barrier disruption in the CNS inflammatory disease – J Clin Invest, 2012, 122(7): 2454-2468

Bach E A, Aguet M, Schreiber R D – The IFN gamma receptor: a paradigm for cytokine receptor signaling – 1997, Ann Rev Immunol, 15,563-591

Balasingam V, Tejada-Berges T, Wright E, Bouckova R, Yong V W – Reactive astrogliosis in the neonatal mouse brain and its modulation by cytokines – 1994, J Neurosci; 14,846-856

Barnum S R, Jones J L, Muller-Ladner U, Samimi A, Campbell I L – Chronic complement C3 gene expression in the CNS of transgenic mice with astrocyte-targeted interleukin-6 expression – 1996; Glia 18, 107-117

Bar-On P, Crews L, Koob A O, Mizuno H, Adame A, Spencer B, Masliah E – Statins reduce neuronal α-synuclein aggregation in *in vitro* models of Parkinson's disease – 2008, J Neurochem 105(5):1656

Barres B A - The mystery and magic of glia: a perspective on their roles in health and disease – 2008, Neuron 60:430–440

Bauer J, Strauss S, Schreiter-Gasser U, Ganter U, Schlegel P, Witt I, et al - Interleukin-6 and alpha-2 macroglobulin indicate an acute-phase state in Alzheimer's disease cortices – 1991, . FEBS Lett.; 285: 111-114

Bauer S, Kerr B J, Patterson P H - The neuropoietic cytokine family in development, plasticity, disease and injury – 2007, Nat Rev Neurosci; 8: 221-232

Benveniste E N, Sparacio S M, Norris J G, Grenett H E, Fuller G M - Induction and regulation of interleukin-6 gene expression in rat astrocytes – 1990, Neuroimmunol; 30: 201-212.

Björkqvist M, Wild E J, Thiele J, Silvestroni A, Andre R, Lahiri N, et al - A novel pathogenic pathway of immune activation detectable before clinical onset in Huntington's disease – 2008, J Exp Med.; 205: 1869-18
77

Bolin L M, Zhaung A, Strychkarska-Orczyk I, Nelson E, Huang I, Malit M, Nguyen Q – Differential inflammatory activation of IL-6 (-/-) astrocytes – 2005; Cytokine 30,47-55

Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank D A, Rozovsky I, Stahl N, Yancopoulos G D, Greenberg M E – Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway – 1997, Science 278, 477-483

Brandner S, Isenmann S, Raeber A, Fischer M, Sailer A, Kobayashi Y, Marino S, Weissmann C, Aguzzi A- Normal host prion protein necessary for scrapie-induced neurotoxicity – 1996, Nature, 1996;379:339–343.

Brown M A, Hural J - Functions of IL-4 and control of its expression – 1997, Crit Rev Immunol; 17:1–32

Brunello A G, Weissenberger J, Kappeler A, Vallan C, Peters M, Rose-John S, Weis J

– Astrocytic alteration in interleukin-6/soluble interleukin-6 receptor alpha double-transgenic mice – 2000; Am J Pathol 157, 1485-1493

Bush T G, Puvanachandra N, Horner C H, Polito A, Ostenfeld T, Svendsen C N, Mucke L,

Bushong E A, Martone M E, Jones Y Z, Ellisman M H - Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains - J Neurosci 2002, 22:183–192

Cabezas R, Avila M, Gonzales J, El-Bacha R S, Baez E, Garcia-Segura L M, Jurado Coronel J C, Capani F, Cardona-Gomez G P, Barreto G E – Astrocytic modulation of blood brain barrier: perspectives on Parkinson's disease – Front Cell Neuro, 2014 8:211

Cadman E D, Naugles D D, Lee C M - cAMP is not involved in interleukin-1-induced interleukin-6 release from human astrocytoma cells – 1994, Neurosci Lett.; 178: 251-254

Callard RE, Matthews DJ, Hibbert L - IL-4 and IL-13 receptors: are they one and the same? - 1996, Immunol Today; 17:108–110

Chaboub L S, Deneen B - Astrocyte Form and Function in the Developing CNS - Semin Pediatr Neurol. 2013; 20(4):

Chai Z, Gatti S, Toniatti C, Poli V, Bartfai T - Interleukin (IL)-6 gene expression in the central nervous system is necessary for fever response to lipopolysaccharide or IL-1 beta: a study on IL-6-deficient mice – 1996, J Exp Med.; 183: 311-316.

Chiang C S, Stalder A, Samimi A, Campbell I L – Reactive gliosis as a consequence of interleukin-6 expression in the brain: studies in transgenic mice – 1994, Dev Neurosci, 16, 212-221

Chikuma T, Yoshimoto T, Ohba M, Sawada M, Kato T, Sakamoto T, Hiyama Y, Hojo H – Interleukin-6 induces prostaglandin E(2) synthesis in mouse astrocytes – 2009; J Mol Neurosci 39,175-184

Christopherson K S, Ullian E M, Stokes C C, Mullowney C E, Hell J W, Agah A, Lawler J, Mosher D F, Bornstein P, Barres B A - Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis – 2005, Cell 120:421–433

Clarke C J P, Hales A, Hunt A, et al - IL-10 mediated suppression of TNF-a production is independent of its ability to inhibit NF-kB activity – 1998, Eur J Immunol; 28:1719–1726

Colby D W, Prusiner S B – Prions – 2011, Cold Spring Harb Perspect Biol 2011;3:a006833

Combrinck M I, Perry V H, Cunningham C – Peripheral infection evokes exaggerated sickness behaviour in pre-clinical murine prion disease – Neurosc 2002;112(1):7-11

CornfieldL J, Sills M A - High affinity interleukin-6 binding sites in bovine hypothalamus – 1991,Eur J Pharmacol.; 202: 113-5.

