IMMUNE MODULATION WITH AMNIOTIC EPITHELIAL CELLS IN PANCREATIC ISLET TRANSPLANTATION

by

KHALID QURESHI

A thesis submitted to the

University of Birmingham

for the degree of

DOCTOR OF MEDICINE

Department of Immunity and Infection

University of Birmingham

April 2012

Abstract

Chronic systemic immunosuppression in pancreatic islet transplantation restricts its clinical application. This study aims to explore the potential of cell-mediated immune-modulation as an alternative to conventional immunosuppressive regimens; specifically investigating the innate immunosuppressive properties of human amniotic epithelial cells (AEC).

Cell constructs composed of human islets and AEC (islet:AEC) were bio-engineered in rotational culture. Insulin secretory capacity and immuno-modulatory potential were characterised using appropriate *in vitro* assays. Fluorescence immunocytochemistry and multiplex arrays was used to identify putative mediators of the immunosuppressive response in isolated AEC monocultures.

Islets and islet:AEC constructs demonstrated sustained, physiologically-appropriate insulin secretion. Resting peripheral blood mononuclear cells (PBMC) were activated on exposure to human islets but this response was significantly ($p<0.05$) attenuated in islet:AEC constructs. Phytohaemagglutinin (5μg/ml)-induced PBMC proliferation was sustained on contact with unmodified islets but abrogated in AEC and islet:AEC constructs. CD4+ and CD8+ T-cell proliferation was responsive to AEC; their in vitro expansion both in response to CD3/CD28 activation and contact with human islets being suppressed by the presence of AEC.

Transplanted islets may thus benefit from an immune-privilege status conferred on them as a consequence of their close proximity to human AEC. Such an approach may diminish the requirement for generalised systemic immunosuppression in islet transplantation.

Dedication

To all my family and friends

Acknowledgements

I would like to thank Mr Richard Downing for his help and encouragement in my career as well as reviewing this thesis and also Dr John Curnow for reviewing this work.

Also a big thank you to Dr Hilary Murray for her help and support in conducting this thesis.

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Abbreviations

ADA	American Diabetes Association
AEC	amniotic epithelial cells
AM	amniotic membrane
AMSC	amniotic mesenchymal stromal cells
ANOVA	one way analysis of variance
ATP	adenosine tri-phosphate
BDNF	brain derived neurotrophic factor
BMI	body mass index
BSA	bovine serum albumin
CD	cluster of differentiation
CIT	cold ischaemia time
CITR	collaborative islet transplant registry
CK19	cytokeratin 19
CM	conditioned medium
CMSC	chorionic mesenchymal stromal cells
CO_2	carbon dioxide
CSC	conventional static culture
CTC	chorionic trophoblastic cells
CTLA	cytotoxic T-lymphocyte associated protein
DC	dendritic cells
DM	diabetes mellitus
DMSO	dimethyl sulfoxide

DTZ	dithizone
ECM	extra cellular matrix
EGF	epidermal growth factor
FACS	fluorescence-activated cell sorting
FasL	fas-ligand
FBS	foetal bovine serum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GAD_{65}	glutamic acid decarboxylase
GDM	gestational diabetes mellitus
GSIS	glucose stimulated insulin secretion
HARV	high aspect ratio vessel
HbA_{1C}	glycosylated haemoglobin
HBSS	Hanks balanced salt solution
HGF	hepatocyte growth factor
HLA	human leucocyte antigen
IBMIR	instant blood mediated inflammatory reaction
IDDM	insulin dependent diabetes mellitus
IDO	indoleamine 2,3 dioxygenase
IEQ	islet equivalents
Ig	immunoglobulin
IGF	insulin-like growth factor
IL	interleukin

INF	interferon
ITN	islet transplantation network
KGF	keratinocyte growth factor
LREC	local research ethics committee
MCP	monocyte chemotactic protein
MHC	major histocompatibility complex
MILR	mixed islet lymphocyte reaction
MMF	mycophenolate mofetil
MODY	mature onset diabetes of the young
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cells
NASA	National Aeronautics and Space Administration
NBS	national blood service
NGS	normal goat serum
NHS	National Health Service
NIDDM	non-insulin dependent diabetes mellitus
NK	natural killer cells
NOD	non obese diabetic
NPH	neutral protamine Hagedorn
NRS	normal rabbit serum
O_2	oxygen
PAK	pancreas after kidney
PBMC	peripheral blood mononuclear cells

PBS	phosphate buffered solution
PEG	polyethylene glycol
PHA	phytohaemagglutinin
pp	pancreatic polypeptide
PPAR-γ	peroxisome proliferator-activated receptor-γ
PTA	pancreas transplant alone
P1	passage 1
RCCS	rotational cell culture system
RLU	relative light units
RT	room temperature
RWV	rotational wall vessel
SI	stimulation index
SPK	simultaneous pancreas and kidney
TGF	transforming growth factor
Th	helper T-cell
TNF	tumour necrosis factor
T-regs	regulatory T-cells
TRITC	tetramethyl rhodamine iso-thiocyanate
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
UKHTB	UK human tissue bank
UW solution	university of Wisconsin solution
VEGF	vascular endothelial growth factor

VNTR	variable number of tandem mini satellite repeats
WHO	world health organisation

CHAPTER 1: INTRODUCTION

CLASSIFICATION, AETIOLOGY, PREVALENCE AND TREATMENT OF DIABETES MELLITUS

1.1. Introduction/Definition and classification of diabetes mellitus (DM)

Diabetes mellitus (DM) is defined as a group of metabolic disorders, characterized by chronic hyperglycaemia with disruption to carbohydrate, fat and protein metabolism, as a consequence of defects in insulin secretion, insulin action, or both (ADA, 2009). The syndrome presents with characteristic symptoms including thirst, polyuria, blurred vision and unexplained weight loss albeit these are often not severe and hyperglycaemia may go undetected for many years. The persistent elevation of plasma glucose, if untreated, heightens the risk of micro-vascular complications including nephropathy, neuropathy and retinopathy (Worrall, 1994, Reichard, 1994, Kilpatrick et al., 2009) and is generally considered to increase the incidence of cardiovascular disease, notably stroke, cardiac failure and peripheral vascular disease resulting in amputation (Stratton et al., 2000, Huysman and Mathieu, 2009).

For the purpose of this thesis, and in its simplest form, DM is classified according to the pathogenic process underlying the hyperglycaemia;

- **Type 1 diabetes** (T1DM) caused by near or complete loss of endogenous insulin production due to pancreatic islet beta (β)-cell destruction which maybe autoimmune or idiopathic
  e.g. with no evidence of anti-islet autoantibodies (Urakami et al., 2002).

- **Type 2 diabetes** (T2DM) a heterogeneous group of disorders characterized by variable degrees of impaired insulin secretion, insulin resistance and loss of regulation of peripheral glucose metabolism.

- **Other specific types of DM** resulting from an underlying defect or disease process which can be identified as disrupting normal β-cell function. Such defects result in a range of different abnormalities including maturity-onset diabetes of the young (MODY) where a genetic defect of beta-cell function results in hyperglycaemia occurring at an early age (usually before 25 years of age). MODY is characterised by impaired insulin secretion with little or no aberration of insulin action (Gat-Yablonski et al., 2006). Other forms of DM in this category include diseases of the exocrine pancreas e.g. fibrocalculous pancreatopathy and endocrinopathies e.g. Cushing's Syndrome.

- **Gestational DM (GDM)** characterized as glucose intolerance occurring or being first observed during pregnancy (Ben-Haroush et al., 2004a). The definition applies regardless of whether the hyperglycaemia is treated with insulin or if it continues post-partum, and does not exclude the possibility of previous undiagnosed hyperglycaemia. GDM is most likely the result of increased peripheral insulin resistance associated with weight gain, hypertension and hyperlipidaemia. In most cases GDM subsides following parturition but women with a history of the condition have a 30-60% chance of developing overt T2DM later in life (Ben-Haroush et al., 2004b).

