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Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Treating ischemic stroke: insulin-like growth factors (IGFs) as a new tool for neuroprotection and neuroregeneration

Aline Brillouet

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :

Prof. dr. Annelies BRONCKAERS

MENTOR :

Mevrouw Lotte ALDERS

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Treating ischemic stroke: insulin-like growth factors (IGFs) as a new tool for neuroprotection and neuroregeneration

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*Running title: IGFs for the treatment of ischemic stroke

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ABSTRACT

Ischemic stroke remains among the leading causes of death and disability worldwide. With current treatments being limited, new therapeutic strategies are needed to meet neuroreparative and -regenerative needs. Insulin-like growth factors (IGFs) have shown a substantial role in the development, plasticity and survival of the central nervous system (CNS) as they promote cellular proliferation, maturation and survival. For this project, we evaluated wild type (WT) IGF-I and its structural variant, Des[1-6] IGF-2, as interventional products for the treatment of ischemic stroke. These compounds were used to target three important aspects of neuroprotection and neuroregeneration: (1) neurogenesis, (2) immunomodulation, and (3) angiogenesis. The neurogenic potential was investigated *in vitro* by analysing the effects on proliferation and migration of neural stem cells (NSCs). Immunomodulatory effects were analysed based on the expression levels of specific inflammatory markers in bone-marrow-derived macrophages (BMDMs). Lastly, the chicken chorioallantoic membrane (CAM) assay was used to study the influence of IGF-2 and its variant on angiogenesis *in ovo*. We demonstrated that exposure to WT IGF-2 promotes dose-dependent proliferation and transmigration of NSCs. In contrast, pre-incubating these cells with WT IGF-2 created an inhibitory effect on migration compared to untreated cells. In addition, immunomodulatory effects were seen as WT IGF-2 reduced the expression of pro-inflammatory markers and slightly increased levels of anti-inflammatory marker expression. In the CAM assay, both variants at 500ng per egg did not induce blood vessel growth. These findings suggest that IGF-2, or one of its derivates, has the potential to promote neuroprotective and -regenerative processes.

INTRODUCTION

Epidemiology and aetiology of stroke - Globally, stroke is among the leading causes of death and severe disability, with an incidence of over 13.7 million new cases each year. This neurovascular disorder is accountable for 5.5 million deaths annually, and it is estimated that 1 in 4 adults (over the age of 25) will experience a stroke once in their lifetime (1). Generally, stroke is defined as an infarction or haemorrhage in a certain part of the brain that is followed by the onset of focal neurological function loss. It can broadly be categorised into ischemic and haemorrhagic stroke, of which the latter includes intracerebral haemorrhage and subarachnoid haemorrhage. This

research paper focuses on ischemic strokes, which account for the majority of all strokes (~ 85%) (2-5). Aetiologically, most ischemic strokes are of thromboembolic origin, of which sources of embolism are commonly large artery atherosclerosis and cardiac diseases such as atrial fibrillation (4, 5). Clinical manifestations of a stroke depend on the lesion site in the central nervous system (CNS) and involve hemiparesis, hemianesthesia, aphasia, homonymous hemianopia, and hemispatial inattention (5, 6).

Pathophysiology of ischemic stroke - Ischemic stroke is accompanied by a series of neurochemical processes, known as the ischemic cascade, triggered by transient or permanent focal cerebral ischemia. Events in this cascade evolve in time and space and are often interrelated since one event can cause or be caused by multiple other events. Moreover, different cells often suffer from a different severity of ischemia and thus undergo different chemical processes. Therefore, the ischemic cascade is a heterogeneous process, but it can generally be summarised as a combination of the following events. The cascade starts with a bioenergetic failure due to focal cerebral hypoperfusion, followed by several cellular and molecular consequences affecting neuronal function, vascular alterations, and neuroinflammation. Neuronal cell function relies on a constant adenosine triphosphate (ATP) supply, which requires oxygen and glucose. Interruption of this supply will lead to the inability of the neuronal cells to maintain their transmembrane gradient due to the failure of energy-dependent ion transporters, resulting in depolarisation as a result of an aberrant influx of sodium (Na^+) and calcium (Ca^{2+}) ions into the cell, combined with the efflux of potassium (K^+) ions. Depolarisation of presynaptic terminals of neurons and glial cells causes the release of excitatory neurotransmitters in the synaptic cleft, mostly glutamate. The release of this excitatory neurotransmitter will activate the glutamate receptors, which will lead to an additional Ca^{2+} influx and a pathological overload that overexcites the cells, causing the release of harmful molecules such as reactive oxygen species (ROS), endonucleases, proteases, and phospholipases. The latter will disrupt the cellular membranes, allowing the entrance of other detrimental molecules, eventually releasing apoptotic signals from mitochondria that trigger the caspase-dependent cell death pathway (5, 7).

Besides neuronal cell damage and death, ischemic stroke is also characterised by a blood-brain barrier (BBB) dysfunction triggered by digestion of the endothelial basal lamina due to the release of proteases by neurons, glial, endothelial cells, and later on by infiltrated neutrophils. Disruption of the BBB allows the entrance of blood components into the brain parenchyma and water due to osmosis leading to vasogenic brain oedema and intracranial hypertension. Finally, the leaky BBB will facilitate the infiltration of inflammatory cells and thus promote a post-ischemic neuro-inflammatory reaction. In the early stages of this reaction, resident microglia are activated by ROS arising from damaged cells, releasing several pro-inflammatory mediators that stimulate the ongoing inflammation. Within 4-6 hours after ischemic onset, circulating leukocytes, including neutrophils and lymphocytes, adhere to the vessel wall and infiltrate the brain. They release additional inflammatory mediators that contribute to secondary injury of potentially salvageable brain tissue. Later on, macrophages participate in the inflammatory reaction by infiltrating the brain parenchyma. However, they can manifest with predominantly pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes, depending on the stage and environmental cues after stroke. The early stages are mainly characterised by M1 macrophages, expressing neuron-damaging factors such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and inducible nitric oxide synthase (iNOS). During the later stages, the phenotype alters to M2 macrophages that typically express the mannose receptor (CD206) and show reduced production of destructive, pro-inflammatory cytokines. In addition, these M2 macrophages are known to clear the cellular debris through phagocytosis and secrete numerous trophic and protective factors that can promote brain repair and regeneration. Given this dual phenotype, macrophages are considered to be potent modulators of CNS regeneration, making them major determinators of the outcome after stroke (5, 8, 9).

Occlusion of intracranial arteries triggers the formation of alternative blood flow pathways, also known as collaterals, that can partially sustain the blood supply to certain regions. The most

clinically relevant source of collateral flow is via leptomeningeal anastomoses. Their presence has been associated with better outcomes and reduced lesion sizes. Given the formation of these collaterals, two main regions can be distinguished after an ischemic stroke; the ischemic core, consisting of irreversibly damaged brain tissue, and the penumbra. The latter includes areas where the blood flow is sufficiently reduced to cause ischemic damage and arrest the electrical and thus physiological function. However, the presence of collateral flow will maintain the metabolic viability in these penumbral regions (5, 10).

Moreover, this penumbra can be salvaged and recover to normal function if reperfusion is established within a couple of hours after stroke. This discovery created the rationale for reperfusion therapy, which consists of intravenous thrombolysis by thrombolytics, such as recombinant tissue plasminogen activator (tPA), or mechanical removal of the thrombus by endovascular thrombectomy. Despite its effectiveness, only 10% of the patients are eligible for reperfusion therapy due to a short therapeutic time window (~ 4.5 hours) and the risk of hemorrhagic complications. Moreover, the currently used treatment solely aims to prevent further damage and thus targets neuroprotection rather than neuroregeneration, leaving most stroke patients with lifelong neurological deficits (5, 11, 12).

Given these limitations, novel treatment strategies targeting both neuroprotection and -regeneration are urgently needed to address these deficits and provide long-term therapeutical potential.

Neuroregeneration after ischemic stroke - For many years, the adult central nervous system has been considered to be limited in its regenerative capacity. More recently, this idea has been revised as it became clear that states of disease or injury can trigger reparative processes in the adult brain, such as neurogenesis. Constitutive generation of neurons occurs in two main regions: the lateral ventricles' subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. Under normal physiological conditions, this phenomenon is known to be involved in cognitive processes, such as learning and memory. However, to play a functional role in the recovery after stroke, the neural stem cells (NSCs) that reside in the brain must proliferate, migrate to the lesion, differentiate and integrate into the local circuit (9, 13-15). Recent studies have revealed that the event of stroke itself triggers the upregulation of neurogenesis. This was seen as the proliferation and presence of neural precursor cells (NPC) in the peri-infarcted zone of rats, where these cells differentiated into a subset of neuronal cells. Besides the evidence in rodents, human post-mortem brain slices have revealed the presence of NPC proliferation and migration after stroke. Although this spontaneous response may not lead to complete brain repair, these findings open a novel therapeutic opportunity to target and upregulate the natural process of restoring lost brain tissue. Regulation of neurogenesis is complex and highly dependent on the environmental state. It involves both local signals from the precursor cells' niche and remote signals derived from the circulation and cerebrospinal fluid (14, 16-19). Furthermore, it was found that macrophage subtypes play a substantial role in the regulation of neurogenesis, of which M1 macrophages impair neurogenesis by the secretion of their destructive, pro-inflammatory factors. In contrast, M2 macrophages have been found to stimulate neurogenesis by promoting NPC proliferation, migration and functional integration (9, 14, 20).

Besides a favourable environment and input of potential new functional cells, angiogenesis is also needed during neuroregeneration to restore the supply of oxygen and nutrients to assure neuronal survival and development. Similarly, as with neurogenesis, it was found that the event of a stroke triggers an angiogenic response, seen as the presence of vascular endothelial cells and microvessels in the peri-infarcted zone of human post-mortem brains. Moreover, it was found that vascular remodelling occurred around newly-born neuroblasts that migrated from the SVZ to the ischemic lesion in mice, indicating the simultaneous recruitment of newborn cells and blood vessels after stroke (15, 21, 22). On top of the importance of macrophages in neuroregeneration, it has been established that the M2 subtype secretes several pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and interleukin 8 (IL-8), again demonstrating the importance of this cell type for the recovery after stroke (9). Given this information, our rationale to stimulate neuroregeneration, using a pharmaceutical

intervention that targets neurogenesis, angiogenesis, and the inflammatory response, emerged.

Insulin-like growth factors (IGFs) in stroke - Insulin-like growth factors (IGFs), including IGF-1 and IGF-2, are members of the insulin-like peptide family and have a substantial role in the development, cell differentiation, plasticity and survival of the CNS as they promote proliferation, maturation, survival and growth of neuronal cells. These peptides are single-chain polypeptides that consist of three domains that are partly homologous to human pro-insulin. Together with insulin, both IGFs are primarily expressed during developmental stages but remain present during adult life at lower levels. Their actions are mediated by three different receptors, including the insulin receptor (IR), IGF-1 receptor (IGF-1R), and IGF-2 receptor (IGF-2R), that show some functional overlap. Both the IR and IGF-1R are tyrosine kinase receptors. Activation of these leads to the phosphorylation of several substrates, including the Ras-mitogen-activated protein kinase (MAPK) and the phosphoinositide 3 (PI-3) kinase pathway that lead to changes in a variety of bioactivities such as metabolic activity, cell growth and differentiation, and protein synthesis (23, 24). The molecular mechanisms of the IGF-2R, also known as the cation-independent mannose-6-phosphate receptor, due to its ability to bind mannose-6-phosphate (M6P) residues on lysosomal enzymes, are less understood (25). This receptor is a type-I transmembrane glycoprotein consisting of a large N-terminal extracellular region, a single membrane-spanning region, and a small cytoplasmic tail. Whereas the interaction of IGF-2 with the IR or IGF-1R mainly promotes cell growth, differentiation, and survival, it is thought that interaction with the IGF-2R lowers IGF-2 bioavailability via internalisation and degradation upon receptor binding (26). While IGF-1 mainly experts its function by interaction with the IGF-1R, it is known that IGF-2 is able to bind all three receptors, however, all with a diverse affinity.