Cuschieri J, Bulger E, Garcia I, Jelacic S, Maier R V - Calcium/ calmodulin-dependent

kinase II is required for platelet-activating factor priming – 2005, Shock 23,99-106

Damiani C L, O'Callaghan J P – Recapitulation of cell signaling events associated with astroglios using the brain slice preparation – 2007, J Neurochem; 100, 720-726

Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB - ATP mediates rapid microglial response to local brain injury in vivo - Nat Neurosci 2005, 8:752-75

Deverman B E, Patterson P H - Cytokines and CNS development – 2009, Cell; 64: 61-78

Devi L A - G-protein-coupled receptor dimers in the lime light – 2000, Trends in Pharm Sci, vol.21, no.9, pp.324– 326

de Waal Malefyt R, Figdor C G, Huijbens R, et al. - Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes – 1993, J Immunol; 151:6370– 6381

Dinarello C A - Biologic basis for interleukin-1 in disease - Blood 1996; 87:2095–2147

Dinarello C A - . Induction of interleukin-1 and interleukin-1 receptor antagonist - Semin Oncol 1997; 24(suppl):S9–81, S9–93

Dinarello C A - Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist -

Int Rev Immunol 1998; 16:457–499

Dinarello C A – Proinflammatory cytokines – 2000, Chest, 118/2

Di Santo E, Alonzi T, Fattori E, Poli V, Ciliberto G, Sironi M, Gnocchi P, Ricciardi-Castagnoli P, Ghezzi P – Overexpression of interleukin-6 in the central nervous system of transgenic mice increases central but not systemic proinflammatory cytokine production – 1996, Brain Res, 740, 239-244

Dong C - Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells - Nature Reviews Immunology, vol. 6, no. 4, pp. 329–333

Dong W, Vuletic S, Albers J J - Differential effects of simvastatin and pravastatin on expression of Alzheimer's disease-related genes in human astrocytes and neuronal cells – 2009, J Lipid Res.; 50(10): 2095–2102

Duffy P, Schmandke A, Schmandke A C, Sigworth C, Shun N, Cafferty W B, et al. - Rho-associated kinase II (ROCKII) limits axonal growth after trauma within the adult mouse spinal cord – J Neurosci, 2009, 29, 15266-15276

Dufour J H, Dziejman M, Liu M T, Leung J H, Lane T E, Luster A D - IFN-gamma-inducible protein 10 (IP10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking – 2002, J Immunol 168: 3195–3204

Durrant D M, Williams J L, Daniels B P, Klein R S – Chemokines referee inflammation

within the central nervous system during infection and disease – 2014, Adv Med,2014:806741. doi: 10.1155/2014/806741. Epub 2014 Sep 30

Eddleston M, Mucke L - Molecular profile of reactive astrocytes—implications for their role in neurological disease – 1993, Neuroscience 54:15–36

Endo A, Kuroda M, Tsujita Y - ML-236A, ML-236B, and ML-236C, new inhibitors of cholesterogenesis produced by *Penicillium citrinium* – 1976, J. Antibiot 1976;29:1346–1348

Eng L F, Ghirnikar R S, Lee Y L - Glial fibrillary acidic protein: GFAP-thirty-one years (1969–2000) – 2000, Neurochem Res 25:1439–1451

Erta M, Quintana A, Hidalgo J – Interleukin-6, a major cytokine in the central nervous system – 2012, Int J Biol Sci, 8(9):1254-1266

Fadok V A, Bratton D L, Konowal A, Freed P W, Westcott J Y, Henson P M - Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-β, PGE2, and PAF – 1998, The Journal of Clinical Investigation, vol. 101, no. 4, pp. 890–898,

Fassbender K, Schneider S, Bertsch T, Schlueter D, Fatar M, Ragoschke A, Kuhl S, Kischka U, Hennerici M - Temporal profile of release of interleukin-1beta in neurotrauma – 2000, Neurosci Lett 284: 135–138.

Fassbender K, Simons M, Bergmann C, Stroick M, Lutjohann D, Keller P, Runz H,

Kuhl S, Bertsch T, von Bergmann K, Hennerici M, Beyreuther K, Hartmann T - Simvastatin strongly reduces levels of Alzheimer's disease β-amyloid peptides Aβ42 and Aβ40 in vitro and in vivo – 2001, Pnas 98,5856-5861

Fattori E, Lazzaro D, Musiani P, Modesti A, Alonzi T, Ciliberto G – IL-6 expression in neurons of transgenic mice causes reactive astrocytosis and increase in ramified microglial cells but no neuronal damage – 1995, Eur J Neurosci, 7, 2441-2449

Fenner F, McAuslan B R, Minis C A, Sambrook J, White D O - Biology of Animal Viruses – 1974, New York, Academic Press

Fitch M T, Silver J – CNS injury, glial scars and inflammation: inhibitory extracellular matrices and regeneration failure – 2008, Exp Neurol 209,294-301

Frei K, Leist T, Meager A, Gallo P, Leppert D, Zinkernagel R, et al - Production of B cell stimulatory factor-2 and interferon gamma in the central nervous system during viral meningitis and encephalitis – 1988, J Exp Med; 168: 449-453.

Frei K, Malipiero U V, Leist T P, Zinkernagel R M, Schwab M E, Fontana A - On the cellular surface and function of interleukin-6 produced in the central nervous system in viral diseases – 1989, Eur J Immunol.; 19: 689-694

Gabay C, Smith M F, Eidlen D, Arend W P - Interleukin 1 receptor antagonist (IL-1Ra) is an acute-phase protein - J Clin Invest. 1997; 99(12): 2930–2940

Gadea A, Schinelli S, Gallo V - Endothelin-1 regulates astrocyte proliferation and reactive gliosis via a JNK/c-Jun signaling pathway – 2008, J Neurosci 28:2394–2408

Gangur V, Simons F E, Hayglass K T - Human IP-10 selectively promotes dominance of polyclonally activated and environmental antigen-driven IFN-gamma over IL-4 responses – 1998, FASEB J 12:705–713

Gerhmann J, Bonnekoh P, Miyazawa T, Hossmann K A, Kreutzberg G W – Immunocyochemical study of an early microglial activation in inschemia – J Cereb Blood Flow Metabol, 12:257-269 , 1992

Ginhoux F, Lim S, Hoeffel G, Low D, Huber T – Origin and Differentiation of Microglia – Front in Cell Neuro, 2013 7:45

Golgi C - On structure of the gray matter of the brain - Gazzetta Medica Italiana Lombardia. 1873;33:244-6.

