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The Immune Response and Implications for Nerve Repair

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Abstract

This chapter aims to provide an overview of the host response to allografts or tissue-engineered constructs containing allogeneic cells in peripheral nerve repair, and potential approaches for promoting the survival of nerve grafts. A large body of current research aims to improve surgical approaches for nerve repair beyond that of the autograft. Potential approaches include nerve allografts, or tissue-engineered nerve constructs which could provide an unlimited source of donor tissue for nerve injury repair. This field requires an interdisciplinary approach to the design of novel therapies, and consideration of the immune response to transplants should not be overlooked. There are many benefits of including living donor cells within transplanted nerve conduits which can improve axonal guidance, vascularization, and promote the release of growth factors to increase regeneration. It is most likely that donor cells or tissues will be of allogeneic origin, with associated implications for eliciting an immune response on transplantation. The following sections provide a summary of the immune response to nerve grafts for consideration in the development of approaches to nerve repair, including some approaches currently under investigation for preventing rejection of transplants.

1 Introduction

Injuries to the peripheral nerves occur frequently as a result of trauma and can have a life-changing impact on individuals, leaving them with disabling motor and sensory deficits. Successful recovery requires the development of a number of cellular and molecular processes distal to the lesion site and toward the denervated target known as Wallerian degeneration (Waller *1850*; Rotshenker *2011*). After degeneration of detached distal axons, myelin and axonal debris are broken down and

phagocytosed by Schwann cells (SCs). These SCs secrete cytokines and chemokines which recruit immune cells into the injured nerve, particularly macrophages, which contribute to myelin clearance and release growth factors leading to the proliferation of SCs and fibroblasts. SCs first degenerate and begin to upregulate regeneration-associated genes. Proliferating SCs then align to form bands of Büngner and provide conduits for regenerating axons (Stoll et al. *1989*). In the distal nerve stump, SCs also secrete extracellular matrix (ECM) molecules, such as laminin, and trophic factors to promote axon growth.

The management of injuries depends on various factors, including the type and location of injury, the size of the nerve deficit, the timing of injury, and any associated soft tissue injury (Palispis and Gupta *2017*). Total disruption of the peripheral nerve (neurotmesis) requires surgical realignment of the nerve stumps, with the primary aim of allowing reinnervation of target organs through guidance of regenerating axons into the distal nerve. Direct epineurial repair is suitable when tension-free coaptation and gross fascicular matching of the proximal and distal nerve ends can be achieved (Grinsell and Keating *2014*). Although surgical advances in peripheral nerve repair have been developed following research into the biology of nerve injury, only partial improvement is still achieved (discussed in the chapter "The History of Nerve Repair"). Sensory recovery has been reported in just 42% of digital nerve repairs, and only 25% with excellent outcomes (Paprottka et al. *2013*). With delayed repair following injury, over time retraction of the stumps will cause an increased gap length for repair, meaning primary suturing of the nerve stumps will cause tension at the coaptation site (Siemionow and Sonmez *2007*). Repair of the nerve gap therefore requires bridging to allow nerve regeneration and restoration of function while avoiding causing tension from direct apposition of the cut ends. Novel strategies for bridging this nerve gap are currently under investigation in order to provide a greater source of potential donor nerve, and improve functional outcomes after repair, through tissue engineering and the use of biomaterials. A number of these strategies require the transplantation of allografts or engineered nerve constructs containing allogeneic cells. While potentially resolving the issues of tissue supply and increasing regenerative potential, this approach may cause immunological rejection of transplanted cells or tissues; thus, a consideration of the host immune response in nerve repair is essential to enable survival and function of implanted tissues to develop feasible approaches for translation. This chapter will discuss the immune response to transplants in the context of nerve repair, and potential approaches to avoid this issue. An additional important consideration in the preparation of engineered tissues for transplantation is the response to the implanted material itself. The foreign body response to implanted materials is discussed briefly but is reviewed more thoroughly elsewhere (Anderson et al. *2008*). Thus, the main focus of this chapter will be on the immune response to allotransplanted cells or tissues.

2 Peripheral Nerve Repair Strategies

Currently, a nerve autograft derived from a different donor site of the patient is the gold standard for clinical repair of nerve gap injuries (Ijkema-Paassen et al. *2004*). The sural nerve is a common donor source as it is easily obtained and can be sacrificed for the repair and reinnervation of more critical muscle. However, despite successful outcomes of the nerve autograft, sacrificing a donor nerve from the patient creates damage in an otherwise healthy area including scarring, sensory loss, and the risk of neuroma formation (Battiston et al. *2017*). In addition to this donor site morbidity, the repair of large defects requires more donor nerve than may be available as autologous grafts. Size and

fascicular matching for autografts is a challenge, and the procedure and injury itself can result in scarring and fibrosis leading to poor regeneration (Ijkema-Paassen et al. *2004*). Clinically, it is estimated that there is a 50% loss of axons at each coaptation site, resulting in successful regeneration of 50% of the original axons through the repair site in primary nerve repair. In a nerve graft with two coaptation sites, this will reduce to 25% successful regeneration through the graft, decreasing further depending on other factors, including the distance to the target (Grinsell and Keating *2014*).

The disadvantages associated with the use of autografts demanded the development of new techniques to provide "off-the-shelf" alternatives to serve as axonal guidance channels. The acellular nerve graft provides an alternative ECM scaffold which can be repopulated by host cells to promote regeneration (Pan et al. *2019*). Evidence has shown successful regeneration across a nerve gap in a rat sciatic nerve injury model (Kim et al. *2004*). There is also evidence for successful regeneration across short gaps (3 cm or below) in patients with digital nerve injuries, following implantation of bioabsorbable polyglycolic acid tubes (Mackinnon and Dellon *1990*). Such acellular allogeneic nerve grafts have been approved for clinical use, including Avance®, produced by AxoGen, Inc. However, although this product showed greater efficacy than a Type 1 collagen guidance conduit (NeuraGen® nerve guide), it did not yield as good regeneration as an isograft when tested in vivo (Whitlock et al. *2009*). The authors concluded that, in a larger gap model, the decellularized allograft may not be able to produce the same results as a nerve isograft; however, it may be suitable for smaller gaps (Whitlock et al. *2009*). Additional approved guidance conduits are mostly fabricated from Type 1 collagen and suitable for small nerve defects, including NeuraGen, NeuroMatrix, NeuroFlex, NeuraWrap, and NeuroMend (discussed in the chapter "Biomaterials and Scaffolds for Repair of the Peripheral Nervous System"). Outcomes following transplantation of these conduits show functional improvement and regeneration, though for long gap injuries (>3 cm), regeneration can be inadequate for recovery and the outcomes following autograft remain superior to nerve conduits (Grinsell and Keating *2014*; Pan et al. *2019*).