It should be stated that whilst attempts are made to classify DM theoretically, in practice it may prove difficult to assign a patient to a specific group due to overlap of the presenting clinical symptoms and an inability to ascertain the underlying pathology.

1.2. Prevalence of DM

DM is a global disease with a rapidly increasing number of individuals being affected. According to the World Health Organisation (WHO), the number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild et al., 2004, Setacci et al., 2009) whilst a more recent and alarming estimate by Shaw *et al* predicts the prevalence at 2030 to be 439 million (Shaw et al., 2009). DM is currently more widespread in developed countries but this is set to change with the greatest relative future increases occurring in the Middle Eastern Crescent, sub-Saharan Africa and India (Setacci et al., 2009, Wild et al.,

2004). Thus, diabetes in the adult population of developing countries is projected to increase by 70% from 2010 to 2030 compared to 20% over the same period in developed countries (Shaw et al., 2009). Factors underlying the massive increase include population growth, the advancing age of the population and urbanization with associated lifestyle changes (Shaw et al., 2009, Setacci et al., 2009, Wild et al., 2004). Together these factors will cause the global incidence of DM to rise by 54% between 2010 and 2030, representing an annual growth of 2.2%: nearly twice the annual growth of the total world adult population (Shaw et al., 2009). Generally the incidence is similar in males and females but gender-dependent (male-dominant) differences may occur following puberty and again in the elderly (higher in females aged above 70) (Setacci et al., 2009).

Table. 1 Prevalence of diabetes – most recent figures comparing England with the United States of America

	England*	**USA****
Adults	2,634,263	25.6 million
Children	26,400	215000

*Figures from the Quality and Outcomes Framework 2010

** Figures from the American Diabetes Association (ADA) 2011 (see http://www.diabetes.org/diabetes-basics/diabetes-statistics/)

1.2.1. UK Prevalence of DM

Estimates for 2011 as provided by the Quality of Outcomes Framework suggest that 4.6% of the UK population have some form of diabetes, with over 2.9 million individuals affected.

Despite these disturbingly high figures, it should be noted that many cases, particularly Type 2 DM, remain undiagnosed, suggesting that the overall UK incidence may be greater still.

1.3. Aetiology and Pathology of DM

1.3.1. Type 1 DM

The underlying pathology in Type 1 DM (T1DM) is a profound loss of beta cell mass, a concomitant cessation of insulin secretion and the onset of chronic metabolic aberration, the most prominent feature being hyperglycaemia (Gerich, 1986). The underlying aetiology in most instances is the autoimmune destruction of β-cells residing in the pancreatic Islet of Langerhans (Nerup and Lernmark, 1981). The process is determined by both genetic and environmental factors. With regard to the genetic component of the disease the most important genes are located at the major histocompatibility complex (MHC) HLA class II region on chromosome 6p21, (once referred to as IDDM1). This region accounts for approximately 45% of the genetic susceptibility to T1DM (Buzzetti et al., 1998). The function of the genes in this region is well characterised (viz. presentation of antigenic peptides to T-lymphocytes) yet their involvement in the aetiology of T1DM is not completely understood. Higher risk of the disease is associated with HLA-DR3 and HLA-DR4 with additional susceptibility linked to the DQα-chains and DQ β-chains. In addition to genes which increase susceptibility to T1DM, other MHC (major histocompatibility complex) halotypes are associated with protection from it.

T1DM is characterised as a heterogeneous and polygenic disorder and approximately 20 non-HLA loci considered to be involved in its pathology have been identified, yet our understanding of the function of most of these is limited. IDDM2 on chromosome 11p5.5 is considered to contribute 10% towards disease susceptibility (Bennett and Todd, 1996), mapping to a variable number of tandem mini-satellite repeats (VNTR). Short class VNTR are associated with a higher incidence of T1DM whilst the long class II alleles provide a protective effect. Additionally, IDDM12 located on 2q33 is involved in the actions of cytotoxic T-lymphocyte associated protein-4 (CTLA-4) which is involved in modulation of the immune response (Nistico et al., 1996) and may thus contribute to β-cell auto-immune destruction. Islet cell auto-antibodies and auto-antibodies to insulin and glutamic acid decarboxylase (GAD_{65}) are markers of immune- mediated β-cell destruction and are found in over 80% of individuals with T1DM (Sandler, 1990).

T1DM has a prolonged sub-clinical phase which may continue for several years prior to diagnosis; but the clinical onset of the disease usually occurs before the age of 30 and in many instances during childhood (Eisenbarth, 1986, Kulmala, 2003). When a genetic component is involved an external event, e.g. a viral infection, is considered to be the trigger for the autoimmune cascade which results in the near obliteration (greater than 90%) of the pancreatic beta-cell population (Haverkos et al., 2003, Kulmala, 2003, Dotta et al., 2007). In the initial stage β-cell damage leads to a loss of first-phase insulin secretion, confirmed by use of an oral glucose tolerance test (OGTT), although normoglycaemia may persist despite the reduction in insulin levels (Bleich et al., 1990, Keymeulen, 2008). As the targeted assault on the beta cell progresses, the resulting impairment of insulin secretion eventually gives

rise to elevated plasma glucose. Once the autoimmune attack is at its height circulating levels of C-peptide become undetectable signifying loss of a functional beta-cell mass.

A degree of pancreatic islet regeneration is thought to occur in Type 1 DM, notable during the initial period following diagnosis (referred to as the “honeymoon” period), but the continued presence of the autoimmune response prevents the recovery of adequate endocrine reserves. As a consequence treatment of the condition relies mainly on the restoration of insulin levels by parenteral routes, or by the re-instatement of endogenous beta-cell mass by transplantation of a donor pancreas or more recently pancreatic islets (Pipeleers et al., 2008, Shapiro et al., 2000).

1.3.2. Type 2 DM

Type 2 diabetes (T2DM) has a higher prevalence than T1DM, and is a chronic, progressive metabolic disorder usually characterized by hyperglycaemia, hyperlipidaemia and insulin resistance. The widespread adoption of a sedentary western lifestyle has transformed the profile of T2DM from a disease of aging (with a later onset of development viz. beyond 40) to one which affects both the old and the relatively young. Rising in parallel with the increased incidence of obesity, T2DM is considered to be fuelled by the imbalance between food intake and energy expenditure (Franks et al., 2007) leading to a decrease in insulin sensitivity. A decrease in the adipose tissue protein adiponectin occurs with obesity which results in an increase in insulin resistance and β-cell dysfunction (Bacha et al., 2004). The incidence of T2DM is also linked to low birth weight and poor nutrition and associated with

the deposition of amyloid plaques although the importance of these factors to the pathology of the condition remains unresolved. In experimental models of T2DM, the expression of islet α-interferon is associated with the development of insulin resistance and glucose intolerance (Koivisto et al., 1989, Huang et al., 1994). As with T1DM, the underlying cause is also thought to involve some hereditary link with an increased risk of developing T2DM in individuals with close relatives (parents, siblings) affected by the disease, although no association between the disorder and specific HLA phenotypes or islet cell cytoplasmic antibodies has been demonstrated (Guven and Kuenzi, 1998). Insulin action is also impaired by nicotinic acid (McCulloch et al., 1991) and glucocorticoids (Gunnarsson et al., 1980).

