Exploring the long term viability of donor Schwann Cells in PEG fused allografts

NEU 365N Term Paper

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Abstract

Since the initial discoveries regarding the fusogenic properties of polyethylene glycol (PEG), many potential clinical applications have been investigated. PEG fusion can be applied to lesions in the peripheral nervous system, repairing transections as well as fusing allografts prior to the onset of Wallerian degeneration. PEG fused allografts have exhibited an unusual phenomenon of immunotolerance despite existing in a non-immune privileged environment in the PNS. This immunotolerance occurs even without the administration of immunosuppressant drugs in laboratory subjects. Conventional PEG fused allografts must be paired with systemic immunosuppression treatment or be sterilized of all cellular elements except for the extracellular matrix in order to be viable. The mechanisms and implications of this immunotolerance phenomenon in PEG fused allografts is not completely understood. Current studies with viable PEG fused allografts do not examine the behavior of glial cells over time. Specifically, it is unknown whether Schwann cell populations in the donor allograft survive over the long term. Schwann cells are the primary glial cell of the PNS involved in myelination of axons as well as mediating the immune response due to their expression of MHC II proteins. This paper examines whether donor Schwann cells are viable over the long term in PEG fused allografts or if they degrade over time and get replaced by local host Schwann cells. In this study, viable sciatic nerve allografts were transplanted and PEG fused from one strain of donor Wistar rats into another strain of host Sprague Dawley rats. After experimental periods ranging from week 1 to week 24, the sciatic nerve complexes were excised and digested by a solution of .05% type 1 collagenase. Each sample was observed using phase contrast micrographs. When compared to a control group with conventional sciatic nerve allografts, the experimental groups were evaluated for population sizes of donor vs. host Schwann cells. This protocol demonstrates whether the donor Schwann cell population survives in the allograft or if it gets slowly replaced by host Schwann cells. Results showed that PEG fused allografts showed significantly increased survival rates for donor SC's when compared to the control group, confirming the hypothesis that PEG fusion of peripheral nerve allografts lead to survival of donor SC populations. This result suggests that host Schwann cells are linked to the immune rejection response in the peripheral nervous system. The theory is supported by the fact that Schwann cells are antigen presenting cells that trigger T cell activation and downstream signaling that leads to inflammation and macrophage activity.

Background

Schwann Cells

Study of the nervous system has shown how Schwann cells have a diverse range of functions, maintaining and supporting neurons in the PNS. As the primary type of glial cell in the PNS, Schwann cells exist alongside axons in all types of peripheral nervous tissues, both motor and sensory.

Origin of Schwann cells

During early embryonic development, the entirety of the nervous system as well as skin tissue is derived from the outermost germ layer, the ectoderm. A monolayer of ectodermal cells eventually forms the neural tube, and its intermediate region is known as the neural crest. The neural crest eventually develops into the entire peripheral nervous system as well as most of the autonomous nervous system. Neural crest tissue gives rise to a diverse range of cell types in the early days of embryonic development, including the precursor cells of Schwann cells. These precursor cells are developed at around E12-13 and are multipotent in their differentiation capabilities. Other radial glial cells in the neural crest are also multipotent. The role of these precursor cells is to proliferate heavily in the developing peripheral nervous system alongside other precursor neuronal cells. This proliferation is induced by signaling cascades during the embryonic development process, indicated by greatly increased levels of cAMP in the precursor cells. Notch signaling is one of the primary signaling pathways responsible for Schwann cell precursor proliferation during embryonic development. This is due to Notch receptors on glial cells activated by Notch ligands on axons. Schwann cell precursors cannot survive outside of the presence of neurons. At around E15-16, these precursor cells differentiate into immature Schwann cells. This can be seen phenotypically with increased expression of Schwann cell specific proteins such as MBP and S100 as well as morphological differences [14]. Immature Schwann cells undergo radial sorting and are produced in a 1:1 ratio with large axons. At this stage of development, Schwann cells can survive independently of neurons. Immature Schwann cells eventually differentiate into various subtypes of mature Schwann cells, either myelinating or non-myelinating. These mature Schwann cells maintain a high degree of plasticity and are able to alter phenotypic expression. They are not completely multipotent as their precursor cells were [12].

There are two subtypes of Schwann cells, the myelinating subtype and non-myelinating subtype. Myelinating Schwann cells wrap around axons in the PNS to form the myelin sheath. The purpose of this is to facilitate faster signal transduction in the peripheral nervous system in both motor and sensory neurons. The musculoskeletal system relies on this function of Schwann cell derived myelination as it increases signal propagation speed by one to two orders of magnitude [26]. Myelinating Schwann cells are the primary subtype of Schwann cell that exists in the peripheral nervous system.

Non-myelinating Schwann cells also play a significant role in the peripheral nervous system. They are important for providing trophic support so that neurons can survive. They also assist in the maintenance and repair of axons. One type of non-myelinating Schwann cell is the Remak Schwann cell, which supports various unmyelinated axons in the PNS. Unmyelinated axons tend to be smaller and exist in bundles, often in volatile environments such as the skin. The high plasticity of Remak Schwann cells is important in the environment of the skin that is prone to constant change and damage. Axons in the musculoskeletal system tend to be long and myelinated, though they are supported by non-myelinating Schwann cells at the neuromuscular junction. Research shows that these terminal Schwann cells help to form synaptic connections at these junctions [6]. While myelinating Schwann cells surround axons, non-myelinating Schwann cells are still located in the nerve fascicle in the endoneurium.

One unique aspect of Schwann cells is that they are phenotypically unstable, unlike their CNS glial cell counterparts (oligodendrocytes) [14]. They are capable of undergoing phenotypic changes and re-differentiating at any time to change their function, which is critical to the role they play in peripheral nervous system repair. Schwann cells myelinate axons in a 1:1 ratio, whereas individual oligodendrocytes myelinate several axons in the CNS. Another hallmark that distinguishes Schwann cells from oligodendrocytes is the presence of basal lamina. Myelinated axons in the CNS are bare at the nodes of Ranvier, whereas Schwann cells still surround the nodes in the PNS [18].

