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Aplicação terapêutica de células endoteliais tratadas com nanopartículas contendo ácido retinóico em modelo experimental de acidente vascular cerebral

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Resumo

O acidente vascular cerebral constitui a principal causa de morte em Portugal e de acordo com a Sociedade Portuguesa do Acidente Vascular Cerebral, Portugal é o país da Europa Ocidental com maior taxa de mortalidade, sobretudo na população com menos de 65 anos de idade. Os tratamentos existentes não são totalmente eficazes e apenas beneficiam uma pequena percentagem de doentes acarretando, na maioria das vezes, efeitos secundários graves. Assim, o desenvolvimento de novas terapias é uma necessidade obrigatória. Esta doença desencadeia uma série de alterações celulares e moleculares que comprometem a viabilidade do tecido nervoso. Neste contexto, a resposta inflamatória desencadeada pela microglia, células do sistema imune inato do Sistema Nervoso Central, dificulta a reparação neurovascular. Após um acidente vascular cerebral, a vasculatura é lesada levando a um comprometimento do suporte trófico de todos os tecidos que irriga, assim como no número de novos neurónios que acorre à área de lesão. Assim, pretendemos acionar mecanismos que modulem a resposta anti-inflamatória da microglia e potenciem a reparação neurovascular. Para tal, propomos o uso do ácido retinóico (um derivado da vitamina A, anti-inflamatório e pro-angiogénico) como agente terapêutico. Para contornar a baixa solubilidade e rápida degradação desta molécula, recorremos ao uso de nanopartículas poliméricas carregadas com ácido retinóico. Após a concretização deste projeto, concluímos que a formulação 1) induz um efeito anti-inflamatório na microglia num contexto isquémico; 2) modula o secretoma das células endoteliais isquémicas de forma a promover uma reposição dos níveis de óxido nítrico e a produzir sinais anti-inflamatórios pela microglia; 3) promove a sobrevivência e reparação neurovascular num contexto inflamatório após a injeção intravenosa e em condições isquémicas. Assim, as nanopartículas poliméricas carregadas com ácido retinóico demostram ser um agente com grande potencial terapêutico para administração intravenosa do acidente vascular cerebral.

Palavras-chave

Acidente vascular cerebral, ácido retinóico, nanomateriais, neuroinflamação, sistema vascular

Resumo alargado

O acidente vascular cerebral ocorre devido a uma interrupção de sangue ao cérebro e afeta uma grande parte da população portuguesa, sendo por isso associado a elevadas taxas de mortalidade e morbilidade. Atualmente distinguem-se dois tipos de acidente vascular cerebral, o hemorrágico (rompimento de um vaso sanguíneo) e o isquémico (obstrução de um vaso sanguíneo). As manifestações clínicas características desta doença variam muito consoante a área do cérebro que se encontra lesada e acarretam várias complicações que desencadeiam uma série de alterações celulares e moleculares que comprometem a viabilidade do tecido nervoso. Neste contexto, gera-se uma resposta inflamatória desencadeada pela microglia, que dificulta a reparação neurovascular; ocorre a formação de edema, a degradação da matriz extracelular, o aumento da permeabilidade endotelial e a formação de espécies reativas de oxigénio que culminam na disrupção da barreira hematoencefálica. Existem diversos tratamentos, aos quais se destaca a administração de agentes trombolíticos que degradam os coágulos de fibrina. Contudo, estes tratamentos apresentam repercussões negativas sobre o tecido lesado, resultantes predominantemente da sua toxicidade. Assim, há uma necessidade emergente de se desenvolverem novas terapias para a reparação da vasculatura cerebral e que sejam administradas de forma eficiente e com poucos riscos para o doente. Estas novas terapias devem considerar a unidade neurovascular uma vez que a ação integrada dos neurónios, das células da glia, dos vasos sanguíneos e de outros tipos celulares regula a homeostasia celular e controla o fluxo sanguíneo. Neste trabalho propomos o uso do ácido retinóico (um derivado da vitamina A, anti-inflamatório e pro-angiogénico) como agente terapêutico para o acidente vascular cerebral. Esta molécula desempenha um papel importante no desenvolvimento do sistema nervoso e é também considerado um indutor da neurogénese uma vez que regula a transcrição de genes pro-neurogénicos e promove a diferenciação das células estaminais neurais. Contudo, quando administrado na sua forma livre apresenta baixa solubilidade em soluções aquosas e rápida degradação. Para contornar estas desvantagens propomos a utilização desta molécula encapsulada em nanopartículas poliméricas. Assim, conseguimos garantir uma libertação eficiente e controlada do ácido retinóico, aumentando a eficácia terapêutica. Curiosamente, esta formulação demonstrou ser cerca de 2500 vezes mais eficiente do que o ácido retinóico na sua forma livre na diferenciação de células neurais estaminais. Assim, esperamos resultados semelhantes no modelo utilizado. No seguimento deste trabalho surgem os seguintes objetivos (1) determinar se as nanopartículas carregadas com ácido retinóico induzem um fenótipo anti-inflamatório sobre as células da microglia num contexto inflamatório clássico ou isquémico; (2) demonstrar se as nanopartículas carregadas com ácido retinóico regulam a libertação de mediadores anti-inflamatórios num contexto isquémico; (3) demonstrar se as nanopartículas carregadas com ácido retinóico representam um potencial agente terapêutico para administração intravenosa num modelo *ex vivo* de acidente vascular

cerebral, restaurando a integridade inflamatória e neurovascular. Neste sentido, após a concretização deste projeto concluímos que: a formulação não compromete a viabilidade da microglia dentro de uma determinada gama de concentrações; inibe a libertação de óxido nítrico e a expressão da enzima que catalisa a síntese de óxido nítrico (iNOS) e promove a expressão de arginase-1 e interleucina-4 pelas células da microglia, num contexto inflamatório e isquémico; modula a morfologia da microglia num modelo *ex vivo* em contexto inflamatório. Numa outra fase do trabalho, concluímos que: o tratamento das células endoteliais com a formulação não apresentou citotoxicidade dentro de uma gama de concentrações que potenciou a proliferação das células endoteliais. Quando as células endoteliais foram sujeitas a um modelo experimental de privação de oxigénio e glucose a formulação demonstrou modular o secretoma das células endoteliais isquémicas de forma a promover uma reposição dos níveis de óxido nítrico e a produzir sinais anti-inflamatórios pela microglia; a formulação promoveu também a sobrevivência e reparação neural num contexto inflamatório após a injeção intravenosa e em condições isquémicas, num modelo *ex vivo*. Assim, as nanopartículas carregadas com ácido retinóico demonstraram ser um agente com grande potencial terapêutico a nível inflamatório, vascular e neural para posterior tratamento intravenoso do acidente vascular cerebral.

Abstract

Stroke constitutes the leading cause of death in Portugal and according to the Portuguese Stroke Society, Portugal is the Western European country with the highest mortality rate, especially in the population under 65 years old. As the treatments available are not fully effective and only benefit a small percentage of patients, leading most often to serious side effects, the development of new therapies is an emerging need. This disease triggers a series of cellular and molecular changes that compromise the viability of the nervous tissue. In this context, the inflammatory response triggered by microglia, the innate immune system cells of the Central Nervous System, accentuates neurovascular damage. Stroke-induced vascular deregulation and/or injury compromises trophic and migratory support of new neurons to the lesion area affecting the final outcome. Accordingly, our goal is to modulate these mechanisms to suppress the inflammatory response and to potentiate neurovascular repair, using retinoic acid (an anti-inflammatory and pro-angiogenic vitamin A derivative) as the therapeutic agent. To bypass the low solubility and the fast degradation of this molecule, we propose the use of retinoic acid-loaded polymeric nanoparticles. In sum, we concluded that our formulation 1) induces an anti-inflammatory phenotype on microglia cells in an ischemic context; 2) modulates the secretome of ischemic endothelial cells towards normalized nitric oxide production and anti-inflammatory cues for microglia cells; 3) promotes survival and neurovascular repair in a suitable inflammatory environment following intravenous injection and ischemia. Thus, this formulation has shown great therapeutic potential for the intravenous treatment of stroke.

Keywords

Stroke, retinoic acid, nanomaterials, neuroinflammation, vascular system

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List of Abbreviations

Arg-1	Arginase-1
BrdU	5-bromo-2′-deoxyuridine
CM	Conditioned media
CNS	Central nervous system
CRABP	Cellular retinoic-acid-binding proteins
DDS	Drug delivery system
DMSO	Dimethyl sulfoxide
FGF	Fibroblast growth factor
HUVEC	Human umbilical vein endothelial cells
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MCAO	Middle cerebral artery occlusion
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric oxide
OGD	Oxygen and glucose deprivation
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PI	Propidium iodide
RA	Retinoic acid
RA-NP	Retinoic acid-loaded polymeric nanoparticles
RBP	Retinol-binding protein
ROS	Reactive oxygen species
RT	Room temperature

Work presented in this thesis has resulted in:

Oral communication on the Portuguese Stroke Society:
- Machado-Pereira M., Santos T., Ferreira L., Bernardino L. and Ferreira R., Anti-inflammatory effect of retinoic acid-loaded nanoparticles in an ischemic context, (February 4-6th, 2016), X Portuguese Stroke Congress, Oporto, Portugal

In this period, the following manuscripts were submitted or are in preparation:

- Machado-Pereira M., Santos T., Ferreira L., Bernardino L. and Ferreira R., Challenging the Great Vascular Wall: can we envision a simple yet comprehensive therapy for stroke? - Journal of Tissue Engineering and Regenerative Medicine **(submitted and under revision)**;

- Machado-Pereira M., Santos T., Ferreira L., Bernardino L. and Ferreira R., Novel approach using retinoic acid-loaded polymeric nanoparticles for M2 microglia polarization **(in preparation)**;

- Machado-Pereira M., Santos T., Ferreira L. Bernardino L. and Ferreira R., Intravenous application of retinoic acid-loaded polymeric nanoparticles for neurovascular repair after stroke **(in preparation)**.

Chapter 1 – Introduction

1.1. Stroke overview

According to "The Atlas of Heart Disease and Stroke", developed by the World Health Organization in collaboration with the United States Centers for Disease Control and Prevention, an estimated 6.7 million people die of stroke and about 17 million strokes occur every year(1). In the European region alone, stroke is the second cause of death and a major cause of neurological and motor disability. This disease, which has a massive social impact, is responsible for 45% of all deaths in Europe, equating to 4 million deaths *per* year. These numbers increase with age (Table 1)(2).

Table 1. Number (n) and percentage of deaths (%) from cerebrovascular disease in Europe by sex and age (adapted from(2)).

	Cerebrovascular disease	
	n	%
Male		
Total deaths (all ages)	415,619	9
Premature deaths—before age 75	190,780	7
Premature deaths—before age 65	92,315	6
Female		
Total deaths (all ages)	598,837	14
Premature deaths—before age 75	145,630	10
Premature deaths—before age 65	53,050	7
Total		
Total deaths (all ages)	1,014,456	11
Premature deaths—before age 75	336,410	8
Premature deaths—before age 65	145,365	6

The impact of stroke morbidity is commonly based on hospital discharge data, detailing prevalence and incidence values. These data show that the population-based rates of hospitalization for stroke have increased, since the early 2000s, in many countries of the European region. However, it should be considered that these rates are not age-standardized, so any increase in hospitalization over time could be the result of ageing(2).

In addition, it is important to be aware that these increasing levels of hospitalization and surgical intervention, alongside high prescription rates may have a high economic and social cost(2). Unfortunately, estimates about the economic cost of many life-changing diseases are often missing and recent data show that brain diseases are frequently much more expensive than anticipated. In 2010, the European total annual cost for stroke was approximately € 64.1

billion, constituting a major health economic challenge(3). To provide context, the current European health program (2014-2020), which devises a health strategy, is € 449.4 million(4).

Generally, a stroke occurs when blood flow to the brain is interrupted and the lack of oxygen and nutrients cause extensive cell death and damage to the brain tissue. There are two types of stroke: ischemic and hemorrhagic stroke. Ischemic stroke occurs when a blood clot blocks a blood vessel and is mainly caused by atherosclerotic disease, cerebral hypoperfusion and cardiac embolism. This type of stroke accounts for about 80% of all stroke cases(5). Hemorrhagic stroke is caused by a blood vessel that bursts, usually small intracerebral arteries that are damaged by chronic hypertension (Figure 1)(6).

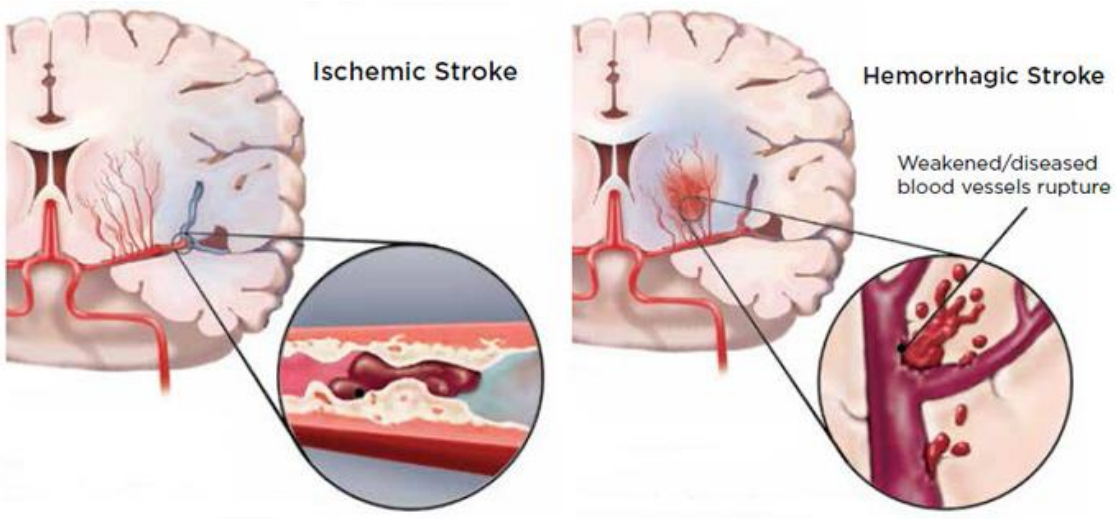


Figure 1. Different types of stroke. Ischemic stroke occurs when a blood clot stops blood flow to a region of the brain, while hemorrhagic stroke occurs when blood leaks into the brain parenchyma (adapted from(7)).