In circulation and the extracellular space, both IGF-1 and IGF-2 are found to be almost completely bound to members of the IGF-binding proteins (IGFBPs). These proteins share structural homology and bind to both IGFs but not to insulin. To the current knowledge, six IGFBPs exist, of which IGFBP-6 is abundantly produced in the CNS. In addition, IGFBP-6 has a 20-100-fold IGF-2 binding preference compared to its family members. The function of IGFBPs remains unclear, but several options have been proposed and include: (1) transportation of the peptides, (2) prolonging the half-life of IGFs in circulation, (3) determination of the tissue- and cell-specific location, and (4) control the biological actions of IGFs by modulating their interaction with the receptors (23, 24, 27-29). Given the potential function of IGFBPs, Des[1-6] IGF-2, a structural variant of IGF-2 lacking the first six amino acids in its N-terminal, was included in this research. This variant has previously been reported to present a lower affinity for IGFBPs, increasing its bioavailability (30). Unfortunately, does the structural difference of the Des[1-6] variant cause up to a 7-fold decrease in the affinity for the IGFs receptors compared to the wild type IGF-2 (30).

Previous research has demonstrated the neuroprotective effects of both IGF-1 and IGF-2 in a rat model (31, 32). Furthermore, a study using IGF-2 knockdown NSCs has identified IGF-2 as a regulator of adult hippocampal neurogenesis by inducing the proliferation of NSCs (33). Similar results were concluded from a study in which peripheral infusion with IGF-1 in healthy rats led to the expansion of NSCs (34). In addition, post-ischemic gene transfer of IGF-1 has already been reported to increase the vascular density in a mouse model (35). However, whether IGFs have the ability to favour neuroprotection and -regeneration at the level of the three aspects explained above remains uninvestigated to our knowledge.

This research study investigated the potential of wild type IGF-2 and Des[1-6] IGF-2 as interventional products to stimulate neuroprotection and -regeneration to treat ischemic stroke. Based on previous knowledge and preliminary data of our research group, we hypothesised that the treatment of stroke with IGFs, including IGF-2 and Des[1-6], targets these processes in three aspects: (1) neurogenesis, at the level of NSC proliferation and migration, (2) immunomodulation, and (3) angiogenesis. These aspects were investigated using several *in vitro, in ovo, and in vivo* assays, in which different models were applied.

EXPERIMENTAL PROCEDURES

Cell culture - All cell types were maintained at 37°C in a 5% CO_2 humidified

atmosphere. Murine neural stem cells (NSCs) that were previously isolated from mice at embryonal day 13-15 (E13-15) and characterised afterwards were used for all following NSC assays. These cells were cultured in Neurobasal-A medium (1088802, Gibco™, Massachusetts, USA) supplemented with 2% B-27™ minus vitamin A (12587010, Gibco™, Massachusetts, USA), 2mM L-glutamine (G7513, Sigma Aldrich, Missouri, USA), 100U/ml penicillin-streptomycin (P4333, Sigma Aldrich, Missouri, USA), 20ng/ml Recombinant Human Epidermal Growth Factor (rh EGF) (11343406, Immunotools, Friesoythe, Germany) and 20ng/ml Recombinant Human Fibroblast Growth Factor-basic (rh FGF-b/FGF-2) (11343623, Immunotools, Friesoythe, Germany). Bovine Fibronectin Protein (1030-FN-05M, Novus Biologicals, Colorado, USA) coating, at a concentration of 5mg/ml, was used for cell culture and each assay involving the use of NSCs to allow adherence. Subculturing was performed when cells reached a confluence of 70-80%. Acutase (11599686, Gibco, Massachusetts, USA) was used for cell detachment.

Bone-marrow derived macrophages (BMDMs) were a kind gift from Dr Jana Van Broeckhoven and were previously isolated from healthy C57BL/6. Cells were cultured, as described before, in RPMI-1640 medium (61870036, Gibco™, Massachusetts, USA) supplemented with 10% FBS and 15% L-cell conditioned media (LCM) for a period of seven days to obtain mature macrophages (M_0) for further experiments (36).

Immunohistochemical stainings (DAB)- NSCs, BMDMs, Human Umbilical Vein Endothelial Cells (HUVECs), and Human Microvascular Endothelial Cells (HMEC-1) were plated in 24-well and allowed to settle and adhere for 24 hours. Afterwards, cells were washed with phosphate-buffered saline (PBS) and fixated with 4% paraformaldehyde (PFA) (in-house made). Protein blocking was performed using 100% Protein Block serum-free (X0909, Dako, California, USA), followed by primary antibody incubation (overnight at 4°C). Primary antibodies included rabbit-anti-IR (NBP2-16970, Novus Biologicals, Colorado, USA), goat-anti-IGF-1R (AF-305-NA, R&D systems, Minnesota, USA), and mouse-anti-IGF2R (NB300-514, Novus Biologicals, Colorado, USA) and were diluted in 100% Protein Block. After incubation, cells were washed and incubated with the corresponding secondary antibody for 30 minutes. Secondary antibodies used included goat-anti-rabbit-HRP (P0448, Dako), rabbit-anti-goat-HRP (P0160, Dako), and rabbit-anti-mouse-HRP (P0260, Dako) and were also diluted in 100% Protein Block. Next, the receptor presence was visualised using the DAB chromogen system (K3468, Dako), followed by hematoxylin counterstaining and permanent mounting. Images were acquired at a magnification of 20x or 40x using a Leica MC170 camera connected to a Leica DM2000 LED microscope.

Neural Stem Cell (NSC) Growth Assay - The protocol for this assay was established after a series of optimisations (see Supplemental Methods and Table S1) to determine the optimal cell seeding density and media composition for compound dilutions. NSCs were plated at 5 000 cells/well in a pre-coated 96-well plate (655180, Greiner Bio-One, Frickenhausen, Germany) in standard growth media and incubated for twenty-four hours to allow adherence. Afterwards, media was replaced with test conditions diluted in standard growth media. Test conditions included; WT IGF-2 (FU100, GroPep, The Barton, Australia) and Des[1-6] IGF-2 (MU100, GroPep, The barton, Australia) at concentrations of 1, 10, 100 and 1000ng/ml, Hydrogen Chloride (HCl) (in-house made) at concentrations corresponding to IGF-2 conditions (0.01, 0.1, 1 and 10µM), to serve as individual negative controls for IGF-2 conditions. Additionally, rh FGF-b (100ng/ml) and standard culture medium were used to serve as a positive and negative control, respectively (n = 11). In addition, were WT and Des[1-6] IGF-2 tested at 1000ng/ml, in combination with IGFBP-6 at concentrations of 100 and 1000ng/ml (n = 2). Cells were then incubated inside the Incucyte® Live Cell Analysis System (Sartorius, Goettingen, Germany) at 36,5°C and at a 5% CO_2 level. Four images were taken in single wells every two hours for four consecutive days using the 10x objective to generate a growth curve. For each run, conditions were tested in triplicate and data of each run was processed by subtracting the net effect of each corresponding HCl control condition to obtain the net effect of each IGF-2 test condition.

Neural Stem Cell (NSC) Transwell Migration Assay - The protocol for this assay was established

after a series of optimisations (see Supplemental Methods and Table S2) to determine the optimal cell seeding density and media composition for compound dilutions. For this assay, growth media consisted of Neurobasal-A medium supplemented with 0.2% B-27™ minus vitamin A instead of 2%. NSCs were plated at 5 000 cells/well in the upper wells of a pre-coated Clearview 96-well Plate for Chemotaxis (4582, Incucyte®, Sartorius, Goettingen, Germany) and allowed to settle. Test conditions were diluted in 0.2% B27 culture media and were added to the bottom wells of the plate. Conditions included WT IGF-2 and Des[1-6] IGF-2 at concentrations of 1, 10, 100 and 1000 ng/ml, HCl at concentrations corresponding to IGF-2 test conditions (0.01, 0.1, 1 and 10µM). For positive controls, both rh FGF-b (100ng/ml) and Recombinant Human Stromal Cell-Derived Factor-1 alpha (rh SDF-1a) (11343363, Immunotools, Friesoythe, Germany) (100ng/ml) were used, while 0.2% B27 growth media was used to serve as a negative control (n = 5). In addition, were WT and Des[1-6] IGF-2 tested at 1000ng/ml, in combination with IGFBP-6 at concentrations of 100 and 1000ng/ml (n = 1). Cells were then incubated inside the Incucyte® Live Cell Analysis System at 36,5°C and at a 5% CO_2 level. Images were taken of both upper and bottom wells every two hours for five consecutive days using the 10x objective. For each run, conditions were tested in triplicate and data of each run was processed to obtain the net effect of each tested condition. For pre-conditioning transwell migration assays, cells were treated twice every 24h with WT IGF-2 (100ng/ml), HCl (1µM), or left untreated. After 48h, cells were collected and plated at 5 000 cells/well in the upper wells, and a transwell migration assay was performed as explained above.

Neural Stem Cell (NSC) Scratch Assay - NSCs were plated in standard growth medium in a pre-coated Incucyte® Imagelock 96-well Plate (4806, Sartorius, Goettingen, Germany) at 50 000 cells/well and were allowed to adhere. When a 95-100% confluence was reached, a scratch was created in each well using the Incucyte® 96-well WoundMaker Tool (4563, Sartorius, Goettingen, Germany). Next, the medium was replaced by test conditions diluted in standard growth media. Conditions included WT IGF-2 at concentrations of 1, 10, and 100 ng/ml, HCl at concentrations corresponding to IGF-2 test conditions (0.01, 0.1, and 1 µM), to serve as individual negative controls for IGF-2 conditions. Both rh FGF-b (100ng/ml) and rh SDF-1a at 100ng/ml were used to serve as positive controls. Standard growth media was additionally included to serve as a negative control. Then, plates were incubated in the Incucyte® Live Cell Analysis System at 36,5°C and a 5% CO_2 level for a period of 24 hours, in which two images were taken in single wells every two hours using the 10x objective to determine cell migration over time. Conditions were tested in triplicate, and data (n = 1) was processed to obtain the net effect of each tested condition.

Bone-marrow derived macrophage (BMDM) differentiation assay - BMDMs were either pre-conditioned with WT IGF-2 at a concentration of 5ng/ml on days 1, 3 and 5 of the maturation process or left untreated during this process. After the maturation, on day 7, cells were seeded at 150 000 cells/well in a 24-well plate and were either left untreated or stimulated for 24h with 200ng/ml lipopolysaccharide (LPS) (437627-5MG, Calbiochem, California, USA) or 33.3ng/ml human interleukin-4 (IL-4) (200-04, New Jersey, USA) to obtain M0, M1, or M2 macrophages respectively. During this cytokine exposure, some cells were simultaneously treated with WT IGF-2 at concentrations of 5 and 1000ng/ml or HCl at concentrations of 0.05 and 10µM to serve as vehicle control. For the remaining cells, medium was replaced with fresh medium containing WT IGF-2 or HCl at the same concentrations and incubated for another 24h. In addition, growth medium was included as a negative control. Afterwards, cells were washed with PBS and collected using QIAzol Lysis Reagent (79306, QIAGEN, Hilden, Germany), and total mRNA was extracted following manufacturer' instructions. Then cDNA was synthesised from the isolated mRNA using qScript cDNA SuperMix (733-1178, Quantabio, Massachusetts, USA) and further used for a quantitative real-time polymerase chain reaction (qRT-PCR) performed on a QuantStudio 3 system (Applied Biosystems™, Massachusetts, USA), using Fast SYBR™ Green Master Mix (4385612, Applied Biosystems™, Massachusetts, USA). Primers were purchased at Integrated DNA Technologies (Iowa, USA), and their sequences are displayed in Table S3. Relative mRNA transcript

levels were standardised to their internal expression of the household genes Cyclin A (CycA) and Hypoxanthine Phosphoribosyltransferase (HPRT) and then to their control conditions.