Gouveia T L, Scorza F A, Silva M J, Bandeira Tde A, Perosa S R, Arganaraz G A, et al - Lovastatin decreases the synthesis of inflammatory mediators in the hippocampus and blocks the hyperthermia of rats submitted to long-lasting status epilepticus – 2011, Epilepsy Behav 20(1):1–5

Gouveia T L, Scorza F A, Iha H A, Frangiotti M I B, Perosa S R, Cavalheiro E A, Silva J A Jr, Feliciano R S, de Almeida A C, Naffah-Mazzacoratti M G - Lovastatin decreases the synthesis of inflammatory mediators during epileptogenesis in the hippocampus of rats

submitted to pilocarpine-induced epilepsy **-** 2014, Epilepsy Behav 36,68-73

Gordon G R, Mulligan S J, MacVicar B A - Astrocyte control of the cerebrovasculature – 2007, Glia 55:1214–1221

Grau G E, Frei K, Piguet P F, Fontana A, Heremans H, Billiau A, et al - Interleukin 6 production in experimental cerebral malaria: modulation by anticytokine antibodies and possible role in hypergammaglobulinemia – 1990, J Exp Med; 172: 1505-1508.

Hampel H, Haslinger A, Scheloske M, Padberg F, Fischer P, Unger J, et al - Pattern of interleukin-6 receptor complex immunoreactivity between cortical regions of rapid autopsy normal and Alzheimer's disease brain – 2005, Eur Arch Psychiatry Clin Neurosci.; 255: 269-278

Hancock W W, Gao W, Csizmadia V, Faia K L, Shemmeri N, Luster A D - Donor-derived IP-10 initiates development of acute allograft rejection – 2001, J Exp Med 193:975–980

Hanisch U K - Microglia as a source and target of cytokine activities in the brain. In: Streit WJ, editor. Microglia in the degenerating and regenerating CNS – 2001; New York: Springer Verlag. p 79– 124.

Hanisch U-K – Microglia as a source and target of cytokines – GLIA 40:140-155 (2002)

Hart P H, Vitti G F, Burgess D R, et al. - . Potential antiinflammatory effects of

interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2 – 1989, Proc Natl Acad Sci USA; 86:3803– 3807

Hashioka S, Klegeris A, Monji A, Kato T, Sawada M, McGeer P L, et al - Antidepressants inhibit interferon-gamma-induced microglial production of IL-6 and nitric oxide – 2007, Exp Neurol.; 206: 33-42

Haviv Y, Avrahami D, Ovadia H, Ben-Hur T, Gabizon R, Sharon R – Induced neuroprotection independently from PrP^{Sc} accumulation in a mouse model for prion disease treated with simvastatin – 2008, Arch neurol, 65(6); 762-775

He F, Ge W, Martinowich K, Becker-Catania S, Coskun V, Zhu W, Wu H, Castro D, Guillemot F, Fan G, de Vellis J, Sun Y E – A positive autoregulatory loop of JAK-STAT signaling controls on the onset of astrogliogenesis – 2005, Nat Neurosci 8, 616-625

Heinrich P C, Behrmann I, Haan S, Hermanns H M, Muller-Newen G, Schaper F - Principles of interleukin (IL)-6-type cytokine signalling and its regulation – 2003, Biochem. J. 374 ; 1-20

Hernandez-Perera O, Perez-Sala D, Navarro-Antolin J, Sanchez-Pascuala R, Hernandez G, Diaz C, Lamas S - Effects of the 3-Hydroxy-3-methylglutaryl-CoA Reductase Inhibitors, Atorvastatin and Simvastatin, on the Expression of Endothelin-1 and Endothelial Nitric Oxide Synthase in Vascular Endothelial Cells – 1998, J Clin Invest 101(12), 2711-2719

Herrmann O, Tarabin V, Suzuki S, Attigah N, Coserea I, Schneider A, et al - . Regulation of body temperature and neuroprotection by endogenous interleukin-6 in cerebral ischemia – 2003, J Cereb Blood Flow Metab.; 23: 406-415.

Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, Kashiwamura S, Nakajima K, Koyama K, Iwamatsu A et al., - Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin – 1986, Nature 324; 73-76

Hirohata S, Miyamoto T - Elevated levels of interleukin-6 in cerebrospinal fluid from patients with systemic lupus erythematosus and central nervous system involvement – 1990, Arthritis Rheum.; 33: 644-9

Huang L, Wu Z-B, ZhuGe Q, Zheng W, Shao B, Wang B, Sun F, Jin K – Glial scar formation occurs in the human brain after ischemic stroke – Int J Med Sci, 2014; 11(4): 344-348

Iadecola C, Nedergaard M - Glial regulation of the cerebral microvasculature – 2007, Nat Neurosci 10:1369–1376

Istvan E S, Palnitkar M, Buchanan S K, Deisenhofer J - Crystal structure of the catalytic portion of human HMG-CoA reductase: insights into regulation of activity and catalysis – 2000, EMBO J 19: 819–830

Istvan E S, Deisenhofer J - Structural mechanism for statin inhibition of HMG-CoA reductase – 2001, Science 292: 1160–1164.