The use of cellular peripheral nerve allografts in the place of autografts would offer an abundant source of nerve for transplantation from cadaveric donors. Allografts can act as viable conduits for transplantation, allowing host sensory and motor axons to successfully grow and reach their targets (Moore et al. *2009*). Allografting also offers the potential for transplantation of the same nerve type, for example, replacing mixed sensory-motor nerves with the same donor nerve to improve recovery. Commonly, the sural nerve (sensory) is used as donor nerve for peripheral nerve autografts, when the nerve being repaired may be motor. Studies in rodents and patients with nerve injury have shown that, unlike autografts, allografts will be rejected by the host immune system. As immunological recognition is due to the recognition of foreign histocompatibility antigens on donor cells within the graft, decellularization of the nerve prior to implantation is possible to provide a conduit along which axonal regeneration can occur. This can be achieved through irradiation, freeze thawing, detergent-processing, and cold preservation (Lin et al. *2013*). However, without the presence of therapeutic SCs within the graft, regeneration is greatly reduced. In addition, there are currently limited options available, with decellularized allografts presently being offered at such a high cost that, despite the associated negatives, the peripheral nerve autograft is still the preferred option. An alternative method to promote the acceptance of a nerve allograft is the use of immunosuppressant drugs, and the use of cadaveric nerve allografts has been explored to restore nerve continuity clinically (Mackinnon et al. *2001*). Immunosuppression for the prevention of allograft rejection requires prolonged systemic administration and can result in significant side effects including nephrotoxicity and hepatotoxicity (Brenner et al. *2002*; Rezzani *2006*; Teh et al. *2011*). As peripheral nerve injury (PNI) is not in itself life-threatening, such severe side effects preclude the use

of systemic immunosuppression in these patients, thus removing the potential for the use of nerve allografts as a source of donor tissue.

An alternative to the grafting of donor nerve is the development of engineered nerve tissues for transplantation. The potential of this approach could increase both supply and therapeutic efficacy, as tissues could be developed incorporating combinations of therapeutic cells, growth factors, and materials specifically designed to improve regeneration. An “ideal conduit” is deemed to have the following requirements: (1) supply a biocompatible or biodegradable tube for integration into the surrounding tissues while supporting axonal regeneration and progression; (2) be of a size and length adequate to connect the stumps of the damaged nerve; (3) contain throughout its length substances exerting chemotactic attraction and allowing axonal progression; and (4) protect regenerating axons against scar invasion (Brunelli et al. *1994*). Commercially available devices have included hollow tubes manufactured from biodegradable polymer/collagen, though their efficacy is restricted to short defects and functional recovery is limited and does not surpass that of autologous nerve grafts (Pabari et al. *2014*). A large body of research has sought to develop tissue-engineered constructs for nerve repair (Pabari et al. *2014*; Faroni et al. *2015*; Carvalho et al. *2019*; Raza et al. *2020*), though there has not yet been a substantial translation to the clinic. The incorporation of therapeutic cells into tissue-engineered constructs is considered to be an important factor in improving regeneration, and a number of options have been tested including SCs, neural stem cells, embryonic stem cells, and bone marrow stromal cells (Carvalho et al. *2019*). As these may be of allogeneic origin, the use of mature cells poses issues of immunological rejection. The development of off-the-shelf acellular products therefore provides an easier route to translation, overcoming issues with variability due to the incorporation of cell products, and regulatory approval of these novel therapeutics. Developing methods to circumvent the problems of immunological rejection would increase the feasibility of translating cell-seeded nerve guidance conduits for peripheral nerve repair.

3 Mechanisms of Rejection

The function of the immune system is to protect against potential threats, from pathogens such as bacteria and viruses. This is achieved through various layers of defense, beginning with physical barriers such as the skin and epithelial linings. Pathogens which breach these barriers meet the innate immune system, so called since it requires no previous exposure to mount a nonspecific response and induce an inflammatory state. Evasion of this innate immune response calls for further defense via the adaptive immune response. Initiated by the ongoing innate response, this adaptive response results in long-lasting immunity. These responses involve coordination between the circulatory system, lymphatic system, lymphoid organs and tissues, and specialized cells which move between these. In the context of transplantation, the consequence of this is detection and directed response against foreign cells. The immune system therefore must have the ability to identify “self” from “nonself” based on antigenic profile, in order to offer protection while preventing autoimmunity. From thorough investigation of skin grafts in humans, Medawar concluded graft rejection to be an immunological phenomenon (Gibson and Medawar *1943*). Subsequent studies in rabbits further investigated this phenomenon through the comparison of autografts and homografts (allografts), confirming that the rejection of foreign skin occurs through “actively acquired immune reactions” (Medawar *1944*). The contribution of the different elements of the immune system to the transplant rejection response is discussed in the following sections. Since these responses will also be affected

by the local resident cell population, and the nature of the transplanted tissues/cells themselves, the response to transplants is then discussed specifically in the context of the peripheral nervous system.

3.1 Innate Immunity

The innate immune response is the first mechanism of defense after injury. This involves a range of cell types such as mast cells, dendritic cells (DCs), basophils, eosinophils, natural killer (NK) cells, neutrophils, and macrophages (Morris et al. *2017*). The main features of innate immunity are the ability to distinguish infectious nonself-molecules from self, and the ability to activate adaptive immune responses to those. Cells of the innate immune system are able to detect both structural pathogen-associated (PAMPs) and damage-associated molecular patterns (DAMPs) via pattern recognition receptors (PRRs), as termed by Janeway (*1989*, *2013*). These consist of four families, Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors, C-type lectin receptors, and RIG-1-like receptors, and can react with broad groups of invasive pathogens. They are also triggered by various physical and metabolic insults, causing a response consisting of various soluble and cellular mediators of inflammation aiming to eliminate the threat (Farrar et al. *2013*). TLRs, the most studied of the PRR families, possess fixed receptor structures, meaning they are capable of binding a limited ligand repertoire, and are highly conserved across evolutionary distant species (Brennan et al. *2010*). Inflammatory cells of the innate immune system have limited clonal expansion and in general do not bring about immunological memory (Oberbarnscheidt et al. *2011*). The innate immune system also includes noncellular mediators of microbial recognition such as complement proteins. The complement and TLR systems of innate immunity therefore demonstrate the integration of soluble and cellular components which provide immune surveillance of tissues (Farrar et al. *2013*). As predicted by Janeway in 1989, mobilization of the innate immune system through PRR activation triggers both inflammation and the initiation of adaptive immunity, meaning it is not only responsible for first-line defense, but also shapes the adaptive immune response (Janeway *1989*, *2013*; Oberbarnscheidt et al. *2011*).

Early on in the immune response to allografts, the nonspecific innate response predominates, followed by the donor-specific adaptive response. Procurement and transplantation of donor organs induces activation of the innate immune system resulting in tissue damage and the death of donor cells within the transplanted organ. This will contribute to innate immune activation such as graft inflammation, and result in tissue damage and the elimination of damaged cells within the graft (Ochando et al. *2019*). Early inflammation independent of the adaptive immune response is considered to be an important factor in graft rejection (Moreau et al. *2013*). Potential DAMPs such as reactive oxygen species and heat shock proteins can be generated by local tissue damage and reperfusion injury, as induced by the physical process of isolation and manipulation of cells and organs for transplantation (Wood and Goto *2012*). Necrotic cell death from reperfusion injury and surgical trauma has been shown to cause a strong inflammatory response, potentially due to mechanisms which have evolved to recognize cell death as an indicator of danger. Since necrotic cell death may be due to an infection, or occurring at a site where microbes may be introduced as a consequence of injury, these threats require rapid response. The instigation of an inflammatory response rapidly mobilizes defenses to mitigate this threat (Rock and Kono *2007*). Hypoxia and reoxygenation of transplanted tissues causes rapid activation of the complement cascade, which can regulate B cells and antibody production, and could also contribute to the modulation of T cell responses (Zhou et al. *2007*). During the early posttransplantation phase, innate immune system activation is a nonspecific response to tissue damage, irrespective of donor and host characteristics.