Figure 1[14]

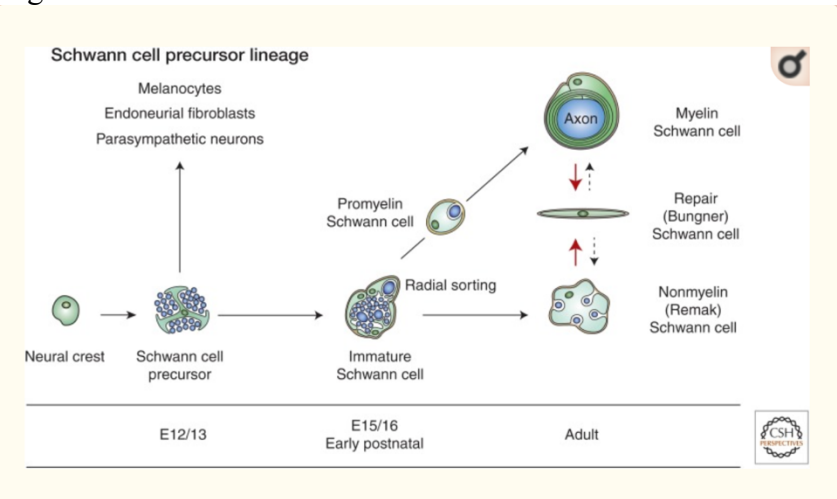


Fig 1. Lineage of differentiation from neural crest to mature Schwann cells, phenotypic plasticity is retained even at mature stage

Role as repair cells

After the onset of traumatic lesions in the peripheral nervous system, Schwann cells are the principle support cells responsible for aiding repair and guiding axonal outgrowth. When axons are transected and the distal segment loses connection with the cell body, the process of Wallerian degeneration initiates cascades of signals within neurons and surrounding cells in the nerve fascicle. The signaling environment of Schwann cells drastically changes as they lose connection to the axon and proximal cell body. Molecules associated with an inflammatory response spread in the surrounding environment, called DAMPs/PAMPs (damage/pathogen associated molecular

patterns). Schwann cells strongly express toll-like receptors that recognize these molecules, signaling them to undergo major transcriptional changes. Normal Schwann cells become what is referred to as “activated” Schwann cells through transdifferentiation. Both myelinating Schwann cells and Remak Schwann cells differentiate into repair cells. Hundreds of genes are up-regulated or down-regulated during this transdifferentiation process causing Schwann cells to phenotypically transform. Morphologically, repair Schwann cells are much longer than regular Schwann cells. The phenotype of repair Schwann cells is regulated by the H3K27 trimethylation mechanism [13].

Activated Schwann cells are partly responsible for helping clear cellular debris during Wallerian degeneration as well as recruiting macrophages to do so, in an immune-like response [27]. They proliferate through cell division triggered by signaling pathways. These signaling mechanisms are similar to those during embryonic development. Activated Schwann cells also have significantly reduced transcription of myelinating proteins, and also aid in the clearing of myelin debris.

Following denervation, Schwann cells maintain basal lamina tubes known as ‘Bands of Bunger’. Repurposed Schwann cells also secrete neurotrophins which help create an environment conducive to growth for regrowing axons. Axonal outgrowth after transections is a slow process and often lacks specificity due to misdirection. Bands of Bunger guide outgrowing axons towards the distal stump and help preserve a specific and directional nature of growth until they form connections at the neuromuscular junction. Outgrowing axons travel through these basal lamina tubes. Chronic denervation causes significant atrophy of the target muscle which ultimately prevents synapses from being re-established at the neuromuscular junction. This muscular atrophy can be delayed through administration of electrical stimulation of muscle tissue. However, this treatment must be administered periodically in order to delay atrophy and only lengthens the window temporarily. This race against time is one of the biggest problems associated with PNS repair by natural axonal outgrowth. The longer it takes, the greater the potential for poor, misdirected outgrowth and much higher chances of atrophy at the neuromuscular junction. This leads to fairly poor outcomes in terms of functional recovery [24]. Chronic denervation has a profound effect on the survival of Schwann cells. After a few months of denervation, only about 50% of repair Schwann cells survive. This hinders regeneration even further as outgrowing axons rely heavily on support from Schwann cells to direct them and increase regeneration. Basal lamina tubes degenerate over the long term, causing misdirection in axonal outgrowth. Thus, chronic denervation creates a negative feedback loop which makes regeneration more and more difficult as time goes on.

Immunogenic properties of Schwann Cells

Recent research on Schwann cells has revealed the significant role that they play in the immune response in the peripheral nervous system. The immune function of Schwann cells initiates during the ‘innate’ stage of the immune response, in response to DAMPs/PAMPs in the environment of the endoneurium. These molecules are broadly associated with pathogens and foreign microbial elements, or cellular damage. They trigger receptors that are expressed on Schwann cells known as toll like receptors that cause Schwann cells to transdifferentiate into “activated” repair Schwann cells. These toll like receptors are often triggered in cases of peripheral nervous system damage. They correspond to the role that Schwann cells play in Wallerian degeneration by recruiting other immunogenic cells to clear cellular debris. Toll-like receptors are

frequently expressed on antigen presenting cells, further demonstrating the immunocompetence of Schwann cells [28].