Jacobson J R, Barnard J W, Grigoryev D N, Ma S F, Tuder R M, Garcia J G - Simvastatin attenuates vascular leak and infl ammation in murine infl ammatory lung injury – 2005, Am J Physiol Lung Cell Mol Physiol 288: 1026–32

Janzer R C, Raff M C - Astrocytes induce blood–brain barrier properties in endothelial cells – 1997, Nature 325, 253–257

Jirik F R, Podor T J, Hirano T, Kishimoto T, Loskutoff D J, Carson D A, et al - Bacterial lipopolysaccharide and inflammatory mediators augment IL-6 secretion by human endothelial cells – 1989, J Immunol.; 142: 144-7

John G R, Lee S C, Brosnan C F - Cytokines: Powerful regulators of glial cell activation – 2003, Neuroscientist 9:10–22

Johnson M H, Sofroniew M V - Leukocyte Infiltration, Neuronal Degeneration, and Neurite Outgrowth after Ablation of Scar-Forming, Reactive Astrocytes in Adult Transgenic Mice – 1999, Neuron, Vol. 23, 297–308

Joss A, Akdis M, Faith A, Blaser K, Akdis C A - IL-10 directly acts on T cells by specifically altering the CD28 costimulation pathway – 2000, European Journal of Immunology, vol. 30, no. 6, pp. 1683–1690

Kandolf R, Sauter M, Aepinus C, Schnorr J J, Selinka H C, Klingel K - Mechanisms and consequences of enterovirus persistence in cardiac myocytes and cells of the immune system – 1999, Virus Res.;62:149–58

Kempster S, Bate C, Williams A – Simvastatin treatment prolongs the survival of scrapie-infected mice – 2007, Neuroph & Neurotox, 18(5);479-482

Kettenmann H, Hanisch U-K, Noda M, Verkhratsky A – Physiology of Microglia – Physiol Rev, 2011 91:461-553

Klein M A, Moller J C, Jones L L, Bluethmann H, Kreutzberg G W, Raivich G – Impaired neuroglial activation in interleukin-6 deficient mice – 1997; Glia 9, 227-233

Klein R S – Regulation of neuroinflammation: the role of CXCL10 in lymphocyte infiltration during autoimmune encephalomyelitis – 2004, J Cell Biochem 92,213-222

Klir J J, Roth J, Szelényi Z, McClellan J L, Kluger M J - Role of hypothalamic interleukin-6 and tumor necrosis factor-alpha in LPS fever in rat – 1993, Am J Physiol Cell Physiol.; 265:512-517.

Kordula T, Rydel R E, Brigham E F, Horn F, Heinrich P C, Travis J – Oncostatin M and the interleukin-6 and soluble interleukin-6 receptor complex regulate alpha1-antichymotrypsin expression in human cortical astrocytes – 1998; J Biol Chem 273, 4112-4118

Lahti A, Jalonen U, Kankaanranta H, Moilanen E - C-jun NH2-terminal kinase inhibitor anthra(1,9-cd)pyrazol-6(2H)-one reduces inducible nitric-oxide synthase expression by destabilizing mRNA in activated macrophages – 2003, Mol pharmacol 64, 308-315

Laurenzi M A, Sidén A, Persson M A, Norkrans G, Hagberg L, Chiodi F - Cerebrospinal fluid interleukin-6 activity in HIV infection and inflammatory and noninflammatory diseases of the nervous system – 1990, Clin Immunol Immunopathol.; 57: 233-421

Lee S C, Liu W, Dickson D W, Brosnan C F, Berman JW - Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1 beta – 1993, J Immunol.; 150: 2659-67

Leung B P, Sattar N, Crilly A, Prach M, McCarey D W, Payne H, Madhok R, Campbell C, Gracie J A, Liew F Y, McInnes I B - A Novel Anti-Inflammatory Role for Simvastatin in Inflammatory Arthritis – 2003, J Immunol 170, 1524–1530.

Levison S W, Jiang F J, Stoltzfus O K, Ducceschi M H - IL6-type cytokines enhance epidermal growth factor-stimulated astrocyte proliferation – 2000, Glia 32:328–337

Lim B-K, Ju E-S, Lao D H, Yun S-H, Lee Y-J, Kim D-K, Jeon E-S - Development of a enterovirus diagnostic assay system for diagnosis of viral myocarditis in humans – 2013, Microbiol Immunol; 57: 281–287

Litterio J J, Roberts A B - TGF-b: a critical modulator of immune cell function – 1997,

Clin Immunol Immunopathol; 84:244–250

Llorente L, Zou W, Levy Y - Role of interleukin 10 in the B lymphocyte hyperactivity and autoantibody production of human systemic lupus erythematosus – 1995, The Journal of Experimental Medicine, vol. 181, no. 3, pp. 839– 844

Lu D, Goussev A, Chen J, Pannu P, Li Y, Mahmood A, Chopp M - Atorvastatin reduces neurological deficit and increases synaptogenesis, angiogenesis, and neuronal survival in rats subjected to traumatic brain injury – 2004, J. Neurotrauma 21 (1), 21–32

Luria S E, Darnell J E Jr, Baltimore D, Campbell A - General Virology. 3rd edition – 1978, New York, John Wiley & Sons

Lust J A, Donovan K A, Kline M P, Greipp P R, Kyle R A, Maihle N J – Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor – 1992, Cytok 4; 96-100

Ma X, Reynolds S L, Baker B J, Li X, Benveniste E N, Qin H - IL-17 enhancement of the IL-6 signaling cascade in astrocytes – 2010, J Immunol; 184: 4898-4906

Macin S M, Perna E R, Farias E F, Franciosi V, Cialzeta J R, Brizuela M, Medina F, Tajer C, Doval H, Badaracco R - Atorvastatin has an important acute anti-inflammatory effect in patients with acute coronary syndrome: Results of a randomized, double-blind, placebo-controlled study – 2005, Amer Heart Jour 149(3), 451-457

Maimone D, Guazzi G C, Annunziata P - IL-6 detection in multiple sclerosis brain – 1997, J Neurol Sci.; 146: 59-65.