Additionally, a transient ischemic attack, also called minor stroke or “mini-stroke” may take place. They are similar to major strokes, but are milder and last only a short period of time. However, it is extremely important to be sensitive to these attacks because most people who have had one or more minor strokes tend to disregard them not knowing they will later have a higher chance of having a major stroke(8). The most common symptoms of stroke are sudden weakness or numbness of the face, confusion, difficulty in speaking or understanding speech, in seeing with one or both eyes, in walking, dizziness, loss of balance or coordination, severe headache with no known cause and fainting or unconsciousness(6).

Thus, in order to minimize the high mortality rates and costs that this disease leads, either to the public health system or families, more efficient treatments are unquestionably needed. Nationwide campaigns for a healthier lifestyle and the creation of specialized stroke units have significantly reduced time of recovery and stroke outcome(1).

The most established therapy is intravenous thrombolysis with recombinant tissue plasminogen activators (e.g. alteplase®) but several other strategies are being discussed and tested in clinical trials, such as endovascular treatment (e.g. Thrombectomy REvascularization of large Vessel Occlusions, TREVO® system) and enhanced intravenous thrombolysis (different combinations of anticoagulants or of glycoprotein IIb/IIIa antagonists; combination of a thrombolytic agent with sonothrombolysis; new thrombolytic agents (tenecteplase®) or lower dosages of standard ones (alteplase®)(1).

Other treatments include antiplatelet agents to prevent blood clots, such as aspirin; anticoagulants or blood thinners, such as warfarin; medicines to control blood pressure, such as calcium-channel blockers and angiotensin converting enzyme inhibitors; and medicines to lower cholesterol(8). The most common side-effects of stroke medicines are allergic reactions that manifest as itching, red or swollen skin, stomach pain and vomiting, diarrhea, high heart rate and dizziness(8).

The mechanisms of ischemic stroke have been studied using *in vitro* and *in vivo* models of cerebral ischemia. Cerebral ischemia models are characterized as global, focal, and multifocal ischemia. Global ischemia happens when cerebral blood flow is reduced throughout most or all of the brain, while focal ischemia occurs by a reduction in blood flow to a very distinct, specific brain region; multifocal ischemia displays a patchy pattern of reduced cerebral blood flow. There are many cerebral ischemia models such photochemical thrombosis, intraluminal filament occlusion, cardiac arrest, aortic occlusion, neck cuff, cephalic artery occlusion and others that may or may not include reperfusion(9). The most common *in vivo* model is produced by occlusion of the middle cerebral artery (MCAO), where damage is more rapid and severe. The *in vitro* models of cerebral ischemia are typically produced by oxygen and glucose deprivation (OGD). In order to perform OGD, cells are usually incubated in glucose-free media under a deoxygenated atmosphere in a hypoxic chamber(10).

Currently, there are no clinical trials directed to vasculature repair and few focus on inflammation reduction. Therefore, new and effective therapies for treating stroke are strictly necessary. However, restoring the damage caused by this disease remains challenging because the brain is the most complex organ.

1.2. The neurovascular Unit

As extensively described, the available treatments for stroke are still somewhat ineffective mainly because they only target one type of cerebral cell rather than a whole functional unit, the neurovascular unit (NVU)(11).

The concept of NVU began to attract more attention in the research area of stroke and it is proven that is crucial to regulate the BBB, contributing to the normal function of the Central Nervous System (CNS)(12-14). Among other things, NVU is essential for protecting neurons and coordinating neuronal activity with supporting cells(12). This guardian of cerebral homeostasis is mainly comprised and responsible for emphasizing the interaction between cerebral microvascular endothelium, pericytes, astrocytes, microglia, neurons and extracellular matrix and more recently, oligodendrocytes (Figure 2) (12, 15-19).

Microvascular endothelium is protected by a basement membrane mainly composed by collagen type IV, laminin, nidogen and proteoglycans. It releases NO, which is responsible for regulating a quiescent, non-thrombogenic and anti-inflammatory phenotype on endothelial cells, and promoting cell adhesion and signaling between the surrounding environment *via* integrins and growth factors(20-22). Pericytes are responsible for enhancing barrier properties and contribute to junctional integrity. However, they express the same proteins (e.g. α-smooth muscle actin, chondroitin sulphate proteoglycan NG2 and vimentin) than other brain cells, which makes their identification a complicated task(19, 22, 23).

Astrocytes also regulate the endothelium phenotype, BBB permeability and, together with microglial cells, mount a large glial barrier, which bridge the gap between the endothelium and neurons(24). In particular, microglial cells, the resident immune cells of the CNS, actively survey the brain parenchyma to maintain it free of pathogens and dead/dying cells but they also impact on outcome of ongoing tissue dysfunction and/or injury. In adverse conditions, microglial cells respond primarily by promoting repair, but they can also have a deleterious effect. The classical activated microglia phenotype (M1) is characterized by high expression of pro-inflammatory and cytotoxic mediators (aggravates damage) while the alternative phenotype (M2) is characterized by high levels of anti-inflammatory and trophic mediators (protects from damage)(25). Neurons are responsible for innervating microvascular endothelium and astrocytes, contribute to BBB permeability, highlighting the relevance of the interaction between the components of the NVU(22, 26).

Several studies have demonstrated that not only neurons suffer from stroke, but also the microvasculature, suggesting that the protection of all elements of the NVU appear to be the

most potential strategy to conquer stroke(17, 27). Recently, NVU is also a great topic of topic for several pharmaceutical companies for CNS drug design and delivery approaches(28).

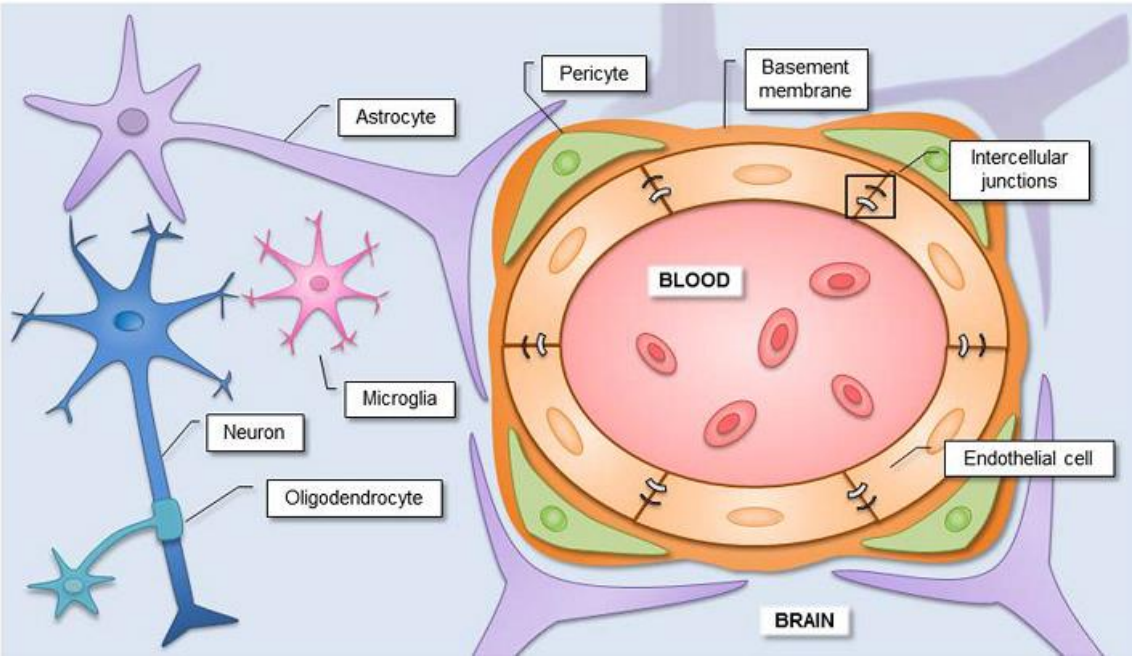


Figure 2. Schematic representation of the components of the neurovascular unit. Tightly connected brain microvascular endothelial cells are covered by pericytes and a protecting basement membrane. A glial barrier is formed by astrocyte endfeet and microglia, which serve as communicating vehicles between the brain endothelium and neuronal cells. More recently, oligodendrocytes were found to have a role in blood-brain barrier permeability and included in the neurovascular unit(adapted from(22)).

1.3. Retinoic Acid (RA)

1.3.1. RA signaling pathway

RA, an established signaling molecule, is the main biologically active derivative of vitamin A (retinol), which plays an important role in neural differentiation, neuronal patterning and axon outgrowth. Currently, RA has been highlighted as a therapeutic option for neurodegenerative disorders, since its signaling pathway is affected in these conditions (29, 30). Humans cannot synthesize vitamin A, so they extract it from diet, in the form of carotenoids and retinyl esters. The transport of these retinoids to cells that require them is performed by retinol, which is released into the bloodstream and circulates bound to retinol-binding protein 4. Retinol is taken up by target cells when it interacts with a membrane receptor for retinol-binding protein 4, STRA6, and enters the cytoplasm, where it binds to cellular retinol-binding protein 1 (RBP1). Then, it is metabolized in a two-step process to *all-trans* RA and two cytoplasmic proteins (cellular retinoic-acid-binding proteins 1 and 2) bind to the newly synthesized RA. There are two types of signaling: paracrine and autocrine. When signaling is paracrine, RA is be released from the cytoplasm (by unknown mechanisms) and taken up by receiving cells. When autocrine signaling occurs, RA enters the nucleus, assisted by cellular retinoic-acid-binding proteins 2, and binds to a transcription complex. This complex has a pair of ligand-activated transcription factors comprising the RA receptor – retinoic X receptor heterodimer, which bind to a deoxyribonucleic acid sequence (retinoic acid-response element). In the end, when *all-trans* RA activates RA receptor, it exits the nucleus and is catabolized by the CYP26 class of P450 enzymes in the cytoplasm (Figure 3)(29). Recently, RA receptors were also found in lipid rafts(31).

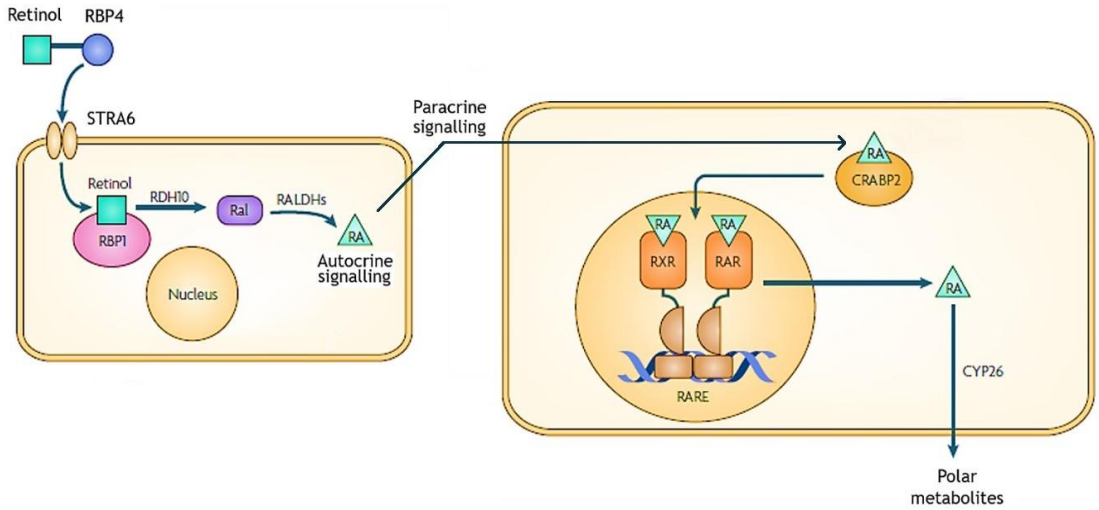


Figure 3. RA signaling pathway depicting generation, action and catabolism of RA. (CRABP2 - cellular retinoic-acid-binding protein 2; CYP26 - retinoic acid-inactivating enzyme; RA – retinoic acid; Ral – retinaldehyde; RALDHs - retinaldehyde dehydrogenases; RAR – retinoic acid receptor; RARE - retinoic acid-response element; RBP1 - retinol-binding protein 1; RBP4 - retinol-binding protein 4; RDH10 - retinol dehydrogenase 10; RXR - retinoid X receptor; STRA6 - stimulated by retinoic acid 6 (membrane receptor for RBP4). (adapted from(29)).

1.3.2. RA in the Central Nervous System

The first role that RA has in the developing CNS is to contribute to both anteroposterior and dorsoventral patterning of the neural plate and neural tube. In the anteroposterior axis of the neural plate, RA, together with WNT proteins (fusion of the Drosophila gene *wingless* and the vertebrate homolog, *integrated* or *int-1*) and fibroblast growth factors, are responsible for the organization of the posterior hindbrain and the anterior spinal cord. Without RA signaling, the posterior hindbrain is missing and the anterior spinal cord is abnormal. In the dorsoventral axis of the neural tube, RA is generated by the newly formed somites, together with sonic hedgehog, bone morphogenetic proteins and fibroblast growth factors (FGF)(29).The second role of RA in the CNS refers to inducing differentiation of various types of neuronal and glial cells by activating gene transcription and for that purpose, some genes or pathways need to be repressed. This regulatory ability of RA can be applied to produce specific neural cell types that can be used for therapeutic cell transplantation(29).

Moreover, it is important to understand how RA induces regeneration or axon regrowth after damage caused by physical trauma or disease. As described, the peripheral nervous system can regenerate after nerve transection, but the CNS cannot(25). There are evidences that in spinal cord injury RA activity is increased by retinaldehyde dehydrogenase 2. In this case, RA acts in paracrine and autocrine fashion, wherein it is responsible to decrease the inflammatory responses of macrophages and to induce the expression of survival factors, such neurotrophins, insulin-like growth factor 2, transforming growth factor-ß, bone morphogenetic protein and FGF(29). So, since CNS regeneration represents a priority in the therapeutic field, RA can be used as an important regulator of this process.

1.3.3. RA-loaded nanoparticles

RA has a great capacity for neuronal differentiation, axon outgrowth and patterning of the neural plate and the neural tube but, unfortunately, it has limitations. RA is unstable, rapidly degraded under physiological conditions and has low solubility in the aqueous phase. Thus, there has been a growing necessity to develop formulations that ensure the efficient intracellular transport and controlled release of RA such as drug delivery systems (DDS)(32).