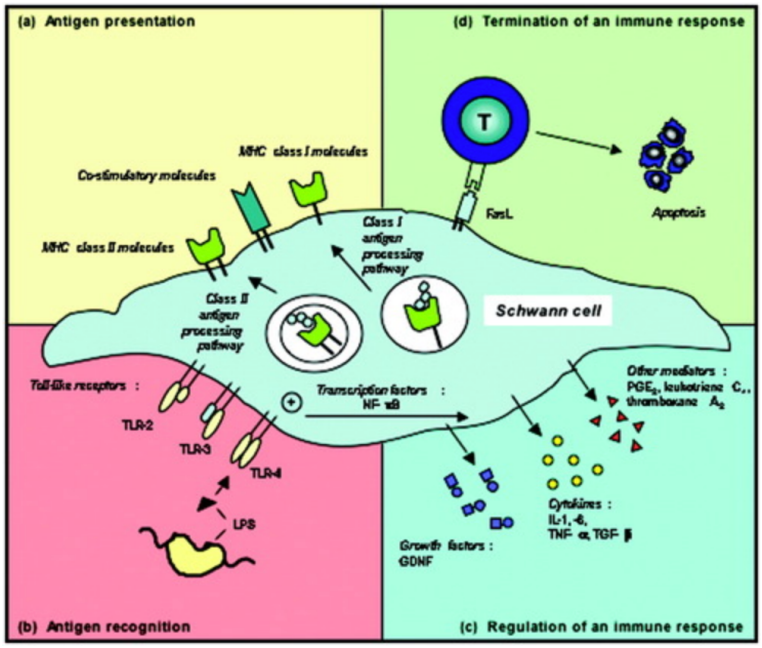


Figure 2 [28]: MHC II proteins expressed on surface of Schwann cells interact with T cells to trigger adaptive immune response.

Schwann cells strongly express major histocompatibility complex (MHC) proteins, specifically MHC II proteins. This is a major hallmark of antigen presenting cells. Antigen presenting cells separate antigens into peptides and express them on the cytoplasmic surface alongside MHC II proteins, which allows them to interact with T cell receptors [3]. MHC II proteins are responsible for presenting antigens to immunogenic T cells to be recognized as foreign bodies and activate the immune response. Schwann cells act as “nonprofessional antigen presenters” and only take on that role for transient periods, unlike professional antigen presenting cells. The role of Schwann cells in T cell activation is critical to recruiting all sorts of immunogenic cells such as macrophages, neutrophils, etc. These cells play a significant role in consuming debris during Wallerian degeneration or consuming any hostile foreign bodies in the nervous system such as pathogens. The ability of the peripheral nervous system to clear debris is one of the reasons why it is conducive to regeneration, unlike the environment of the CNS [13]. Unfortunately, certain disorders can cause misrecognition of host nervous tissue as pathogenic and initiate an autoimmune response that leads to degeneration of the peripheral nervous system. Guillain-Barré syndrome is one such autoimmune disorder.

The immune response is thought to be self-regulating, as inflammatory cells secrete cytokines. Cytokines are agents that mediate the reaction of immunogenic cells, modulating the communication between immune cells. This intercellular communication plays a significant role in creating a negative feedback loop to prevent an uncontrolled cascade. Schwann cells are capable of producing cytokines and other immunosuppressive mediators, as well as pro-inflammatory agents.

This suggests that they play a significant role in regulating the immune response in the peripheral nervous system. Beyond mediation, Schwann cells can also trigger the completion of the immune response by signaling T lymphocytes to undergo apoptosis through interactions between FAS receptors on their cell surfaces [11].

Conventional Nerve Allografts

Purpose and usage

When axons or entire nerves get transected or ablated, the standard clinical practice is to surgically attach the proximal and distal stumps together. Even though the distal stump will undergo Wallerian degeneration, connecting the nerve ends helps to guide outgrowing axons towards the neuromuscular junction. This treatment marginally increases the specificity of regeneration by outgrowth. Proximal and distal stumps are often aligned and connected together through microsuture surgery, though research has shown that the administration of fibrin glue can be a more effective method [1]. In a number of cases, it can be difficult for the proximal and distal ends to meet together even with administration of microsuture surgery, due to an uneven and sizable gap between the nerve ends. This complication makes regeneration by axonal outgrowth even more difficult due to misdirection and poor specificity. The nerve gap is often longer than the ablated segment due to the retraction of both ends. The elastic nature of nerve fibers causes them to retract when tension is disrupted due to transection or ablation. Researchers have tried to overcome this complication by elongating the nerve segments at both ends in order to suture them together. However, this is often ineffective due to the high traction forces at the suturing site [21]. To resolve this issue, researchers have used a grafted nerve segment to ‘bridge’ the two ends together in order to guide the outgrowing axons back towards the neuromuscular junction.

Types of Nerve Conduits

One option that has been used in clinical settings is a nerve autograft: a nerve segment excised from within the subject’s own body and transplanted to the damaged region. This method produces a number of complications. Excision of the autograft segment creates a neural deficit elsewhere in the subject [22]. Additionally, if the nerve gap is long enough and large in diameter, there is a lack of analogous nerve segments that can be excised from elsewhere in the subject without creating excess morbidity. For these reasons, allografts have become a very popular option in clinical settings. Allografts are nerve segments taken from another organism and transplanted into the host subject. The biggest adverse consequence of using allografts over autografts is the emergence of an immune response to foreign tissue in the host body.

Another option that has gained popularity is the use of commercially produced artificial nerve conduits. These synthetic conduits have often been used in cases where an extensive amount of nervous tissue has been ablated. If the size of the ablation and number of damaged nerves exceeds the clinical threshold for the use of autografts, then synthetic grafts are favored. These synthetic nerve conduits are often made of collagen to mimic the extracellular matrix of a real nerve segment. Various synthetic nerve tubes have been approved by the FDA for lengths that generally do not exceed ~3cm. This is due to the fact that synthetic nerve conduits lack Schwann

cells, trophic factors, and other supporting elements that help induce regeneration by outgrowth. Researchers have made progress in the development of synthetic nerve tubes to increase the effective maximum length beyond the previous 3cm limit. These efforts often involve harvesting autologous Schwann cells to populate the synthetic nerve graft in order to aid regeneration of axons [15].

Types of Allografts and Immune Rejection

Many researchers have performed studies to assess the most effective kind of allograft to improve clinical nerve regeneration outcomes. One type of allograft is an unmodified cadaveric allograft. Cadaveric allografts are simply excised from the donor subject and transplanted into the subject, bridging the nerve gap. The advantage of this method is that the existing cellular mass in the nerve graft is highly supportive of regeneration by outgrowth. This due to the presence of viable Schwann cells and trophic factors that aid regrowing axons. Blood vessels are also key as they provide transport for endothelial cells conducive to regeneration. However, the presence of large quantities of foreign cells in this type of graft tissue triggers a severe immune response in the host subject that can be fatal if the allograft tissue is significant in size. This requires the use of systemic immunosuppression through medication to inhibit the immune response. Preservation of allografts at cold temperatures can also help mitigate the immune response. Reduced temperatures decrease the activity of immunogenic cells and decrease inflammation [30].