Chicken Chorioallantoic Membrane (CAM) Assay - The angiogenic capacity of WT and Des[1-6] IGF-2 was determined in an in ovo CAM assay as previously described (37). Briefly, fertilised leghorn chicken eggs *(Gallus gallus)* were incubated in a humidified atmosphere at 37°C for three days. On day 3 of the embryonic development (E3), 3-4ml of albumin was removed to detach the developing CAM from the eggshell and fertilisation was visually confirmed. On E9, CAMs were incubated with plastic discs containing 20-30µl of the investigated conditions, including WT IGF-2 and Des[1-6] IGF-2 at a concentration of 500ng, and their corresponding concentration of HCl (500µM) to serve as a negative control. Compounds were diluted in HCl or PBS first and further dissolved in growth factor-reduced Matrigel™ (356231, Corning, New York, USA), which was further allowed to solidify at 37°C. Eggs were then incubated for three consecutive days at 37°C for further development of the CAM, which was dissected from the eggs at E12 to analyse angiogenesis. Images were taken using a Sony HDR-XR350VE handycam camera (Sony Corporation, Tokyo, Japan) and quantified by drawing two concentric circles (radiuses of 3 and 4mm) over the plastic discs on the picture and quantifying intersecting blood vessels. Quantifications were performed by three independent researchers in a blinded manner.

Statistical Analysis - Statistical analysis was performed using Prism9 (GraphPad Software, California, USA). All data are presented as mean ± standard error of mean (SEM). Outliers were identified using the Grubb's test at a significance level of 0.05 and excluded if needed. Normality and equality of variances were confirmed using the Shapiro-Wilk normality test and the Brown-Forsythe test, respectively. The means of multiple groups were compared using an ordinary one-way ANOVA, followed by a Dunnett's multiple comparisons test. A two-way ANOVA or mixed-effects model was used to compare multiple groups over time, followed by a Dunnett's multiple comparisons test. Differences with p values < 0.05 were considered significant.

RESULTS

The neurogenic potential of wild type and Des[1-6] IGF-2 - To achieve a neuroregenerative response after stroke, NSCs are required to replace the neurons lost due to ischemic damage. Before they can become functional new neurons, the stem cells that reside in the brain first have to successfully proliferate, migrate, differentiate and integrate within the existing neuronal network. We started by investigating the effects of IGFs on the proliferation and migration of NSCs as these are the two most essential aspects of initiating neurogenesis. IGFs are known to exert their actions through interaction with one of their receptors, therefore, was their presence on NSCs evaluated using immunohistochemical stainings. All three receptors (IR, IGF-1R, and IGF-2R) were present on NSCs, indicating their potential responsiveness to IGF-2 and its variants (Fig. 1A). Next, the proliferative capacity of both WT and Des[1-6] IGF-2, on NSC was tested. Growth curves of various concentrations were generated and are displayed in Figure 1. Starting from 48h, WT IGF-2 showed to promote cell growth in an upward trend at a concentration of 100ng/ml. This trend showed to be dose-dependent as other concentrations did not influence the expansion of NSCs (Fig. 1B). Moreover, these proliferative effects could not be seen when treating NSCs with the Des[1-6] variant at concentrations of 100 and 1000ng/ml (Fig 1C). Since IGF-2 shows to be almost completely bound to IGFBPs in the circulation and extracellular space, an additional assay was performed to determine the combinatory effect of WT or Des[1-6] IGF-2 and IGFBP-6. Administration of IGFBP-6 alone showed little to no effect on the expansion of NSCs in the beginning phase of the experiment but a downward trend could be observed later on (Fig. 1D-E). However, it should be pointed out that these experiments were only conducted twice and a substantial amount of variation in these conditions existed. Although WT IGF-2 did not show a positive effect on the NSC proliferation at a concentration of 1000ng/ml, co-administration with IGFBP-6 did lower the expansion in a dose-dependent manner (Fig. 1D). Contradictory results could be seen when the Des[1-6] variant is simultaneously administered with IGFBP-6. Here a lower concentration of

IGFBP-6 decreased the effect of Des[1-6] IGF-2 more than a high concentration (Fig 1E).

Besides the proliferation of NSCs, which provides a large pool of potential newborn neurons, these cells require to reach the lesion site where the damage occurred. Therefore, the migratory capacity of WT and Des[1-6] IGF-2 was investigated using several transwell migration assays that are displayed in Figure 2. Representative images of the whole bottom wells at 24h and 72h already gave an indication of differences between the negative control and WT IGF-2 (Fig. 2A). Quantification of these images confirmed the visual presumptions as

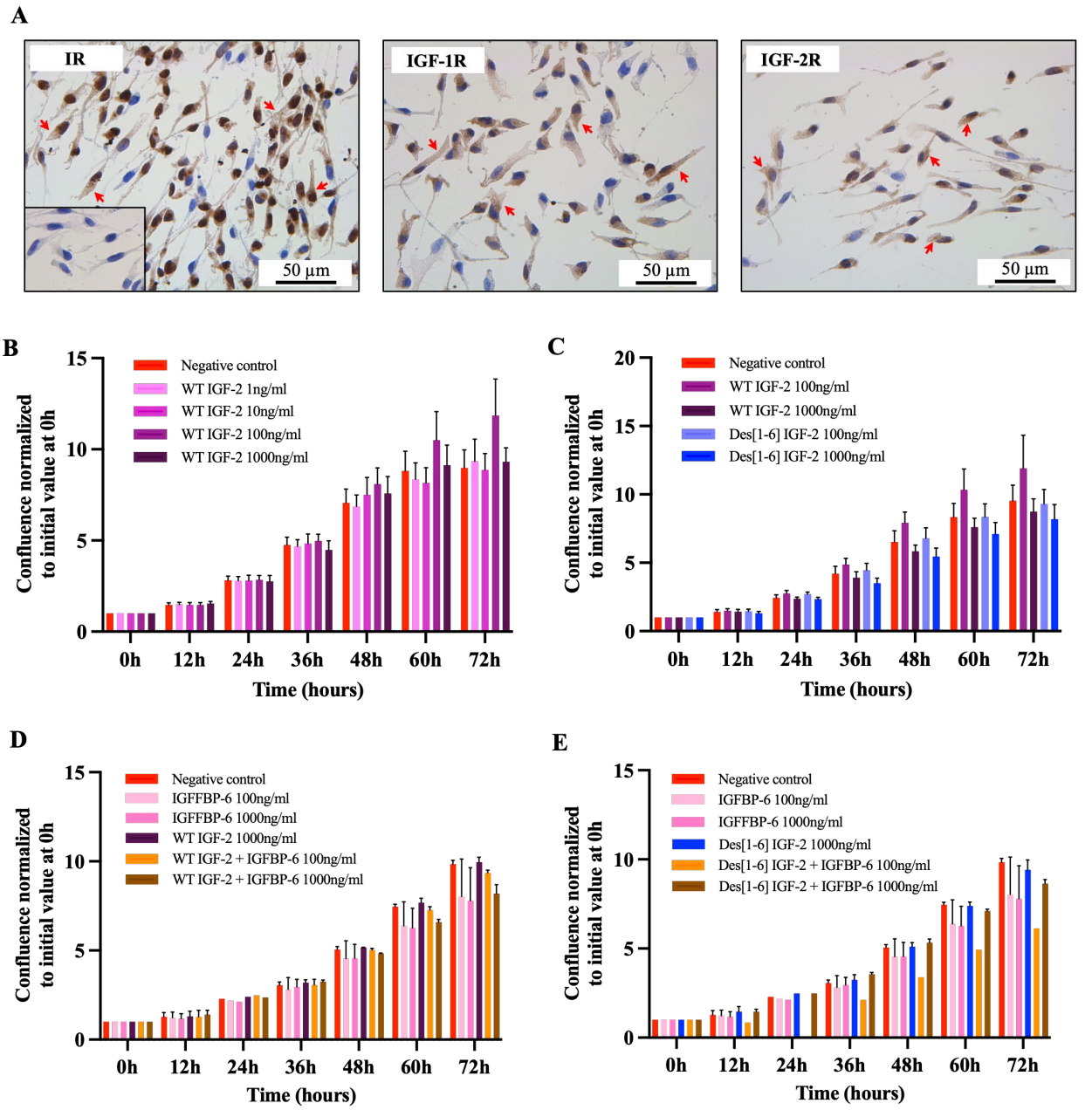

Figure 1. The neurogenic potential of WT and Des[1-6] IGF-2. (A) Immunohistochemical staining on NSCs showing the presence of the IR (left panel), IGF-1R (middle panel), and the IGF-2R (right panel). Insert shows the negative control. Receptor presence is indicated by red arrows. NSC growth assay showing the proliferative capacity of **(B)** WT IGF-2 at 1, 10, 100, and 1000ng/ml (n = 11) **(C)** Des[1-6] IGF-2 at 100 and 1000ng/ml (n = 5) **(D)** combination of IGFBP-6 (100 and 1000ng/ml) with WT IGF-2 (1000ng/ml) (n = 2) and **(E)** the combination of IGFBP-6 (100 and 1000ng/ml) with of Des[1-6] IGF-2 (1000ng/ml) (n = 2). Scale bar = 100µm. Data are expressed as mean ± SEM. IR: insulin receptor; IGF-1R: IGF-1 receptor; IGF-2R: IGF-2 receptor; WT: wild type; IGF-2: insulin-like growth factor 2.