The resulting release of proinflammatory cytokines which identify the transplant as a site of injury and inflammation triggers adaptive immunity, leading to the maturation of antigen-presenting cells (APCs), upregulation of costimulatory molecules, and further secretion of proinflammatory cytokines (Wood and Goto *2012*; Moreau et al. *2013*). The complement system, comprising soluble and cell surface proteins, also plays a role in the inflammatory response and host defense, as well as linking the innate and adaptive arms of the immune response (Zhou et al. *2007*). Complement activation can occur through three different pathways leading to the clearance of immune complexes, invading pathogens, and injured cells. All three pathways result in the activation of the plasma protein C3 leading to inflammation, leukocyte recruitment, cytokine and chemokine release, oxygen radical production, and increased blood vessel permeability. There is evidence for a role of the complement cascade in complications during allograft transplantation, causing recruitment and activation of neutrophils and monocytes from the circulation into the allograft resulting in cell apoptosis and necrosis (Ochando et al. *2019*).

3.2 Adaptive Immunity

The adaptive immune system includes T and B lymphocytes, which express highly specific antigen receptors which are diverse due to somatic gene rearrangement. For B lymphocytes, these surface receptors are antibodies (immunoglobulins), and for T lymphocytes they are T cell receptors (TCRs) which recognize nonself antigens. T cells expand clonally during antigen recognition, contributing to immunological memory (Yatim and Lakkis *2015*). These antigen receptors allow recognition and targeting of nonself antigens which occurs through extensive lymphocyte proliferation and differentiation into specialized subsets. B lymphocytes become plasma cells which can produce antibody, and T lymphocytes into helper and effector/cytotoxic subsets which secrete a distinct set of cytokines. A distinct and specialized population of T cells are regulatory T cells (Tregs), characterized by expression of CD4, CD25, and FoxP3. These cells contribute to the suppression of T cell responses, in order to maintain self-tolerance within the organism (Sakaguchi *2000*). Although, following a response, the majority of antigen-specific lymphocytes ultimately die following this proliferation and differentiation, some memory lymphocytes remain to ensure that a second encounter with the same foreign antigen results in rapid targeting and elimination (Mueller et al. *2013*).

3.3 The Major Histocompatibility Complex

The major histocompatibility complex (MHC) encodes the major antigens responsible for eliciting rejection, with transplantation of cells and tissues between MHC-identical individuals being readily accepted as opposed to between MHC-mismatched individuals, which are rejected in the absence of immunosuppression (Ayala-García et al. *2012*). In humans, MHC antigens are collectively termed human leukocyte antigens (HLA) and are divided into three classes (I, II, and III) based on tissue distribution, structure, and function. The function of HLA class I and II surface molecules is to present peptides to T lymphocytes from either intracellular proteins, or proteins sampled from the extracellular environment, thus providing an extracellular representation of intracellular invasion (Germain *1994*). Class III genes encode components of the complement system. In addition to playing a key role in antigen presentation, HLA molecules are recognition elements for immune

cells surveying the body, allowing the determination of "self" and "nonself." Immune cells will recognize specific peptides presented by APCs which bear identical MHC molecules to those expressed by the lymphocytes themselves, known as "MHC restriction" (Bolton and Bradley *2013*). In the context of transplantation, immune cells respond to "nonself" MHC molecules with the initiation of a rejection response through antigen processing and presentation, described further below. To reduce the risk of rejection and the amount of immunosuppression administered, transplantation therefore requires close matching of donor and recipient HLA types. However, the large variability in potential HLA molecules expressed creates a challenge in matching between donor and host.

HLA class I and II have different functions reflected in their cellular distribution and have evolved to present cytoplasm-derived peptides or intracellular parasites (mainly viruses) via HLA class I, or bind peptides from extracellular proteins via class II. These are recognized by the two main T cell subsets, $CD8^+$ (cytotoxic) and $CD4^+$ (T helper) T cells, respectively (Germain *1994*). All nucleated cells express varying levels of class I antigens, depending on the tissue, whereas class II antigens are normally expressed only by immune cells such as B cells, activated T cells, macrophages, DCs, and thymic epithelial cells (Klein and Sato *2000*; Bolton and Bradley *2013*). Importantly, upregulation of class II antigens on other cell types can be induced by the presence of proinflammatory cytokines such as interferon-γ (IFN-γ) (Klein and Sato *2000*).

3.4 Antigen Processing and Presentation

The antigen-specific adaptive immune response follows later than the innate response via alloantigen presentation by APCs, allowing recognition by recipient T cells. All cells which can express MHC Class II are able to present antigen to T cells; however, different cell types are able to process and present antigen at different efficiencies. Therefore, certain cells are considered to be "professional APCs," including macrophages, DCs, and B lymphocytes (Schneider and Sercarz *1997*; Trombetta and Mellman *2005*). The so-called "immunological synapse" refers to these T cell-APC interactions (Norcross *1984*) and can include any interface between lymphocytes or NK cells, and the cells which are being recognized (Huppa and Davis *2003*).

In transplantation, due to the presence of both donor and host antigen presentation via MHC class I and II, the recognition of tissues can be mediated by "direct" and "indirect" antigen recognition. Direct recognition describes the recognition of intact MHC class II molecules on the surface of donor cells by host CD4 T cells. As these are recognized via MHC class II expression, this requires the presence of donor APCs. CD4 T cells will recognize and be tolerant to self HLA class II whereas the recognition of nonself antigen present on donor APCs, along with costimulatory molecule interaction, will activate the T cell. Indirect recognition allows host APCs, typically DCs, to internalize and process donor peptides and present their fragments for recognition by T cells and, therefore, does not require the presence of donor APCs (Gould and Auchincloss *1999*; Siu et al. *2018*). Activation and rapid expansion of T cells is initiated in response to the detection of alloantigen, requiring various related signals. The activation of naïve CD4 T cells has been suggested to be the most important event in initiating adaptive immunity, resulting in cytokine secretion, initiation of the adaptive immune response, and the induction of effector mechanisms leading to graft destruction.

Antigen processing and presentation requires the formation of MHC/antigen peptide complexes bound to the TCR to induce T cell activation, known as "signal 1." This interaction determines

which T cells will be activated, since each TCR will respond to a particular antigen (Janeway and Bottomly *1994*). However, this signal alone is not sufficient for T cell activation; additional signals are required via interaction of costimulatory molecules on the T cell surface with their ligands on APCs, known as “signal 2” (Lafferty and Cunningham *1975*). Stimulation of the TCR in the absence of costimulation will instead result in anergy, or apoptosis of the responding T cells (June et al. *1990*; Schwartz *1990*). There are a number of different costimulatory molecules which may support or inhibit T cell activation, classified into four distinct groups based on their structure, which are variably expressed on T cells (Kinnear et al. *2013*). For example, the first pathway to be defined and most well characterized is the B7/CD28/CTLA-4 pathway. CD28 is constitutively expressed on around 80% of human naïve T cells (50% of CD8 and all CD4), with increased expression following T cell activation (June et al. *1990*; Lenschow et al. *1996*).

The CD28 ligands, the B7 molecules CD80 and CD86, are expressed on APCs and are upregulated on T cell activation (Greenwald et al. *2005*). Following TCR stimulation (signal 1), costimulation via CD28/B7 ligands lowers the threshold for activation and increases the expression of IL-2 (signal 2), promoting growth and proliferation of T cells into effector T cells (Lenschow et al. *1996*; Wood and Goto *2012*). CTLA-4 is an additional B7 receptor, upregulated on T cells following activation and with a higher binding affinity for CD80/CD86. Following upregulation on activated T cells, this creates competition for ligation with the B7 molecules, limiting CD28/B7 interaction and resulting in decreased IL-2 secretion, thus attenuating the T cell response (Walunas et al. *1994*). Therefore, a balance between costimulation from CD28 and CTLA-4 is required for T cell activation and preventing continuation of this response (Alegre and Najafian *2006*). After T cell activation, a number of factors can influence T cell differentiation via their effect on cytokine release from transplanted cells or tissues. These factors have been described in more detail above and include the immune status of the recipient, the degree of ischemia-reperfusion injury, donor-host mismatch, or immunosuppressive treatment used to prevent rejection (Wood and Goto *2012*).