Another type of allograft used clinically is an acellular allograft. This type of allograft is mostly sterilized. This means that most of the axonal cell mass, blood vessels, and other debris are removed from the graft tissue. Glial cells such as Schwann cells are also removed. The only remaining element is the extracellular matrix that acts as a skeletal structure for regenerating axons to grow through. The advantage of this method is that it avoids major rejection from the immune system and only elicits a minor immune response, due to the fact that all the cell mass is removed. However, the disadvantage is that due to the lack of endogenous Schwann cells or trophic factors, axonal regeneration through the graft is slow and limited compared to a cadaveric allograft. It takes time for the host Schwann cells to populate the allograft matrix and period greatly delays outgrowth. Due to this delay, acellular allografts are often limited to segments no more than 3cm long, similar to synthetic nerve grafts [22]. There are few differences between an acellular nerve allograft and a synthetic nerve conduit.

Different types of hybrids between the two main models of allografts have also been studied. Scientists have found some success populating acellular allografts with vascular endothelial growth factor, and Schwann cells [9]. Most of these models, however, used Schwann cells taken from the host subject, due to the idea that donor Schwann cells would be rejected [7]. Using host Schwann cells helps regeneration by outgrowth to occur at an acceptable rate, whereas the lack of Schwann cells and trophic factors would severely inhibit any outgrowth at all.

Behavior of donor Schwann Cells in conventional cadaveric allografts

Few studies have been conducted to observe and model the behavior of donor Schwann cells in conventional allografts over the long term. This is due in part to the fact that most

commonly used clinical allografts are acellular allografts and not cadaveric allografts that come with viable Schwann cells and supporting blood vessels. One such study by Midha et. al thoroughly tested and observed the immune rejection of donor Schwann cells in sciatic nerve allografts under different conditions of immunosuppression. This provides a basis of comparison for further study of donor Schwann cell behavior when the allograft is PEG fused.

Study Methodology

Midha et. al modelled donor Schwann cell behavior using an animal model of Shiverer mice, a strain of mice with a knockout mutation for myelin basic protein (MBP). MBP is expressed in all wild type Schwann cells, and thus the knockout mutation gave them the ability to distinguish between donor vs. host cells for the allografts in the study. After performing transplants of sciatic nerve allografts they observed all the subjects over 14 weeks under various immunosuppressive conditions. One of the most common immunosuppression drugs used in clinical tissue transplantation is Cyclosporin A. It is highly bioavailable and can be administered orally, and does not exhibit myelotoxicity [16]. One group was given continuous immunosuppression treatment via Cyclosporin A. The other experimental group was given temporary CsA treatment for only 6 weeks. These two test groups were compared against a negative control group that received no immunosuppression treatment.

After the observation periods, all subjects had the entire sciatic nerve complex excised and analyzed with stains using immunohistochemistry against the primary antibody MCA 409. Any tissue that was “positive” for the stain would indicate the presence of MBP, whereas negative tissue would indicate a lack of MBP consistent with the donor Schwann cells of the knockout Shiverer mice. Cross sections of the nerve segments were also stained with toluidine blue and observed for morphometric features. The purpose of this was to observe the myelination of axons distal to the transection site. The researchers assessed how immunosuppression affected the longer term myelination of outgrowing axons. This is significant due to the fact that it reveals whether host Schwann cells are largely responsible for myelination during outgrowth or if donor Schwann cells also play a significant role.

Results

Figure 2[19]

TABLE 1
Summary of Histologic Results within Nerve Allograft Segment with MBP Immunohistochemistry* and Inflammatory Cell (IC) Infiltrate**

	No CsA	Continuous CsA	Temporary CsA
Week 6			
n	5	5	
MBP results	3N, 1S, 1I	5S	
# with IC	2	5	
Week 10			
n	5	5	5
MBP results	5N	3S, 2N	4N, 1S
# with IC		3	
Week 14			
n	5	5	5
MBP results	5N	2S, 2N	4N
# with IC		1	2

* MBP results expressed as positive or normal (N), negative or Shiverer (S) and intermediate (I), both positive and negative, staining characteristics.
** Refers to the samples where large numbers of mononuclear inflammatory cells were seen in the nerve allograft segments.

The histologic study demonstrated that with temporary or no immune suppression treatment, donor Schwann cells in a cadaveric allograft eventually get replaced entirely by the host Schwann cell population. Even with continuous immune suppression treatment, 50% of the donor Schwann cell population gets replaced over 14 weeks and this likely continues past that point as even a suppressed immune system detects and attacks the foreign tissue.

Figure 3[19]

TABLE 2
Mean (±SD) Number of Myelinated Axons*

	No CsA	Continuous CsA	Temporary CsA
Week 6	602 (275)	332 (242)	
Week 10	985** (126)	306** (257)	586 (480)
Week 14	1,234** (840)	650** (365)	773 (495)

* Refers to the mean number of myelinated fibers in the host nerve distal to the allograft segment.
** Significantly different from each other ($p < 0.05$) at the stated week end-points by ANOVA and *post hoc t*-tests.

The morphometric study demonstrated a strange phenomenon: axons were re-myelinated twice as much in the control group than the continuously suppressed experimental group. This contradicts with the fact that immunosuppression treatment is necessary and beneficial when cadaveric allografts are used. Researchers hypothesized that this was due to a sustained inflammatory response given the fact that the foreign cells survived longer in the Continuous CsA group. Host Schwann cells are likely to be active in expressing myelination proteins whereas donor Schwann cells are likely inactive or ‘dormant’. Thus, repopulation of the graft with host Schwann

cells corresponds to an increase in myelination. Ultimately, immunosuppression treatment did not guarantee donor Schwann cell survival over the long term but only delayed and elongated the period of immune rejection. In this case, inflammation and a high presence of macrophages would inhibit the rate of re-myelination [19].