There are many types of DDS, like liposomes, polymeric nanoparticles, polymeric micelles and dendrimers with different physical and chemical characteristics. These characteristics (size, shape, charge and others), individually, have major roles in the biodistribution and cellular internalization of therapeutic agents(33). The DDS that is commonly used to ensure the efficient intracellular transport and controlled release of RA is RA-loaded polymeric nanoparticles (RA-NP)(32).

In general terms, polymeric nanoparticles, compared with others DDS, have more advantages such as high biocompatibility and flexibility in their structures, which can be modified for desired characteristics. The polymers of these nanoparticles can be modified for various applications in a single delivery system and they show great promise in preclinical studies. Although RA encapsulation in solid lipid nanoparticles is low, it clearly facilitates the stability, solubility, and cellular uptake of RA(32, 33). We have recently developed polymeric RA-NP that have demonstrated an approximately 2,500 fold higher pro-neurogenic effect that the solubilized molecule (in dimethyl sulfoxide, DMSO) *in vitro* and *in vivo*(34, 35). The formulation has approximately 200 nm in diameter, positive net charge, and disassembles preferentially at acidic pH values. The mechanisms of RA-NP internalization are not known. RA-NP uptake can occur by endocytosis, micropinocytosis or phagocytosis. Once internalized, RA is gradually released by desorption, diffusion or nanoparticle erosion in a pH-dependent process(34).

1.3.4. RA in neurogenesis and angiogenesis

The generation of new neurons from stem cells (neurogenesis) has been associated with the formation of new blood vessels (angiogenesis). However, their underlying molecular mechanisms are not yet fully known. Neurogenesis can occur in the adult brain, in a few discrete regions like the rostral subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG)(36).

Particularly, in the SGZ of the hippocampus, an association between angiogenesis and neurogenesis has been reported. This association suggests that both endothelial cells and neural stem and progenitor cells respond to mutual mitogenic signals inducing their simultaneous mitotic expansion(37).

Additionally, the expansion of hippocampal endothelial and neuronal progenitor cells is mediated by potent factors, like vascular endothelial growth factor, epidermal growth factor, FGF-2 and nitric oxide (NO). These factors have been found to be upregulated in ischemia and brain injury and are believed to be an angiogenic response to these noxious stimuli(37). Moreover, recent data showed that the same mediators are associated with ischemia-induced neurogenesis, like FGF-2, stem cell factor, brain-derived neurotrophic factor and erythropoietin(36).After injury and/or cerebral ischemia, reactive neurogenesis is increased in the SGZ, in the gerbil and mouse(36). And, in this context, damaged astrocytes cooperate with the vascular endothelium to request the resident progenitor pool. Here, the main role of astrocytes is to release bursts of adenosine triphosphate, which promotes inter-glial calcium waves and in turn trigger further adenosine triphosphate release in the astrocytic

communication network(37). The brain vasculature can also act as a physical scaffold to allow the arrival of immature neurons (or neuroblasts) to the lesion site. Hence, brain endothelial cells are important for both trophic support and neuroblast migration. Under pathological conditions, we have previously found that RA-NP promote the survival of ischemic endothelial cells and enrich their secretome with pro-survival, proliferation and differentiation cues for neural stem cells (38).

1.3.5. RA in neuroinflammation

Mechanisms of neurogenesis and angiogenesis adapt to pathological conditions to promote survival. In this context, glial cells in the CNS may respond by actively proliferating, migrating and by mounting a response to injury that affects the outcome of neuronal and vascular regeneration(36). Typically, astrocytes, whose endfeet communicate directly with the endothelium, and microglia, who act as innate immune cells, will release a series of inflammatory mediators that can be protective in the early stages of injury, but ultimately compromise recovery.

Previous studies have examined the effects of RA on gliosis, which is induced in cerebral ischemia in the penumbral region, and they showed that this treatment reduced gliosis by approximately 50%(36). In addition, retinol-deprived rats showed increased reactive astrocytosis, and RA inhibited the release of inflammatory cytokines by invading macrophages(29). Microglial cells also have a fundamental role in stroke. After OGD, microglial cells become activated by injured neurons and engage in close cell-cell contact allowing the recognition and fast phagocytic removal of these dying/dead cells. Moreover, microglial cells provide trophic support by releasing growth factors that promote neuronal survival(39). Another protection mechanism conferred by microglia resides in their interaction with endothelial cells to prevent focal blood-brain barrier disruption(40). Also, in rats subjected to MCAO, transplantation of human microglial cells reduced ischemic deficits and apoptosis(41).

1.4. Aims

Stroke is the leading cause of death and disability in adulthood worldwide and triggers many cellular and molecular changes that compromise the viability of the nervous system. In this context, an inflammatory response predominantly mediated by microglia is generated, which difficults neurovascular repair. As the treatments available are not fully effective and may have serious side effects, the aim of this project was to trigger mechanisms that suppress the inflammatory response and effectively repair the ischemic brain in its vascular and neuronal components. For this purpose, we propose the use of retinoic acid (RA), a metabolite of vitamin A with anti-inflammatory and pro-angiogenic properties as the therapeutic agent. This molecule is delivered *via* polymeric nanoparticles previously produced by our group, retinoic acid-loaded nanoparticles (RA-NP).

To achieve this main goal, we propose to:

- determine if RA-NP can induce an anti-inflammatory phenotype (M2) on microglial cells in a classical inflammatory (lipopolysaccharide challenge) or in an ischemic environment;

- demonstrate if RA-NP can regulate endothelial cell activity and the release of anti-inflammatory cues to shift microglial activation towards a protective phenotype;

- show if RA-NP represent a potential therapeutic agent for intravenous administration in an *ex vivo* model of organotypic slice cultures subjected to ischemia, by restoring inflammatory and neurovascular integrity.

Chapter 2 - Materials and Methods

All experiments were performed in accordance with protocols approved by national ethical requirements for animal research, the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Union Directive number 2010/63/EU) for the care and use of laboratory animals.

2.1. Microglia cell cultures

Murine N9 microglia cell line was maintained at 37ºC in a 95% atmospheric air and 5% CO_2 humidified atmosphere in RPMI medium (Sigma, St. Louis, MO, USA) supplemented with 5 mM glucose (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Barcelona, Spain), and 5% fetal bovine serum (Millipore, Berlin, Germany). Microglial viable cells were accounted for by counting trypan blue-excluding cells and were then plated at a density of 2×10^4 cells *per* well in 24-well trays (immunocytochemical studies), plated at a density of 5×10^5 cells *per* well in 6-well trays (NO quantification), or plated at a density of 5×10^3 cells *per* well in 96-well trays (MTT assay).

2.2. Endothelial cell cultures

Human umbilical vein endothelial cells (HUVEC) were maintained at 37ºC in a 95% atmospheric air and 5% CO_2 humidified atmosphere in EBM-2 medium and onto fibronectin-coated surfaces (5 µg cm^2, Merck Millipore, Nottingham, UK). As previously described by us(38), EBM-2 medium is supplemented with supplied factors, including 0.04% hydrocortizone, 0.1% recombinant human vascular endothelial growth factor (rhVEGF), 0.1% ascorbic acid, 0.1% recombinant human epidermal growth factor (hEGF), 0.4% recombinant human fibroblast growth factor-B (rhFGF-B), 0.1% gentamicin sulphate/amphotericin-B, 0.1% recombinant long R insulin-like growth factor-1 (R3-UGF-1), 0.1% heparin and 2% fetal bovine serum (FBS) (all from Lonza, Walkersville, MD, USA)). HUVEC were used until passage 10 and trypan blue-excluding cells were counted as viable cells.

2.3. Nanoparticle synthesis

RA-NP was previously developed by us(34). Briefly, RA (2% w/v in DMSO) was added to poly-ethylenimine (PEI; 1% w/v in borate buffer, pH 8.0). Afterwards, dextran sulphate solution (1% w/v) and 1 M zinc sulphate were added (Figure 4A). RA-NP were then centrifuged in 5% mannitol solution at 14 000g for 20 min. The resulting nanoparticles were lyophilized and stored at 4 °C (Figure 4B). Blank nanoparticles were prepared similarly in the absence of RA.

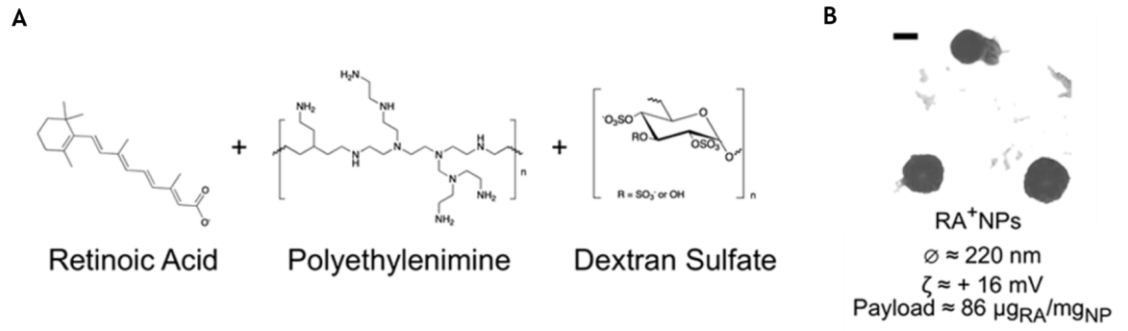


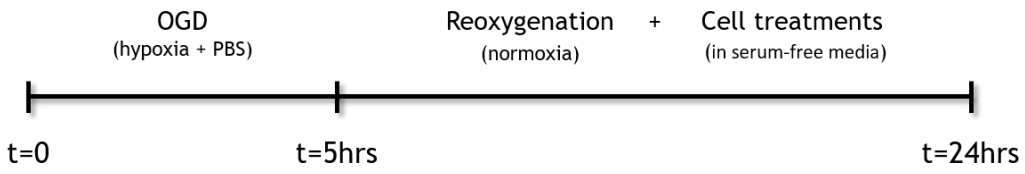
Figure 4. RA-NP characteristics. A) RA-NP composition. B) Representative image of the physical properties of RA-NP. (Ø – diameter; ζ – zeta potential; NP – nanoparticles; RA – retinoic acid; $RA^{+}NPs$ – retinoic acid-loaded nanoparticles). (adapted from(35)).

2.4. Cell treatments

To determine the impact of RA-NP on microglia polarization, N9 cells were exposed to different concentrations of RA-NP and blank NP (not containing RA) (3-100 μg/ml), in the presence or absence of lipopolysaccharide (LPS) (100 ng/ml) (Sigma) for 24 hours and in an ischemic environment (for 5 hours). RA-NP and blank NP were previously developed by us(34). Controls with free all-trans RA (0.12 and 10μM) (Sigma) and DMSO (0.01%) (Merck Millipore, Darmstadt, Germany), responsible for solubilizing free RA (non-encapsulated), were also performed. Moreover, to determine the impact of RA-NP on endothelial cells, HUVEC were exposed to different concentrations of RA-NP and blank NP (3-100 μg/ml), in a physiological and ischemic environment.

2.5. Oxygen and glucose deprivation (OGD)

N9 and HUVEC cells were kept at 37 °C in a 5% CO_2 and 95% N_2 gas environment (0.1% O_2) for 5 hours, in a MIC-101 modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA, USA). In these conditions, glucose-containing media were replaced by 0.15M phosphate-buffered saline (PBS) and after this period cell treatments occurred in serum-free media (Scheme 1). These procedures that simulate the ischemic environment have been previously described by us(38).



Scheme 1. Timeline for N9 and HUVEC cell treatments under OGD. (OGD - Oxygen and glucose deprivation; PBS - Phosphate-buffered saline).

2.6. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

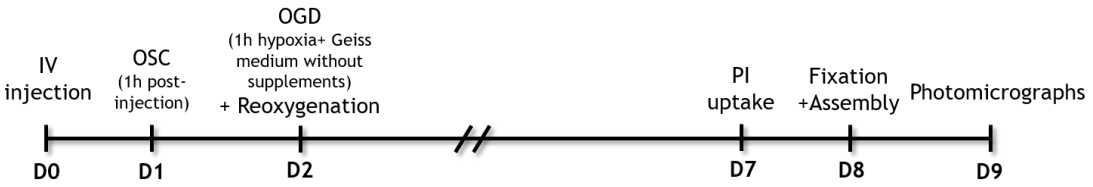
After cell treatments, MTT solution (5 ng/ml) was added to cells for 4 hours. Afterwards, 10% sodium dodecyl sulfate (SDS) in 0.01M PBS was added to dissolve formazan crystals. Cytotoxicity was determined by measuring optical density at 630 nm. Data were normalized relative to control (untreated cells).

2.7. Griess assay

After cell treatments, lysis mixture solution (137mM NaCl, 20mM Tris-HCl, 1% Triton X-100, 10% glycerol, 1mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5mM sodium vanadate (all from Sigma), pH 8.0) was added to cells. Griess reagents were added (1:1) to each well: 0.1% N-1-naphthylenediamine dihydrochloride at room temperature (RT) for ten minutes and 1% sulfanilamide in 5% phosphoric acid (Promega, Madison, WI, USA) also at RT for ten minutes. NO production was determined through the formation and accumulation of its stable metabolite product (nitrite (NO_2)) by measuring optical density at 540 nm in an enzyme-linked immunosorbent assay plate reader (SPECTRA max 384 Plus, Molecular Devices). The total amount of protein was quantified using the bicinchoninic acid assay (BCA) (Thermo Scientific). Data were normalized relative to control (untreated cells).

2.8. Cell death

HUVEC incorporated propidium iodide (PI; 3 µM; Sigma-Aldrich, St Louis, MO, USA) 10 min before the end of cell treatments and cells were fixed with 4% paraformaldehyde (PFA) as previously described by us(38). Then, cells were stained with Hoechst-33342 (2 µg/ml; Invitrogen) and mounted in Dakocytomation fluorescent medium (Dakocytomation Inc., CA, USA). Photomicrographs were recorded using digital camera (Axiocam HRC, Carl Zeiss) coupled to an Axioskop 2 Plus fluorescent microscope (Carl Zeiss). The percentage of PI-positive cells was calculated from cell counts in five independent fields *per* coverslip. On organotypic hippocampal slice cultures, after five days in culture, PI (3 µM in serum Optimem; Sigma-Aldrich) was added for 24 hours and then slices were fixed with 4% PFA and mounted in Dakocytomation fluorescent medium (Dakocytomation Inc.) (Scheme 2). Photomicrographs were also recorded using digital camera coupled to an Axioskop 2 Plus fluorescent microscope and the percentage of PI-positive cells was calculated from cell fluorescence intensity.