Marchant A, Bruyns C, Vandenabeele P, et al - Interleukin-10 controls interferon-g and tumor necrosis factor production during experimental endotoxemia – 1994, Eur J Immunol; 24:1167–1171

Mastorakos G, Chrousos G P, Weber J S - Recombinant interleukin-6 activates the hypothalamic-pituitary-adrenal axis in humans – 1993, J Clin Endocrinol Metab.; 77: 1690-1694.

Michlmayr D, McKimmie C S – Role of CXCL10 in central nervous system inflamation – 2014, Int Journ Interf, Cytok Med Resear, 6, 1-18

Milner R, Campbell I L – Increased expression of the beta4 and alpha5 integrin subunits in cerebral blood vessels of transgenic mice chronically producing the proinflammatory citokines IL-6 or IFN-alpha in he central nervous system – 2006, Mol Cell Neurosci, 33, 429-440

Miziorko H M – Enzymes of the mevalonate pathway of isoprenoid biosynthesis – 2011, Arch Biochem Biophys 505(2): 131–143

Mogi M, Harada M, Narabayashi H, Inagaki H, Minami M, Nagatsu T - Interleukin (IL)-1 beta, IL-2, IL-4, IL-6 and transforming growth factor-alpha levels are elevated in ventricular cerebrospinal fluid in juvenile parkinsonism and Parkinson's disease – 1996,

Neurosci Lett; 211: 13-16

Mok S W F, Thelen K M, Riemer C, Bamme T, Gultner S, Lutjohann D, Baier M – Simvastatin prolongs survival times in prion infections of the central nervous system – 2006, Biochem Biophys Res Comm, 348;697-702

Monier A, Adle-Biassette H, Delezoide A L , Evrard P, Gressens P, Verney C - Entry and distribution of microglial cells in human embryonic and fetal cerebral cortex – J Neuropathol Exp Neurol 2007 (5):372-82

Moon G J, Kim S J, Cho Y H, Ryoo S, Bang O Y - Antioxidant Effects of Statins in Patients with Atherosclerotic Cerebrovascular Disease – 2014, J Clin Neurol;10(2):140-147

Morillas M, Swietnicki W, Gambetti P, Surewicz WK - Membrane environment alters the conformational structure of the recombinant human prion protein - 1999; J Biol Chem 274: 36859–36865

Morimoto K, Janssen W J, Fessler M B, McPhillips K A, Borges V M, Bowler R P, Xiao Y-Q, Kench J A, Henson P M, Vandivier R W - Lovastatin Enhances Clearance of Apoptotic Cells (Efferocytosis) with Implications for Chronic Obstructive Pulmonary Diseas e – 2006, J Imm, 176: 7657–7665

Mullberg J, Schooltink H, Stoyan T, Gunther M, Graeve L, Buse G, Mackiewicz A, Heinrich P C, Rose-John S – The soluble interleukin-6 receptor is generated by shedding – 1993, Eur J Immunol 23, 473-480

Na Y J, Jin J K, Kim J I, Choi E K, Carp R I, Kim Y S - JAK-STAT signaling pathway mediates astrogliosis in brains of scrapie-infected mice – 2007, J Neurochem 103; 637-649

Naito M, Takahashi K, Nishikawa S - Development, differentiation, and maturation of macrophages in the fetal mouse liver – J Leokoc Biol 1990 (1):27-37

Navarra P, Pozzoli G, Brunetti L, Ragazzoni E, Besser M, Grossman A B - Interleukin-1 beta and interleukin-6 specifically increase the release of prostaglandin E2 from rat hypothalamic explants in vitro – 1992, Neuroendocrinology ; 56: 61-68.

Neary J T, Zimmermann H - Trophic functions of nucleotides in the central nervous system – 2009, Trends Neurosci 32:189–198

Nimmerjahn A, Kirchhoff F, Helmchen F - Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo – Science 2005, 308:1314-1318

Norris J G, Tang L P, Sparacio S M, Benveniste E N - Signal transduction pathways mediating astrocyte IL-6 induction by IL-1 beta and tumor necrosis factor-alpha – 1994, J Immunol.; 152: 841-850.

Oberheim N A, Goldman S A, Nedergaard M - Heterogeneity of Astrocytic Form and Function - Methods Mol Biol. 2012 ; 814: 23–45

O'Callaghan JP, Sriram K, Miller DB – Defining “Neuroinflammation” - Ann N Y Acad Sci. \ 2008 Oct;1139:318-20

Opal S M - Interleukin-1 receptor antagonist in sepsis - In: Bodmer M, Henderson W, eds. Therapeutic modulation of the cytokines. London, UK: CRC Press, 1996; 196–219

Opal S M, DePalo V A – Anti-inflammatory cytokines - Chest 2000; 117:1162–1172

Pagliusi S R, Schachner M, Seeburg P H, Shivers B D - The Adhesion Molecule on Glia (AMOG) Is Widely Expressed by Astrocytes in Developing and Adult Mouse Brain – 1990, Eur J Neurosci.; 2: 471-80

Pahan K, Sheikh F G, Namboodiri A M S, Singh I - Lovastatin and Phenylacetate Inhibit the Induction of Nitric Oxide Synthase and Cytokines in Rat Primary Astrocytes, Microglia, and Macrophages – 1997, J Clin Invest 100(11), 2671-2679

Pang Y, Fan L W, Zheng B, Cai Z, Rhodes P G – Role of interleukin-6 in lipopolysaccharide-induced brain injury and behavioral dysfunction in neonatal rats – 2006; Neurosci 141, 745-755