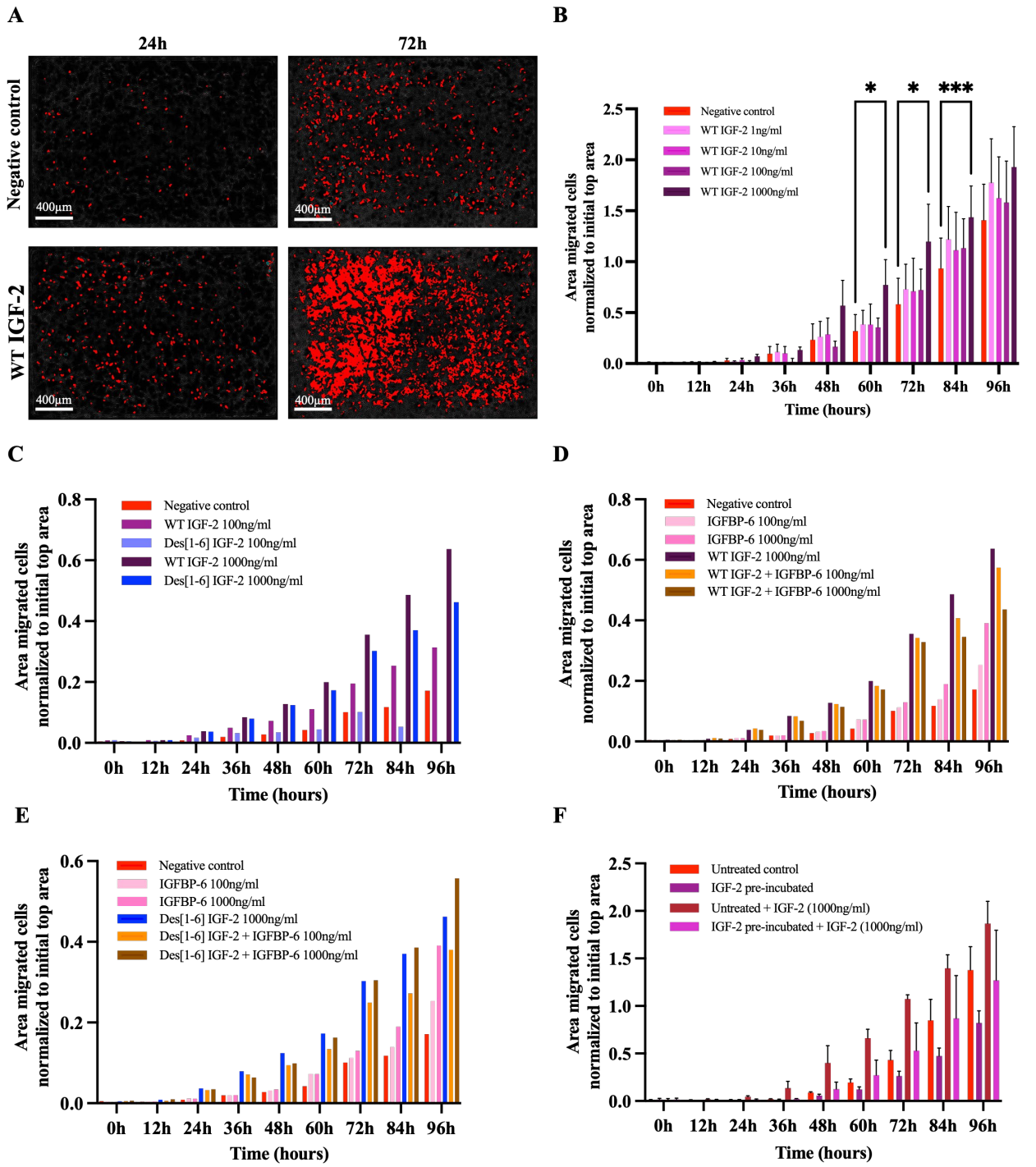

Figure 2. The potential of WT and Des[1-6] IGF-2 as chemoattractant for the migration of NSCs. (A) Representative images of the bottom well of the negative control and WT IGF-2 (1000ng/ml) condition at 24h and 72h. Images were processed with ImageJ to indicate migrated cells in red**.** NSC transwell migration assay showing the migratory potential of **(B)** WT IGF-2 at 1, 10, 100, and 1000ng/ml on NSCs (n = 5) **(C)** Des[1-6] IGF-2 at 100 and 1000ng/ml on NSCs (n = 1) **(D)** combination of IGFBP-6 (100 and 1000ng/ml) and WT IGF-2 (1000ng/ml) (n = 1) and **(E)** combination of IGFBP-6 (100 and 1000ng/ml) on the migratory potential of Des[1-6] IGF-2 (1000ng/ml) (n = 1) **(F)** Transwell migration assay showing the effect of WT IGF-2 (5ng/ml) pre-incubation on NSC migration (n = 2). Scale bar = 400µm. WT: wild type; IGF-2: insulin-like growth factor 2. Data are expressed as mean ± SEM. * $p \leq 0.05$, *** $p \leq 0.001$ as determined by a mixed-effect analysis followed by a Dunnett's multiple comparisons test.

WT IGF-2 showed to significantly promote migration at a concentration of 1000ng/ml compared to the negative control at 60h (0.772 ± 0.248 vs. 0.319 ± 0.162, p = 0.0295), 72h (1.198 ± 0.367 vs. 0.583 ± 0.254, p = 0.0200), and 84h (1.436 ± 0.307 vs. 0.935 ± 0.297, p = 0.0010) (Fig. 2B). The Des[1-6] variant's migratory capacities were investigated in the same transwell migration assay and were compared to the WT in Figure 2C. At a concentration of 1000ng/ml, Des[1-6] IGF-2 seemed to promote migration in a similar manner as the WT compound. However, this trend diminished after 60h where Des[1-6] IGF-2 showed to be less potent compared to the WT. Next, an additional transwell migration assay was performed to determine the combinatory effect of WT or Des[1-6] IGF-2 and IGFBP-6. When administered alone, the administration of IGFBP-6 showed little to no effect on the migration of NSCs in the early stages of migration. At later stages, however, a pro-migratory trend in both concentrations of IGFBP-6 could be observed (Fig 2D-E). Compared to WT IGF-2 (1000ng/ml) alone, de addition of IGFBP-6 showed a dose-dependent and downward trend of NSC migration (Fig. 2D). In contrast to the effect on WT IGF-2, did the addition of IGFBP-6 to Des[1-6] IGF-2 not lead to a reduction in migrated cells (Fig. 2E). Additionally, the effect of pre-conditioning NSCs with WT IGF-2 (5ng/ml) on the migration of these cells was investigated. Results from these assays are displayed in Figure 2F. Although pre-incubation evoked an overall downward trend in migrated cells, it could be observed that the administration of 1000ng/ml WT

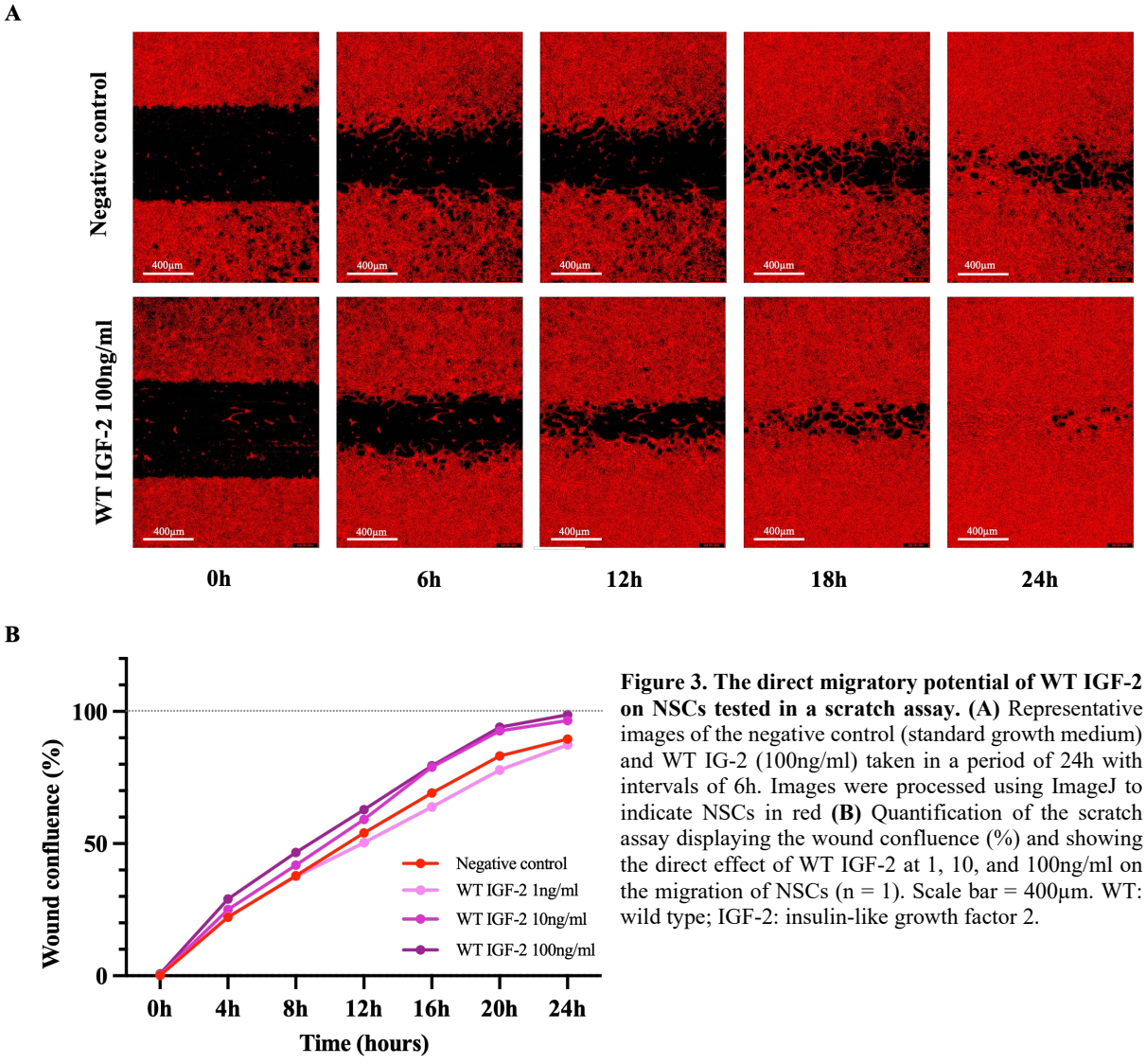

Figure 3. The direct migratory potential of WT IGF-2 on NSCs tested in a scratch assay. (A) Representative images of the negative control (standard growth medium) and WT IG-2 (100ng/ml) taken in a period of 24h with intervals of 6h. Images were processed using ImageJ to indicate NSCs in red **(B)** Quantification of the scratch assay displaying the wound confluence (%) and showing the direct effect of WT IGF-2 at 1, 10, and 100ng/ml on the migration of NSCs (n = 1). Scale bar = 400µm. WT: wild type; IGF-2: insulin-like growth factor 2.

IGF-2 was more or less able to restore this trend to the levels of untreated cells.

To further investigate the direct effect, rather than the chemoattractant potential, of WT IGF-2 on the migration of NSCs, a scratch assay was performed (Fig. 3). When inspected visually, it could already be observed that both in the negative control and IGF-2 (100ng/ml) condition NSCs started to migrate, aiming to close the scratch wound. Although migration occurred in both conditions, it was noticeable that NSCs migrated more rapidly when exposed to IGF-2 and were able to close the wound completely after 24h (Fig. 3A). Quantification of these data confirmed these visual findings. Moreover, was IGF-2 able to elicit the same upward trend in NSC migration at a lower concentration of 10ng/ml, while 1ng/ml failed to create any upward trend compared to the negative control (Fig. 3B).

Immunomodulatory properties of wild type IGF-2 - Besides neuroregeneration, providing neuroprotection by preventing further damage, is of importance in the treatment of ischemic stroke. The strong inflammatory reaction, which is mainly led by pro-inflammatory macrophages (M1), is known to exacerbate the primary damage caused by ischemia. Therefore, we aimed to provide an immunomodulatory effect by administering WT IGF-2, which was investigated in BMDMs. Immunohistological stainings of these cells revealed the presence of all three receptors and thus potential responsiveness for IGF-2 (Fig. 4A). To investigate the effect of IGF-2 on BMDM polarisation, gene expression levels of specific M1 and M2 macrophages genes were determined in three different experiments. First, the effect of incubating immature BMDMs with IGF-2 during the maturation process, on the response to LPS exposure, was determined. Overall, no large differences in gene expression could be observed. Although minor downward trends in the expression of the M1 genes CD86 and CD38 could be seen, these trends were not consistent throughout the other pro-inflammatory genes. Moreover, the expression level of all the M2 genes (Arg1, FIZZ1, Ym-1, and CD206) remained unchanged when BMDMs were treated with IGF-2 during their maturation (Fig. 4B). Next, the impact of simultaneous exposure to IGF-2 and LPS or IL-4 on the gene expression was established. The gene expression, normalised to untreated control cells