3.5 Transplant Rejection

Organ transplant rejection takes place through hyperacute, acute, and chronic rejection according to its time-course. Hyperacute rejection develops minutes to hours after the transplantation of vascularized grafts due to presensitization to donor tissue and is usually mediated by alloantibody and complement (Colvin and Smith *2005*). This occurs due to the presence of antidonor antibodies in the recipient prior to transplantation. This type of rejection can be avoided by matching donor and recipient for blood type (ABO compatibility) and excluding the presence of antidonor HLA antibodies in vitro (Moreau et al. *2013*). Hyperacute rejection is also found in phylogenetically distant xenotransplantation, for example, primates (including humans) possess antibodies to the α-galactosyl epitope expressed by many porcine cell types, making immunosuppression essential for such xenografts (Sandrin et al. *1993*). More recently, the genetic modification of pigs has aimed to develop a potential xenogeneic source of donor tissue for transplantation by deleting xenoantigens to which humans have preformed antibodies, and by transgenic expression of protective proteins such as human complement proteins (Cooper et al. *2021*). Solid organ xenotransplants from Galα1-3Galβ1-4GlcNAc (Gal) knockout pigs and baboons require either additional genetic manipulation, or immunosuppressive treatments, to promote survival (Zhong *2007*). Long-term survival of genetically multimodified α1,3-galactosyltransferase knockout pig hearts expressing human CD46 (complement) and thrombomodulin pig hearts following transplantation into baboons has been demonstrated recently; however, recipient animals also required extensive immunosuppressive

treatment (Längin et al. *2018*). These requirements for further treatments to promote the survival of organ xenografts mean, as yet, genetically modified porcine donor tissue is unlikely to be a suitable source of tissue for peripheral nerve transplantation. As well as preformed natural antibodies, potential transplant recipients may possess antibodies developed due to prior exposure to foreign antigens (Auchincloss and Sachs *1998*). Potential prior antigen exposure, for example, through pregnancy or previous transplant, is therefore an important consideration in the selection of transplant recipients. Acute rejection occurs from 1 week to several months after transplantation and is thought to result from two immunological mechanisms, either alone or in combination. These are alloimmunity, following the development of antigen-specific T cells for donor MHC, and a B-cell-dependent process resulting in acute humoral rejection through the development of donor-specific antibodies (Colvin and Smith *2005*; Moreau et al. *2013*).

Chronic rejection, now the leading cause of rejection of solid organ transplants, develops over months to years and can also be mediated by humoral or cellular mechanisms (Moreau et al. *2013*). Circulating HLA-specific antibodies are often found in patients with long-term organ allografts, and the presence of these antibodies greatly increases the risk of chronic rejection (Colvin and Smith *2005*). If the recipient has not previously been exposed to the transplant alloantigen, B cells do not directly participate in the acute rejection response. However, after sensitization from, for example, a previous transplant or blood transfusion, antibodies will be produced which can cause hyperacute and accelerated rejection. This may also contribute to chronic rejection of transplants, due to the development of alloantibody (Sayegh and Turka *1998*). Alloantibodies target foreign MHC molecules, but antibodies specific for minor histocompatibility antigens, endothelial cells, and blood group antigens can also contribute to rejection. Although the main alloantigens responsible for inducing transplant rejection are MHC molecules, host or recipient MHC can also present minor histocompatibility antigens and result in rejection even in MHC-matched transplants (Peugh et al. *1986*).

4 Peripheral Nervous System Immunology

As for the central nervous system (CNS), the peripheral nervous system (PNS) is considered a site of relative “immunological privilege.” These areas are separated from the periphery by barriers which can limit the passage of immune cells, and lack a resident population of immune cells themselves; however, this privilege is not absolute. The blood-nerve-barrier (BNB) in endoneurial blood vessels prevents the movement of substances including circulating immune cells via tight junctions. The perineurium, composed of layers of perineurial cells connected by tight junctions, offers the main barrier between the endoneurium and extrafascicular tissues (Peltonen et al. *2013*). Tight junctions comprise a complex network of transmembrane and peripheral proteins to restrict the flow of ions and molecules into the endoneurium, including claudins, occludins, junctional adhesion molecules, and zonula occludens complexes (Richner et al. *2019*). The BNB and perineurial barriers act as diffusion barriers preventing access to the endoneurial compartment; however, this restriction is not complete. Soluble factors and cellular elements can enter the PNS in areas where the barrier does not exist, including root entry and exit zones where CNS and PNS segments meet, and nerve terminals (Kieseier et al. *2006*). In addition, a lack of lymphatic drainage and limited passage of lymphocytes separates the endoneurial environment, reducing foreign antigen presentation in the peripheral lymphoid organs. Similar to the CNS, along with these physical barriers, cells in the nerve parenchyma generally lack constitutive MHC expression and are not professional APCs (Hughes

1992). Professional APCs are required to process and present antigen to T cells via MHC molecules in order to initiate an immune response (Wekerle et al. *1987*).

However, as for the CNS, immunological privilege in the PNS is no longer considered to be absolute. Constant immunological surveillance by patrolling T and B lymphocytes is operative, and following activation, for example, in a disease state, these cells can cross the BNB and increase permeability of the barrier thus also allowing the access of antibodies irrespective of the specificity of antibodies or T cells (Pollard et al. *1995*; Spies et al. *1995*). This disruption has been shown to include loss of tight junctions and separation of endothelial cells (Powell et al. *1991*). The BNB is also compromised after PNI, often via a breach at the injury site, and additionally following axonal degeneration, the barrier is compromised along the length of the nerve distal to the injury up to at least 4 weeks postinjury (Gray et al. *2007*). The peak inflammatory response occurs after 4–7 days, and this corresponds with maximal perineurial permeability (Weerasuriya and Hockman *1992*). Thus, increased permeability is also induced following the administration of proinflammatory cytokines such as TNF-α (Spies et al. *1995*). Increased permeability under these circumstances will allow cells and blood-borne factors to enter the nerve and facilitate tissue repair. In the steady state, local resident macrophages make up around 2–9% of the endoneurial population in peripheral nerves (Monaco et al. *1992*; Mueller et al. *2003*). Resident macrophages express MHC molecules and complement receptors, allowing them to perform antigen presentation and surveillance.

Although transplanted nerve grafts do not contain MHC class II expressing professional APCs, the secretion of inflammatory cytokines will cause its upregulation on donor cells within the transplanted tissue thus permitting direct presentation to host lymphocytes. Endoneurial macrophages show up-regulation of MHC class II following the secretion of IFN- γ (Hughes *1992*). Similarly, SCs constitutively express low levels of MHC class I but no MHC class II molecules. However, they have the capacity to upregulate MHC class I and express class II following stimulation by IFN-γ and tumor necrosis factor α (TNFα), or in coculture with activated T cells. Consequently, upregulation is also observed in vivo in an inflammatory environment due to exposure to these cytokines (Hartlehnert et al. *2017*). Additionally, molecules such as ICAM-1 which can exert costimulatory functions are also upregulated by cytokines (Gold et al. *2006*). Thus, SCs have been identified as inducible APCs, capable of presenting antigen to T cells, including presenting antigenic epitopes of myelin components following myelin phagocytosis by SCs (Wekerle et al. *1986*). In addition, SCs are a source of proinflammatory cytokines including interleukin-1 (IL-1), IFN-γ, and TNFα, showing a role in the modulation of local immune reactions in the PNS. They are also able to modulate local immune reactions in the PNS via the release of humoral factors which can inhibit or stimulate T cells (Gold et al. *2006*).