Patidar A K, Jeyakandan M,Mobiya A K, Selvam G – Exploring potential of quinoxaline moiety – 2011, Int J PharmTech Res, 3(1);386-392

Paul W E - Interleukin-4: a prototypic immunoregulatory lymphokine – 1991, Blood; 77:1859–1870

Pereira J A, Pessoa A M, Cordeiro M N D S, Fernandes R, Prudencio C, Noronha J P,

Vieira M - Quinoxaline, its derivatives and applications: A State of the Art review – 2015, Eur J Med Chem, 97;664-672

Perry V H, Teeling J - Microglia and macrophages of the central nervous system: the contribution of microglia priming and systemic inflammation to chronic neurodegeneration - Semin Immunopathol (2013) 35:601–612

Piermartiri T C B, Figueiredo C P, Rial D, Duarte F S, Bezerra S C, Mancini G, de Bem A F, Prediger R D S, Tasca C I - Atorvastatin prevents hippocampal cell death, neuroinflammation and oxidative stress following amyloid-β1–40 administration in mice: Evidence for dissociation between cognitive deficits and neuronal damage – 2010, Exp Neur 226, 274-284

Pizzi M, Sarnico I, Boroni F, Benarese M, Dreano M, Garotta G, et al - Prevention of neuron and oligodendrocyte degeneration by interleukin-6 (IL-6) and IL-6 receptor/IL-6 fusion protein in organotypic hippocampal slices – 2004, . Mol Cell Neurosci.; 25: 301-311

Pogue A I, Cui J G, Li Y Y, Zhao Y, Culicchia F, Lukiw W J – Micro RNA-125b (miRNA-125b) function in astrogliosis and glial cells proliferation – 2010; Neurosci Lett, 476, 18-22

Pontieri G.M. - Patologia Generale & Fisiopatologia Generale – Piccin Edit, 2012

Poulsen C B, Penkowa M, Borup R, Nielsen F C, Caceres M, Quintana A, et al. - Brain

response to traumatic brain injury in wild-type and interleukin-6 knockout mice: a microarray analysis – 2005, J Neurochem.; 92: 417-432

Powell E M, Geller H M - Dissection of astrocyte-mediated cues in neuronal guidance and process extension – 1999, Glia 26:73–83

Pruefer D, Makowski J, Schnell M, Buerke U, Dahm M, Oelert H, Sibelius U, Grandel U, Grimminger F, Seeger W, Meyer J, Darius H, Buerke M - Simvastatin Inhibits Inflammatory Properties of Staphylococcus aureus -Toxin – 2002, Circul 106, 2104-2110

Purves D, Augustine G J, Fitzpatrick D et al – Neuroscience, 2nd edition – Sinauer Associates, 2001

Qiu Z, Gruol D L - Interleukin-6, beta-amyloid peptide and NMDA interactions in rat cortical neurons – 2003, J Neuroimmunol.; 139: 51-57.

Quintana A, Molinero A, Borup R, Nielsen F C, Campbell IL, Penkowa M, et al - Effect of astrocyte-targeted production of IL-6 on traumatic brain injury and its impact on the cortical transcriptome – 2008, Dev Neurobiol.; 68: 195-208

Ramesh G, MacLean A G, Philipp, M T - Cytokines and Chemokines at the Crossroads of Neuroinflammation, Neurodegeneration, and Neuropathic Pain - 2013, Med of Inflamm

Reis P A, Estato V, da Silva T I, d'Avila J C, Siqueira L D, Assis E F, Bozza P T, Bozza F A, Tibirica E V, Zimmerman G A, Castro-Faria-Neto H C - Statins Decrease

Neuroinflammation and Prevent Cognitive Impairment after Cerebral Malaria – 2012, PLoS Pathog 8(12): e1003099. doi:10.1371/journal.ppat.1003099

Reyes T M, Fabry Z, Coe C L - Brain endothelial cell production of a neuroprotective cytokine, interleukin-6, in response to noxious stimuli – 1999, Brain Res, 851:215-220

Rezaie P, Male D K – Colonization of the developing human brain and spinal cord by microglia: a review – Microsc. Res. Tech 45, 1999 359-382

Rezaie P, Lantos P L – Microglia and the pathogenesis of spongiform encephalopathies – Bra. Res. Rev, 35 2001 55-72

Rezaie P, Dean A, Male D, Ulfig N - Microglia in the cerebral wall of the human telencephalon at second trimester – Cereb cortex 2005 Jul;15(7):938-49

Riesner D – Biochemistry and structure of PrP^{C} and PrP^{Sc} – 2003, Br Med Bull (2003) 66 (1): 21-33

Riesner D, Kellings K, Post K et al - Disruption of prion rods generates spherical particles composed of four to six PrP 27–30 molecules that have a high α-helical content and are non-infectious - 1996; J Virol 70: 1714–22

Righi M, Mori L, De Libero G, Sironi M, Biondi A, Mantovani A, et al - Monokine production by microglial cell clones - Eur J Immunol.; 19: 1443-8.