Scheme 2. Protocol to assess PI uptake on mouse organotypic hippocampal slice cultures under OGD. (D – day; IV - Intravenous; OSC - Organotypic hippocampal slice cultures; OGD - Oxygen and glucose deprivation; PI -Propidium iodide).

2.9. Cell proliferation

HUVEC-proliferation induced by retinoic acid-loaded nanoparticles was acquired by incorporation with 5-Bromo-2′-deoxyuridine (BrdU; 10 μM; Sigma-Aldrich), 4 hours before the end of cell treatments. Then, cells were fixed in 4% PFA and BrdU was unmasked following 10 min in 1% Triton X-100 (Sigma-Aldrich) at RT and 30 min in 1 M HCl at 37 °C. Then, HUVEC cells were washed with PBS and incubated for 30 min at RT in blocking solution (3% bovine serum albumin (BSA; Sigma-Aldrich) and 0.3% Triton X-100). Cells were incubated overnight at 4°C with rat anti-BrdU (1:50; BioRad, Carlsbad, CA, USA) and then incubated with the respective secondary antibody, anti-rat Alexa Fluor 546 (1:200; Invitrogen). Nuclei were stained with 2 μg/ml Hoechst 33342 (Invitrogen) and cells were mounted in Dakocytomation fluorescent medium (Dakocytomation Inc.). Photomicrographs were recorded using a digital camera (Axiocam HRC, Carl Zeiss) coupled to an Axioskop 2 Plus fluorescent microscope (Carl Zeiss) and percentage of BrdU-positive cells was obtained from cell counts in five independent fields *per* coverslip.

2.10. Detection of reactive oxygen species (ROS) production by N9 cells exposed to HUVEC-conditioned media (CM)

HUVEC-CM were collected from healthy or ischemic endothelial cells to evaluate the impact of RA-NP-modulated secretome on microglia cells. Endothelial cells were previously exposed to OGD and then treated with 3 and 10 μg/ml RA-NP, and 10 μM free RA. Positive control was conducted with 100 ng/ml LPS in EBM-2 medium. Previously, microglia were exposed to RPMI and EBM-2 media to assess whether endothelial media (EBM-2) *per se* induced ROS production. No significant changes were observed and remaining experiments were performed using EBM-2 medium by default. HUVEC-CM were collected after 24 hours, clarified by centrifugation for 20 minutes at 14000 rpm and applied to microglial cells for additional 24 hours. Dihydroethidium (DHE, 0.5 μM) is a superoxide indicator and was added to N9 cells for 20 minutes in PBS. Cells were washed once in PBS and fluorescent signal was measured in a plate reader (SpectraMax Gemini EM, Molecular Devices, Sunnyvale, CA, USA) at excitation wavelength of 518 nm and emission wavelength of 605 nm.

2.11. Immunocytochemistry

Cells were fixed with 4% PFA (Sigma), washed with PBS and placed in blocking solution (0.3% BSA) in 0.1% Triton X-100 solution (Sigma)) for 20 minutes at RT to prevent nonspecific binding. Cells were incubated overnight at 4°C in a primary antibody solution and then in the corresponding secondary antibody solution for 1 hour at RT. Antibodies were used as following: purified mouse anti-iNOS/NOS type II (1:500) (BD Transduction Laboratories, BD Biosciences), purified rat anti-mouse IL-4 (1:100) (BD Biosciences) and purified mouse anti-arginase I (1:400) (BD Transduction), Alexa Fluor 546 donkey anti-mouse, Alexa Fluor 488 donkey anti-mouse (Life Technologies), Alexa Fluor 594 donkey anti-mouse (Life Technologies) (all 1:200). Nuclei were stained with Hoechst 33342 (4µg/ml) (Molecular Probes). Cell preparations were mounted in Dakocytomation fluorescent medium (Dakocytomation Inc.) and corresponding images were acquired by confocal microscopy (LSM 510 Meta, Carl Zeiss, Gottingen, Germany).

2.12. Organotypic hippocampal slice cultures

Two distinct culture protocols were performed. In the first work stage, to evaluate microglia morphology, slice cultures were obtained from 7-day-old C57BL/6 mice as previously described by us(42). Briefly, brains were removed to isolate both hippocampi in Gey's Balanced Salt Solution (GBSS) (Biological Industries, Israel), under sterile conditions. Hippocampi were cut into 350 µm-thick slices using a tissue chopper (McIlwain) and transferred to 0.4 µm porous insert membranes (Millipore Corp., Bedford, MA), which were placed in six-well plates containing culture medium (composed of 25% heat-inactivated horse serum, 50% Opti-MEM minimal essential medium, 25% Hank's Balanced Salt Solution (HBSS), 25nM D-glucose (Merck, Darmstadt, Germany) and 50 U/ml penicillin and 50µg/ml streptomycin (all from Invitrogen, Carlsbad, CA, USA). Each membrane contained six slices and was kept in a humidified atmosphere (5% CO_2) at 37°C and media were refreshed every 2 days. After 6 days, slice cultures were exposed to LPS (100 ng/ml) and cell treatments (with different concentrations of RA-NP and free RA) for 24 hours. Then, in the second work stage, to deliver RA-NP intravenously, slice cultures were obtained from 2-day-old C57BL/6 mice. As described by Lampe and colleagues, intravenous injection was performed in the temporal vein (which is just anterior to the ear bud and only visible for the first two days after birth) with 20 µl of different doses of RA-NP (Figure 5)(43). To carry out the injection, animals were anesthetized with ice. One hour after injection, the organotypic hippocampal slice cultures were performed as described above.

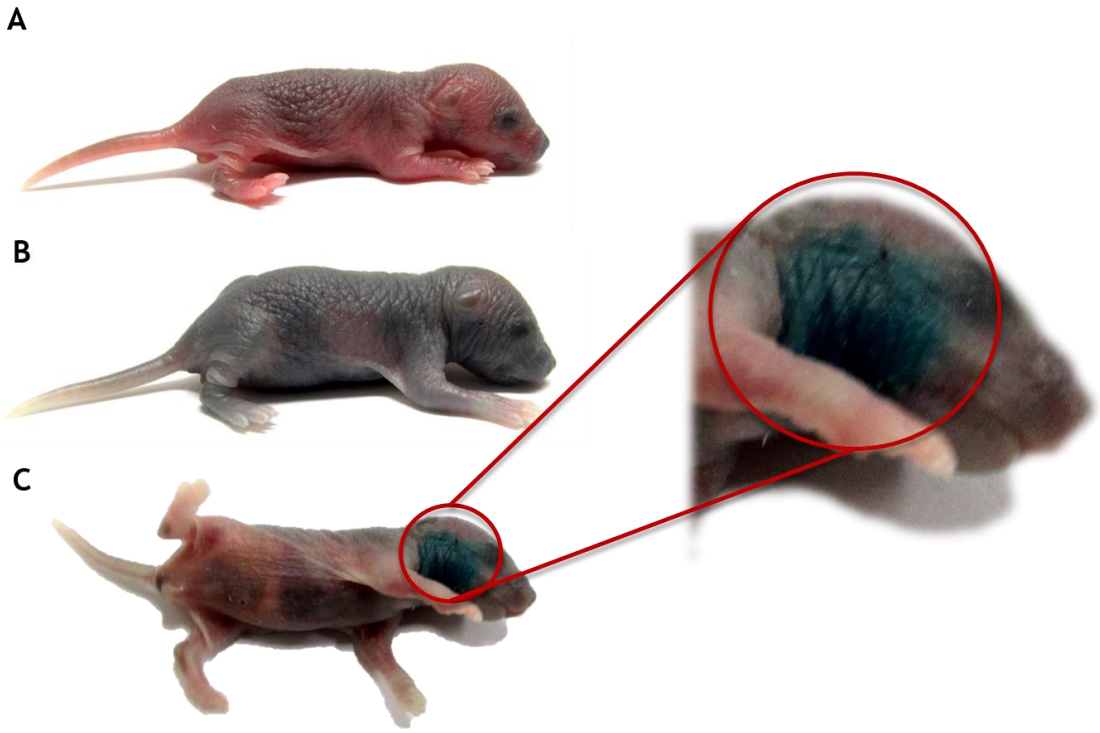


Figure 5. Intravenous injections in mice. A) Healthy P2 mice prior to the injection. B) Properly injected mice; uniform blue coloration is visible throughout the whole animal. C) Mice not injected properly; edema is visible at the site of injection while the rest of the body is not colored. These animals were not considered for experiments. The RA-NP solution was prepared in a blue food dye for visualization purposes.

2.13. Immunohistochemistry

In the first work stage , cultures were fixed with PFA overnight at 4°C, washed with PBS and placed in blocking solution (2% heat-inactivated horse serum and 0.3% Triton X-100 in PBS) for 1 hour at RT. Slice cultures were incubated overnight at 4°C in a primary antibody solution and the corresponding secondary antibody solution for 2 hours at RT. Antibodies used were rat monoclonal anti-CD11b (1:500) (AbD Serotec) in 0.3% Triton X-100 and Alexa Fluor 488 donkey anti-rat (1:500) (Life Technologies). Nuclei were stained with Hoechst 33342 (4 µg/ml) (Molecular Probes). Cell preparations were mounted in Dakocytomation fluorescent medium (Dakocytomation Inc.) and corresponding images were acquired by confocal microscopy (LSM 510 Meta, Carl Zeiss, Gottingen, Germany). In the second work stage we performed the staining protocol described by Gogolla and colleagues(44). Briefly, 1 ml of cooled 4% PFA solution was added above and beneath the membrane insert for 5 minutes. Afterwards, PFA solution was replaced by 1 ml of cooled 20% methanol/PBS solution above and beneath the insert for another 5 minutes. After this procedure, slices were detached from the membrane and permeabilized with 0.5% Triton X-100 in PBS overnight at 4°C. The permeabilization solution was removed on the following day and blocking solution (2% heat-inactivated horse serum and 0.3% Triton X-100 in PBS) was added for 1 hour at RT. Then, slice cultures were

incubated for 4 hours at RT in the blocking solution and in the corresponding secondary antibody solution for another 4 hours at RT. Primary antibodies used were rat monoclonal anti-CD11b (AbD Serotec), mouse anti-CD31 (Novocastra, Leica Biosystems, Nussloch GmbH, Germany) and goat anti-DCX (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (all 1:500). Secondary antibodies used were Alexa Fluor 488 donkey anti-rat, Alexa Fluor 546 donkey anti-mouse and Alexa Fluor 546 donkey anti-goat (all 1:500, Life Technologies), respectively. Nuclei were stained with Hoechst 33342 (4 µg/ml) (Molecular Probes). Cell preparations were mounted in Dakocytomation fluorescent medium (Dakocytomation Inc.) and corresponding images were acquired by confocal microscopy (LSM 510 Meta, Carl Zeiss, Gottingen, Germany).

2.14. Statistical analysis

Experimental conditions were performed at least in three sets of independent experiments (n), unless stated otherwise, and performed in duplicate. For immunocytochemistry analysis, 5 microscopy fields were acquired *per* coverslip (with approximately 40 cells *per* field). Statistical analysis was performed using GraphPad Prism 6.1 (GraphPad Software, San Diego, CA). Statistical significance was determined using Student's t test or one-way analysis of variance followed by Dunnett's or Bonferroni's Multiple Comparison Test and was considered relevant for p values < 0.05. Data are demonstrated as a mean ± standard error of mean (SEM).

Chapter 3 - Results

3.1. RA-NP do not compromise microglia cell viability

To evaluate the therapeutic potential of RA-NP on microglia activity, we evaluated its impact on cell viability, NO production, release of pro- and anti-inflammatory mediators, and cell morphology. These parameters were measured using an *in vitro* model, namely a murine N9 microglial cell line. Later in the work, data was collected from an *ex vivo* organotypic slice culture model to portray more complex intercellular interactions.

Initially, RA-NP cytotoxicity was evaluated by MTT reduction assay to assess cell physiology impairment (Figure 6A). Treatments with 3, 10, 30 µg/ml RA-NP and 30 µg/ml blank NP did not affect impair microglia cell metabolic activity (3RA-NP= 98.90 ± 3.61%; 10RA-NP= 85.41 ± 3.95%; 30RA-NP= 91.14 ± 1.78%; 30blank NP = 104.10 ± 4.22%; $p < 0.05$, $p < 0.01$, n=3) compared to untreated cells. The amount of RA contained in 3, 10 and 30 µg/ml RA-NP is equivalent to 0.12, 0.40 and 1.20 µM free RA, respectively. To better depict nanoparticle internalization over the course of time, the highest non-toxic concentration of RA-NP was chosen (30 µg/ml). Nanoparticle distribution was visualized by confocal microscopy because the formulation contains a green fluorophore, fluorescein isothiocyanate (Figure 6B).