4.1 The Immune Cell Response to Nerve Injury

The innate immune system plays an important role in recovery from PNI. The injury site is invaded by various immune cells, with phagocytic neutrophils and monocyte-derived macrophages arriving quickly following injury (hours to days), and lymphocytes accumulating in the distal stump after a week or so (Gaudet et al. *2011*). Regeneration is dependent on Wallerian degeneration in nerve segments distal to the lesion site, through which severed axons grow back to target tissues. Axons regenerate readily after crush but not transection, resulting in variation between the cellular and molecular events following a crush injury or a cut. Wallerian degeneration is characterized by the breakdown of axons, recruitment and activation of macrophages, to remove degenerated axons and

myelin along with resident SCs and resident endoneurial macrophages (Mueller et al. *2003*; Rotshenker *2011*). As described previously, cytotoxicity and cell death pathways are important components of the response to infection, disease, or injury. In the context of nerve injury and pain, inflammation and cytotoxicity are key mechanisms of the cellular immune response, designed to facilitate the repair of injured tissue (Davies et al. *2020*). Following trauma, local cytokine and chemokine release by immune and nonimmune cells (e.g., fibroblasts) leads to the accumulation of bone-marrow-derived macrophages, which are not normally present in the intact PNS (Shamash et al. *2002*). The first phase is characterized by proinflammatory cytokine (e.g., TNFα,) production, and the second phase by anti-inflammatory cytokines (such as IL-10) and macrophages of the M2 phenotype, involved in tissue repair (Rotshenker *2011*). Macrophages, along with SCs, are important for the removal and degradation of myelin debris during Wallerian degeneration and exert cytotoxicity via phagocytosis (Stoll et al. *1989*). Degeneration of the peripheral nervous system requires this immune response and predicates successful regeneration. This debris clearance is also facilitated by complement, with components synthesized by SCs to attract macrophages to the injury site, and a reduction in autoimmunity through the clearance of apoptotic cells (Cashman and Hoke *2017*). The interaction between different arms of the immune response (complement, innate, adaptive) and the cell types concerned is complex, and the exact roles of each in debris clearance are still under investigation. However, it has been shown that deficiencies in adaptive immunity, as tested in immunodeficient mice, do not prevent degeneration or debris clearance, showing that the innate immune response is dominant in the clearance process early after injury (Cashman and Hoke *2017*).

4.2 The Immune Response to Peripheral Nerve Grafts

The presence of therapeutic cells within nerve grafts greatly improves the outcomes of nerve repair; however, if donor cells elicit a rejection response from the host immune system, this can have a negative effect on regeneration (Gulati and Cole *1994*). Although clinical studies can provide information about the efficacy of peripheral nerve autografts and allografts, preclinical studies include grafts of varying genetic disparity. The peripheral nerve autograft is commonly used as well as “isografts” between different donor animals from within an inbred strain, which are assumed to not differ genetically. These can be compared against mismatched allografts between rats of differing inbred strains. In the literature, outbred rat stocks are often frequently used, which will increase variability in transplant studies between even littermates due to potentially inconsistent differences between animals. While no immune response is elicited to peripheral nerve autografts/isografts, allografts induce a response initiated by the recognition of foreign histocompatibility antigens on donor tissue and result in destruction of the graft via the mechanisms described above (Gulati *1998*). Transplanted nerves are, however, classified as tissues of low immunogenicity, as the immune response to allografts has been reported to be lower than in other tissues such as muscle and skin (Hettiaratchy et al. *2004*; Siemionow and Sonmez *2007*).

As previously described, transplanted nerve tissue contains a range of different cell types, which may or may not be recognized by the host immune system. The cellular components of the endoneurium have been concluded to be the immunogenic component of the graft. Among these, SCs have been suggested to have the highest antigenicity, due to the upregulation of MHC Class II following transplantation and their subsequent role as APCs (Wekerle et al. *1986*; Hughes *1992*; Siemionow and Sonmez *2007*). As decellularization of nerve allografts reduced the immune response to transplanted peripheral nerve, living donor cells within the implanted nerve must be

capable of inducing an immune response (Gulati and Cole *1994*). After transplantation, the BNB is broken down, and rapid revascularization of the allograft occurs from the proximal and distal segments of the host nerve via direct anastomosis between the host and graft vessels or neovascularization. Revascularization allows progressive invasion of blood-borne macrophages and lymphocytes into the donor nerve, initiating local inflammatory responses and allowing recognition of donor-specific antigens (Gulati *1998*; Siemionow and Sonmez *2007*). Established long-term rat allografts in immunocompromised or immunosuppressed hosts have been shown to be protected by normal permeability barriers, which were assumed to be from donor perineurium and endoneurial vasculature (Zalewski et al. *1993*). However, lymphocyte infiltration into nerve allografts through compromised barriers will occur in immunocompetent hosts (Gulati *1998*; Hellenbrand et al. *2016*).

Living SCs within peripheral nerve grafts can act as APCs and have been considered the main target in nerve allograft rejection, though the relative contribution of the direct and indirect pathways is not clear. Ray et al. (*2011*) identified a more significant contribution of the indirect pathway in allorecognition than previously assumed, via allotransplantation of MHC−/− mouse sciatic nerve grafts into wild type hosts and vice versa, thus isolating the indirect and direct pathways (Ray et al. *2011*). The authors found that the elimination of the indirect pathways reduced the host response, improving regeneration compared to allografts. However, eliminating only the direct pathways yielded no improvement in comparison to allografts. This seems to suggest a predominance of the indirect pathway, though this may be due to the relative lack of professional APCs in nerve allografts. Since SCs are facultative APCs, indirect presentation of antigen may contribute to a larger portion of the host response compared to grafts containing, for example, professional APCs such as DCs (Ray et al. *2011*). Although different cell types, such as SCs, can present antigen, it is known that the efficiency of antigen presentation can be very different. Thus, some cell types are considered professional APCs, including B lymphocytes, macrophages, and DCs. Of these, B cells and DCs are effective APCs which function for antibody secretion and the initiation of T cell responses, respectively. Although, in addition to MHC-1, macrophages express MHC-II and costimulatory molecules, these are at much lower levels, and therefore macrophages are less efficient at antigen presentation than B cells or DCs (Trombetta and Mellman *2005*).

Transplantation of nerve isografts has been shown to produce superior regeneration to nerve allografts (Gulati and Cole *1994*; Kvist et al. *2007*). Early research sought to quantify the immune response to nerve allografts and identify the associated immunological mechanisms using rat allograft models with mismatching RT1 (rat MHC) histocompatibility (Mackinnon et al. *1982*). Nerve allografts were found to be less immunogenic than skin allografts with only minor differences in donor/host histocompatibility, which may explain the success of some clinical nerve allograft cases. The response was shown to be composed of dense infiltration with activated lymphocytes and macrophages first in the epineurial tissues, while the perineurium conferred a temporary barrier to these cells before their infiltration. Such infiltration was not observed in isografts, or in the intact nerve (Mackinnon et al. *1982*). When matching for major or minor histocompatibility antigens in nonimmunosuppressed rats, it was found that host nerve fibers regenerated functionally through 2 cm but not 4 cm nerve allografts, irrelevant of the level of matching (Zalewski and Silvers *1980*). This suggests a sufficient immune response to prevent functional regeneration through a longer graft, even from minor antigens.