Ringheim G E, Burgher K L, Heroux J A - Interleukin-6 mRNA expression by cortical neurons in culture: evidence for neuronal sources of interleukin-6 production in the brain – 1995, J Neuroimmunol.; 63: 113-23

Riol-Blanco L, Lazarevic V, Awasthi A et al. - IL-23 receptor regulates unconventional IL-17-producing T cells that control bacterial infections – 2010, The Journal of Immunology, vol. 184, no. 4, pp. 1710–1720

Rolls A, Shechter R, Schwartz M - The bright side of the glial scar in CNS repair - Nat Rev Neurosci. 2009; 10: 235-41

Romagnani S - Biology of human TH1 and TH2 cells – 1995, Journal of Clinical Immunology, vol. 15, no. 3, pp. 121–129

Rossi D, Zlotnik A – The biology of chemokines and their receptors – 2000, Annu Rev Immunol, 18,217-242

Rotbart H A - Enteroviral infections of the central nervous system – 1995, Clin Infect Dis.;20:971–81

Sanna G, Farci P, Busonera B, Murgia G, La Colla P, Giliberti G - Antiviral properties from plants of the Mediterranean flora – 2015, Nat Prod Res; 23: 1-6

Scheller J, Grotzinger J, Rose-John S - Updating IL-6 classic- and trans-signaling – 2006, Signal Transduct, 6; 240-259

Schreuder H, Tardif C, Trump-Kallmeyer S, et al - A new cytokine-receptor binding mode revealed by the crystal structure of the IL-1 receptor with an antagonist - . Nature 1997; 386:194–200

Schöbitz B, Pezeshki G, Pohl T, Hemmann U, Heinrich P C, Holsboer F, et al - Soluble interleukin-6 (IL-6) receptor augments central effects of IL-6 in vivo – 1995, FASEB J.; 9: 659-664

Silver J, Miller J H – Regeneration beyond the glial scar – Nat Rev Neurosc 5, 146-156, 2004

Silverthorn D U – Human physiology: an integrated approach, 5th edition – Pears Educ, 2010

Simms J E, Gayle M A, Slack J L, et al - Interleukin-1 signaling occurs exclusively via type 1 receptor - Proc Natl Acad Sci USA 1993; 90:6155–6159

Sofroniew M V - Molecular dissection of reactive astrogliosis and glial scar formation – 2009, Trends Neurosci 32:638–647

Sofroniew M V, Vinters H V – Astrocytes: biology and pathology - Acta Neuropathol (2010) 119:7–35

Sommeijer D W, MacGillavry M R, Meijers J C M, Van Zanten A P, Reitsma P H, Ten

Cate H –Anti-inflammatory and anticoagulant effects of pravastatin in patients with type 2 diabetes – 2004,Diab care, 27(2)468-473

Sporeen A, Kolmus K, Laureys G, Clinckers R, De Keyser J, Haegeman G, Gerlo S – Interleukin-6, a mental cytokine – 2011, Brain Res Rev 67;157-183

Sriram K, Benkovic S A, Hebert M A, Miller D B, O'Callaghan J P – Induction of gp130-related cytokines and activation of JAK2/STAT3 pathway in astrocytes precedes up-regulation of glial fibrillary acidic protein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of neurodegeneration: key signaling pathway for astrogliosis in vivo? - 2004; Biol Chem, 279, 19936-19947

Stancu C, Sima A - Statins: mechanism of action and effects – 2001, J.Cell.Mol.Med. 5(4), 378-387

Streit W J, Hurley S D, McGraw T S, Semple-Rowland S L - Comparative evaluation of cytokine profiles and reactive gliosis supports a critical role for interleukin-6 in neuron-glia signaling during regeneration – 2000, J Neurosci Res; 61: 10-20.

Streit W J, Mrak R E, Griffin W S T – Microglia and Neuroinflammation: a Pathological Perspective – Journ of Neuroinfl, 2004 1:14

Su D-L, Lu Z-M, Shen M-N, Li X, Sun L-Y - Roles of Pro- and Anti-Inflammatory Cytokines in the Pathogenesis of SLE – Journal of Biomedicine and Biotechnology, vol.

2012, Article ID 347141, 15 pages, 2012. doi:10.1155/2012/347141

Sui Y, Potula R, Dhillon N, Pinson D, Li S, Nath A, Anderson C, Turchan J, Kolson D, Narayan O, et al.- Neuronal apoptosis is mediated by CXCL10 overexpression in simian human immunodeficiency virus encephalitis – 2004, Am J Pathol, 164(5):1557-1566

Sui Y, Stehno-Bittel L, Li S et al. - CXCL10-induced cell death in neurons: role of calcium dysregulation – 2006, Eur Journ Neurosci, 23;957-964

Takemoto M, Liao J K - Pleiotropic e□ects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors – 2001, Arterioscler Thromb Vasc Biol 21, 1712–1719

Takeshita Y, Ransohoff R M – Inflammatory cell trafficking across the blood-brain barrier: chemokine regulation and in vitro models – 2012, Immunological Reviews, vol. 248, pp. 228–239

Terblanche M, Almog Y, Rosenson R S, Smith T S, Hackam D G - Statins and sepsis: multiple modifi cations at multiple level – 2007, Lancet Infect Dis 7, 358-368

Thomas D M, Francescutti-Verbeem D M, Kuhn D M - Gene expression profile of activated microglia under conditions associated with dopamine neuronal damage – Faseb J 2005 Mar;20(3):515-7

Tilg H, Trehu E, Atkins M B, Dinarello C A, Mier J W – Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor

necrosis factor receptor p55 – 1994, Blood 83; 113-118

Tilgner J, Volk B, Kaltschmidt C – Continuous interleukin-6 application in vivo via macroeca psulation of interleukin-6-expressing-COS-7 cells induces massive gliosis – 2001; Glia, 35,234-245

Tobert J A - Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors – 2003, Nat Rev Drug Discov 2, 517-526

Trepicchio W L, Wang L, Bozza N, et al - Interleukin-11 regulates macrophage effector function through the inhibition of nuclear factor-kB – 1997, . J Immunol; 159:5661–5669

Tribouillard-Tanvier D, Striebel J F, Peterson K E, Chesebro B – Analysis of protein levels of 24 cytokines in scrapie agent-infected brain and glial cell cultures from mice differing in prion protein expression levels – 2009, J Virol, 83(21):11244-11253

Tribouillard-Tanvier D, Race B, Striebel J F, Carroll J A, Phillips K, Chesebro B - Early Cytokine Elevation, PrPres Deposition, and Gliosis in Mouse Scrapie: No Effect on Disease by Deletion of Cytokine Genes IL-12p40 and IL-12p35 – 2012, J Virol.; 86(19): 10377–10383.