In addition to impaired insulin action, it is now appreciated that individuals with Type 2 diabetes also experience significant β-cell loss. Impaired glucose tolerance is associated with approximately 40% loss of β-cell mass, and a 60-70% decline in β-cell numbers is observed at the time of diagnosis of Type 2 diabetes, as found at post-mortem (Butler et al., 2003). It is likely that a combination of genetic factors and the adverse metabolic environment in which the islet resides leads to a gradual deterioration in β -cell function, leading to the loss of first phase insulin secretion and impaired/ delayed second phase insulin response. With the ensuing onset of hyperglycaemia, a diagnosis is made and oral hypoglycaemics are prescribed. It is thought that the success of this treatment i.e. restoration of normoglycaemia is due to the replenishment of functional β -cell mass and decreased insulin resistance, especially when combined with increased physical activity and weight loss. However, in many cases this therapy alone proves unsuccessful due to the progressive nature of the β -cell depletion and, as with T1DM, exogenous insulin therapy becomes the

only effective treatment option for the management of the condition and prevention/delayed progression of the associated complications.

1.4. Management of DM

The goals of therapy for T1 and T2 DM are to:

- Eliminate symptoms related to hyperglycaemia
- Reduce or eliminate the long-term microvascular and macrovascular complications
- Allow the patient to achieve as normal a life-style as possible

There are a number of pharmaceutical agents that are used in the management of DM; examples of the main groups are presented in the following sections.

1.4.1. Management of T1DM

As individuals with this form of disease lack endogenous insulin production, administration of exogenous insulin is essential for its management.

(i) Insulin Preparations

Current human insulin and insulin analogues are manufactured using recombinant DNA technology although porcine and bovine insulins remain available. Human insulin analogues are further formulated to provide distinctive pharmacokinetics to mimic physiologic insulin secretion. Insulin preparations can be classified according to their plasma half-lives into

- Rapid-acting analogues e.g. insulin lispro, insulin aspart, insulin glulisine
- intermediate acting insulins e.g. isophane protamine insulin, NPH
- long acting insulin e.g. insulin detemir, insulin glargine
- Biphasic preparations containing mixtures of rapid and intermediate acting insulin

(ii) Insulin Regimens

In all regimens, intermediate or long-acting insulins supply basal insulin, whereas the short acting provides prandial insulin. One commonly used regimen consists of twice-daily injections of intermediate-acting insulin mixed with short-acting insulin before the morning and evening meal. Such regimens usually prescribe two-thirds of the daily insulin dose in the morning (with about two-thirds given as intermediate insulin and one-third as short acting) and one-third before the evening meal (with approximately one-half given as intermediate and one-half given as short acting insulin). The drawback of such a regimen is that it enforces a rigid schedule on the patient, in terms of daily activity and the content and timing of meals. If the patient's meal pattern or content varies or if physical activity is increased, hyperglycaemia or hypoglycaemia may result.

Multiple component insulin regimens refer to the combination of basal insulin, pre-prandial short acting insulin and changes in short acting insulin doses to accommodate the results of frequent blood glucose monitoring. Such regimens offer the patient more flexibility in terms of life style and the best chance for achieving near normoglycaemia. This regimen is further

improved by use of continuous subcutaneous insulin infusion systems. Sophisticated insulin infusion devices (insulin pumps) can accurately deliver small doses of short-acting insulin (microlitres per hour) with additional doses to compensate increased glucose levels at mealtimes. Effective use of insulin pumps requires a health professional with considerable experience and frequent interactions with the diabetic management team. Insulin infusion devices present unique challenges, such as infection at the site of infusion (which is less common with the newer devices), hyperglycaemia because the infusion set becomes obstructed or diabetic ketoacidosis if the pump becomes disconnected or otherwise malfunctions.

(iii) Disadvantages of insulin therapy

Despite the combination of insulins employed or the route of their administration they can never perfectly mimic the profile of endogenous hormone release. Individuals with diabetes may at times experience episodes of hyperglycaemia (especially after meals) or conversely hypoglycaemia which can develop without warning and with a severity which may result in hospitalisation.

Additionally, current insulin regimens have immediate access to the systemic circulation, whereas endogenous insulin is secreted into the portal venous system. Thus, exogenous insulin administration exposes the liver to sub-physiological insulin levels, affecting hepatic function and further disrupting glucose homeostasis (Lewis et al., 1996).

1.4.2. Management of T2DM

The management of type 2 diabetes begins with changes to lifestyle, mainly to the nutritional intake and level of physical activity. If after 3 to 4 weeks of this approach the glycemic target is not achieved then pharmacological therapy is indicated. Pharmacological interventions include both oral glucose lowering agents (oral hypoglycaemics) and insulin.

(i) Glucose lowering agents

Oral glucose lowering agents are sub-divided into agents that increase insulin secretion, reduce glucose production/absorption or increase insulin sensitivity.

- **Insulin secretagogues (sulphonylureas) -** stimulate insulin secretion by interacting with the ATP-sensitive potassium channel on the beta cell. These drugs are most effective in individuals with recent onset disease (<5 years), who tend to be obese with residual endogenous insulin production. Sulphonylureas increase insulin acutely and should therefore be taken shortly before a meal. Sulphonylureas are generally well tolerated but have the potential to cause profound and persistent hypoglycaemia. Their hepatic metabolism and renal clearance preclude use in individuals with liver or kidney impairment. Examples of this group are tolbutamide, glimepride and glipizide.

- **Biguanides** reduce hepatic glucose production and improve peripheral glucose utilisation. Although the precise mechanism(s) of action of the biguanides is not

known, reports suggest a role in reducing hepatic glucose production by inhibition of gluconeogenesis (Hundal and Inzucchi, 2003, Hundal et al., 2000). The occurrence of lactic acidosis poses the main risk to this group of compounds but is largely prevented by avoiding its use in patients with renal insufficiency, any form of acidosis, congestive heart failure, liver disease or severe hypoxia. A widely used and effective drug belonging to this class is metformin.

- **α- glucosidase inhibitors** diminish postprandial hyperglycaemia by delaying glucose absorption but do not affect glucose utilization or insulin secretion. This group is unique because it reduces the postprandial glucose even in individuals with type 1 DM. Examples of this group are acarbose and miglitol.

- **Thiazolidinediones** reduce insulin resistance by binding to the PPAR-γ (peroxisome proliferator-activated receptor-γ). Circulating insulin levels fall with the use of these drugs indicating a reduction in insulin resistance. These drugs are contraindicated in patients with liver disease or congestive heart failure (type III or IV). The major drug in this class, rosiglitazone has recently been withdrawn from use in the UK and Europe due to an increased cardiovascular risk associated with its use (Nissen and Wolski, 2010, Graham et al., 2010).

(ii) Insulin therapy in type 2 DM

Insulin should be considered as the initial therapy in type 2 DM, particularly in lean individuals or those with severe weight loss, in individuals with underlying renal or hepatic insufficiency that precludes oral glucose lowering agents, or in individuals who are hospitalized or acutely ill. Insulin therapy is ultimately required by a substantial number of individuals with type 2 DM because of the progressive nature of the disease and the relative insulin deficiency (due to β-cell loss) that develops in patients with long standing diabetes. Treatment with insulin is not without its side effects. Some of the disadvantages of insulin therapy have been mentioned in section 1.4.1. All of the adverse effects of insulin therapy can be potentially eradicated by the use of β-cell replacement therapy.

1.5. Strategies to restore endogenous insulin secretion in T1DM

1.5.1. Pancreas transplantation

The restoration of endogenous insulin secretion by means of pancreatic transplantation has been carried out for many years. The first pancreatic transplant was conducted in 1966 at the University of Minnesota Hospital **,** USA (Kelly et al., 1967)**,** since when, over 23,000 pancreatic transplants have been reported through the International Pancreatic Transplant Registry. The different types of pancreatic transplants are:

- Simultaneous pancreas and kidney transplant (SPK) - accounting for 80% of total performed
- Pancreas after kidney transplant (PAK) – accounting for 10% of total performed
- Pancreas transplant alone (PTA)
- Simultaneous pancreas-kidney transplant from live donors

There was an exponential rise in the number of pancreatic transplants performed worldwide in the 1990's following which the number has reached a plateau. In Europe the number of transplants is decreasing, presumably due to better medical management of diabetes.