The time line for the rejection response to peripheral nerve allografts varies between reports. Some show an acute rejection response, peaking around 7–10 days posttransplantation, with full clearance of donor cells by 21 days (Ansselin and Pollard *1990*). Others find an ongoing increase in infiltration of immune cells within the donor nerve up to at least 4 weeks posttransplantation (Gulati *1998*).

When investigating the contribution of different arms of the immune response to peripheral nerve allografts in mice, Ishida et al. found a peak in cytotoxicity due to cytotoxic T cells at around 11 days posttransplantation. Specific antibody production peaked in serum at 3–4 weeks after grafting (Ishida et al. *1990*). These data suggest the cellular immune response is involved in the early stages of rejection, with the humoral response due to the development of specific serum antibodies developing later. Strong activity to both MHC class I and class II antigens was also reported in the study, suggesting expression of both was present in the grafted peripheral nerve tissues. Antibody was detected for up to 3 months posttransplantation, suggesting antibody-induced cytotoxicity to host antigen can remain active for long periods in vivo (Ishida et al. *1990*).

Lassner et al. studied the cellular mechanisms of rejection in rat-peripheral nerve allografts, finding highly elevated donor-specific MHC class I and II expression 1 week after transplantation, with specific expression on donor myelin and interstitial cells close to the coaptation sites. After 2 weeks, this had largely reduced and was no longer detectable by 6 weeks. Finally, after 12 weeks, signs of regeneration were evident. The authors suggest that nerve allograft rejection is directed at vascular endothelium and myelin as a product of SCs, and that donor-derived SCs are no longer present in rejected allografts at 6 weeks. Subsequently, the graft acts as an acellular allograft, with intact tissue architecture, permitting some regeneration (Lassner et al. *1989*). More recently, Roballo and colleagues sought to evaluate the immune response to 1 cm nerve autografts and allografts in the sciatic nerve of immunocompetent rats, by characterizing cellular processes at various timepoints posttransplantation. The authors reported a significant elevation in immune cell density within autografts 3 days after transplantation compared to allografts, in particular of macrophages, NK cells, and cytotoxic ($CD8^+$ T cells) (Roballo and Bushman *2019*). This subsequently reduced up to 28 days posttransplantation, except for the regulatory T cell (Treg) marker CD25, which spiked at day 7. In allografts, no consistent elevation of effector T cell density was found compared to autograft. This is suggested to be due to the immunological privilege of the peripheral nerve. It is unclear why this would be the case given the ability of SCs within the graft to express MHC-II and act as APCs to present antigen to T cells accumulating within the graft. In addition, no increase in $CD25^+$ Tregs was found in autografts; therefore, the lack of rejection could not be mediated by the repression of effector T cells by Tregs (Roballo and Bushman *2019*). Indeed, it has been shown that peripheral nerve cells and tissues display several immunological hallmarks, all nucleated cells would express MHC Class I within the peripheral nerve, and resident macrophages and DCs would be capable of presenting antigen via MHC Class I and II. Mouse-peripheral nerve cells have been shown to be robustly immunogenic in vitro (Ishida et al. *1990*). A confounding factor in this experiment is the use of donor tissue from an outbred rat strain, which may increase variability in the data due to chance potential tissue compatibility between donor and host (Evans et al. *1994*).

The immune response to peripheral nerve allografts also poses a challenge for the development of novel tissue engineering solutions for peripheral nerve repair. Tissue-engineered constructs with a cellular component have been shown to produce greater functional outcomes than nerve conduits alone (Carvalho et al. *2019*), though this has implications for the immunogenicity of the graft. Although it is possible that autologous cells could be used to develop grafts, a more cost-effective solution for an “off the shelf” living nerve construct would be using allogeneic cells. The potential for the generation of cell banks of different haplotypes to attempt to HLA match transplants to individuals has been proposed (Taylor et al. *2012*), though it has been shown that HLA matching only may be insufficient without the use of further immunosuppression. Even delivering matched transplants to the brain, another “immunologically privileged site” does not afford sufficient protection from the immune response (Aron Badin et al. *2019*). Reports have suggested that only very low levels of MHC class I and II are expressed on immature cells (Odeberg et al. *2005*) and on

embryonic stem cells (ESCs) (Drukker et al. *2002*), and therefore that these donor cells may be less susceptible to rejection on transplantation. However, subsequent differentiation of cells in culture was shown to cause upregulation of MHC class I expression, and the addition of IFN-γ also induced MHC class II upregulation suggesting these cells are likely still immunogenic on transplantation (McLaren et al. *2001*; Odeberg et al. *2005*; Drukker et al. *2006*). Variable immunogenicity has also been demonstrated in induced pluripotent stem cells (iPSCs), with autologous iPSC transplant rejection observed in mouse hosts (Zhao et al. *2011*). Following the rejection of cellular components of nerve allografts, tissue architecture remains intact, allowing function as a nerve conduit. However, the regenerative potential of such a conduit is reduced compared to a graft containing viable therapeutic cells such as SCs (Pan et al. *2019*). These findings indicate that transplantation of cellular nerve grafts is likely to require host immunosuppression, or an alternative approach, to prevent the rejection of grafts and allow regeneration.

An additional consideration for the use of artificial nerve guidance conduits is the response to the biomaterials themselves. The use of natural materials such as decellularized tissues and tissue-derived hydrogels may elicit a foreign body response and adaptive immune responsive due to the presence of native molecules resident in the tissue (Morris et al. *2017*). The foreign body response is initiated by implantation of material as a response to the injury, following a similar but altered response to normal wound healing and repair mechanisms. This begins with protein adsorption to the material surface, where a provisional matrix forms at the tissue/material interface comprising a thrombus/blood clot (Anderson et al. *2008*). This provisional matrix contains chemoattractants, cytokines, and growth factors which induce proliferation and activation of macrophages and other cell populations involved in the inflammatory response. Acutely, this inflammatory response is characterized by the invasion of neutrophils and mast cells which interact with surface-adsorbed proteins and release inflammatory cytokines. Depending on the extent of injury at the implant site, this acute inflammatory response usually resolves in less than a week. Recruited macrophages infiltrate and become the predominant cell type in the peri-implant space. Ultimately, these begin to fuse and become multinucleated foreign body giant cells, which further release cytokines and growth factors to promote the invasion of fibroblasts and induce fibrotic deposition of ECM, indicating a chronic inflammatory response. Resolution of the acute and chronic response by around 2 weeks can occur with biocompatible materials. By around 4 weeks, this response results in the encapsulation of implants in a largely avascular collagenous capsule (Morris et al. *2017*).