Other research on cadaveric allografts

Studies on cadaveric PNS allografts in larger animal models such as sheep or pigs have demonstrated less successful outcomes. Larger allografts induce a more pronounced immune response and subsequently demand more potent immune suppression. Researchers identified FK506 as a more effective immunosuppressant than Cyclosporin A for peripheral nerve allografts, highlighted by decreased inflammation. However, in all conventional allografts, donor Schwann cells are eventually replaced by host cells even with immunosuppression. This process of host Schwann cell repopulation corresponds to regeneration of axons, suggesting that the presence of host Schwann cells is necessary for outgrowth to effectively occur [17].

PEG Fused allografts

PEG fusion and change in allograft behavior

Polyethylene glycol (PEG) is a hydrophilic compound that is capable of acting as a cell membrane fusogen. Researchers have applied PEG fusion to peripheral nerve injuries to fuse proximal and distal nerve segments prior to the onset of Wallerian degeneration. Axonal continuity remains preserved and nerve function is restored very quickly. This is a stark contrast to regeneration by axonal outgrowth, in which axonal continuity is not maintained and the distal stump undergoes Wallerian degeneration. The proximal end regenerates at a rate of no more than 2mm per day and ultimately takes months or even years to reach the neuromuscular junction in larger mammals. This outgrowth is often accompanied with ample axonal misdirection and poor functional recovery due to chronic denervation [2]. One complication of PEG fusion is that it fuses membranes indiscriminately. This can create problems as different types of axons are fused together without specificity [4].

For peripheral nerve allografts, PEG fusion can be used to fuse the graft segment to the proximal and distal nerve ends and bridge the nerve gap. This process is also applied before the onset of Wallerian degeneration, fundamentally changing the nature and behavior of the allograft [5]. Conventional nerve allografts are only meant to act as a conduit for the outgrowing axon to be guided through as the distal segment undergoes Wallerian degeneration; PEG fused allografts retain full continuity of axons and signal transduction. The distal stump does not degenerate.

Immunotolerance phenomenon

Research regarding PEG fused allografts is very recent and left with a multitude of unanswered questions. One unexpected consequence of the application of PEG fusion to peripheral nerve allografts is that PEG fused allografts are not rejected by the immune system. This is despite

the fact that the peripheral nervous system is not an immune privileged environment [20]. Various areas of the body are immune privileged in order to avoid excessive inflammation that an immunogenic environment produces. The eye is one example of such an environment [10]. It is an exceedingly unique occurrence as almost all foreign cellular tissue introduced into a host organism is recognized by both the innate and adaptive immune system and rejected. This is why almost all organ transplants, including conventional nerve allografts, require the administration of immunosuppression drugs to ensure clinical viability. Due to the fact that PEG fusion circumvents the immune response in allografts, fully viable cadaveric allografts can be used with no modification to the cell mass. This negates the need for acellular allografts that lack regeneration-inducing elements such as Schwann cells and blood vessels. PEG fused allografts are also fundamentally different from autografts or synthetic nerve conduits, as they maintain axonal continuity. There is no upper limit to the length of the segment that can be used, nor is there an added morbidity of having to create a neural deficit elsewhere in the clinical subject.

Studies observing the immunotolerance phenomenon have shown a number of different observations of reduced immune activity, both innate and adaptive, in the environment of the PEG fused allograft. The reduced expression of MHC II proteins indicates that there is no antigen presentation activity occurring via Schwann cells, thus T cells would not be activated. Reduced T cell infiltration confirms this and further evidence of the lack of immunogenic activity can be seen with the reduced expression of cytokines. Most importantly, macrophages and other phagocytes do not infiltrate the allograft tissue, as the primary immunogenic cells that consume foreign bodies or debris. These observations demonstrate that no immune response takes place to reject the allograft even though it is not tissue matched [25].

Figure 4[25]

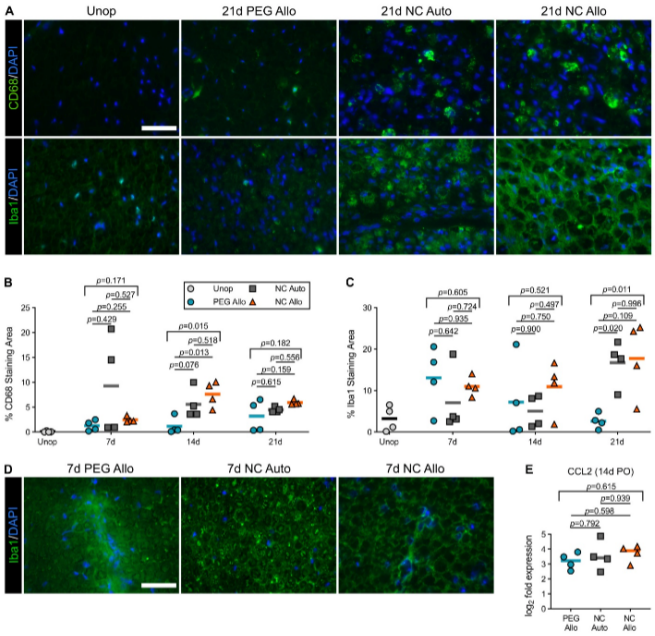


Fig 4. Reduced infiltration of macrophages seen in the PEG allograft 21 days p.o compared to normal allograft. Key indicator of reduced immune activity due to lack of upstream signals

Experimental Design

Introduction

Current studies regarding PEG fused allografts have used fully viable allografts with cell mass and found no immune rejection response. However, no long term studies were performed to observe whether or not host Schwann cells repopulate the allograft tissue as one would see in conventional allografts, replacing donor cells. That prompts the principle research question of this study: Are donor Schwann cells viable over the long term in PEG fused allografts or do they get replaced by local host Schwann cells? I hypothesize that host Schwann cells do not repopulate the allograft segment and replace donor Schwann cells in PEG fused allografts.