Pancreatic transplantation is effective in maintaining normoglycaemia without the risks of inducing hypoglycaemia. In this respect it has clear advantages over the use of exogenous insulin. In addition, evidence suggests that it may prevent the long term complications of diabetes and reverse or alleviate pre-existing complications of the condition e.g. diabetic nephropathy (Fioretto and Mauer, 2011).

Pancreatic graft rejection is prevented by the use of chronic systemic immunosuppressive drugs which counteract the allogeneic reaction to the donor tissue. Both the short term and long term survival rates of pancreas grafts have improved as a result of technical advances and improvement of immunosuppressive regimens (Sutherland and Gruessner, 2007). SPK has the highest graft survival rate at 86% for the pancreas and 93% for the kidney at one year. The overall patient survival is over 95% at one year and 83% at 5 years post transplantation (Gruessner, 2011). Despite the high success rates, pancreatic transplantation is a major procedure which carries significant morbidity in comparison to pancreatic islet transplantation which offers a less invasive means of β-cell replacement.

1.5.2. Clinical Islet Transplantation

Whole pancreas transplantation restores endogenous insulin production but, as individuals with T1DM usually have normal exocrine pancreatic function, islet transplantation represents a less invasive and thus more attractive option (Srinivasan et al., 2007). Islet transplantation is not only an option for the management of T1DM but has the potential, in the long-term, to become a curative procedure. According to the Diabetes Control and Complications Trial in 1993, intensive insulin therapy improved glycaemic control and outcomes in terms of secondary complication rates but at the cost of more frequent hypoglycaemic reactions in a significant proportion of patients (Srinivasan et al., 2007). The ultimate goal of islet transplantation is to liberate patients from the extensive and potentially life-limiting subcutaneous exogenous insulin regimen, whilst delivering sustained improvements to glycaemic control and thereby halting the progression of secondary

complications, chiefly end-stage organ failure. Current candidates for this procedure are limited to those with hypoglycaemic unawareness and those who already require immunosuppressive therapy due to a previous renal transplant (Ichii and Ricordi, 2009, Digon, 2009, Srinivasan et al., 2007).

1.5.3. Historical Overview of Clinical islet Transplantation

The concept of islet cell replacement pre-dates the discovery of insulin. Watson-Williams and Harshant performed a xenotransplantation of sheep pancreas into a 15 year old boy in 1893 who presented with the symptoms of diabetic ketoacidosis (Watson-Williams, 1894). Unprocessed sheep pancreatic grafts were transplanted subcutaneously in the patient's chest and abdominal wall in the belief that extracts from this organ were able to counteract the clinical symptoms of glycosuria. Whilst the premise for the procedure was correct, supposedly based on the earlier findings of von Mering and Minkowski connecting pancreatectomy with the occurrence of glycosuria (Mering and Minkowski, 1889), the approach was bold and doomed to failure, with the patient succumbing to his illness 3 days later. Other attempts of pancreatic grafts, including human tissue from cadaveric donors were of minimal success, and following the groundbreaking research of Banting, Best, Macleod and Collip and the subsequent isolation and purification of insulin in 1921 the concept of islet replacement was virtually forgotten. For many years insulin remained the only treatment for T1DM, prolonging the lives of those affected. In time, as these patients lived longer it became evident that insulin was unable to counter the life-threatening secondary complications of diabetes to which many diabetic individuals did, and still do,

eventually succumb. In response, attempts to perform a vascularised pancreas transplantation were made (Kelly et al., 1967), and as a natural progression the possibility of replacing the islets alone was explored.

Interest in islet replacement therapy was stimulated by the results of pancreatic islet autografts. A report in 1980 by Najarian *et al* described the outcome of a series of 10 patients who underwent total pancreatectomy and subsequent autologous islet transplantation for chronic pancreatitis; 5 patients experienced varying periods of insulin-independence (Najarian et al., 1980). In another study later that year (Largiader et al., 1980), 4 patients had combined renal and pancreatic transplantation in which pancreatic micro fragments were implanted into the spleen of which one remained insulin-free up to $9^1/_2$ months following transplantation. These autografts were considered as "proof-of-concept" for islet cell replacement and were followed by numerous reports of allogeneic islet transplantation in humans. However, the overall success rate from these studies in terms of insulin independence at 1 year was less than 10% (Srinivasan et al., 2007). Indeed, of the 267 allografts transplanted between 1990 and 2000, only 12.4% resulted in insulin independence for periods of more than one week, and only 8.2% remained insulin independent for more than one year (Shapiro et al., 2000).

A paradigm shift occurred in 2000 with the publication of a trial of clinical islet transplantation conducted by researchers in Canada (Shapiro et al., 2000). Seven patients with a history of Type 1 diabetes of over five years, erratic blood glucose measurements despite being on insulin therapy and who experienced recurrent severe hypoglycaemia with

coma or metabolic instability received multiple infusions of allogeneic islets into the liver via the portal vein and were maintained under a glucocorticoid-free immunosuppression regimen (sirolimus, tacrolimus and daclizumab). All seven patients became insulin independent and remained so at a median follow-up of 11.9 months (range, 4.4 to 14.9) (Shapiro et al., 2000). The protocol (now widely referred to as The Edmonton Protocol) was subsequently conducted as an international, multicentre trial in 2006 at nine locations to evaluate its reproducibility (Shapiro et al., 2006). Thirty six patients who met the required criteria were enrolled onto the study and the results showed that at 1 year after transplantation, 44% of the patients were insulin independent and 31% of these patients remained insulin independent at 2 years post transplantation. The results at 5 years indicated attrition of graft function with time as only 10% remained insulin-free. However, persistent graft function even without insulin independence appears to provide protection against severe hypoglycaemia and to improve glycaemic stability with the potential, therefore, to limit the progression of secondary complications (Ryan et al., 2005).

1.6. Current clinical outcomes for Clinical Islet Transplantation

According to the latest reports from the Collaborative Islet Transplant Registry (CITR, 2011), there have been 571 allogeneic islet transplant recipients between 1999 and 2009 who have received 1072 infusions from 1187 donors. 481 of these transplants were islet alone whilst 90 were islets with or after kidney transplantation.

Insulin independence was seen in 65% of recipients in the first year post first infusion and by year 2 this was 75%. Despite loss of insulin independence over long-term follow up, the 10 year data indicates overall increase in graft survival rates, presumably due to further refinements to the technique. Thus, the insulin independence period was significantly greater in the graft recipients treated during the period 2004-2007 compared to those receiving implants from 1999-2003. The rates of graft survival can differ with rates of 40% to 80% at 5 years being recorded in the registry. Glycosylated haemoglobin (HbA_{1c}), an indication of glycaemic control, is reduced in islet transplant recipients and in addition a decreased incidence of severe hypoglycaemia is observed even in those recipients who return to insulin therapy and have a partially functioning graft.

1.6.1. Risks of Islet Transplantation

Amongst the 571 islet graft recipients, 29 incidences of malignant neoplasms have been observed involving 27 patients. There have been 18 reported deaths for islet recipients resulting from infection (5), cerebral haemorrhage (3), cardiovascular (2), acute respiratory distress syndrome (1), diabetic ketoacidosis (1), lung carcinoma (1), multi-organ failure (1), acute toxicity (1) and unknown causes (3).

The overall conclusions drawn from the CITR report suggest that islet transplantation is an evolving treatment option for diabetes, which continues to show improved long-term benefits. The technique is minimally invasive with few infusion related complications and a comparatively low incidence of cancer and death.