Ucar M, Mjorndal T, Dahlqvist R - HMG-CoA reductase inhibitors and myotoxicity – 2000, Drug Saf 22:441-457

Ulich T R, Yin S, Guo K, Yi E S, Remick D, del Castillo J – Intratracheal injection of endotoxin and cytokines. Interleukin-6 and transforming growth factor beta inhibit acute

inflammation – 1991, Am J Pathol, 138; 1097-1101

Ullian E K, Sapperstein S K, Christopherson K S, Barres B A - Control of synapse number by glia – 2001, Science 291:657– 661

Valerio A, Ferrario M, Dreano M, Garotta G, Spano P, Pizzi M - Soluble interleukin-6 (IL-6) receptor/IL-6 fusion protein enhances in vitro differentiation of purified rat oligodendroglial lineage cells – 2002, Mol Cell Neurosci.; 21: 602-15

Vallières L, Rivest S - Regulation of the genes encoding interleukin-6, its receptor, and gp130 in the rat brain in response to the immune activator lipopolysaccharide and the proinflammatory cytokine interleukin-1beta – 1997, J Neurochem.; 69: 1668-83.

Vannier E, Miller M C, Dinarello C A - Coordinated antiinflammatory effects of interleukin4: interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and synthesis of interleukin 1 receptor antagonist – 1992, . Proc Natl Acad Sci USA; 89:4076–4080

Vetrugno V, Di Bari M A, Nonno R, Puopolo M, D'Agostino C, Pirisinu L, Pocchiari M, Agrimi U – Oral pravastatin prolongs survival time of scrapie-infected mice – 2008, J Gen Vir, 90;1775-1780

Vilcek J - “The cytokines: an overview,” in The Cytokine Handbook - ,W. Angusand M. T. L. Thomson, Eds., pp.1–18, Academic Press, San Diego, Calif, USA, 4thedition, 2003

Vinet J, Van Weering H R J, Heinrich A, Kalin R E, Wegner A, Brouwer N, Heppner F L, Van Rooijen N, Boddeke H WG M, Biber K – Neuroprotective function for ramified microglia in hippocampal excitotoxicity – 2012, Journ Neuroinf, 9:27

Virchow R - Cellular pathology: as based upon physiological and pathological histology. Twenty lectures delivered in the pathological institute of Berlin during the months of February, March and April, 1858 [in German] - First edition ed. Berlin: August Hirschwald; 1858

Vorberg I, Chan K, Priola S A - Deletion of β-Strand and α-Helix Secondary Structure in Normal Prion Protein Inhibits Formation of Its Protease-Resistant Isoform – 2001, J Virol, 75(21); 10024-10032

Woiciechowsky C, Schoning B, Stoltenburg-Didinger G, Stockhammer F, Volk H D – Brain-IL-1-beta triggers astrogliosis through induction of IL-6: inhibition by propanolol and IL-10 – 2004, Med Sci Monit; 10, BR325-BR330

Wong WW-L, Dimitroulakos J, Minden M D, Penn L Z - HMG-CoA reductase inhibitors and the malignant cell: the statin family of drugs as triggers of tumor-specific apoptosis – 2002, Leukemia 16, 508–519

Xia M Q, Bacskai B J, Knowles R B, Qin S X, Hyman B T – Expression of the chemokine receptor CXCR3 on neurons and the elevated expression of its ligand IP-10 in reactive astrocytes: in vitro ERK1/2 activation and role in Alzheimer's disease – 2000, J Immunol, 108(1-2); 227-235

Yang Y, Higashimori H, Morel L - Developmental maturation of astrocytes and pathogenesis of neurodevelopmental disorders – Journ neurodevel disord, 2013, 5:22

Yasukawa K, Hirano T, Watanabe Y, Muratani K, Matsuda T, Nakai S, et al. - Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene – 1987, EMBO J.; 6: 2939-45

Youssef S, Stuve O, Patarroyo J C, Ruiz P J, Radosevich J L, Hur E M, Bravo M, Mitchell D J, Sobel R A, Steinman L, Zamvil S S - The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease – 2002, Nature 420(6911), 78-84

Zeinstra E, Wilczak N, Chesik D, Glazenburg L, Kroese F G M, De Keyser J - Simvastatin inhibits interferon-γ-induced MHC class II up-regulation in cultured astrocytes – 2006, J Neur , 3:16 doi:10.1186/1742-2094-3-16

Zhang J-M, An J – Cytokines, inflammation and pain – 2007, Int Anesthesiol Clin. ; 45(2): 27–37

Zhang P L, Izrael M, Ainbinder E, Ben-Simchon L, Chebath J, Revel M - Increased myelinating capacity of embryonic stem cell derived oligodendrocyte precursors after treatment by interleukin-6/soluble interleukin-6 receptor fusion protein – 2006, Mol Cell Neurosci; 31: 387-398

Zhang PL, Levy A M, Ben-Simchon L, Haggiag S, Chebath J, Revel M - Induction of neuronal and myelin-related gene expression by IL-6-receptor/IL-6: a study on embryonic dorsal root ganglia cells and isolated Schwann cells – 2007, . Exp Neurol.; 208: 285-296.

Zhong J, Dietzel I D, Wahle P, Kopf M, Heumann R - Sensory impairments and delayed regeneration of sensory axons in interleukin-6-deficient mice – 1999, J Neurosci.; 19: 4305-4313

Zhong Q, Yang Z, Liu Y, et al. - Antiviral activity of arbidol against Coxsackie virus B5 in vitro and in vivo – 2009, ArchVirol,154(4):601-607.

Zurawski G, de Vries J E - Interleukin 13, an interleukin-4- like cytokine that acts on monocytes and B cells, but not on T cell – 1994, Immunol Today; 15:19–26