Although the focus of this chapter is on the host response to allografts or transplants containing allogeneic cells, with the use of biomaterials in tissue-engineered constructs or for growth factor/drug delivery, it is important to consider that the innate response to biomaterials can have an influence on the adaptive response to associated antigens (Sefton et al. *2008*). Biomaterials may be used as vehicles for the delivery of cells to the peripheral nervous system, and therefore may potentiate the immune response toward the cells via the adjuvant effect of the biomaterial (Babensee *2008*). The main mechanism for this is through the maturation of APCs, specifically DCs. DCs have been shown to infiltrate and interact with biomaterials and to mature in their presence, increasing the expression of costimulatory and MHC Class II molecules and secretion of proinflammatory cytokines and inducing T cell proliferation in vitro (Yoshida and Babensee *2004*). This effect has been shown to be dependent on contact with biomaterial, and maturation of DCs differs depending on the type of biomaterial (Babensee *2008*). The recognition of biomaterials by DCs is thought to occur via the same mechanisms as the response to pathogens, through PRRs which may recognize molecular patterns associated with biomaterials, similar to PAMPs. In addition, the protein layer adsorbed to the biomaterial surface may provide ligands which serve as “danger signals” or DAMPs. These can prime the system for an enhanced immune response which will be targeted at the

antigenic component of the transplant, the donor cells. Thus, the avoidance of these signals through minimizing tissue injury during implantation and optimal biomaterial selection should reduce DC maturation and the resulting adaptive immune response to transplanted cells.

5 Strategies for Preventing Rejection of Peripheral Nerve Transplants

A number of strategies have been investigated for preventing allograft rejection. Preventing the immune response to transplants could be achieved through inhibiting the host immune response, or modification to lower the immunogenicity of the transplanted tissue itself (Ishida et al. *1990*). A simple approach to reducing the immunogenicity of nerve allografts has been achieved through decellularization, removing the cellular component which is responsible for inducing the immune response to grafts, and leaving the nerve bridge structure intact.

5.1 Pharmacological Immunosuppression

In organ transplantation, the introduction of cyclosporine A (CsA) yielded great improvements in the preservation of graft function as well as prolonging patient survival. Transplantation became a possibility for not only acutely life-threatening conditions, but also those which adversely affected quality of life and longevity (Brenner et al. *2002*). To prevent the rejection of nerve transplants through suppression of the immune response, chronic systemic administration of immunosuppressant drugs is required, associated with secondary risks of toxic effects including nephrotoxicity and hepatotoxicity (Rezzani *2006*; Teh et al. *2011*). Treatment also requires long-term treatment at high doses to achieve efficacy (Rustemeyer et al. *2010*). The aim of peripheral nerve transplantation is to restore function and thus improve quality of life. Since this is not treating a life-threatening condition, the risks associated with systemic immunosuppression may not be warranted (Mackinnon et al. *1992*). Benefits of allografting and the potential outcomes must therefore be carefully weighed against the risks associated with immunosuppression. However, unlike other transplants, the function of a nerve graft is to provide a conduit to facilitate axonal regeneration across a nerve gap. Once host axons have traversed the nerve conduit and achieved functional connections with target end organs, living donor cells within the graft may be replaced with host cells including vasculature and SCs (Mackinnon et al. *1992*). This scenario gives the possibility of temporary immunosuppressive treatments for peripheral nerve allografts or engineered nerve grafts containing allogeneic cells (Midha et al. *1993*).

Cyclosporine A (CsA) is an immunophilin ligand which binds cyclophilin, blocking the phosphatase activity of calcineurin which is essential in T cell activation and therefore prevents immune response initiation (Ho et al. *1996*). CsA or other immunosuppressant drugs can be administered individually, or in combination to promote transplant survival. Effective regeneration across peripheral nerve allografts following immunosuppression with CsA and FK506, an alternative immunophilin ligand with higher potency, has been demonstrated in rodents. Successful immunosuppression resulted in comparable regeneration in allografts as compared to autografts/isografts (Bain et al. *1988*; Udina et al. *2003*). Treatment with FK506 has also been shown to be effective in rescuing rejecting rat peripheral nerve grafts, albeit within a specific window of 10–14 days posttransplantation (Feng et

al. *2001*). In addition to its immunosuppressive action, FK506 has been shown to accelerate regeneration both in vitro and in vivo in rodent models of peripheral nerve injury even after short-term administration (Gold et al. *1994*, *1995*; Rustemeyer et al. *2010*; Yan et al. *2012*), discussed in the chapter "Drug Therapies for Peripheral Nerve Injuries". However, due to chronic side effects associated with systemic delivery of FK506 and global immunosuppression, this is not in regular clinical use for the treatment of PNI.

During regeneration across a nerve allograft, donor-antigenic components within the graft such as SCs are gradually lost and replaced by host components, suggesting only temporary immunosuppression may be required to achieve successful regeneration across a nerve allograft (Midha et al. *1993*, *1994*). Temporary immunosuppressive treatment, with CsA in rats for 8 weeks after nerve allografting, resulted in graft rejection at 14 weeks associated with a decrease in function. However, long-term assessment (14–20 weeks) showed improvement in motor function and morphological parameters, demonstrating that although initial graft rejection resulted in short-term functional decline, good long-term functional regeneration was achieved (Mackinnon et al. *1992*). Clinically, immunosuppressive treatment with CsA or FK506 of individuals receiving peripheral nerve allografts has also been shown to sustain nerve function after discontinuation of treatment (Mackinnon et al. *2001*). Additionally, local delivery of immunosuppression is a potential option which could allow the survival of allogeneic cells for peripheral nerve repair, while avoiding associated side effects. Developments in the field of materials science have yielded various drug delivery devices which can provide sustained, targeted, and controlled release of drugs to specific tissues while reducing systemic toxicity. For example, controlled release formulations have been developed for delivery of calcineurin inhibitors Cyclosporine A (CsA) and FK506, in the form of micro−/nanoparticles or hydrogels, which have shown suppression of T cell proliferation in vitro and in vivo in various rodent allograft models (reviewed in (Fisher et al. *2015*)). Recently, improved regeneration through fresh rat sciatic nerve allografts was shown following the application of PLGA FK506 microspheres suspended in a fibrin hydrogel over the superficial surface of the length of the nerve graft (Zuo et al. *2021*), and some evidence of efficacy in vivo (Fisher et al. *2015*; Dzhonova et al. *2018*).

5.2 Costimulation Blockade

As previously described, two signals are required to initiate an immune response, with the formation of the MHC-T cell receptor complex and the interaction of costimulatory molecules with their ligands. This suggests that blocking these costimulatory signals during TCR stimulation could prevent the T cell response, allowing long-term allograft survival (Sayegh and Turka *1998*). Therefore, a body of research aims to manipulate this interaction via costimulatory blocking to produce tolerance to transplants and avoid adverse effects of immunosuppressant drugs. Blocking costimulatory signals is an approach to manipulate the immune system to promote allograft survival which has been investigated previously in organ transplantation. A number of experiments have reported successful tolerance to transplants following costimulatory pathway blocking. A dual treatment with CD40L antibody following an injection of allogeneic donor splenocytes into mouse hosts was shown to promote skin graft survival for at least 100 days without further immunosuppression, though alloresponsiveness was reported to have increased at this point (Markees et al. *1997*). As no chimerism was reported, the authors proposed a state of "split tolerance" had been achieved. In the context of nerve transplants, even temporary accommodation of a graft would be acceptable to allow survival until regeneration across a nerve gap could be

achieved. Further successful costimulatory blocking has shown that administration of CTLA4-Ig, which blocks the CD28-mediated costimulatory signal, inhibits the immune response to vascularized cardiac allografts in mice for over 100 days. In contrast to the response described by Markees et al., the authors reported donor-specific transplant tolerance when tested with donor-specific or third-party skin grafts (Pearson et al. *1994*). Pearl et al. successfully prevented the rejection of xenogeneic human ESCs and iPSCs after intramuscular injection in adult murine hosts using a combination of three costimulatory receptor-blocking antibodies (CTLA4-Ig, anti-LFA-1, and anti-CD40L). Outcomes were comparable to those treated with immunosuppressant drugs, and mice were found to be tolerant to donor cells, showing specific T cell anergy with no detrimental effects on the hosts' immunity to other cell types (Pearl et al. *2011*).