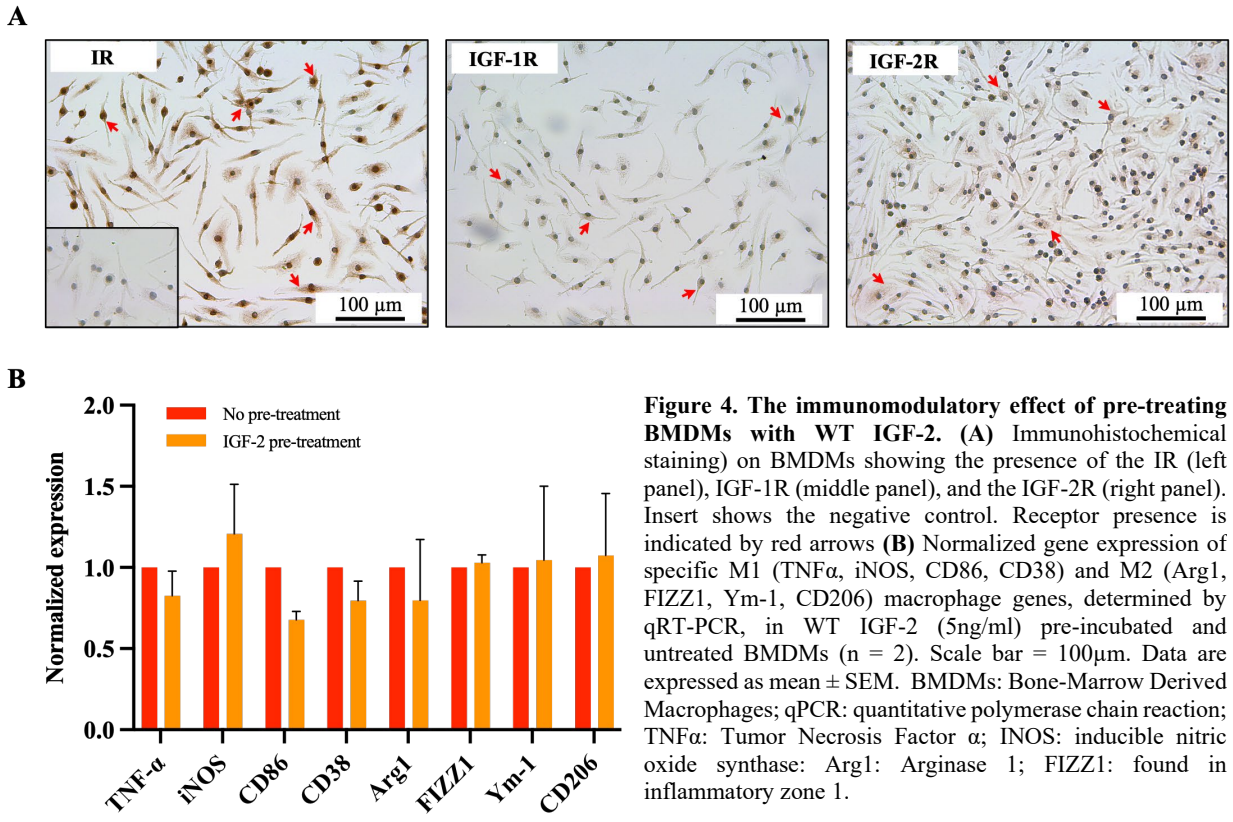

Figure 4. The immunomodulatory effect of pre-treating BMDMs with WT IGF-2. (A) Immunohistochemical staining) on BMDMs showing the presence of the IR (left panel), IGF-1R (middle panel), and the IGF-2R (right panel). Insert shows the negative control. Receptor presence is indicated by red arrows **(B)** Normalized gene expression of specific M1 (TNFα, iNOS, CD86, CD38) and M2 (Arg1, FIZZ1, Ym-1, CD206) macrophage genes, determined by qRT-PCR, in WT IGF-2 (5ng/ml) pre-incubated and untreated BMDMs (n = 2). Scale bar = 100µm. Data are expressed as mean ± SEM. BMDMs: Bone-Marrow Derived Macrophages; qPCR: quantitative polymerase chain reaction; TNFα: Tumor Necrosis Factor α; INOS: inducible nitric oxide synthase: Arg1: Arginase 1; FIZZ1: found in inflammatory zone 1.

(M0), of the specific M1 and M2 targets is displayed in Figure 5. In general, LPS exposure led to an increased expression of M1 genes in all conditions, indicating a successful M1 macrophage polarisation. It should be taken into consideration that in the following result, HCl served as vehicle control. Therefore the effect of 0.05µM and 10µM HCl should be taken into account for IGF-2 conditions at 5ng/ml and 1000ng/ml, respectively. Overall, the treatment with IGF-2 at a concentration of 5ng/ml led to a downward trend in the expression of pro-inflammatory markers when cells were simultaneously exposed to LPS. A similar trend could be observed when cells were treated with a higher concentration of IGF-2 (1000ng/ml). However, a higher concentration of IGF-2 (1000ng/ml) demonstrated less anti-inflammatory effect compared to 5ng/ml IGF-2 as the reduction in gene expression seemed to be mostly induced by HCl (10µM) (Fig. 5A). On the contrary, the expression of anti-inflammatory targets showed less consistency as only the expression of the genes Arg1 and CD206 were upregulated when exposed to 5ng/ml IGF-2 (Fig 5B). When cells were stimulated with IL-4, gene expression of the M2 target overall increased (data not shown), indicating a successful M2 macrophage polarisation. Moreover, it can be noticed that the simultaneous exposure to IL-4 and IGF-2 (5ng/ml) led to an upward trend in the gene expression of the M2 targets FIZZ1 and Ym-1compared to the IL-4 treated control (Fig. 5C).

A similar experiment was performed in which BMDMs were stimulated first with either LPS or IL-4 and afterwards treated with IGF-2. Trends seen for simultaneous stimulation, did here not return for the gene expression of M1 targets when cells were treated with 5ng/ml of IGF-2. In contrast, the treatment with a higher concentration of IGF-2 (1000ng/ml) created a descending trend in the

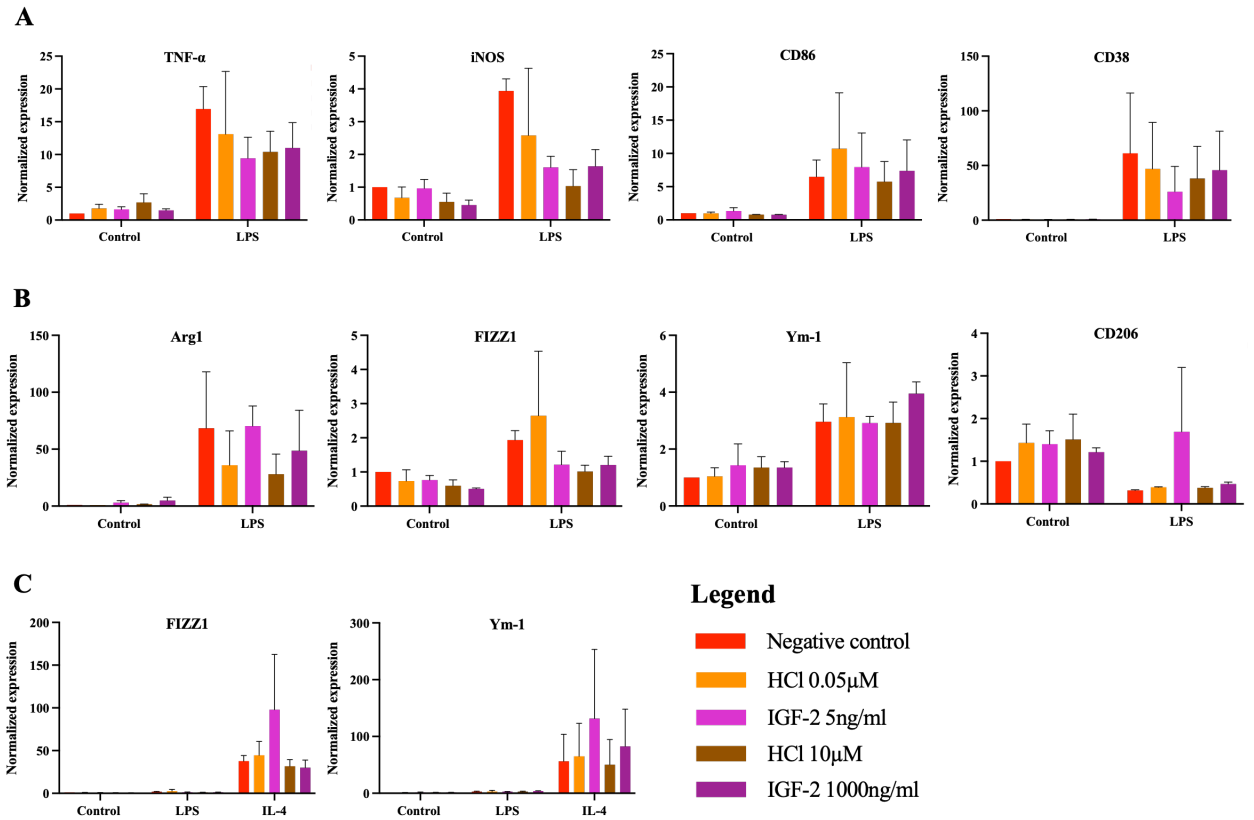

Figure 5. The immunomodulatory effect of simultaneous WT IGF-2 exposure to BMDMs (A) Normalised gene expression, determined by qRT-PCR, of specific M1 macrophage genes in BMDMs simultaneously exposed to LPS and test compounds (n = 2) **(B)** Normalised gene expression, determined by qPCR, of specific M2 macrophage genes in BMDMs simultaneously exposed to LPS and test compounds BMDMs (n =2) **(C)** Normalised gene expression, determined by qPCR, of specific M2 macrophage genes in BMDMs simultaneously exposed to IL-4 and test compounds (n = 2). Data are expressed as mean ± SEM. Bone-Marrow Derived Macrophages; qPCR: quantitative polymerase chain reaction; TNFα: Tumor Necrosis Factor α; INOS: inducible nitric oxide synthase: Arg1: Arginase 1; FIZZ1: found in inflammatory zone 1.

expression of M1 genes TNFα, CD86, and CD38 (Fig. 6A). For the expression of M2 genes, the treatment with a low concentration IGF-2 only created a slight upward trend in the expression of Arg1 and Ym-1 (Fig. 6B).

Angiogenic evaluation of wild type and Des[1-6] IGF-2 - Another aspect of neuroregeneration is the formation of new blood vessels, or angiogenesis, to supply the newly formed neurons with oxygen and other nutrients. Immunohistological stainings confirmed the presence of all three receptors for IGF-2 on both HMEC-1 and HUVECs (Fig. 7A). Next, three independent CAM assays evaluated the paracrine potential of both WT and Des[1-6] IGF-2 to stimulate angiogenesis. Therefore fertilised eggs were incubated with Matrigel droplets containing the test compounds on their CAM. Afterwards, eggs were photographed, and the number of blood vessels growing towards the test compounds was determined at a radius of 4mm. For these repetitions, different compound-Matrigel ratios and total volumes were tested. It should be taken into consideration that for these experiments, HCl served as vehicle control for IGF-2 conditions, and therefore the effect of HCl should be taken into account. First, a 1:3 ratio and a total volume of 20µl were tested. Results from his first experiment showed an upward trend in angiogenesis compared to the negative control when CAMs were incubated with Des[1-6] IGF-2 (Fig. 7C). However, a similar trend could be observed for the HCl condition, creating the suspicion that HCl itself interfered with our compound and/or angiogenesis. Therefore a pH experiment was performed (data not shown) that revealed a considerably low pH (2 - 3.5) for HCl-containing conditions. Consequently, pH levels were buffered (± 7) for the following experiments by diluting compounds in PBS first. Next, a 1:1 ratio in combination with a total volume of 20µl was tested, which led to a minor upward trend in angiogenesis for both WT and Des[1-6] IGF-2 when taking the counteracting effect of HCl into account (Fig. 7D). Results from the last experiment, in which a 1:1 ratio was tested in combination with a total volume of 30µl, showed no trend in any of the tested conditions (Fig. 7E).