Methods

Experimental Protocols

Test Group	Number of Subjects	Protocol	Sampling Time
Experimental Group: PEG Fused Allograft	120 (10 per sampling period)	PEG fused allograft, excised after observation period	1, 2, 4, 8, 14, 24 weeks
Positive Control: Conventional Allograft	120 (10 per sampling period)	Conventional microsutured allograft, excised after observation period	1, 2, 4, 8, 14, 24 weeks
Negative Control: Excised Wistar rat sciatic nerve	120 (10 per sampling period)	Non-allografted sciatic nerve excised from Wistar rat	1, 2, 4, 8, 14, 24 weeks
Negative Control: Excised Sprague Dawley rat sciatic nerve	120 (10 per sampling period)	Non-allografted sciatic nerve excised from Sprague Dawley rat	1, 2, 4, 8, 14, 24 weeks

Experimental Model

This study will be conducted using an animal model to observe the behavior of donor and host Schwann cells in a PEG fused allograft. The usage of an animal model is expected to have sufficient predictive validity regarding the behavior of PEG fused allografts in a clinical setting in larger mammals. The animal species to be used are two strains of rats, Wistar rats and Sprague-Dawley rats. These are two common strains of rats used in laboratory research, particularly in

neurobiology studies. Literature has demonstrated that these two rat strains express phenotypic differences in Schwann cells that can be distinguished through observations of cell morphologies. These morphological differences are strongly accentuated by digestion of the nerve tissue in a solution of 0.05% concentrated type 1 collagenase that isolates Schwann cells [29]. Wistar rat Schwann cells transform drastically to show a round morphology after treated with type 1 collagenase while Sprague Dawley rats maintain an elongated, spindle morphology.

Experimental Groups

The study will consist of 1 experimental group of Sprague Dawley rats who receive a sciatic nerve allograft from Wistar rats via PEG fusion. The positive control group will consist of Sprague Dawley rats who receive a sciatic nerve allograft from Wistar rats via conventional microsuture surgery. Both of the groups will receive a fully viable cadaveric allograft with Schwann cells, blood vessels, and other nerve tissue. Neither group will undergo administration of immunosuppression therapy. The experimental subgroups will segregate the rats that have the allograft complex excised at different time intervals post implant: 1 week, 2 weeks, 4 weeks, 8 weeks, 14 weeks, and 24 weeks. There will be 2 negative control groups in which the non grafted sciatic nerve is excised from normal Wistar and Sprague Dawley rats. These negative control groups account for any unknown variables in the collagenase digestion process that affect Schwann cell populations. This brings the total up to 24 test groups between the subjects who receive PEG fused or conventional allografts.

Surgical Procedure

Wistar rats are not part of the in-vivo test group and thus will be euthanized in order to dissect and extract a section of sciatic nerve tissue for allograft transplant. The live subject Sprague Dawley rats will have an equal sized segment of sciatic nerve tissue excised to create the nerve gap to be filled by the Wistar sciatic nerve allograft.

The extracted Wistar rat sciatic nerve segment will be transplanted into the sciatic nerve complex of the Sprague Dawley subjects. In the control group, this allograft transplant will be executed using conventional methods of microsuture surgery. In the experimental group, the allograft transplant will be executed using PEG fusion methods. After both experimental groups receive their respective allografts, they will not be subjected to immune suppression treatment in order to see the full development of the innate immune response to each type of allograft. After the time intervals have passed for each experimental subgroup (ranging from 1 – 24 weeks), the live Sprague Dawley subjects will be euthanized. The entire sciatic nerve complex will be excised from each subject for analysis.

Analytical procedure

After the sciatic nerve complex is excised from each subject, it will be preserved using CO_2. The sciatic nerve tissue will be digested through a solution of 0.05% (w/v) type 1 collagenase

and then observed for 14-21 days to see the morphological transformation of both donor and host Schwann cell types in the allograft. At this point, the donor Wistar Schwann cell types will show a circular morphology while the host Sprague Daley Schwann cell types will maintain spindle morphology.

Using phase contrast microscopy, the isolated Schwann cells will be observed for each experimental subgroup. Both types of Schwann cells will be counted using computer methods for each sample, and then the ratio of donor vs. host Schwann cells will be calculated.

The quantitative results will be analyzed using a paired t-test at each time interval. This will determine if the difference in survival of donor Schwann cells between the experimental and control groups is statistically significant.

Results

Overview

The results of the study show that the ratio of donor/host Schwann cells remains relatively stable all the PEG fused allograft subgroups. Donor Schwann cell populations did not change significantly over the 24 weeks of observation, suggesting long term survival. In the control group, the ratio of donor/host Schwann cells decreased dramatically over the first 8 weeks until only host Schwann cells remained after the 14 week observation period.

The results of the paired t-test show that the difference in experimental conditions for both test groups yields a statistically significant result, supporting the hypothesis. The donor to host ratio was significantly different between the PEG fused allograft group and the conventional allograft group on average.

Subjectively, the animals in the controlled group showed clear signs of an elevated immune response. The excised sciatic nerve complexes from the control group showed high levels of inflammation. The sciatic nerve complexes from the PEG fused allograft group did not show the same visual signs of inflammation or immune rejection upon excision.

Figure 5[29]