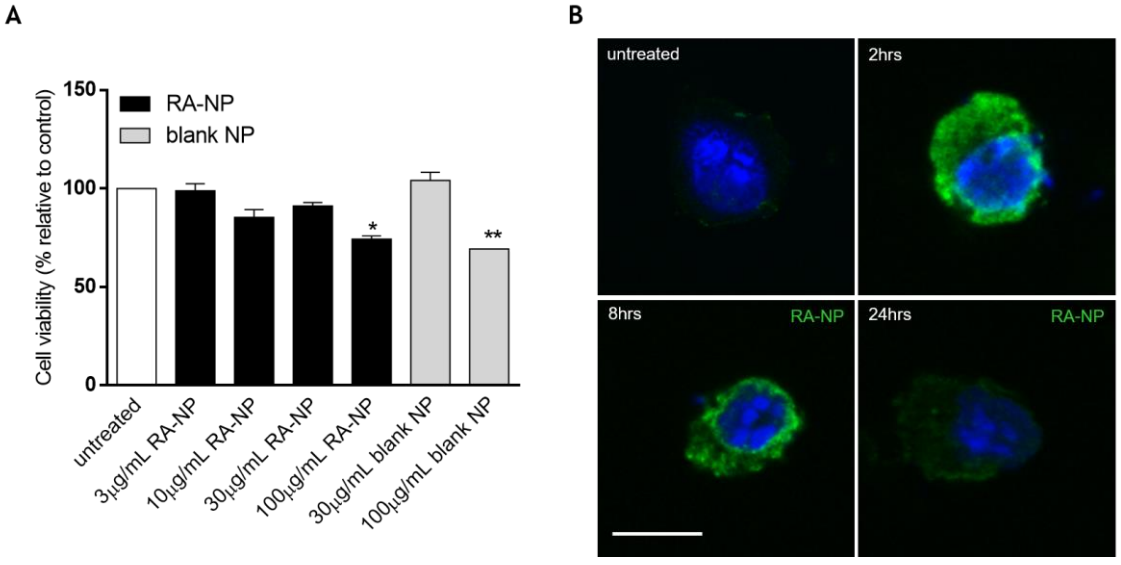


Figure 6. RA-NP promote microglia cell viability. A, N9 microglial cells were treated with RA-NP and blank NP (24 hours) to assess toxicity, using MTT assay. RA-NP and blank NP induced toxicity at concentrations starting at 100 µg/ml (n=3; *p<0.05, **p<0.01 compared to untreated cells). B, RA-NP (30 µg/ml) were internalized by N9 microglial cells. RA-NP internalization was observed by confocal microscopy over the course of 24 hours.

3.2. RA-NP prevent NO production and decrease iNOS expression by microglial cells after an inflammatory challenge

Classically activated microglia release a large range of pro-inflammatory and neurotoxic mediators, including cytokines (e.g. TNF-α, IL-1β), free radicals (e.g. NO, superoxide) and others metabolites(45, 46). Accordingly, we began by quantifying the levels of NO produced by microglial cells in a physiological and in an inflammatory context (100 ng/ml LPS, for 24 hours as previously demonstrated by us(47)) (Figure 7A and 7B, respectively). Using the Griess assay, which measures nitrite production, we demonstrated that the RA-loaded or void formulation (blank NP) and the free molecule *per se* did not change basal NO production (Figure 7A). LPS-stimulated cells produced approximately two times more NO as compared to control (untreated cells) (LPS= 228.20 ± 22.97%; **p < 0.01, n=4-5). After RA-NP treatment this effect was reverted since our formulation could significantly inhibit NO production and more evidently than 0.40 μM RA (LPS+10RA-NP= 109.30 ± 21.21%; LPS+0.4RA= 128.30 ± 16.54%; #p < 0.05, ##p < 0.01, n=3-4).

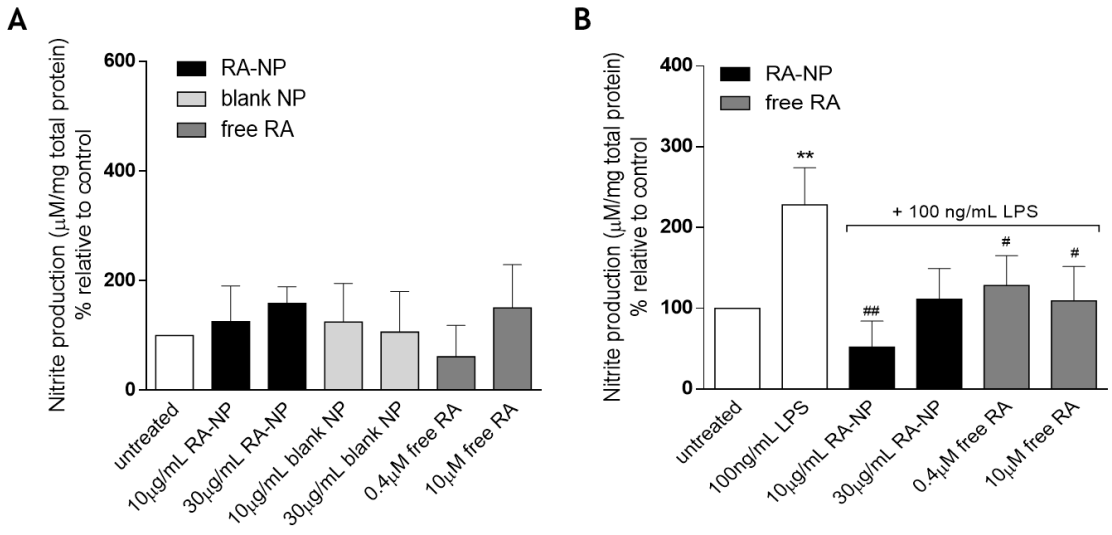


Figure 7. RA-NP prevent NO production by microglial cells after an inflammatory challenge. A) RA-NP, the RA-void formulation (blank NP), or free RA did not induce any effect in a physiological context. B) RA-NP inhibited NO production in an inflammatory context (100ng/mL LPS, 24 hours). Cell treatment with 10 μg/ml RA-NP or 0.4 μM free RA prevented LPS-induced nitrite production (n=4; **p<0.01 compared to untreated cells; #p<0.05, ##p<0.01 compared to LPS).

We also tested whether RA-NP affected the synthesis of inducible nitric oxide synthase (iNOS), the main enzyme expressed by microglia responsible for enabling NO production. By immunocytochemical studies we observed that cells exposed to an inflammatory environment demonstrated the strongest expression of iNOS (LPS= 386.20 ± 85.08%; *p < 0.05, n=3). When these cells were treated with RA-NP, iNOS expression was inhibited (LPS+10RA-NP= 148.10 ± 0%; n=1) compared to LPS-stimulated cells. Free RA did not change significantly iNOS expression in the presence of LPS (LPS+0.4RA= 362.90 ± 111.70%; #p < 0.05, n=3) (Figure 8A

and 8B). These results suggest the effectiveness of our formulation since 10 µg/ ml RA-NP contain the equivalent to 0.40 µM RA.

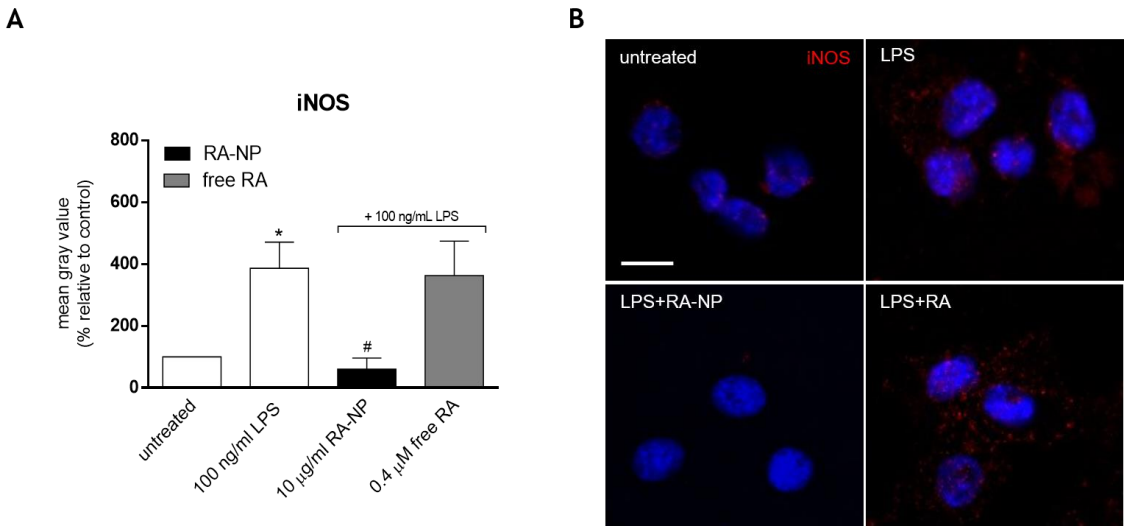


Figure 8. RA-NP decrease iNOS expression by microglial cells after an inflammatory challenge. A) Cell treatment with 10 µg/ml RA-NP prevented LPS-induced iNOS expression while free RA had no effect (n=3-6; *p<0.05 compared to untreated cells, #p<0.05 compared to LPS). B) Representative confocal images depicting iNOS (in red) overexpression after LPS treatment and its inhibition after RA-NP treatment. Nuclear staining in blue.

3.3. RA-NP increase Arg-1 and IL-4 expression by microglial cells after an inflammatory challenge

To further elucidate the effect of RA-NP under inflammatory conditions, we evaluated the expression of classic anti-inflammatory mediators of the M2 phenotype (namely Arg-1 and IL-4)(25). By immunocytochemical studies we observed that cells treated with 100 ng/ml LPS demonstrated a weak expression of Arg-1 (LPS= 30.72 ± 12.39%; **p < 0.01, n=5) and when cells were treated with RA-NP (10µg/ml), Arg-1 expression was almost completely restored (LPS+10RA-NP= 86.04 ± 14.34%; #p < 0.05, n=3). A low Arg-1 expression was obtained in free RA-treated cells under inflammatory conditions, similarly to LPS-treated cells (LPS+0.4RA= 12.04 ± 8.42%; n=3) (Figure 9A and 9B).

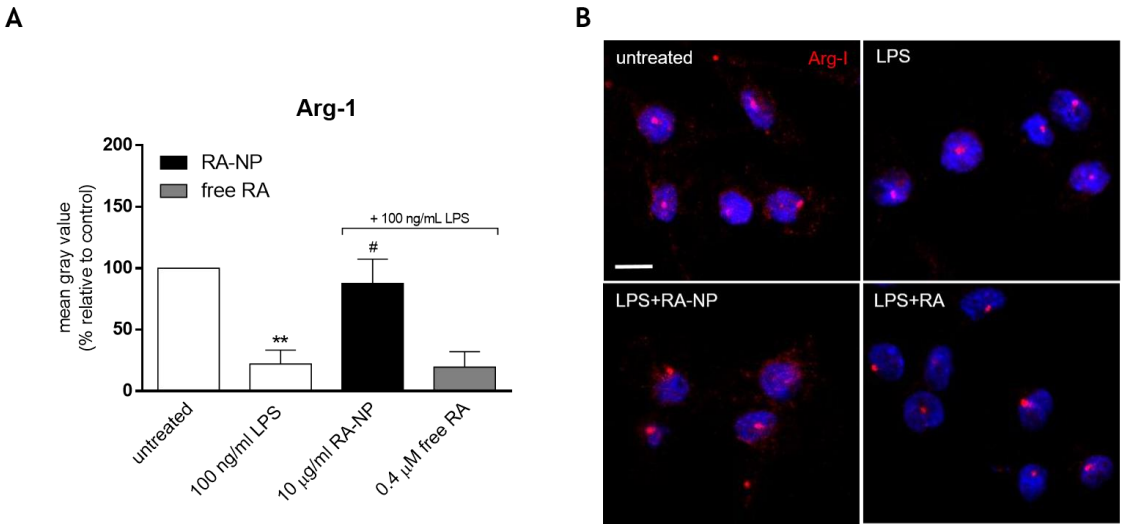


Figure 9. RA-NP increase Arg-1 expression by microglial cells after an inflammatory challenge. A) Cell treatment with 10 µg/ml RA-NP promoted LPS-inhibited Arg-1 expression while free RA had no effect (n=3-6; **p<0.01 compared to untreated cells, #p<0.05 compared to LPS). B) Representative confocal images depicting Arg-1 (in red) overexpression after LPS treatment and its inhibition after RA-NP treatment. Nuclear staining in blue.

Similar results were obtained for IL-4 expression (LPS= 39.31 ± 9.23%; LPS+10RA-NP= 103.90 ± 5.19%; LPS+0.4RA= 40.87 ± 33.24%; ##p < 0.01, ***p < 0.001, n=3-5) (Figure 10A and 10B). These results suggest again the efficacy of RA-NP compared to free RA and its ability to promote a protective M2 phenotype on microglial cells.

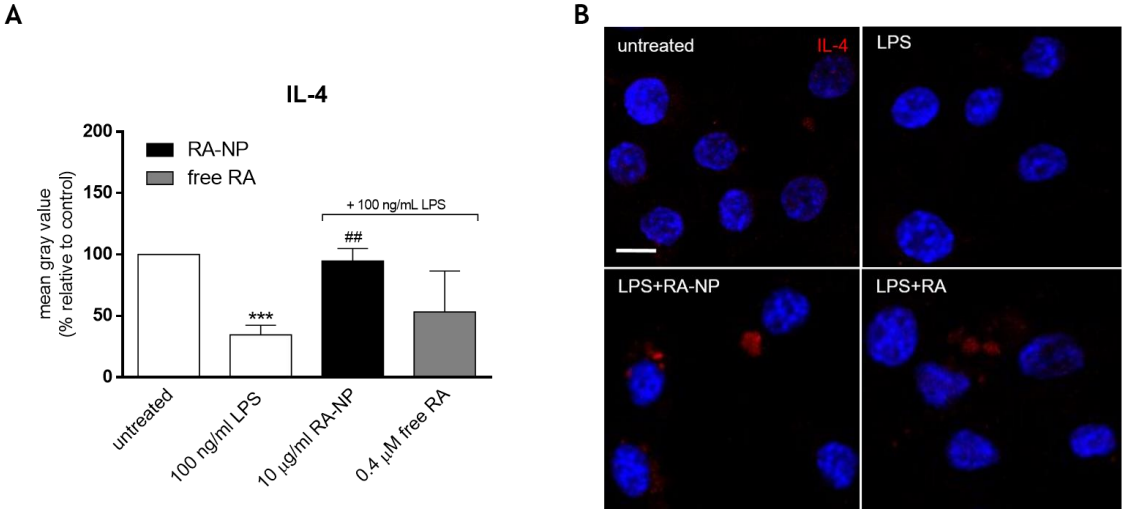


Figure 10. RA-NP increase IL-4 expression by microglial cells after an inflammatory challenge. A) Cell treatment with 10 µg/ml RA-NP promoted LPS-inhibited IL-4 expression while free RA had no effect (n=3-6; ***p<0.001 compared to untreated cells, ##p<0.01 compared to LPS). B) Representative confocal images depicting IL-4 (in red) overexpression after LPS treatment and its inhibition after RA-NP treatment. Nuclear staining in blue.