DISCUSSION

Stroke still remains one of the leading causes of death and severe disability. This condition affects 13.7 million people worldwide, of which the majority are ischemic strokes (1). The currently available treatments for ischemic stroke fail to reach

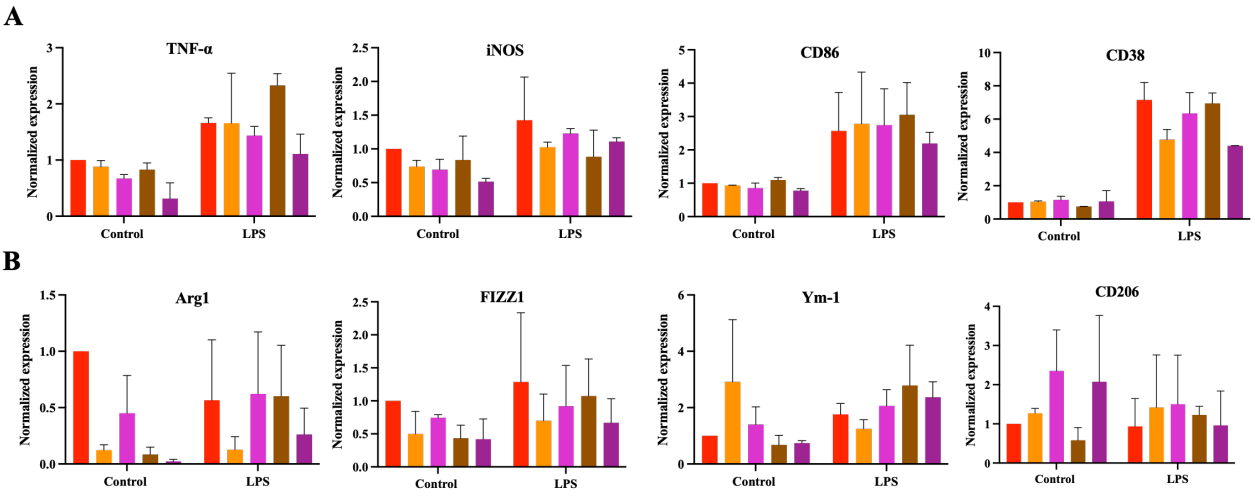

Figure 6. The immunomodulatory effect of WT IGF-2 exposure to BMDMs after cytokine stimulation. (A) Normalised gene expression, determined by qPCR, of specific M1 macrophage genes in BMDMs exposed to LPS followed by exposure to test compounds (n = 2) **(B)** Normalised gene expression, determined by qRT-PCR, of specific M2 macrophage genes in BMDMs exposed to LPS followed by exposure to test compounds BMDMs (n = 2). Data are expressed as mean ± SEM. Bone-Marrow Derived Macrophages; qPCR: quantitative polymerase chain reaction; TNFα: Tumor Necrosis Factor α; INOS: inducible nitric oxide synthase: Arg1: Arginase 1; FIZZ1: found in inflammatory zone 1.

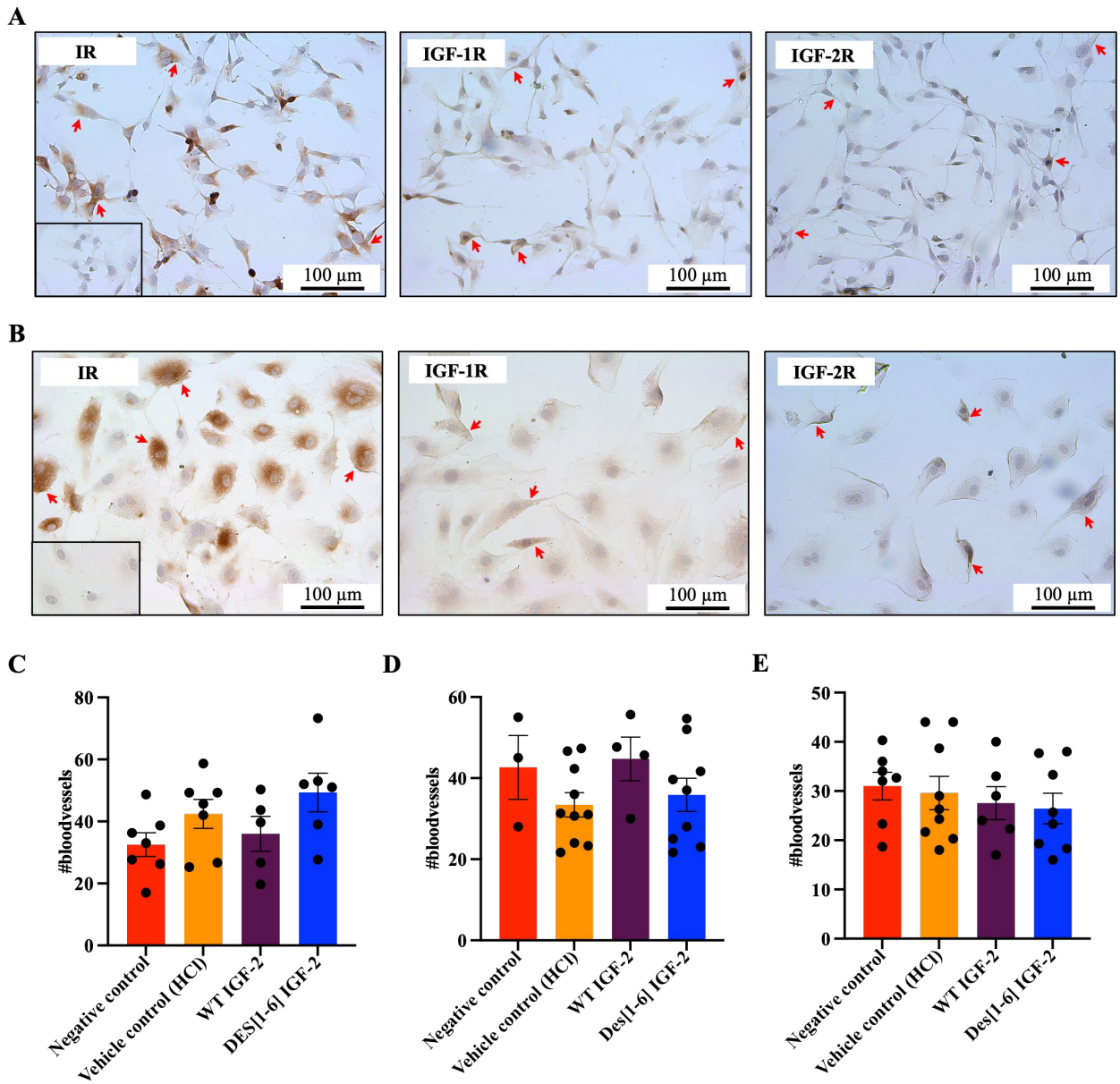

Figure 7. The angiogenic potential of WT and Des[1-6] IGF-2 (A) Immunohistochemical staining on HMEC-1, showing the presence of the IR (left panel), IGF-1R (middle panel), and the IGF-2R (right panel). Insert shows the negative control. Receptor presence is indicated by red arrows **(B)** Immunohistochemical staining on HUVECs, showing the presence of the IR (left panel), IGF-1R (middle panel), and the IGF-2R (right panel). Insert shows the negative control. Receptor presence is indicated by red arrows **(C)** Quantification of the number of blood vessels intersecting a circle with 8mm diameter. Compounds were added in a 1/3 compound-Matrigel ratio, and a total volume of 20µl was loaded on a plastic disc and placed on the developing CAM. Compounds included sterile water as negative control (n = 7), HCl (n = 7), WT IGF-2 (n = 5), and Des[1-6] IGF-2 (n = 6) **(D)** Quantification of the number of blood vessels intersecting a circle with 8mm diameter. Compounds were added in a 1/1 compound-Matrigel ratio, and a total volume of 20µl was loaded on a plastic disc and placed on the developing CAM. Compounds included sterile water as negative control (n = 3), HCl (n = 10), WT IGF-2 (n = 4), and Des[1-6] IGF-2 (n = 9) **(E)** Quantification of the number of blood vessels intersecting a circle with 8mm diameter. Compounds were added in a 1/1 compound-Matrigel ratio, and a total volume of 30µl was loaded on a plastic disc and placed on the developing CAM. Compounds included sterile water as negative control (n = 7), HCl (n = 9), WT IGF-2 (n = 6), and Des[1-6] IGF-2 (n = 8) Scale bar = 100µm. D*ata are expressed as mean ± SEM*. CAM: chicken chorioallantoic membrane assay.

all patients due to the short therapeutic window and potential risk factors. Moreover, these treatments are missing to target neuroregeneration and thus only provide neuroprotection by preventing further

damage. Therefore, the current study focused on investigating IGF-2 and its structural variant Des[1-6] IGF-2 to serve as interventional products for a neuroprotective and -regenerative treatment for ischemic stroke. More specifically, these compounds were evaluated based on their neurogenic, immunomodulatory, and angiogenic potential.

To study the neurogenic capacity of WT and Des[1-6] IGF-2, murine NSCs were used in several *in vitro* assays. NSC proliferation provides a pool of neural progenitors necessary to give rise to sufficient mature neurons to replace the lost ones. The involvement of the IGF system in NSC maintenance and proliferation has already been demonstrated in numerous studies. Ziegler et al., for example, established the importance of the IR in the self-renewal of NSCs and the capacity of IGF-2 to induce this effect via the IR (38). These findings were corroborated by a recent study by Chidambaram et al., who found a reduced number of NSCs in the SVZ of IR-deleted adult mice accompanied by an aberrant olfactory bulb neurogenesis (39). In addition, Lehtinen et al. showed that CSF-induced growth of NSCs is highly IGF-2-dependent (40). Additional research by Ziegler et al. suggested that the proliferative, next to the self-renewing capacity effect of IGF-2, was primarily mediated through the IR (41). The same research group verified the importance of IGF-2 in the maintenance of NSCs in the SVZ and SGZ, as IGF-2 deletions led to a considerable reduction of the adult stem cell pool, including the NSCs of both niches (42). Results from this current study can confirm the possibility that the effects of IGF-2 are mediated through multiple receptors, as we demonstrated that all three known receptors for this compound are present on NSCs in culture. Moreover, WT IGF-2 administration creates a trend of NSC expansion in a dose-dependent manner. Although an obvious trend can be seen, no significant differences could be obtained, which potentially could be attributed to the high variation among the separate runs.

Following the expansion of the NSCs pool, the migration of these cells will be key to potentially providing a neuroregenerative response (14). The involvement of IGF-1 in this process has previously already been demonstrated. Research on olfactory bulb neurogenesis, for example, has observed an enhanced neuroblast migration in murine rostral migratory stream (RMS) explants when exposed to IGF-1. Moreover, it was seen that IGF-1 deficient mice accumulated neuroblasts in their SVZ. This emphasises the role of IGF-1 in the migration of neuroblasts and suggests that IGF-1 is not essential for the proliferation of these cells (43). These results are supported by a study conducted in the neuroblastoma cell line (SH-SY5Y cells). Here, IGF-1 promoted neuroblast chemotaxis in a dose-dependent fashion. In addition, did selective inhibition of the IGF-1R lead to reduced cellular motility, suggesting that activation of the IGF-1R triggers pro-migratory downstream cascades (44). However, the migratory potential of IGF-2 has not been reported to our knowledge. With the current study, we were the first to demonstrate that WT IGF-2, at a high concentration, serves as a potent chemoattractant to promote the migration of NSCs. In contrast, pre-incubation with WT IGF-2 created a downward trend in migration compared to untreated cells. It is known that ligand binding with the IGF-2R leads to rapid internalisation and degradation of this receptor (26). Therefore it can be hypothesised that pre-incubating of our cells created a negative feedback loop, limiting the receptor presence and thus lowering cellular responsiveness for IGF-2 at a later stage. Furthermore, it was discovered in cancer research that overexpressing the IGF-2R led to a decreased motility of cancer cells, while the reduction of IGF-2R led to increased cellular motility (45, 46). It is plausible that these findings rely upon the proposed IGF-2 scavenging function of the IGF-2R. Moreover, these findings indicate that the migratory effects of IGF-2 are primarily mediated by interaction with the IR and IGF-1R and not with the IGF-2R. Additional cancer research found evidence of IGF-2 stimulating the motility of sarcoma cells through interaction with the isoform A of the IR, independently of the IGF-1R (47). Secondly, results from an additional migration assay (scratch assay) provided evidence that besides intervening as a chemoattractant, WT IGF-2 directly interacts with NSCs to promote their migration. Nevertheless, should it be taken into consideration that this experiment was only conducted once, and more repetitions must be performed to confirm this assumption. Moreover, the mechanism by which IGF-2 exerts this effect remains unknown. Further research should elucidate which receptors are addressed during IGF-2 mediated migration of NSCs and unravel the

involved intracellular cascades. Given the findings in cancer research and the role of the IGF-2R in regulating the bioavailability of IGF-2, priority should be given to the IR and IGF-1R in following investigations. Nevertheless, it should be confirmed first whether selectively disabling the IGF-2R in NSCs promotes cellular motility, as seen in sarcoma cells, to exclude its involvement in NSC migration (46). Moreover, IGF-2 analogues that selectively bind the IGF-2R, such as [Leu^{27}] IGF-2, could be used to support previous findings. Next, NSC motility, in response to exogenous IGF-2, should be compared when the IR and IGF-1R are individually inhibited. Receptor inhibition could be obtained by using specific receptor antibodies that prevent receptor-IGF-2 interaction. Receptor involvement should be further confirmed by investigating their activation by determining the phosphorylation status of the IR, IGF-1R, and their downstream mediators such as p44/42 MAPK and PI-3 kinase. These experiments will give insight into which receptor is addressed when administering a range of IGF-2 concentrations. Lastly, it would be of interest to investigate the effect of treating NSCs with exogenous IGF-2 while blocking the IGF-2R activation. This would provide insights into the way that this receptor influences IGF-2 bioavailability.