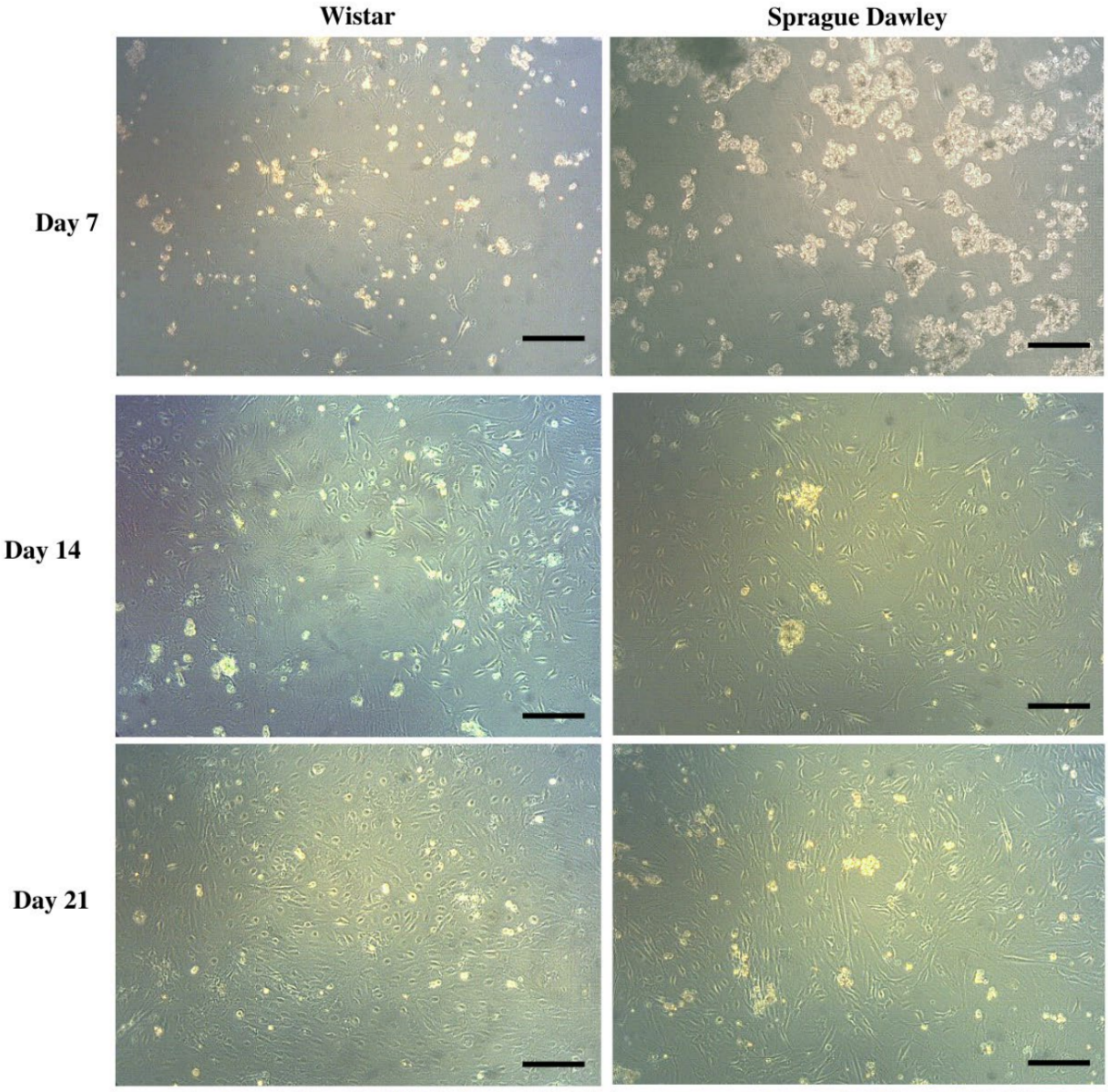


Figure 5: Panels show transformation of isolated Schwann cells digested under .05% type I collagenase over period of weeks. The samples are fully digested and distinguishable by Day 21.

Figure 6 [29]

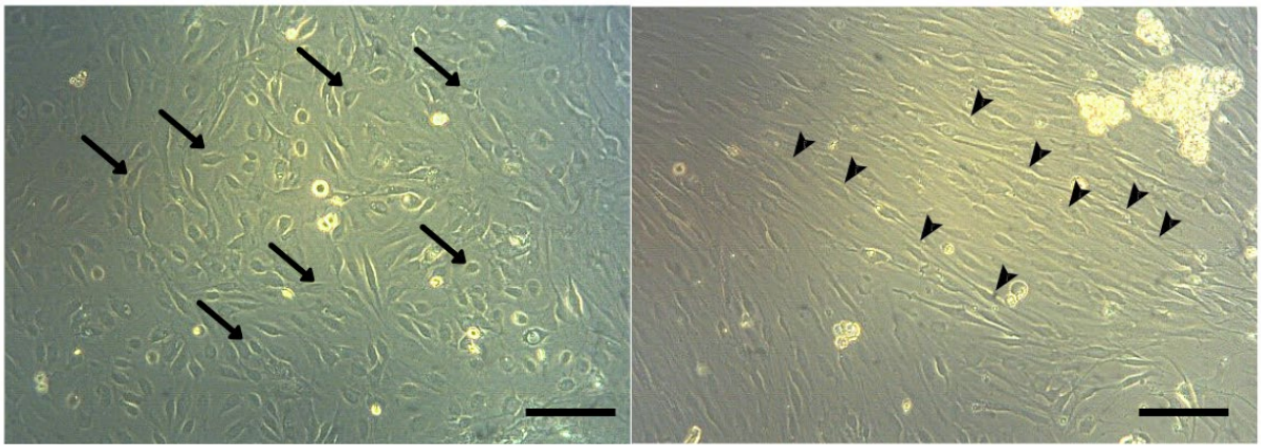
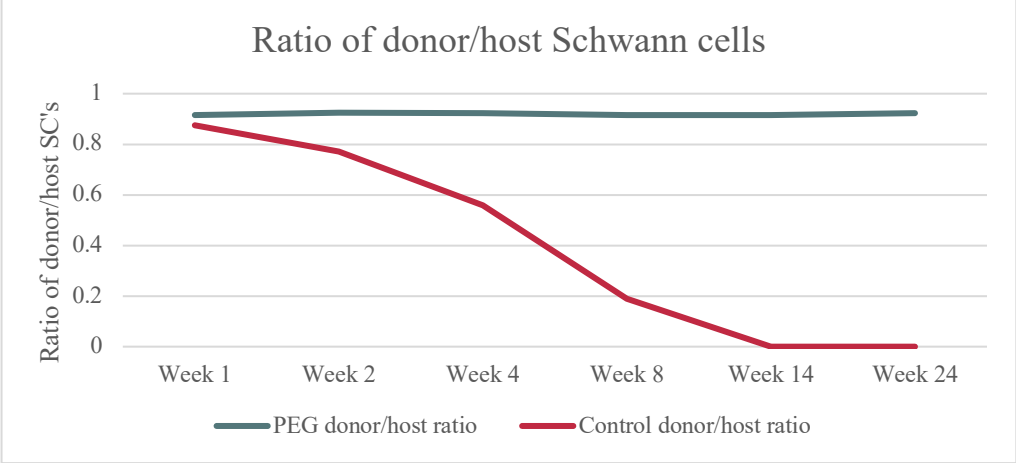


Figure 6: Left panel shows PEG fused sciatic nerve allograft sample in which donor Wistar Schwann cells remain viable after 24 week sampling period. Wistar cells are indicated by circular morphology. Right panel shows conventionally microsutured sciatic nerve allograft sample in which all donor Schwann cells are replaced by host Sprague-Dawley Schwann cells. Sprague Dawley cells are indicated by elongated, spindle morphology.