Costimulatory blockade via the same triple treatment (CTLA4-Ig, anti-LFA-1, and anti-CD40L) successfully suppressed the immune response to mouse nerve allografts, though with no effect on short-term nerve regeneration (up to 9 days), with better regeneration still observed in animals receiving nerve isografts (Kvist et al. *2007*). Subsequent investigation of the effects on long-term regeneration (49 days) confirmed continued suppression of the immune response and, although no difference in regeneration was observed compared to isografts again, the authors reported increased myelination in treated animals (Kvist et al. *2008*). Additional research has found suppression of the immune response and improvements in regeneration of mouse allografts using anti-CD40L antibody treatment to block costimulatory binding, which was validated in two different mismatched allograft models, though again outcomes did not exceed that of the nerve isograft (Jensen et al. *2004*; Brenner et al. *2004*). As reported previously with anti-CD40L treatment (Markees et al. *1997*), tolerance to allografts was not conferred, and subsequent immunological challenge with a repeated allograft resulted in a strong immunological response (Brenner et al. *2004*). Further investigation tested treatment with increasing degrees of costimulatory blockade, CD40/CD40L or CD28 pathway blocking alone (anti-CD40L antibody or CTLA4-Ig), double treatment with both anti-CD40L and CTLA4-Ig, or triple treatment with the addition of ICOS/ICOSL pathway blocking agent anti-ICOSL (Tai et al. *2010*). Increasing degrees of costimulatory blockade were shown to reduce the host response to donor antigen with a clear effect on regeneration showing that triple treatment could improve regeneration comparable to isografts. This depended on the addition of ICOS pathway blocking, suggesting optimal nerve allograft function requires a higher immunosuppressive requirement than allograft survival alone. Blocking this pathway has a specific effect on activated T cells, which may achieve this increased immunosuppressive requirement (Tai et al. *2010*).

Clinically, costimulatory blocking agents are used, though in combination with pharmacological immunosuppressive treatments to reduce repeated dosing and risks of nonspecific immunosuppression (Tai et al. *2010*). Data suggest that blocking multiple pathways for costimulation may be a promising approach for peripheral nerve allografting. It also is an appropriate method of temporary immunosuppression well suited to the requirements for nerve allografts, allowing prolonged unresponsiveness to transplanted tissues or cells while regeneration and repopulation of the graft with host cells occurs.

5.3 Cotransplantation of Regulatory T Cells

Under certain conditions, activation of $CD4^+$ T cells can secrete cytokines which may not be associated with a rejection response. For example, $CD4^+ CD25^+ FoxP3^+$ Tregs play a role in maintaining tolerance to self-proteins and avoiding the development of autoimmunity and can

secrete the anti-inflammatory cytokine IL-10. Therefore, it is hypothesized that balancing Tregs over effector cells could determine transplantation tolerance in the host. This could be achieved either through the in vivo induction and expansion of Tregs, or the infusion of ex vivo expanded cells (Safinia et al. *2015*). It has been shown that Tregs are required for the induction of tolerance to allogeneic antigens, including through costimulatory blocking (Taylor et al. *2001*). The use of Tregs to induce tolerance to transplants allows the possibility of genetic reprogramming of Tregs to tune subpopulations, or expand alloantigen-specific cells (Singer et al. *2014*; Safinia et al. *2015*). The establishment of long-term tolerance requires adoptively transferred Tregs to survive and expand within the recipient, or for the a tolerogenic phenotype to be induced on other T cells within the host.

Transplantation of allogeneic Tregs along with peripheral nerve allografts in rats has been attempted as a method of local, temporary immunosuppression, by delivery around grafts via a degradable hydrogel (Santos Roballo et al. *2019*). The authors aimed to ensure sufficient numbers of Tregs at the graft site through incorporation into a hydrogel which could be administered across the whole site of the nerve transplant and polymerized in situ with UV light. It was shown in vitro that 84% of Tregs encapsulated in hydrogels escaped over 14 days and remained viable, and this was hypothesized to be sufficient to provide immunomodulation during the period of infiltration of nerve grafts with immune cells. After 21 days in vivo, data suggested that implanted Tregs had infiltrated and integrated within the transplanted nerves, and a reduction in infiltration of host CD4+ T cells was also reported. After 20 weeks, improved regeneration was reported in allografts delivered with Treg hydrogels compared to vehicle or untreated allografts, measured with compound muscle action potential recordings and quantification of axons (Santos Roballo et al. *2019*).

5.4 Modifying the Immunogenicity of Donor Cells

Using genetic manipulation to avoid the rejection of transplanted cells is a potential approach to generating a “universal donor” cell. MHC expression is the largest contributor to the rejection response, with higher levels of MHC Class I expression relating to increased transplant rejection and lower with transplant survival (Mason et al. *1986*). Therefore, deletion of both classes has been attempted as a method of evading immunological detection of transplanted cells (Bradley et al. *2002*). Low levels of MHC Class I and II expression in undifferentiated human ESCs have been reported, though subsequent differentiation is found to elevate MHC-I expression. The upregulation of both MHC-I and II is also observed following the addition of IFN-γ (Drukker et al. *2002*). Therefore, although lower MHC expression in undifferentiated ESCs may indicate lower immunogenicity, following differentiation (likely to occur prior to transplantation to avoid tumorigenicity) and upon transplantation and induction of the inflammatory response and associated cytokine release, MHC will be upregulated and increase the chance of recognition and elimination by the host immune system. However, the level of MHC expression is not the only factor relating to rejection, and a lack of MHC expression on allogeneic cells has been shown to activate NK cells which subsequently target transplanted cells for rejection (Phillips et al. *2013*). There is currently little information on the potential contribution of minor histocompatibility antigens in the rejection of hESC-derived cells, though it is accepted that donor cells should be as genetically similar as possible to the recipient. Male hESC lines should not be used as donor cells for transplantation into females, as proteins encoded by the Y chromosome are not present in the host (Roopenian et al. *2002*).

Advances in cell engineering and gene delivery offer the potential for genetic modification of graft cells to reduce the potential for immunological rejection following transplantation, as opposed to manipulating the host through immunosuppression. Engineering cells to reduce or eliminate HLA expression could provide a method to achieve this and offer a “universal” cell line for transplantation. Silencing or deletion of essential molecules for HLA expression, discussed in Cicciarelli et al., can be achieved through various methods (Cicciarelli et al. *2013*). Donor cells can also be modified by the inhibition of costimulatory pathways, and ectopic expression of immunosuppressive molecules. However, as yet, immune tolerance of “universally compatible” donor cells has not been demonstrated in humans, and these cells are also potentially still susceptible to some types of immune responses, including the NK cell response described previously (Zheng et al. *2016*).

6 Conclusions

The development of novel approaches to surgical nerve repair requires a consideration of the host immune response to transplants, as well as the development of the cellular and material components of the nerve transplant. Important consideration should be taken to the inflammatory response elicited by transplantation, the potential foreign body response associated with the use of tissue-engineered constructs, and the immunological response to donor cells within the grafts. It is essential to consider the choice of biomaterial and associated response, and achieve noninvasive delivery of tissue-engineered constructs to reduce tissue damage and avoid increasing the adaptive response to implanted cells. Developing strategies to overcome immunological rejection of allografts, or to modify transplants to reduce immunogenicity, could provide a sustainable source of donor nerve for transplantation. This would alleviate the issues associated with autografting, and improve functional outcome by providing a supply of sufficient donor nerve of the relevant size for repair.

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