1.7. Factors influencing Islet Transplant Availability and Outcome

Despite the success of the Edmonton Protocol and an increasing interest in the field of pancreatic islet transplantation, the limited availability of donor organs and progressive loss of graft function adversely restricts the wider use of this approach for the management of DM. The factors most likely to limit wider clinical application include:

- Shortage of donor organs
- Injury to the islet due to the isolation process
- Impact of the site of transplantation
- Risk of recurrent autoimmune destruction
- Use of chronic systemic immunosuppression

1.7.1 Shortage of donor supply

The marked disparity between potential graft recipients and available donor organs limits the clinical application of all major transplant therapies, and this is also the case in islet transplantation. Whilst it has been reported that only 50% of available cadaveric pancreata enter the donor pool (McCall and James Shapiro, 2012), it is widely accepted that the ever increasing number of patients suffering with T1DM will always far exceed the number of clinical islet transplants that may be carried out. The situation is exacerbated by the fact that, at present, whole pancreas transplantation is viewed as the preferred treatment option for re-instating endogenous insulin reserves, further reducing the number of organs available for islet replacement therapy. Also each graft recipient may require islet infusions from up to 3 pancreata to achieve insulin-independence, placing a greater burden on the restricted donor pool.

Widespread adoption of tissue/cell-based treatments for diabetes will, therefore, rely heavily on the successful development of strategies to either obtain or generate large numbers of appropriately functional, readily transplantable and ideally immune-tolerant β-cells. Current research in the field has, to date, failed to identify a reliable source of surrogate β-cells which adequately substitute for those obtained from cadaveric donors. At present the major lines of investigation include the potential use of xenogeneic islets, human embryonic stem cells (hESC) and human induced pluripotent cells (iPSC) which are fully differentiated somatic cells with the capacity for re-programming to a β-cell phenotype.

In regard to xenogeneic transplantation, research has focussed on the use of porcine islets due to their physiological similarity to human islets. Pre-clinical trials are presently being conducted at several centres using encapsulated porcine-derived islet cells with promising preliminary results (Hering et al., 2006).

Several groups have reported on the in-vitro differentiation of embryonic stem cells, adopting defined culture protocols in an attempt to produce stable insulin-secreting cells (Kroon et al., 2008). However at present relatively low numbers of cells have been generated by these methods and those produced lack the characteristics of fully mature and physiologically appropriate insulin-secreting cells; well-defined “proof of concept” studies relating to their ability to sustainably reverse hyperglycaemia are yet to be performed. Furthermore, the use of cells from embryos continues to raise objections on ethical grounds.

Human induced pluripotent stem cells (iPSC) circumvent the controversy associated with the use of embryonic stem cells and furthermore offer the possibility of generating patient-specific cells for use in regenerative therapy. Indeed, certain reports suggest the feasibility of deriving iPSC from the fibroblasts of diabetic individuals which are subsequently reprogrammed towards a pancreatic lineage (Maehr et al., 2009). However, the technique is at present expensive and inefficient and the use of viral vectors and/or oncogenic factors will always be a cause for concern.

Thus, whilst all the above-mentioned techniques may, in time, represent alternatives for use in clinical islet transplantation, at present they remain largely experimental and are not likely to contribute significantly to the wider use of cell replacement therapy in the near future.

1.7.2. Impact of isolation process

Islet isolation is imperative to clinical islet transplantation but the process has deleterious effects on islet viability. It is generally considered that the functions of the endocrine pancreas are closely related to its micro-architecture and therefore metabolic control depends not only on the integrity of the islets but also on the interaction of different cell types within and around the islet (Hopcroft et al., 1985). Disruption of the anatomical relationship between different pancreatic cell populations as a consequence of the isolation process is therefore thought to contribute to loss of islet function. Additionally, the pancreas has a complex vascular and neuronal network that is destroyed during the islet isolation process and is another factor contributing to the loss of functioning islets and subsequent graft failure (Correa-Giannella and Raposo do Amaral, 2009). Ischaemia of the islets occurring during the isolation process causes further insult (Dionne et al., 1993). It has been suggested that the pre-transplant culture of islets may lead to their rehabilitation and also allow time to confirm cell viability and sterility. The original Edmonton Protocol involved the infusion of freshly isolated islets (Shapiro et al., 2000) but in subsequent studies published by the Edmonton group and others islet culture has been shown to enhance islet morphology and overall viability (Murray et al., 2005) despite a slight decline in overall beta cell mass (Kin et al., 2008).

1.7.3. Site of transplantation

Data from the Islet Transplant Registry indicates that 90% of clinical islet transplantations performed worldwide involve portal infusion into the liver (Merani et al., 2008). Historically the liver has been viewed as a suitable site for transplantation based on evidence from experimental studies such as those described by Kemp *et al* showing that fewer numbers of islets are required to reverse hyperglycaemia in rats when located in the liver compared with intraperitoneal and subcutaneous sites (Kemp et al., 1973). The site is advantageous mainly for two reasons: access to the portal vein can be achieved relatively easily under ultrasonic or fluoroscopic guidance (Owen et al., 2003, Goss et al., 2003) and the hepatic site permits physiological delivery of insulin as pancreatic β cells secrete the hormone directly into the portal circulation (Kemp et al., 1973, van der Windt et al., 2008).

Despite the advantages described above there are several reasons to view the liver as a less than hospitable site, contributing to the poor long-term outcome of islet transplantation when compared to that of whole organ. The oral immunosuppressive agents used to prevent graft rejection are present in higher concentrations in the portal blood (prior to the first-pass effect), and are known to have toxic effects on transplanted islets (van der Windt et al., 2008). The portal route places the islets in direct contact with blood making them vulnerable to the 'instant blood mediated inflammatory reaction' (IBMIR) which is characterised by activation of the coagulation cascade resulting in significant cellular damage and a profound reduction in functional graft mass. Also an adaptive immune response may be induced as a result of the early inflammatory changes brought about by resident macrophages (Kupffer cells). Chronic hyperglycaemia results in oxidative stress in tissues due to the induction of

reactive oxygen species. Islets in the liver are exposed to high glucose concentrations in the portal blood and as a result are vulnerable to such an assault (Robertson and Harmon, 2006). Additionally, toxins absorbed by the gastrointestinal tract travel to the liver via the portal vein and thus expose the islets to these potentially harmful substances (Robertson, 2004). Hepatically located islets demonstrate a lower oxygen tension compared to native islets (Carlsson et al., 2001) and disruption of hepatic homeostasis may occur due to a loss of counter regulation mechanisms whereby islets in the liver fail to recognise the metabolic cues to produce glucagon in response to hypoglycaemia (Paty et al., 2002b). Finally, relating to diagnosis and cause of graft failure, taking biopsies of transplanted islets after intraportal transplantation is extremely difficult because the islets randomly distribute throughout the liver sinusoids, parenchyma and portal vascular system; efforts to confirm loss of functional mass by histological means is therefore hampered.

As a consequence to the limitations of the hepatic approach, alternative sites for islet transplantation are being examined including subcutaneous sites which offer easy access for monitoring and graft retrieval. However, subcutaneously implanted islets present insulin to the liver via the systemic circulation rather than the preferred portal route, and with time are thought to become necrotic, indicative of reduced oxygenation and nutrition supply (Shapiro et al., 2000). The gastric submucosa is also being considered as a substitute, offering endoscopic islet delivery, and preliminary experimental studies in large mammals have yielded some promising results (van der Windt et al., 2008). Other sites which have been considered but largely rejected for clinical use include the sub-renal capsule (more generally used in experimental transplant studies), the spleen (previously used for islet auto-

grafts) the pancreas and the omental pouch which offer portal insulin delivery. Intramuscular sites for implantation have been proposed following a report of autologous islet transplantation into the brachioradialis (forearm muscle) of a young girl treated for hereditary pancreatitis. In this instance the graft was fully functional after 2 years (Rafael et al., 2008) but this site is associated with significant leucocytic infiltration (Merani et al., 2008).