3.4. RA-NP modulate microglia morphology in murine organotypic hippocampal slice cultures

To further characterize the ability of RA-NP to induce a protective phenotype on microglial cells, we used analyzed microglia morphology in an *ex vivo* organotypic hippocampal slice culture model. In addition to the expression of pro- and anti-inflammatory mediators, microglia morphology is an important hallmark of its polarization (48). For this purpose, we evaluated CD11b expression, a surface marker that highlights cell morphology changes. As expected, RA-NP (10µg/ml) treatment changed microglia morphology from enlarged and amoeboid (LPS-activated state) to small and ramified one, in an inflammatory context, while free RA (0.40 µM) had no effect (Figure 11).

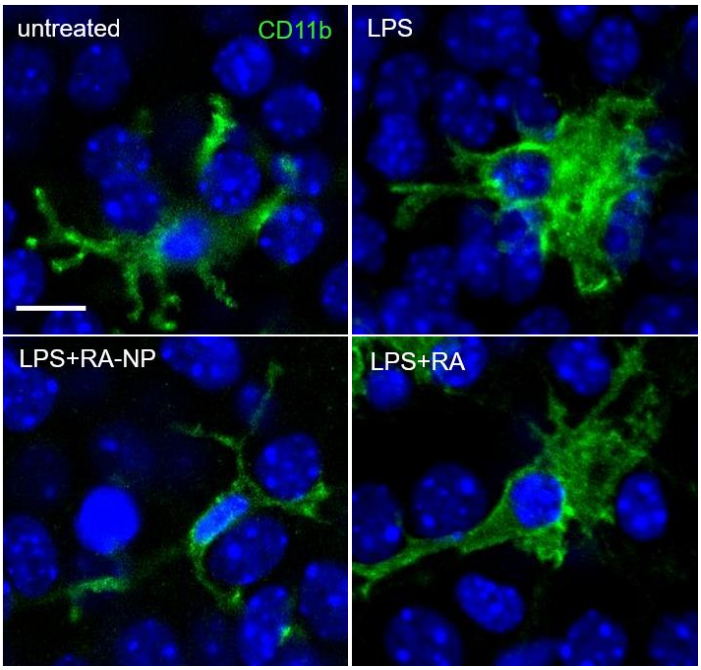


Figure 11. RA-NP modulate microglia morphology in organotypic hippocampal slice cultures. Murine organotypic hippocampal slice cultures (P7) were treated with RA-NP (10 µg/ml) or free RA (0.40 µM). RA-NP treatment changed microglia morphology from enlarged and amoeboid (LPS-activated state) to small and ramified, even in an inflammatory context (100 ng/mL LPS, 24 hours) while free RA treatment had no effect. Representative brain slices were stained for CD11b (green), a microglial/macrophage marker, and nuclei are stained in blue.

3.5. RA-NP prevent NO production and decrease iNOS expression by microglial cells after ischemia

We also quantified the levels of NO produced by microglial cells in an ischemic environment (under oxygen and glucose deprivation for 5 h). In that sense, untreated ischemic cells

produced significantly more NO (135.60 ± 9.26%; *p < 0.05; n=3) as compared with control (untreated non-ischemic cells). After RA-NP treatment (3 and 10 µg/ml) this effect was robustly inhibited and more significantly than free RA (10 µM) (3RA-NP= 72.73 ± 25.37%; 10RA-NP= 95.65 ± 7.01%; 10RA= 108.20 ± 24.99%; #p < 0.05, ##p < 0.01, n=3-5) (Figure 12).

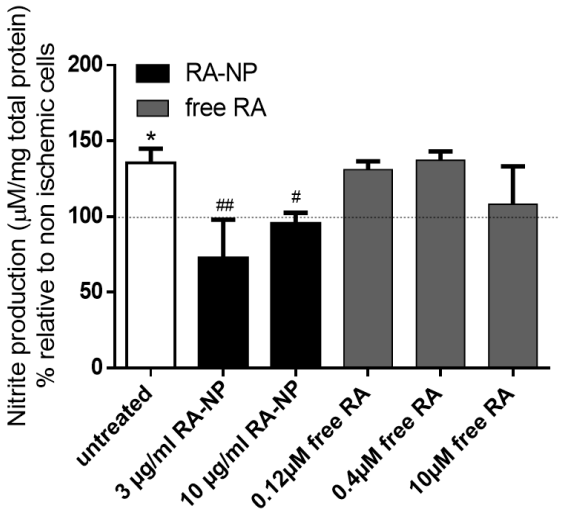


Figure 12. RA-NP inhibited nitric oxide production by microglial cells in an ischemic context. Cell treatment with 10 µg/ml RA-NP prevented OGD-induced nitrite production while free RA had no effect (n=3-5; *p<0.05 compared to healthy cells; #p<0.05, ##p<0.01 compared to untreated cells). Dashed line represents values from non-ischemic cells.

By immunocytochemical studies we observed that cells exposed to an ischemic environment demonstrated the strongest expression of iNOS and when these cells were treated with RA-NP (3 and 10µg/ml), iNOS expression was inhibited (preliminary data) (Figure 13). These results suggest that RA-NP may be relevant to the maintenance of physiological levels of NO by significantly reducing the expression of iNOS in ischemic environment.

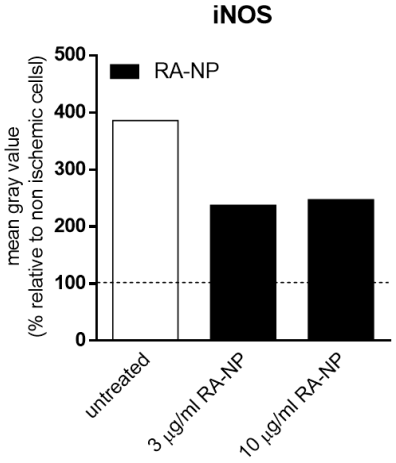


Figure 13. RA-NP inhibited iNOS expression after ischemia. Cells treated with 3 µg/mL and 10 µg/mL RA-NP showed less iNOS expression after being exposed to OGD for 5 hours (n=1). Dashed line represents values from non-ischemic cells.

3.6. RA-NP increase Arg-1 and IL-4 expression by microglial cells after ischemia

To further characterize the effect of RA-NP on microglial phenotype under ischemic conditions, we also evaluated the expression of Arg-1 and IL-4. By immunocytochemical studies we observed that cells under ischemic conditions demonstrated a weak expression of Arg-1 and IL-4 levels similar to non-ischemic cells. When cells were treated with RA-NP (3 and 10 µg/ml), Arg-1 and IL-4 expression was increased compared to untreated ischemic cells (preliminary data) (Figure 14A and 14B).

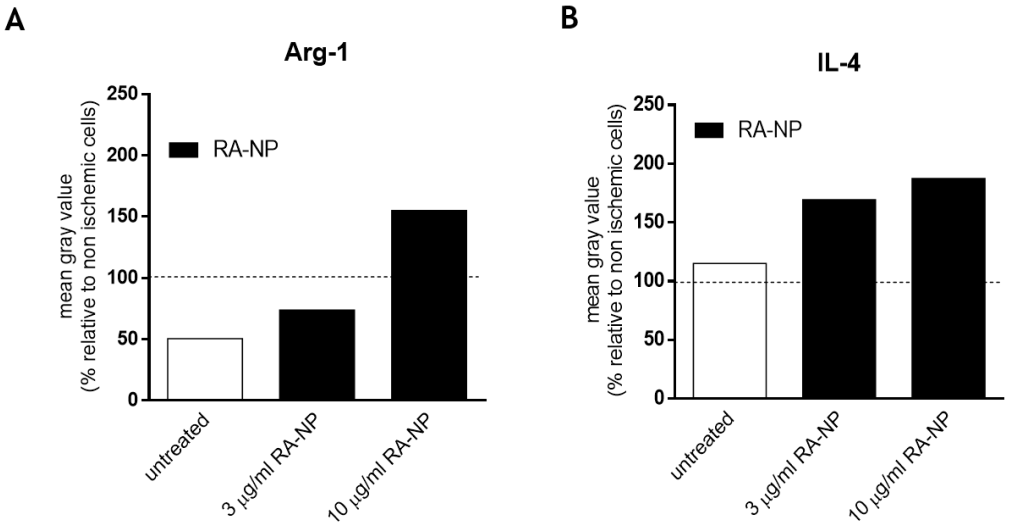


Figure 14. RA-NP increase Arg-1 and IL-4 expression by microglial cells after ischemia. Cells treated with 3 µg/mL and 10 µg/mL RA-NP showed higher levels of anti-inflammatory markers after being exposed to OGD for 5 hours (n=1). Dashed line represents values from non-ischemic cells. Panel A depicts Arg-1 expression levels while panel B illustrates IL-4 expression levels.

3.7. RA-NP did not compromise endothelial cell viability

After assessing the anti-inflammatory potential of RA-NP on microglial cells, we tested the therapeutic potential of RA-NP on endothelial cells to evaluate the possibility of 1) delivering our formulation intravenously to the brain parenchyma and 2) testing the effect of a RA-NP-treated vasculature influence the ischemic brain tissue. For that purpose, we first evaluated crucial parameters of vascular activity, namely cell death, proliferation and NO production. Treatments up to 30 µg/ml RA-NP and up to 10 µg/ml blank NP did not affect endothelial cell viability (3RA-NP= 1.43 ± 0.74%; 10RA-NP= 3.23 ± 1.07%; 30RA-NP= 9.37 ± 2.63%; 3blank NP= 5.44 ± 2.11%; 10blank NP= 7.96 ± 3.31%; $^{**}p < 0.01$, n=3) compared to untreated cells (Figure 15A). For time-course experiments, the highest non-toxic concentration of RA-NP was chosen

(30 µg/ml uptake for 1 hour, 4 and 24 hours) and nanoparticle internalization was demonstrated by confocal microscopy (Figure 15B).

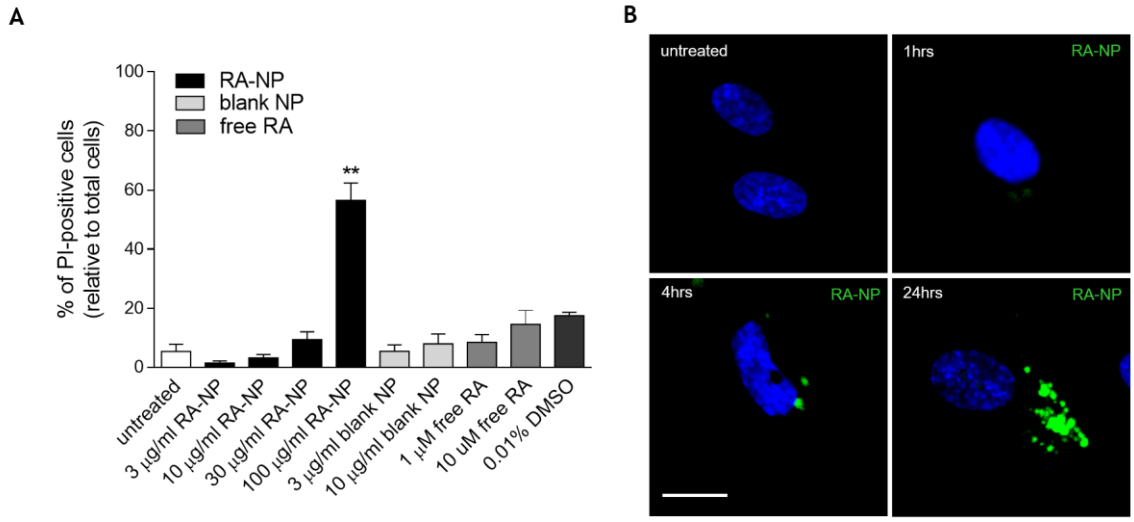


Figure 15. RA-NP did not compromise endothelial cell viability. A) Cell treatments (24 hours) with RA-NP or free RA were used to assess toxicity. RA-NP induced toxicity only at concentrations superior to 30 µg/ml, as assessed by propidium iodide uptake (n=3-5; **p<0.01, compared to untreated cells). B) RA-NP (30 µg/ml) were internalized at different time points and visualized by confocal microscopy due to the presence of a green fluorophore in the formulation (FITC). Signal detection was maximal at 24 hours.

3.8. RA-NP promote endothelial cell proliferation

Treatment with 10 µg/ml RA-NP and 10 µM free RA significantly increased proliferation (10RA-NP= 25.53 ± 6.74%; 10RA= 20.00 ± 6.61%; *p < 0.05, **p < 0.01, n=3) compared to untreated cells (5.70 ± 0.15%) (Figure 16). Blank formulations and free RA vehicle (DMSO) had no effect.

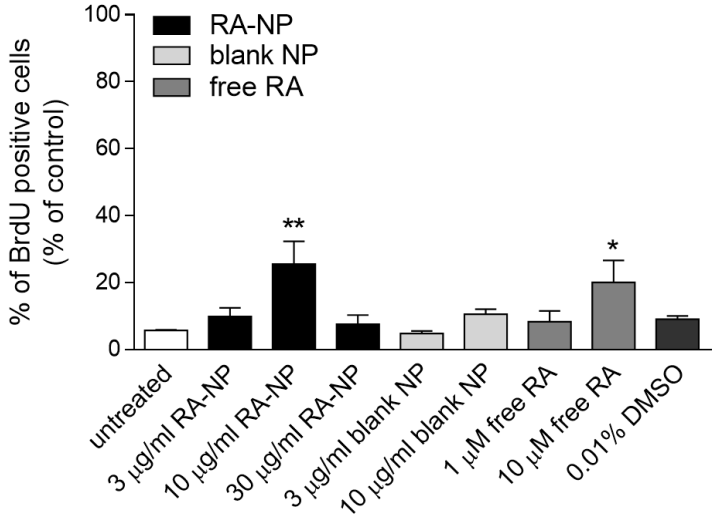


Figure 16. RA-NP promote proliferation of human endothelial cells. Cell treatments (24 hours) with free RA or RA-NP were used to assess proliferation. Cell proliferation was significantly enhanced by 10 µg/ml RA-NP and 10 µM free RA; RA-void formulation (blank NP) or free RA vehicle (DMSO) had no effect (n=3; *p<0.05, **p<0.01, compared to untreated cells).