IGFs occur naturally almost completely bound to one of the IGFBP family members. Although the exact function of these proteins remains unclear, it can be suggested that they bind IGFs to modulate or prevent the interaction with their receptors (28). Our results give an indication for this IGFBP function, as co-administration of WT IGF-2 and IGFBP-6 led to both a decrease in NSC proliferation and transmigration compared to when WT IGF-2 was administered alone. Additionally, this decrease showed to be dependent on the given dose of IGFBP-6. Again, it should be noted that this experiment was only conducted once or twice, and further repetitions should confirm the consistence of the given results. Given the possible function of IGFBPs, Des[1-6] IGF-2, a structural variant lacking the first six amino acids in its N-terminal, was included in this research. Due to this structural difference with WT IGF-2, this variant is ought to exist in the circulation in an unbound state and is thus expected to be freely available (30). Despite this, Des[1-6] IGF-2 did not show the same potential to promote either NSC proliferation or migration as the WT. One can suggest that these differences arise due to a decreased receptor binding, which has already been reported previously by Francis et al.. They determined the effective dose for half-maximal competition to binding of the tracer (ED_{50}) with the IGF-1R and IGF-2R in L6 myoblasts. These experiments demonstrated that Des[1-6] IGF-2 exhibits an ED_{50} almost twofold of the WT ED_{50} (4.1 vs. 2.09 nM). Similarly, Des[1-6] IGF-2 showed to be less than half as potent as WT IGF-2 in binding the IGF-2R, with ED_{50} values of 17 nM and 6.6 nM, respectively. In addition, for the receptor binding potency of the IR, tested in H35B hepatoma cells, Des[1-6] IGF-2 was more than seven times less potent than the WT (30). Similar results of reduced receptor binding of IGF-2 variants were seen in a study by Luthi et al.. Here, the receptor affinity of Des[1-3 IGF-2] for the IGF-2R was only 25% of the affinity of the WT compound. In IGF-1R binding assays, this variant showed a sixfold lower affinity compared to WT IGF-2. They attributed this to a local disturbance in a specific region (A-domain region) of the IGF-2 molecule that is involved in receptor binding (48). Another explanation for our results could be found in the possibility of a compromised stability of the used compound.

The co-administration of Des[1-6] IGF-2 and IGFBP-6 resulted in some contradictory results, as the addition of IGFBP-6 at a low dose (100ng/ml) led to a decrease in both NSC migration and transmigration compared to Des[1-6] IGF-2 alone. Besides this, was there an effect on proliferation (negative) and migration (positive) compared to the negative control when IGFBP-6 was administered alone. Although IGFBPs were initially thought to interact with IGFs solely, these proteins have been reported to have IGF-independent actions. Examples are found where IGFBP-6 entered the nucleus, subsequently altering cell fate in terms of cell proliferation, migration, and survival (49-51). Again, experiments concerning IGFBP-6 were only performed once or twice and should be repeated to identify consistent results before any conclusions can be made.

Besides stimulating the generation of new neurons, providing neuroprotection to restrict further injury to the brain parenchyma, remains of importance. This can be achieved by reducing the strong neuroinflammatory reaction which is observed after cerebral ischemia. The acute neuroinflammation causes secondary damage,

provoking cellular death, while in the more chronic phase after stroke, it has beneficial effects on the recovery by initiating reparative processes (9, 52). One of the key players in determining the path of this reaction are macrophages. These cells are known to dynamically present themselves in two distinct phenotypes; pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages that secrete several factors capable of promoting the repair and regeneration of cerebral tissue (8). Previous studies have already sought to use IGF-2 as a stimulus to polarise macrophages into the anti-inflammatory phenotype. Du et al., for example, reported that IGF-2 had a critical role in pre-programming macrophages during their maturation process, shaping their anti-inflammatory properties. Expression levels of the pro-inflammatory markers Interleukin 1ß (IL-1ß) and iNOS were reduced while programmed death-ligand 1 (PD-L1) expression increased. However, these results could not be seen in mature macrophages (53). The current study was unsuccessful in obtaining similar outcomes for pre-programmed macrophages as little to no differences in expression levels of pro- and anti-inflammatory markers were seen. Moreover, were we unable to validate other experiments from Du et al., which showed similar effects of IGF-2 on the expression profile of maturing THP-1 cells (data not shown, Supplemental Methods). It could be argumented that other genes of interest were investigated in our study and that IGF-2 interferes with other signalling pathways causing the up- or down-regulation of only a specific subset of macrophage markers. Another study, performed by Wang et al., demonstrated dual opposing roles for IGF-2 in determining the macrophage phenotype. In this study, the influence of both low and high concentrations of IGF-2 on macrophage expression profiles was determined. Exposure to low concentrations created an anti-inflammatory status of macrophages, characterised by reduced expression of pro-inflammatory markers and an enhanced expression of anti-inflammatory markers. On the other hand, a high concentration of IGF-2 created opposing results as it promoted the expression of some pro-inflammatory markers (not all) and hardly influenced the expression of anti-inflammatory markers. Additionally to these findings, they established that the effects of a high IGF-2 concentration and thus promotion of pro-inflammatory macrophages are mediated through activation of the IGF-1R, while the effects of low IGF-2 concentrations are mediated through IGF-2R activation (54). Results from our study confirmed that primary macrophages could be responsive to IGF-2 as all three receptors are highly expressed. Although effects of IGF-2 exposure in our study resulted in less pronounced effects compared to the study by Wang et al., we could observe similar effects when cells were simultaneously treated with IGF-2 and LPS. Treatment with low-dose IGF-2 (5ng/ml) did induce a downward trend in the expression level of pro-inflammatory markers, while the expression of anti-inflammatory markers seemed to be upregulated. High-dose IGF-2 (1000ng/ml) on the other hand, showed little to no or contradictory results compared to the low dose. These effects were rather diminished when cells were treated with IGF-2 after LPS exposure. This could possibly be attributed to intrinsic regulation of the cells' restoring gene expression and therefore attenuating potential effects of IGF-2.

Dose-dependence of the IGF-system was additionally seen in an *in vivo* study performed by Mackay et al., investigating the neuroprotective effects of IGF-1 and IGF-2 administration after ischemic stroke in rats. Although IGF-1 was previously already reported to elicit neuroprotective effects *in vivo*, this study was the first to establish a similar effect with the treatment of IGF-2. Intracerebroventricular (ICV) administration of IGF-2 showed to be as effective as IGF-1 at reducing the total lesion volume. Interestingly, the most effective dose showed to be different for both IGFs. While IGF-1 had the most favourable effects at a high dose (50µg), while a low dose of IGF-2 (5-10ng) showed the largest reduction in lesion volume (55). For future research by our research group, these results will be validated in a mouse model of ischemic stroke. Still, these results again demonstrate the importance of IGF dosage *in vivo*. Nevertheless, more research will be necessary to unravel the complex interplay of IGFs, their receptors, and IGFBPs. For further investigations of immunomodulatory effects of IGF-2 specifically, the current experiment of simultaneously treating BMDMs with LPS or Il-4 and IGF-2 should be repeated with a broader range of IGF-2 concentrations and including the Des[1-6] variant, both in combination with IGFBP-6. Besides qRT-PCR analysis of specific macrophage markers,

analysis of the conditioned media could be performed. For example measuring the production of the pro-inflammatory mediator, nitric oxide (NO), using the Griess assay. This could provide a better understanding of how IGF-2 can influence the inflammatory secretion behaviour of macrophages. Previously it was described that M2 macrophages themselves produce IGF-1 and create an autocrine pathway to maintain this subtype through the interaction with the IGF-1R (56, 57). Whether these macrophages also produce IGF-2, however, remains unknown to our knowledge. With the current study we showed that IGF-2 treatment maintains the M2 expression profile and even upregulates some M2 markers. Therefor it would be of interest to compare secreted levels of IGF-2 in IL-4 stimulated macrophages with those of unstimulated macrophages. Another aspect would be to determine the effect of the IGF-2R presence on the bioavailability of IGF-2 and immunomodulatory effects after its exogenous administration. This could be achieved by simultaneously blocking the IGF-2R and repeating the current experimental setup. Analysing the conditioned medium afterwards with an ELISA, should determine possible differences of the IGF-2 bioavailability. Additionally, receptor involvement could be demonstrated by determining the phosphorylation status of the IR, IGF-1R, and their downstream mediators through Western Blot.

Lastly, the formation of new blood vessels in the process of angiogenesis will be key to supporting neuroregeneration. Post-mortem research of ischemic stroke patients emphasised the importance of angiogenesis as increased neovascularisation was correlated with longer survival of patients (22). Multiple studies have already indicated the potential of IGFs and their receptors to promote key events in angiogenesis. An *in vivo* study showed that postischemic gene transfer of IGF-1 enhanced the vascular density, associated with improved perfusion, in the peri-infarcted area (35). In addition, Zhang et al. established that the IGF-1R is required to mediate the angiogenic potential of a plant-derived compound (58). The importance of the IGF-1R already indicates a possible role for IGF-2, as its ligand, in promoting neovascularization. Dallinga et al. investigated the involvement of IGF-2 and the IGF-1R in a key step of angiogenesis, called sprouting. They discovered that knocking down these genes both led to a reduced fraction of tips cells (essential for sprouting) with diminished sprouting as a consequence, both *in vitro* and i*n vivo* (59). However, reports concerning the influence of exogenous administration of IGF-2 on angiogenesis by other groups are non-existing to our knowledge. Previous research by our group already demonstrated the capacity of IGF-2 to promote angiogenesis *in ovo* using the CAM assay (60). With the present study, however, these results could not be reobtained as IGF-2 did not significantly enhance the formation of new blood vessels. Nonetheless, it should be brought to attention that methods slightly differed as IGF-2 previously was diluted in concentrated Dulbecco's Modified Eagle Medium (DMEM) before diluting in Matrigel. The possibility exists that the stability of IGF-2 was compromised in the current study, and potential effects were therefore not detected. Future CAM assay should be performed where both methods are applied and compared. In addition, alternative delivery systems, such as the use of methylcellulose disks or fibrin matrices, should be explored (61). Lastly, the current study did show the presence of all three receptors for IGF-2 on both HUVECs and HMEC-1 cells, indicating their possible responsiveness for this compound and its derivates. Future investigations should be conducted to determine the effect of IGF-2 administration on these cells' proliferation, migration, and tube formation, as these processes are key to providing an angiogenic response.

CONCLUSION

In this project, the potential of IGF-2 and its structural variant Des[1-6] IGF-2 to provide a neuroprotective and -regenerative response. We demonstrated the dose-dependence of these compounds in NSC expansion and migration, both key processes for neurogenesis. The same dose-dependence recurred in immunomodulatory effect of WT IGF-2. Moreover, the finding of IGFBP-6 lowering WT IGF-2 neurogenic potential, opens opportunities of intervening at the level of these proteins in future investigations. In conclusion, our results provide fundamental evidence for future use of IGF-2 and its derivates in the treatment of ischemic stroke. However, extensive research is further needed to confirm and validate these results, elucidate the mechanism of actions, and the exact effective dose in each separate process.