1.7.4. Recurrence of the auto-immune response

The mechanism(s) underlying auto-immune-mediated destruction of islet beta cells are not fully understood with existing knowledge being derived mainly from post mortem studies, tissue taken from pre-diabetic and recently diagnosed patients and experimental models of diabetes. It is generally accepted that both CD4+ and CD8+ T-cells are involved in the process of islet insulitis but their relative contributions to β-cell destruction are poorly understood, as are the precise identities of the molecular effector mechanisms which mediate cell death. In addition to T-lymphocytes a myriad of other immune cells including macrophages, dendritic cells (DC) and B lymphocytes work together to induce and sustain the immune assault. Antigen-presenting cells, such as macrophages and DC are the first cells to initiate a response and recruit T-cells via the release of chemokines which infiltrate the islet, closely followed by natural killer cells (NK) and B lymphocytes. Infiltrating macrophages serve to activate cytotoxic CD8+ cells which are known to execute β-cell destruction by the release of cytolytic agents; granzymes and perforins. CD4+ T-cells may also act indirectly through B-cell activation and the generation of complement fixing antibodies. The process is further

stimulated by pro-inflammatory cytokines released by macrophages including interleukin (IL)-12 which activate Th1-type CD4+ T-cells. In turn activated CD4+ T-cells secrete IL-2, interferon-γ, and TNF-α which maximise CD8+ activation. Reactive oxygen species such as nitric oxide may also be released by activated macrophages at the site of islet infiltration. Additionally, the Fas-Fas Ligand pathway has also been implicated in CD8+ T-cell mediated cell death although it has also been suggested that this pathway may play a role in graft tolerance (Yolcu et al., 2011) presumably by directing the apoptosis of Fas bearing T-cells.

In addition to the action of T-cells B lymphocytes are also involved in the pathology of T1DM. Thus, as part of the auto-immune response anti-islet cell antibodies are produced which specifically target the insulin-secreting β-cells. These auto-antibodies persist in many individuals with T1DM even when all of the insulin-containing cells have been destroyed. Replacing these with islets from a donor re-introduces the target antigen, albeit now from an allogeneic source. It is therefore possible that graft recipients will experience a recurrence of the auto-immune response targeting the transplanted tissue in addition to the allo-immune response that would occur in the absence of immunosuppressive therapy. This has been demonstrated in pancreas transplantation as reported by Sutherland *et al* (Sutherland et al., 1984, Sibley et al., 1985, Sutherland et al., 1989). In this series of studies living donor pancreas transplants between HLA-identical twins and siblings performed in the absence of immunosuppression were characterised by a rapid return of hyperglycaemia and evidence of insulitis but no indication of overt acute graft rejection. It was concluded that the damage was caused due to recurrence of the auto-immune response, supported by the detection of circulating islet cell antibodies corresponding with the return of hyperglycaemia. High islet

cell antibody titers may also be detected in pancreas and islet graft recipients, correlating with poor graft function (Thivolet et al., 2000).

1.7.5. Chronic Systemic Immunosuppression – current regimens versus adverse effects

Immunosuppression in clinical islet transplantation aims to reduce two major immune responses likely to be directed towards the graft; (i) the recurrence of the auto-immune destruction of β-cells as described above and, (ii) specific alloantigen mediated rejection. Whilst most immunosuppressive regimens exhibit a degree of success in achieving these aims, they bring with them a range of adverse effects which compromise the health of both the recipient and the islet graft.

Long term immunosuppression is associated with a deterioration of pre-existing morbidity (renal and liver impairment) (Hafiz et al., 2005, Ryan et al., 2005) and the development of new complications (rashes, ulcerations, organ toxicity, infections and tumours) which may be life-threatening (Ryan et al., 2004). Prior to the Edmonton Protocol, islet transplant protocols relied heavily on steroid-based immunosuppression consisting chiefly of antibody induction with an anti-lymphocyte globulin combined with cyclosporine, azathioprine and/or glucocorticoids (Shapiro et al., 2000) many of which were in themselves diabetogenic. The Edmonton Protocol heralded the start of the "steroid-free" era with induction using a monoclonal antibody against IL-2 receptor (Daclizumab), and maintenance with a combination of sirolimus (rapamycin – target 7-10μg/l) and tacrolimus (FK506 – target 3-6 μg/L) (Ichii and Ricordi, 2009, Shapiro et al., 2000) which is thought to have contributed to

improved clinical outcomes (i.e. 54% of recipients insulin-independent at 3years). Despite this success, there remain major problems associated with the new immunosuppressive drugs employed. Reports suggest that sirolimus frequently causes dyslipidaemia, myelotoxicity, hypertension and increases the occurrence of skin rashes, oral mucosal ulceration and symptomatic small bowel ulceration (Molinari et al., 2005). Less frequent side effects of sirolimus include interstitial pneumonitis and proteinuria which resolve on withdrawal (Digon et al., 2003, Senior et al., 2005). Tacrolimus is diabetogenic and its use is associated with neurotoxicity, nephrotoxicity (Gruessner, 1997), and less frequently severe atopic dermatitis and alopecia (Ponte et al., 2007a, Zuk et al., 2011). Recent reports suggest that premenopausal female islet recipients using a sirolimus and tacrolimus immunosuppression regimen have a high incidence of developing ovarian cysts and other gynaecological abnormalities (Del Olmo Garcia et al., 2011, Alfadhli et al., 2009). This drug combination has also been linked to an increased incidence of lymphoma but no definitive relationship has been established (Shapiro and Shaw, 2007), and may also result in abnormal renal and liver function tests, haematological abnormalities and electrolyte imbalance. Mycophenolate Mofetil (MMF – 1000mg/BID) has proven to be an effective alternative to tacrolimus and/or sirolimus and its use reduces the occurrence of adverse reactions (Senior et al., 2005, Hafiz et al., 2005, Hering et al., 2005). Since the Edmonton Protocol was first introduced, this drug along with newer alternatives to the standard induction therapies i.e. alemtuzumab or rabbit anti-thymocyte globulin have been employed demonstrating similar results to that seen with the standard regimen on early follow-up.

In time it may be possible to minimize the side effects associated with chronic systemic immunosuppression but it is less likely that their impact on graft function can be totally eliminated. As previously mentioned the hepatic location of transplanted islets places them squarely in the path of high concentrations of orally-administered immunosuppressive drugs which has direct relevance to long-term islet graft survival. MMF and tacrolimus are both associated with hyperglycaemia and have been shown to inhibit glucose-stimulated insulin release in both primary islets and insulin-secreting cell lines (Paty et al., 2002a). Tacrolimus is considered to directly decrease insulin synthesis and secretion, as evidenced by loss of secretory granules and reduced β-cell density. Reports also suggest that tacrolimus causes dose-dependent and reversible cytoplasmic swelling, and vacuolization of islets leading to apoptosis which is reversed if the drug is discontinued (Drachenberg et al., 1999). Sirolimus inhibits β-cell proliferation and prevents islet regeneration (Zahr et al., 2007, Niclauss et al., 2011) also having a deleterious effects on islet engraftment and function (Marzorati et al., 2009). Of note, sirolimus has been shown to decrease islet cell viability following implantation by both a direct effect on islet integrity and by preventing graft re-vascularisation through inhibition of vascular endothelial growth factor (VEGF) production (Laugharne et al., 2007, Cross et al., 2007). It is evident that the chronic use of immunosuppressive drugs may not be compatible with long-term β-cell survival in clinical islet transplantation and alternative approaches to graft immuno-protection must be devised.

1.8. Strategies to Circumvent Chronic Systemic Immunosuppression

Non-pharmacological methods of preventing islet allo-graft rejection seek to eliminate the harmful consequences of chronic systemic immunosuppression both for the recipient and the islet graft. This field has grown alongside that of clinical islet transplantation itself and, with an increased understanding of immune-tolerance, new and more sophisticated means of delivering graft immune-protection have been developed and clinically evaluated.