Figure 7

Average donor vs. host SC count by time interval								
	PEG donor SC Count	PEG host SC Count	PEG donor/host SC ratio	Positive Control donor SC count	Positive Control host SC count	Control donor/host ratio	Wistar Negative Control SC count	Sprague-Dawley Negative Control SC count
Wk 1	121	132	.9166	112	128	.8750	118	134
Wk 2	123	133	.9248	101	131	.7710	119	132
Wk 4	122	132	.9242	77	138	.5580	115	133
Wk 8	119	130	.9154	29	153	.1895	121	128
Wk 14	120	131	.9160	0	172	.0000	123	131
Wk 24	121	131	.9237	0	212	.0000	117	133

Figure 8



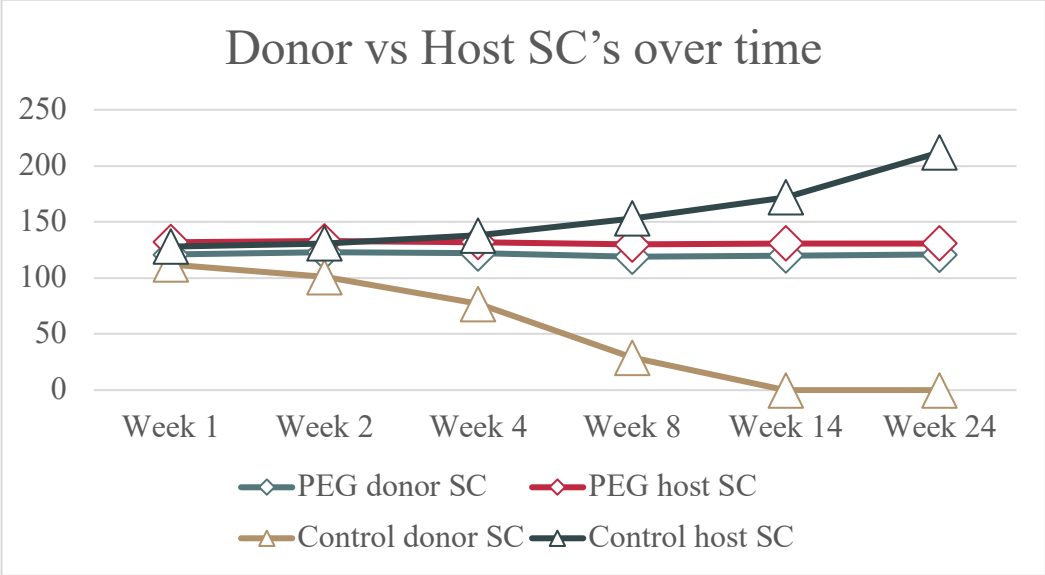


Figure 8: In the PEG fused allograft groups, the populations of both the donor and host Schwann cells remain stable over all sampling periods. This is contrasted by the positive control group results, which show an increase in host Schwann cells in the samples over time. The donor cells in this group are completely eradicated and replaced by week 14.

Average paired t-test p-value	.0081

Discussion

The results of the study further reaffirm the phenomenon shown in prior research that PEG fused allografts do not illicit an immune rejection response as seen in conventional allografts. Furthermore, these results confirm the hypothesis that PEG fusion of peripheral nerve allografts lead to the survival of donor Schwann cells. Host Schwann cells do not repopulate the allograft as seen in conventional allografts.

This conforms with what we know about Schwann cells and the immune environment of the peripheral nervous system. It has been demonstrated in many ways that Schwann cells are immunogenically active, antigen presenting cells that heavily express MHC type II proteins. Since PEG fused allografts restore axonal continuity, Wallerian degeneration does not occur and the DAMP/PAMP molecules do not proliferate in the PNS environment. Toll like receptors on host Schwann cells are not activated without the presence of these molecules, so the innate immune response is not inherently triggered.

The results also suggest that the survival of donor Schwann cells also play a role in 'escaping' the adaptive immune response. Since donor Schwann cells survive in the allograft segment, they recognize the tissue as endogenous and not foreign or hostile. This allows the allograft segment to 'escape' the immune recognition from the host and avoid rejection, because host Schwann cells do not populate the graft and process antigens to recognize it as foreign tissue.

Researchers have observed that host Schwann cell proliferation of a conventional allograft is a precursor to axonal outgrowth. In previous studies, the onset of inflammatory response and other symptoms of rejection coincided with the replacement of donor Schwann cells with host Schwann cells. Axonal regeneration follows this repopulation of host Schwann cells [9]. Due to this, it can be understood that there is a correspondence between the rejection response and proliferation of host Schwann cells in allograft tissue. PEG fused allografts do not necessitate the proliferation of host Schwann cells because regeneration of axons is completely bypassed. It is likely that donor Schwann cells remain in the graft tissue and 'hide' the foreign tissue from the immune system in the PNS. Other factors also mediate the immune response in peripheral nerves, but it is likely that the lack of innate and adaptive immune response triggered in host Schwann cells plays a significant role in inhibiting the rejection of PEG fused allograft tissue.

Alternative Results

If the results were to show that there is no statistically significant difference in the survival of donor Schwann cells between the experimental group and the control group, it would invalidate the hypothesis. This would contradict the theory that the survival of donor Schwann cells correlates with the unexpected ability of PEG fused allografts to escape the immune response. Researchers would have to hypothesize that there is some other unknown factor that distinguishes PEG fused allografts from conventional allografts, causing the immunotolerance phenomenon.

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