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SUPPLEMENTAL METHODS

Optimisations for NSC growth and migration assays - A series of optimisations were performed to identify the optimal combination of cell seeding density and medium composition. More specifically, the medium compositions for the compound dilutions were alternated at the concentration of B-27 (-VitA), and the growth factors rh EGF and rh FGF-2. For the NSC growth assays, cell seeding densities of 5 000 and 10 000 cells/well were tested in combination with growth media supplemented with 0.2% or 2% B-27 and the full dose of growth factors (20ng/ml). The transwell migration assay was optimised by alternating cell seeding densities of 3 000 and 5 000 cells/insert in combination with growth media supplemented with 0.2% or 2% B-27 and containing either the full dose (20ng/ml), half dose (10ng/ml), or no growth factors. The optimal conditions were then selected based on cell viability, growth and migration in the negative medium control condition. An overview of the optimalisation processes of both the growth and transwell migration assays can be found in Tables S1 and S2.

THP-1 Cell differentiation and gene expression - THP-1 cells, derived from an acute monocytic leukaemia patient, were a kind gift from the research group of Prof. Dr. Jerome Hendriks. These cells were cultured in RPMI-1640 medium (R5886, Sigma Aldrich, Missouri, USA), supplemented with 10% Fetal Bovine Serum (FBS) (s181B, Biowest, Nuaillé, France), and 100U/ml penicillin-streptomycin (P4333, Sigma Aldrich, Missouri, USA). Subculturing was performed when cells reached a concentration of 1-2 million cells/ml. THP-1 cells were plated at 1 x 10^6 cells per well in a 6-well plate. In general, THP-1 cells were stimulated to form resting macrophages (M0) by 24h exposure to 5ng/ml phorbol 12-myristate 13-acetate (PMA) (1201, Tocris Bioscience, Bristol, UK). Treatment, with test conditions, of the cells occurred either before (pre-programming) or after PMA stimulation. Test conditions included 5ng/ml IGF-2 (11343575, Immunotools, Friesoythe, Germany) and 50µM HCl, and were added for 48h. After PMA stimulation, cells were stimulated with either 250ng/ml LPS and 20ng/ml recombinant human interferon-gamma (IFN-γ) (300-02, PeproTech, New Jersey, USA), or 20ng/ml IL-4 for a period of 6h to induce differentiation to M1 or M2 macrophages, respectively. In addition, sterile water was included as a test condition to evaluate the success of macrophage differentiation later on. After cytokine stimulation, cells were collected, and total RNA was isolated using the RNeasy Mini Kit (74104, Qiagen, Hilden, Germany) following the manufacturer's instructions. Then cDNA was synthesised from the isolated RNA using qScript cDNA SuperMix and further used for a quantitative polymerase chain reaction (qPCR) performed on a QuantStudio 3, using Fast SYBR™ Green Master Mix. Primers were purchased at Integrated DNA Technologies (Iowa, USA) and their sequences are displayed in Table S2. Relative mRNA transcript levels were standardised to their internal expression of the household genes TATA-Box-Binding Protein (TBP), 60S ribosomal protein L37a (RPL37A), and beta-actin (act-ß) and then to their control conditions.

SUPPLEMENTAL FIGURES

Table 1: Optimalisation process determining optimal conditions for NSC growth assays

Date	Cell origin	# seeded cells	Medium conditions	Tested conditions	Remark	Conclusion
7/12/2021	P19#24	5k and 10k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1µM); IGF-2 (Gropep) (1, 10, 100ng/ml)	Cells were not in a optimal condition	Full run excluded
14/12/2021	P18#11	5k and 10k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1µM); IGF-2 (Gropep) (1, 10, 100ng/ml)	Cells were not in a optimal condition	Full run excluded
11/01/2022	P18#22	5k and 10k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml) IGF-2 (Immunotools) (1, 10, 100, 1000ng/ml)	Both cell seeding densities show similar growth curves + 1000ng/ml IGF-2 shows larger differences than lower concentrations	Exclude the seeding density of 10k to limit overusing cells
25/01/2022	P18#13	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); IGF-2 (Immunotools) (1, 10, 100, 1000ng/ml)	HCl controle conditions were not included	Full run excluded
18/02/2022	P18#15	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); IGF-2 - HCl compensated (1, 10, 100ng/ml); IGF-2 (Immunotools) (1, 10, 100, 1000ng/ml)	IGF-2 (Immunotools) conditions have no influence in 0.2% B27 medium conditions	Retest all conditions
24/02/2022	P18#12	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); IGF-2 - HCl compensated (1, 10, 100ng/ml); IGF-2 (Immunotools) (1, 10, 100, 1000ng/ml)	IGF-2 (Immunotools) conditions are inconsistent + cells were not in a optimal condition from the start on	Full run excluded
24/02/2022	P18#15	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); IGF-2 - HCl compensated (1, 10, 100ng/ml); IGF-2 (Immunotools) (1, 10, 100, 1000ng/ml)	IGF-2 (Immunotools) conditions are inconsistent	Excluded Immunotools for future runs
3/03/2022	P18#13	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml)	/	Repeat current conditions
16/03/2022	P19#22	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml)	Majority of the cells died	Full run excluded
18/03/2022	P19#16	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml)	Cells were not in a optimal condition and did not reach 100% confluence like other runs	Full run excluded
8/04/2022	P18#17	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); Des[1-6] IGF-2 (100; 1000ng/ml)	Starting density was higher and inconsistent compared to other runs and creates misleading results	Full run excluded
12/04/2022	P18#18	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); Des[1-6] IGF-2 (100; 1000ng/ml)	Both 0.2% and 2% B27 medium show to be suitable for growth assays but 2% B27 seems to evoke a more desired growth curve including a exponential and plateau phase	Repeat current conditions to determine consistency
12/04/2022	P19#23	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); Des[1-6] IGF-2 (100; 1000ng/ml)	Difference in growth curve course between 0.2% and 2% B27 remains conisitent through the runs	Exclude 0.2% B27 runs
19/04/2022	P18#20	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); Des[1-6] IGF-2 (100; 1000ng/ml)	Difference in growth curve course between 0.2% and 2% B27 remains conisitent through the runs	Exclude 0.2% B27 runs
19/04/2022	P19#25	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); Des[1-6] IGF-2 (100; 1000ng/ml)	Difference in growth curve course between 0.2% and 2% B27 remains conisitent through the runs	Exclude 0.2% B27 runs
25/04/2022	P18#22	5k	2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); Des[1-6] IGF-2 (100; 1000ng/ml);	/	Repeat and determine effect of IGFBP-6
13/05/2022	P18#16	5k	2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); Des[1-6] IGF-2 (100; 1000ng/ml); 1000ng/ml of WT and Des[1-6] in combination with IGFBP-6 (100; 1000ng/ml)	/	/
17/05/2022	P18#17	5k	2% B27, full dose of growth factors	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); Des[1-6] IGF-2 (100; 1000ng/ml); 1000ng/ml of WT and Des[1-6] in combination with IGFBP-6 (100; 1000ng/ml)	/	/

Table 2: Optimalisation process determining optimal conditions for NSC Transwell migration assay

Date	Cell origin	# seeded cells	Medium conditions	Pre-incubated	Tested conditions	Remark	Conclusion
10/12/2021	P19#25	3k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	/	Negative control (growth medium); HCl (0.01, 0.1, 1µM); IGF-2 (Gropep) (1, 10, 100ng/ml)	Cells died or did not migrate in any condition	Number of seeded cells could be too low as cells require contact. Test a higher number of seeded cells. Full run excluded
7/03/2022	P19#20	5k	0.2% B27, full dose of growth factors; 2% B27, full dose of growth factors	/	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml)	Cells in 2% B27 growth medium died	Exclude 2% B27 growth medium conditions in future runs
28/03/2022	P18#15	5k	2% B27, full dose of growth factors	Untreated IGF-2 (100ng/ml) HCl (1µM)	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml)	/	/
28/03/2022	P19#20	5k	2% B27, full dose of growth factors	Untreated IGF-2 (100ng/ml) HCl (1µM)	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml)	/	/
4/04/2022	P18#16	5k	2% B27, full dose of growth factors	/	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml)	/	/
4/04/2022	P19#21	5k	2% B27, full dose of growth factors	/	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml)	All cells died	Full run excluded
13/05/2022	P19#20	5k	2% B27, full dose of growth factors	/	Negative control (growth medium); HCl (0.01, 0.1, 1, 10µM); IGF-2 (Gropep) (1, 10, 100, 1000ng/ml); Des[1-6] IGF-2 (100; 1000ng/ml); 1000ng/ml of WT and Des[1-6] in combination with IGFBP-6 (100; 1000ng/ml)	/	/

Table S3: Primer sequences for qPCR analysis of BMDMs

Gene	***Forward sequence (3' -- 5')***	***Reverse sequence (5'-- 3')***
iNOS	*CCC TTC AAT GGt TGG TAC ATG G*	*ACA TTG ATC TCC GTG ACA GCC*
TNFα	*GTC CCC AAA GGG ATG AGA AGT*	*TTT GCT ACG ACG TGG GCT AC*
CD86	*GAG CGG ATA GT AAC GCT GA*	*GGC TCT CAC TGC CTT CAC TC*
CD38	*ACT GGA GAG CCT ACC ACG AA*	*TGG GCC AGG TGT TTG GAT TT*
Arg1	*GTG AAG AAC CCA CGG TCT GT*	*GCC AGA GAT GCT TCC AAC TG*
FIZZ1	*TCC AGC TAA CTA TCC CTC CAC TGT*	*GGC CCA TCT GTT CAT Agt CTT GA*
Ym1	*GGG CAT ACC TTT ATC CTG AG*	*CCA CTG AAG TCA TCC ATG TC*
CD206	*CTA CAA GGG ATC GGG TTT ATG GA*	*TTG GCA TTG CCT AGT AGC GTA*
CycA	*TAT CTG CAC TGC CAA GAC TGA GTG*	*CTT CTT GCT GGT CTT GCC ATT CC*
HPRT	*CTC ATG GAC TGA TTA TGG ACA GGA C*	*GCA GGT CAG CAA AGA ACT TAT AGC C*

Table S4: Primer sequences for qPCR analysis of THP-1 cells

Gene	*Forward sequence (3' -- 5')*	*Reverse sequence (5'-- 3')*
RPL37A	*ATT GAA ATC AGC CAG CAC GC*	*AGG AAC CAC AGT GCC AGA TCC*
Act-B	*ATT GCC GAC AGG ATG CAG AA*	*GCT GAT CCA CAT CTG GAA*
PD-L1	*ACT GTG AAA GTC AAT GCC CC*	*TGC TCC AGA TGA CTT CG*
iNOS	*CCT GAG CTC TTC GAA ATC CCA*	*CCC GAA ACC ACT CGT ATT TGG*
IL-1B	*ATG ATG CGT TAT TAC AGT GGC AA*	*GTC GGA GAT TCG TAG CTG GA*
IL-6	*ACT CAC CTC TTC AGA ACG AAT TG*	*CCA TCT TTG GAA GGT TCA GGT TG*
HLADR	*GAG CAA GAT GCT GAG TGG AGT C*	*CTG TTG GCT GAA GTC CAG AGT G*
CD206	*CTA CAA GGG ATC GGG TTT ATG GA*	*TTG GCA TTG CCT AGT AGC GTA*
Arg1	*GTG GAA ACT TGC ATG GAC AAC*	*CCT GGC ACA TCG GGA ATC TTT*
FIZZ	*AAC CCG GGG AGT ACT CAG TGT*	*GCA CAG CCA GTG ACA GCC AT*
TBP	*TAT AAT CCC AAG CGG TTT GC*	*GCT GGA AAA CCC AAC TTC TG*

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