3.9. RA-NP promote NO production by endothelial cells after ischemia

Ischemia compromises the integrity and activity of blood vessels that supply the surrounding tissues. In an ischemic environment, we quantified the levels of NO produced by endothelial cells and we demonstrated that untreated ischemia-stimulated cells significantly produced less NO as compared to control (untreated non-ischemia-stimulated cells) (ischemic-stimulated cells= 10.87 ± 4.54%; **p < 0.01, n=3-4), consistent with data presented by Ma and colleagues(49). After RA-NP treatment (3 µg/ml), we demonstrated that our formulation could increase NO production by ischemia-stimulated cells (3RA-NP ischemic-stimulated cells= 40.98 ± 4.90%; #p < 0.05, compared to untreated ischemia-stimulated cells, n=3-4). Interestingly, only with 83 times more free RA (10 µM free RA) we could demonstrate the increase of NO production (10RA ischemic-stimulated cells= 53.55 ± 1.49%; ##p < 0.01, compared to untreated ischemia-stimulated cells, n=3-4) (Figure 17). The conditions shown in figure 17 were selected based on data obtained by our group showing that only 3 µg/ml RA-NP and 10 µM free RA promoted survival and proliferation of ischemic endothelial cells (unpublished; data not shown).

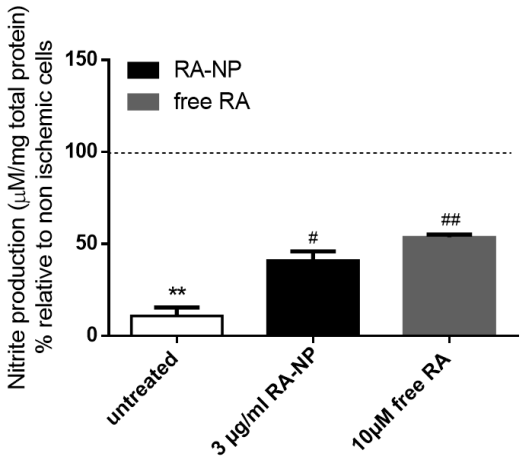


Figure 17. RA-NP inhibited NO production in an ischemic context. Cells treated with 3 µg/ml RA-NP or with 10 µM free RA significantly stimulates OGD-inhibited nitrite production (n=3-4; **p<0.01, compared to healthy cells; #p<0.05, ##p<0.01 compared to untreated ischemic cells). Dashed line represents values from non-ischemic cells.

3.10. HUVEC-CM decrease the production of superoxide by microglia cells

After demonstrating that RA-NP restore NO production by endothelial cells, we evaluated the impact of endothelial cell-conditioned media (after OGD and 10 µg/ml of RA-NP treatment) on microglial cells. For that purpose and because of the essential role of ROS in the mechanisms by which microglia cause neuronal damage, we evaluated ROS production by

these cells. We demonstrated that microglia produced less ROS when in contact with ischemic endothelial cell-conditioned media after 3 µg/mL RA-NP treatment (Figure 18). None of the other treatments were successful. Thus, RA-NP could modulate the secretome of ischemic endothelial cells in order to lower microglial ROS production.

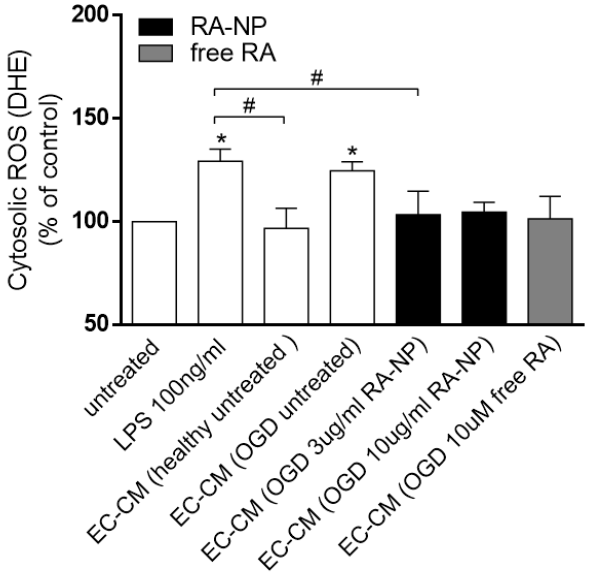


Figure 18. Microglial cells produced less ROS when in contact with ischemic endothelial cell-conditioned media after 10 µg/mL RA-NP treatment. Cells were incubated with dihydroethidium (DHE) probe (5 µM) to detect superoxide formation (n=2-5).

3.11. RA-NP promote murine organotypic hippocampal slice culture viability after ischemia

The therapeutic potential of RA-NP on mouse organotypic hippocampal slice cultures after ischemia was assessed by us. Slice cultures include an increased number of cellular interactions and different cell types (including neurons, microglia, astrocytes and oligodendrocytes) that enable the creation of a more realistic cellular environment. In this sense and in an ischemic environment, slice cultures were treated with different concentrations of RA-NP to assess their role on cell viability. We demonstrated that RA-NP only induced toxicity at 100 µg/g while concentrations ranging from 10 to 40 µg/g reduced cell death (10RA-NP= -0.04 ± 0.02; 20RA-NP= -0.03 ± 0.01%; 40RA-NP= -0.02 ± 0.01%; 100RA-NP= 0.07 ± 0.02 %; n=3-5), compared to untreated ischemic cells (Figure 19A). Then, we demonstrated that these potentially therapeutic concentrations significantly reduced basal cell death (10RA-NP= -0.03 ± 0.00; 20RA-NP= -0.05 ± 0.04%; 40RA-NP= -0.08 ± 0.01%; n=2-3), compared to untreated cells (Figure 19B). Representative images depicting cell death on organotypic hippocampal slice cultures after OGD and 10 µg/g RA-NP treatment are shown in

Figure 19C. These results are relevant for the design of a therapy consisting of intravenous administration since treatment with be in contact with both healthy and ischemic cells.

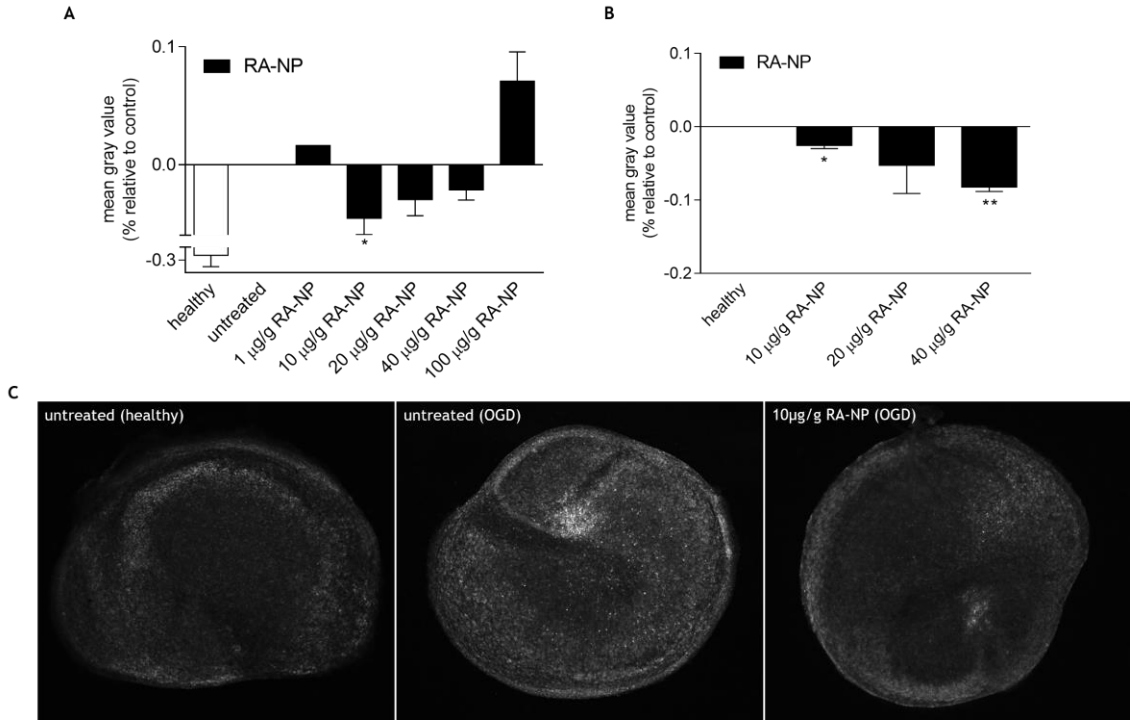


Figure 19. RA-NP promote organotypic hippocampal slice culture survival after ischemia. A) RA-NP protected from ischemic cell death using with doses ranging between 10 to 40 µg/g RA-NP. Above that dose, RA-NP aggravated cell death in an ischemic environment (n=3-5). B) RA-NP did not induce toxicity on organotypic hippocampal slice cultures in the therapeutic range (10 µg/g to 40 µg/g). (n=3-4; *p<0.05, **p<0.01, compared to healthy cells). Cell death was assessed by propidium iodide uptake. C) Representative images depicting cell death on organotypic hippocampal slice cultures after OGD and RA-NP treatment (10 µg/g).

3.12. Effect of RA-NP on neurovascular repair and neuroinflammation on ischemic organotypic hippocampal slices

To determine the therapeutic effect of RA-NP on the neurovascular unit, we analyzed the expression of vascular, neuronal and microglia markers, namely cluster of differentiation 31 (CD31), doublecortin (DCX) and CD11b, on mice organotypic hippocampal slice cultures after ischemia. CD31 is a transmembranar adhesion molecule present in endothelial cell intercellular junctions, mainly responsible for leukocyte transmigration (50). DCX is a specific marker for migratory or immature neurons in the brain (51, 52). CD11b is a surface marker that emphasizes microglial activation and that enables the diapedesis process of leukocytes through the endothelium(47, 53). We observed that intravenous injection of 10 µg/g RA-NP prior to the ischemic lesion 1) reduced the number and changed the morphology of microglia towards an anti-inflammatory phenotype (Figure 20, first panel); 2) did not appear to change the number and density of the microvasculature (Figure 20, middle panel); 3) increased the

number and induced an unipolar morphology typical of migrating neuroblasts (Figure 20, bottom panel). This pattern of migration seemed to resemble blood vessels because immature neurons use the vasculature for migration purposes (n=1).

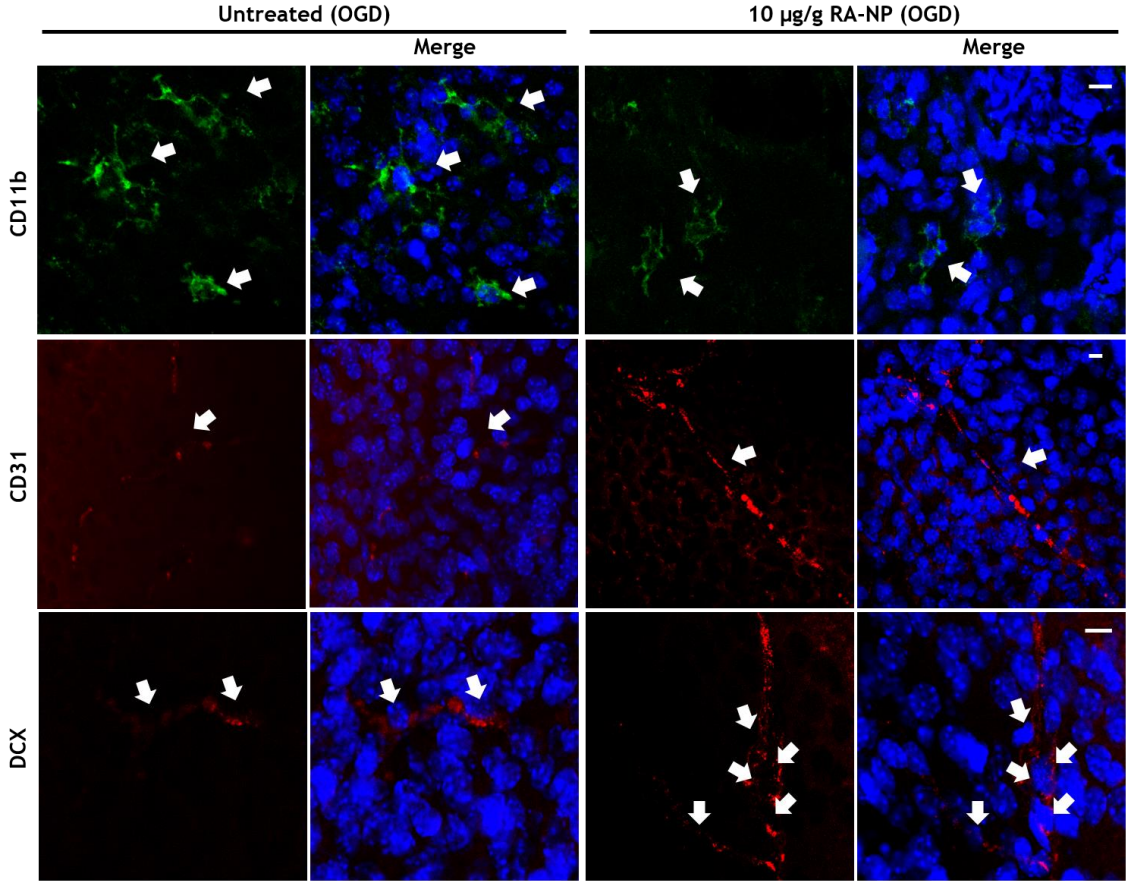


Figure 20. RA-NP modulated neurovascular repair and neuroinflammation on the hippocampal slices in an ischemic context. Representative confocal images of DCX- (red), CD31- (red), CD11b-positive cells (green) (white arrows) were obtained in organotypic hippocampal slices previously treated for 1 hour with 10 µg/g RA-NP and exposed to OGD for 1 hour (preliminary data). Nuclear staining in blue. Scale bar is 10 µm.

Alternatively, we considered the intravenous administration of RA-NP-treated human endothelial progenitor cells isolated from ischemic patients in an experimental stroke model (MCAO). The aim was supported by recently published data in which we showed that 3 µg/ml RA-NP potentiated cell proliferation, among other effects(54). Our goal was to assess if RA-NP treatment could potentiate vascular repair and then compare both approaches (direct RA-NP intravenous administration *versus* indirect effect *via* RA-NP-treated endothelial progenitor cell injection). However, technical difficulties pertaining to the immunohistochemistry technique prevented data collection.

Data obtained in this work are summarized in table 2 for a better understanding.