1.8.1. Immune-Isolation: Islet encapsulation to reduce allograft recognition

To date, the most widely explored technique for the immune-protection of grafted cells is micro-encapsulation; the process of embedding islets within a semi-permeable membrane (de Vos et al., 2010, Beck et al., 2007, de Vos et al., 2002, Gray, 2001). The process aims to minimize the allo- immune response, chiefly by reducing the ability of the host to recognise surface antigens expressed by the graft. Coating of the islets with a biocompatible material also serves to provide a physical barrier between the host immune system and the transplanted cells, thus preventing direct cellular attack. The masking of surface antigens also makes encapsulation relevant to the field of xenotransplantation; clinical trials are currently underway to determine the efficacy of encapsulated porcine islet grafts which have lent insight into the potential of this technology (Valdes-Gonzalez et al., 2010, Valdes-Gonzalez et al., 2007, Valdes-Gonzalez et al., 2005).

Whilst the theory underlying micro-encapsulation is valid, in practise it has posed challenges, particularly in terms of the materials used. In order to maintain capsule integrity and reduce

the possibility of fibrotic overgrowth encapsulation materials must be biocompatible, robust, endotoxin-free and of a sufficient permeability (porosity) to permit access of vital nutrients and oxygen and the release of endocrine hormones, whilst preventing passage of immune cells. In early studies most capsule materials failed to supply the highly metabolic islet with sufficient nutrients and oxygen conducive to its survival and graft-derived cytokines escaped the capsule to provoke an inflammatory assault (Schrezenmeir et al., 1994, Zekorn et al., 1993) both leading to a significant loss of islet viability. However, more recent improvements including the modification of materials used (e.g. increasing the purity of alginates, poly-lysine, poly(ethylene glycol) (PEG) diacrylates) has led to enhanced islet transplant outcomes as demonstrated in animal studies (Figliuzzi et al., 2006, Langlois et al., 2009).

A further major limitation associated in the technique of encapsulation relates to capsule size which dictates clinical application. Thus, at present, infusion of microencapsulated islets into the liver is precluded by the high risk of venous occlusion and therefore the technique is not compatible with the current clinical islet transplantation protocol. Alternative suitable sites are being evaluated in pre-clinical studies; the peritoneal cavity would be able to accommodate larger transplant volumes but may be unfavourable in terms of its poor potential for graft re-vascularisation. In contrast the omental pouch receives a significant arterial supply, provides appropriate surgical access and displays unique reconstructive capabilities (Berman et al., 2009).

Encapsulation technology may prove most effective when combined with short-term, low-dose immunosuppression which has been shown to reduce capsular overgrowth. Of interest, the use of sirolimus and belatacept (a CD28-CD154 co-stimulation blockade regimen) has proven effective in preventing the rejection of encapsulated porcine islets transplanted into primates, an advance for the use of xenografts (Cardona et al., 2007, Cardona et al., 2006). Nano-capsule devices, formed with multiple layers of biocompatible polymer applied directly to the islet surface markedly reduce bead diameter thus circumventing the problem of restricted nutrient and hormone transfer. When impregnated with a low-dose immunosuppressive agent (e.g. cyclosporine) nano-capsules provide camouflage to allogeneic islets whilst actively creating a local site of immune-privilege (Lee et al., 2006). This process has obvious potential but is technically challenging and incurs significant beta cell loss which is especially critical in light of the shortage of suitable organs for use in clinical islet transplantation. Additionally, it is not yet proven that the nano-capsules have sufficient integrity to withstand the transplantation procedure itself, or to remain intact long-term at the implant site. Energies are now focussed on standardizing the methods for micro-and nano-capsule production including the grade of the components, capsule size, diffusivity, permeability, and structural integrity in an attempt to advance their clinical effectiveness.

1.8.2. Immune-modulation: Anatomical immune-privileged sites and tissues which support allogeneic transplantation

The concept of immune-privilege as proposed by Peter Medawar (Medawar, 1948), has long been the fascination of immunologists and transplant surgeons. Anatomical locations within the body including the eye, testis, pregnant uterus and brain exhibit a rare ability to evade the actions of the immune system and, as such are considered to hold the key to successful allograft transplantation. These sites are considered to be immune-privileged as implanted foreign tissue, placed for example in the anterior chamber of the eye, is capable of survival and thus fails to abide by the normal rules governing immune rejection (Medawar, 1948). To date, a clear explanation as to the special status of these sites has not been fully elucidated although the lack of lymphatic drainage and the presence of a physical blood:tissue barrier are considered to be likely mechanisms, creating "immunological ignorance" (Medawar, 1948). Whilst these physical features may have some significance, especially in the eye, it is now known that immune-privileged sites remain fully accessible to immune cells and that a number of physical and chemical properties work in concert to bring about the diminution of the immune response seen in these tissues. The net effect is down-regulation of the immune system by (a) alteration of antigen accessibility (b) modification of antigen presentation, and (c) modulation of immune cell activity at the site of the graft. The term immune-privilege is now associated both with anatomical sites e.g. areas of the body able to accommodate an allogeneic graft in the absence of systemic immunosuppression, and tissue i.e. groups of cells which survive as allogeneic grafts at anatomical sites where normal tissues would immediately be rejected.

Immune-privileged tissues generally have common features which underlie their inertness including the lack of expression of class I and class II MHC molecules and the expression of class Ib molecules (e.g. HLA-G in the placenta and amniotic membrane). Some tissues also demonstrate unique physical properties where their parenchymal cells are held together by an extensive series of tight junctions as is the case for Sertoli cells (SC) and retinal pigmented epithelial cells. Additionally, cells exhibiting immune-privilege characteristics have been shown to secrete a myriad of immunosuppressive cytokines including interleukin (IL) 4, IL-10 and TGF-β. Furthermore, the constitutive expression of the pro-apoptotic protein Fas Ligand (FasL) has also been reported in a number of immunomodulatory tissues including testis, retina and placental membranes.

In the context of clinical islet transplantation anatomical sites which offer a degree of immune privilege would be beneficial. Sites such as the thymus and testis have been proposed as implantation sites where islets experience a degree of immune-protection. As the thymus is the site for T-cell maturation, including negative selection, the thymic infusion of islets could theoretically expose developing T-cells to islet alloantigens, the net result being deletion of allogeneic islet-specific T-cells (Merani et al., 2008). This would be a significant immunological advantage, yet experimental studies indicate that implantation to the thymus requires a large number of islets if glucose homeostasis is to be achieved (Levy et al., 2002).

The testis provides a second immune-privileged site for islet infusion where experimental studies demonstrate that intra-testicular islet transplantation results in decreased memory CD8+ T-cell infiltration to the site of the graft and induces more antigen-specific CD4+CD25+ regulatory T-cells (Nasr et al., 2005). The use of Sertoli cells for co-transplantation with islets is seen as a natural progression to intratesticular islet transplantation and has been enthusiastically explored both experimentally and in small clinical trials (Yin et al., 2009, Valdes-Gonzalez et al., 2007, Valdes-Gonzalez et al., 2010, Valdes-Gonzalez et al., 2005, Kin et al., 2008) although some controversy surrounds the clinical efficacy of this approach (Wang et al., 2005, Sykes et al., 2006, Sykes and Cozzi, 2006). More recently the anti-inflammatory and immuno-modulatory properties of bone marrow derived mesenchymal stromal cells have been harnessed for clinical use; notably for the treatment of graft versus host disease and autoimmune arthritis, and are now being evaluated for ability to prevent allograft rejection in experimental islet transplant models (Duprez et al., 2011, Ding et al., 2010). It is evident that being able to create immune-privilege either in or within the vicinity of the islet graft would be a major positive step towards improving the clinical outcome and therefore applicability of islet transplantation. The use of “bystander” immunosuppression as suggested by the co-transplantation studies with Sertoli and mesenchymal stromal cells add credence to this approach; its widespread adoption awaits greater understanding of the underlying mechanism(s) for the observed immuno-regulatory effects and detailed studies on the long-term efficacy and safety.