Table 2. Results summary. (↑↓ - change observed in the result; Ø - no effect; ≈ no differences; N/D – not determined; Arg-1 – arginase-1; CD11b – cluster of differentiation 11b; CD31 – cluster of differentiation 31; DCX – doublecortin; EC-CM – endothelial cell-conditioned media; IL-4 – interleukin-4; iNOS – inducible nitric oxide synthase; LPS – lipopolysaccharide; NO – nitric oxide; OGD – oxygen and glucose deprivation).

	Therapeutic agent	
	RA-NP	Free RA
Microglial cells		
Cell viability	Safe up to 30 μg/ml	N/D
NO production	↓ (LPS) 10 μg/ml	↓ (LPS) 0.40 μM
	↓ (OGD) 3 μg/ml	Ø
iNOS expression	↓ (LPS) 10 μg/ml	Ø
	↓ (OGD) 3-10 μg/ml	N/D
Arg-1	↑ (LPS) 10 μg/ml	Ø
	↑ (OGD) 3-10 μg/ml	N/D
IL-4	↑ (LPS) 10 μg/ml	Ø
	↑ (OGD) 3-10 μg/ml	N/D
Morphology in organotypic cultures	↓ (LPS) 10 μg/ml	Ø
Endothelial cells		
Cell viability	Safe up to 30 μg/ml	Safe up to 10 μM
Cell proliferation	↑ (basal) 10 μg/ml	↑ (basal) 10 μM
NO production	↑ (OGD) 3 μg/ml	↑ (OGD) 10 μM
EC-CM on superoxide production	↑ (OGD-CM) 3 μg/ml	Ø
Organotypic slice cultures		
Cell viability	↑ (OGD) 10 μg/g	N/D
	↑ (basal) 10-40 μg/g	N/D
Neuroblast labeling (DCX)	↑ (OGD) 10 μg/g	N/D
Endothelial labeling (CD31)	≈ (OGD)	N/D
Microglial labeling (CD11b)	↓ (OGD) 10 μg/g	N/D

Chapter 4 - Discussion

Stroke is a major cause of death and long-term disability worldwide, and for that reason, the reduction of mortality and morbidity is undoubtedly a high-priority(55). Unfortunately, the available treatments only benefit a small percentage of patients and are not always able to ensure full neurologic and/or sequelae-free recovery to stroke survivors. Stroke is characterized as a sudden loss of neurological function because of vascular damage; moreover, inflammatory mechanisms mainly triggered by microglial cells are involved in the pathophysiology of the disease and may affect its final outcome(56, 57). Microglial cells are the major immune cells involved in the defense against brain damage and are extremely important because their morphology and functional changes are related with the release of danger signals induced by stroke and impact on tissue recovery. When excessively activated by damage molecular patterns following stroke, microglia cells respond acting as phagocytic cells and are able to release inflammatory mediators (e.g. cytokines, chemokines and reactive oxygen species) that disrupt neural cells and the blood-brain barrier, influencing neurogenesis and neuronal survival(47, 57, 58). In this sense, the development of effective mechanisms which are able to modulate cell responses under these adverse conditions is an emerging need. Our group has been working extensively with RA contained in a polymeric nanoformulation (RA-NP) and has obtained promising results towards neuronal and vascular recovery(30, 35, 38). Thus, we propose the therapeutic use of RA-NP as a means to modulate microglia response towards an anti-inflammatory phenotype and to enhance vascular regulation of neuronal replenishment and repair in ischemia.

In this work, we demonstrated for the first time that RA-NP can act as a key modulator of the inflammatory reaction, acting as a microglial activation repressor. Firstly, we demonstrated that RA-NP do not compromise microglia cell viability in a wide range of biocompatible concentrations (up to 30 μg/ml). We evaluated RA-NP cytotoxicity by PI uptake (a marker of necrotic and late apoptotic cells) because we have previously shown that our formulation only induces death by necrosis(34, 38). Polymeric RA-NP are composed by poly-ethylenimine and dextran sulphate and integrate a fluorescent molecule (fluorescein isothiocyanate) to facilitate visualization by confocal microscopy. RA-NP are quickly internalized by microglial cells and their signal only begins to fade after 24 hours, when they are possibly degraded intracellularly in the phagosome or because they are released by exocytosis (38). Then, to assess the anti-inflammatory potential of RA-NP in microglial cells, we evaluated a shift in the microglia polarization phenotype (M1 polarization – the classical microglia phenotype characterized by high expression of pro-inflammatory mediators and M2 polarization – the alternative phenotype characterized by high levels of anti-inflammatory mediators), under inflammatory and ischemic conditions(25, 47). In accordance with previous works reported by our group and others, LPS challenge stimulated NO production(47, 59) and RA-NP were able to prevent NO production and iNOS expression. The highest reduction in NO and iNOS was

observed with RA-NP. Although NO levels were reduced in the presence of free RA (0.4 μM), this effect was not so extreme and hence the impact on iNOS was not as noticeable with free RA treatment. A possible explanation for this result is that the formulation allows a constant release of RA (maintaining NO and iNOS levels consistently low) while free RA treatment implies a single pulse (enabling iNOS levels to start rising again). Testing other time points (36 and 48 hours) could help to elucidate this matter. Furthermore, RA-NP treatment after LPS challenge led to the increase of the expression of anti-inflammatory mediators (namely IL-4 and Arg-1). As previously characterized, cells that display an M1 phenotype will more likely produce cytotoxic NO *via* iNOS while cells that present an M2 phenotype produce little NO but more ornithine from the same substrate (L-arginine) *via* arginase (60). The levels of iNOS and Arg-1 affect the inflammatory responses in an opposite form. NO obtained from iNOS inhibits cell proliferation and kills pathogens, while ornithine promotes cell proliferation and repairs damaged tissue(61-64). Additionally, several studies have demonstrated that Arg-1 is widely used as a marker of M2 phenotype and contributes to the resolution of inflammation and tissue repair(63). Its expression is induced by a variety of stimuli (e.g. IL-4, IL-13, high cAMP and TGF-β)(64-68). Moreover, we showed that RA-NP increased IL-4 expression, under inflammatory and ischemic conditions (although basal IL-4 expression was not different from untreated ischemic cells). The later results have only been performed once, and for that reason, we cannot draw any definite conclusions. In recent years, both RA isoforms (all-trans and 9-cis) were shown to promote IL-4 synthesis, while decreasing pro-inflammatory mediator expression (IFN-γ and TNF-α) by activated human T cells(69). Recent work has also demonstrated that RA significantly enhances wound healing and in combination with IL-4, activates Arg-1, in M2 macrophages(70). A similar approach (combination of IL-4 plus RA treatment) was shown to inhibit the production of pro-inflammatory cytokines(71). Ultimately, RA-NP were more effective than free RA at the equivalent concentration (0.40 μM RA is the amount found in 10 μg/ml RA-NP). In organotypic slice cultures, a model that presents complex cellular interactions and different cell types (including neurons, astrocytes, microglia and oligodendrocytes), RA-NP treatment changed microglia morphology from enlarged and amoeboid (LPS-activated state) to small and ramified in an inflammatory context(47). This result suggests the ability of RA-NP to promote an anti-inflammatory phenotype in microglial cells even in a more complex *ex vivo* context. Although we did not quantify this effect, RA-NP treatment seemed to reduce the number of microglial cells in the ischemic slice suggesting that our formulation may also affect microglia proliferation. Moreover, treatments were applied to the whole slice, so we cannot conclude that the effect of RA-NP on microglia is direct. RA activates the transcription of many genes (e.g. cell signaling molecules, structural proteins, enzymes and cell-surface receptors) in other cell types (e.g. astrocytes) and, therefore, microglia phenotype could be modulated indirectly(29). To pinpoint the direct effect of RA-NP on microglia, we could use a model of microglia depletion (saporin treatment) on organotypic slices, which aggravates ischemia-induced neurodegeneration(72), followed by RA-NP-treated microglia cell grafting. These

experimental models may however introduce variables (e.g. number of grafted cells, trypsin-induced microglia activation prior to grafting, tissue responses caused by microglia depletion) that may compromise data interpretation and result in information without biological significance. In summary, our work determined a novel anti-inflammatory role for RA-NP under both a classical inflammatory challenge and after ischemia.

Next we evaluated the ability of RA-NP to enhance vascular regulation and neuronal repair in ischemia. For that purpose, we initially used a human endothelial cell line (HUVEC) to perform *in vitro* studies and then, to better mimic complex cellular interactions we used mice organotypic slice cultures. As supported in this work, RA-NP did not compromise endothelial cell viability in a wide range of biocompatible concentrations (up to 30 μg/ml) and were internalized within 24 hours, as assessed by confocal microscopy. We also demonstrated that RA-NP promoted endothelial proliferation in physiological conditions and this parameter was enhanced by 10 μg/ml RA-NP treatment. As described by Otsuka and colleagues, vascular endothelium generally produces antithrombotic molecules (e.g. tissue plasminogen activator, prostacyclin, thrombomodulin, heparin-like molecules and tissue factor pathway inhibitor) and one of them, namely NO is undoubtedly crucial for vascular activity(73). This mediator is produced by the endothelium through enzymatic conversion of L-arginine by endothelial nitric oxide synthase (eNOS) and when endothelium dysfunction occurs, higher levels of NO can be protective(74). Besides having a number of vasoprotective effects (vascular relaxation, endothelial regeneration and leukocyte chemotaxis inhibition), NO inhibits platelet aggregation and blood coagulation, which is relevant in an ischemic context(75). Endothelial cells exposed to OGD suffer a considerable decrease in NO production(76) but after RA-NP treatment (3 μg/ml) we stimulated the replenishment of basal NO levels. Interestingly, RA-NP treatment had previously reduced LPS- and ischemia-induced NO levels in microglia cells but then potentiated NO production in ischemic endothelial cells, emphasizing its regulatory effect on the inflammatory microenvironment, and its complex mechanism of action. Moreover, RA-NP-treated ischemic endothelial cells secreted molecules that inhibited superoxide production by microglial cells, suggesting that a repaired endothelium may significantly contribute to resolve parenchymal inflammation. Finally, in organotypic slice cultures we observed that up to 40 μg/ml RA-NP, our formulation not only did not induce toxicity but also decreased basal and ischemic cell death (10 μg/g RA-NP induced the higher protection against OGD-induced death). Herein, it is important to note the difficulty that this protocol entails, particularly in the injection of the formulation in the middle temporal vein(43). Hence, animals that showed edema due to misplacement of the needle were not included in the study. Nevertheless, we show for the first time that RA-NP can be administered systemically, reach both the brain vasculature and can affect the brain parenchyma. Preliminary data suggest that RA-NP modulate neuronal differentiation by increasing DCX expression, an indicator of undergoing repair mechanisms. We also observed a reduction on the number of microglia as well as a change in morphology from large and amoeboid to small and ramified. These data indicate the potential of RA-NP in providing a

suitable inflammatory environment for neuroblast survival. Neuroblast migration and trophic support depend on the integrity of blood vessels but at this point we were not able to observe significant changes in vascular integrity. These data were affected by technical difficulties, possibly related to the fixation and permeabilization procedures. Nevertheless, data collected thus far support that RA-NP could be tested intravenously in several models of vascular dysfunction. For instance, a group has recently developed urinary biomarkers to detect thrombin activity in animals subjected to thromboplastin-induced pulmonary embolism. These biomarkers are administered intravenously providing a positive correlation with the amount of clots formed in the lungs(77). RA-NP could be administered intravenously on thrombotic animals to verify whether the number of clogs could diminish (compared to healthy and untreated thrombotic animals).

Chapter 5 - Conclusion

The present research project represents undoubtedly a potential clinical approach to neurological and vascular repair in stroke, and possibly in other vascular and inflammatory diseases. In the first work stage we demonstrated that RA-NP promoted an anti-inflammatory M2 phenotype in microglial cells, in a classical inflammatory environment (*in vitro* and *ex vivo*) and under ischemia. In the second work stage, the formulation demonstrated a promising therapeutic role by modulating the secretome of endothelial cells subjected to OGD without impacting on basal cell viability and proliferation. The endothelial secretome of RA-NP-treated ischemic cells inhibited a pro-inflammatory response by microglial hinting that vascular repair may restore the inflammatory milieu of the injured brain parenchyma. Moreover, intravenous administration of RA-NP appeared to potentiate neuronal differentiation and possibly migration while inducing a suitable environment provided by anti-inflammatory microglia, in ischemic organotypic slice cultures. Altogether, our data support the use of RA-NP as a therapeutic agent suitable for intravenous administration highlighting the translational potential of this formulation for cerebrovascular disorders.

Supplementary experiments and further studies and are necessary to confirm the potential of RA-NP treatment on neurovascular repair. Firstly, we will conclude the experiments that characterize the effect of RA-NP on microglial phenotype under ischemic conditions to demonstrate that RA-NP significantly decrease iNOS expression and increase Arg-1 and IL-4 expression. We will compare this effect to the application of free RA. Our aim will be to determine if RA-NP can induce a protective M2 phenotype in LPS- and ischemia-activated microglia. Additionally, we will evaluate the effect of RA-NP on microglial proliferation (using the BrdU incorporation assay in the *in vitro* and/or *ex vivo* model). In parallel, since infection increases the vulnerability to ischemic tissue damage, we will test the effect of RA-NP treatment on organotypic slices submitted to ischemia alone and to a combination of ischemia and LPS treatment. Additionally, we will optimize the immunolabeling protocol for CD11b, CD31 and DCX markers (different times and/or concentrations of the fixation and permeabilization solutions need to be tested), evaluate other markers (e.g. GFAP labeling for astrocytes) and include data for healthy slices. The last phase of this work will be performed using two different approaches in an *in vivo* model of stroke (MCAO): 1) the intravenous application of RA-NP and 2) the intravenous application of human endothelial progenitor cells previously treated with the formulation, which will be accompanied by behavioral tests to assess cognitive and motor recovery, and additional immunohistological data to exclude nanoparticle toxicity to peripheral organs. Endothelial progenitor cells are bone marrow-derived cells that can be mobilized into circulation when needed (e.g. ischemia). These cells

are able to reach distant targets and to differentiate into mature endothelial cells and incorporate the growing wall of pre-existing blood vessels (angiogenesis) or to induce the formation of new ones (vasculogenesis). We expect RA-NP treatment to potentiate this effect and to indirectly induce vascular repair, which will also reflect on a higher number of migrating neuroblasts and a more sustainable inflammatory environment. The markers mentioned above will also be evaluated in this model. If successful, RA-NP represent a promising treatment for ischemic stroke and for other vascular and inflammatory disorders.

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