1.8.3. Human Amniotic Epithelial Cells

Recent studies suggest that human foetal membranes provide an alternative source of cells which may also induce localised immunosuppression (Wolbank et al., 2007, Parolini et al., 2008) but without the limitations of availability and standardisation experienced with Sertoli and mesenchymal cells. Human amniotic membrane has current clinical use as a temporary dressing for wounds, burns and as an adjunct to ophthalmic surgery (Sheridan and Moreno, 2001, Mermet et al., 2007, Gomes et al., 2005) where it is grafted without rejection. The cells comprising its inner lining, amniotic epithelial cells (AEC), possess innate anti-inflammatory and immunomodulatory characteristics; secreting soluble factors with the ability to inhibit local activation/migration of neutrophils and macrophages (He et al., 2008, Li et al., 2006a, Tseng et al., 2004, Li et al., 2005) and suppress the activation and graft-destroying actions of immune T-cells (Wolbank et al., 2007, Li et al., 2005). Human AEC also express soluble mediators associated with the creation of immune-privilege including FasL, HLA-G, and IL-10 (Parolini et al., 2008, Wolbank et al., 2007, Bertelli and Bendayan, 2005, Lefebvre et al., 2000). Preliminary data indicates that AEC exhibit a potent and robust suppression of mitogen-induced lymphocyte proliferation whilst exhibiting immune-inertness (Parolini et al., 2008, Wolbank et al., 2007). Their ability to extend this immune-privilege to other, co-transplanted cells (viz. as observed with Sertoli and mesenchymal cells) forms the basis of this thesis.

1.9. Aim of this Study

It is widely considered that islet transplantation offers a unique opportunity to treat T1DM and reduce the incidence of its debilitating secondary complications. The results of several clinical trials including those performed in the UK suggest that a degree of metabolic stability is achieved by islet transplantation which far exceeds that attainable using even the most strict exogenous insulin regimen. However, the widespread use and long-term survival of the islet graft is hampered by a number of factors, including the impact of chronic immunosuppressive drug use.

The unique feature of islet transplantation compared to whole organ treatments is the relatively small volume and cellular nature of the graft which provides the opportunity to use a more defined/discrete and novel means of immune-protection. Microencapsulation in a semi-permeable membrane to evade the immune system is one such approach. Equally credible is a method of actively suppressing the immune system at the site of the grafted cells – effectively creating localised immune-privilege.

1.9.1. Hypothesis

Current literature suggests that human amniotic epithelial cells possess certain unique, inherent immunosuppressant properties which may be exploited to provide a biocompatible method of modifying the immune response. This study proposes that these properties may be extended to provide immune protection to transplanted islets thereby reducing the risks of chronic systemic immunosuppression faced by those undergoing islet transplantation. The present study investigates the immunomodulatory properties of human amniotic cells and their ability to alter the immunogenicity of islet cells as observed in an in vitro co-culture system.

Chapter 2: HUMAN ISLET ISOLATION AND ASSESSMENT OF BETA (β)-CELL FUNCTION IN THE POST-ISOLATION SETTING

2.1. Introduction-Isolation of Pancreatic islets for Research Purposes

The isolation of functionally viable human islets is a prerequisite of both successful clinical islet transplantation and transplant-related research. The technique of islet isolation has evolved over the last 5 decades, culminating in the development of a semi-automated method, capable of providing high quality, clinical grade islets, suitable for use in transplantation. Of note, improvements to the method of organ procurement, the design of a refined collagenase enzyme blend (Liberase, Roche), development of a ductal route of enzyme delivery and custom-made apparatus for organ digestion (The Ricordi Chamber) ensure complete pancreas dissociation. Furthermore, modifications made to the COBE 2991 cell processor have enabled its effective use in islet purification using a linear continuous density gradient. Yet despite these advances, successful islet isolation using the semi-automated method still relies heavily on the procurement of pancreata which meet strict criteria both in terms of donor characteristics and the condition of the organ at the time of retrieval. Added to this, the relative lack of multi-organ donors in the UK means that all pancreata which fulfil these criteria are automatically assigned to the clinical pancreas and islet transplantation program with only those which fail to do so being made available for research.

The islet research laboratory in Worcester, along with others, have devised manual methods of islet isolation whereby additional interventions can be made for the unpredictable variables associated with marginal organs, including multiple sampling during the digestion phase and the use of discontinuous gradients for separation of islets. Interestingly, such manual methods use fewer resources yet are still capable of producing islet yields of up to 300,000 islets per isolation which compare favourably with that achieved using the semi-automated method (400-500,000 islets per isolation). Furthermore, manual islet isolation allows researchers to fully utilize organs rejected for clinical transplantation, producing viable islets in 90% of isolations attempted which contrasts with the semi-automated method where the main islet isolation centres have reported successful clinical grade islet isolations from 25-75% of pancreata procured (Paget et al., 2007). These modifications have enabled viable retrieval of islets from sub-optimal donor pancreata thus making best use of scarce human tissue to further advance the field of islet transplantation and other areas of diabetes research.

Aim of the Chapter

The following is a detailed description of the manual method of islet isolation employed in this study; presenting data regarding the morphological and functional characteristics of the islet preparations obtained and discussing their suitability for use in research.

2.2. Materials and Methods

2.2.1 Pancreas procurement

Human pancreata were obtained through the UK Human Tissue Bank (UKHTB), De Montfort University, Leicester, from multi-organ donors. These organs are deemed unsuitable for clinical use (whole organ or islet transplantation) due to the donor exclusion criteria applied or because of the physical characteristics of the organ (e.g. fatty, fibrous, damage to the capsule or associated vessels). Organs were normally referred within 10 hours of retrieval. For the purpose of the present study the following donor criteria were considered when accepting a pancreas for the purpose of islet isolation.

- **Age-** In most instances organs were accepted from donors aged between 18 and 65. In our experience pancreata from younger donors (i.e. < 15 yrs), whilst yielding islets with superior function, routinely produce a lower yield due to the difficulty in separating islets from the surrounding acinar tissue, as reported by others (Ichii and Ricordi, 2009).

- **Body mass index (BMI)** - Organs referred generally came from donors with a higher (>25) BMI. Studies have shown that such donors yield a higher number of viable islets (Sakuma et al., 2008, Ponte et al., 2007b, Ichii and Ricordi, 2009).

- **Cold ischaemia time (CIT) -** Organs were transported by road from the retrieval centre (or the transplanting centre which had subsequently rejected the organ for clinical use) to our islet isolation laboratory which would often prolong the cold ischaemia time. Routinely we would seek to process organs with a CIT of less than 12 hours. Prolonged CIT adversely affects islet yield and purity (Shapiro et al., 2006).

- **Brain-death versus non-heart-beating donor**- Most organs referred were from brain-dead donors. The degree of warm ischaemia would determine whether we accept organs from non heart beating donors which happened in exceptional circumstances. Such organs have been shown to yield viable islets (Zhao et al., 2007).

- **Time in ITU-** Long periods in intensive care negatively impact islet isolation usually giving a poor yield. In this study only pancreata from donors who had spent less than 3 days in ITU were accepted.

- **Degree of fatty infiltration**- Moderately fatty pancreata are associated with a higher islet yield (Ponte et al., 2007b) although longer digestion periods may be required.

- **Degree of fibrosis** – Highly fibrotic organs resist enzymatic digestion and produce a low yield. Severely fibrotic glands were therefore excluded from this study.

2.2.2. Pancreas digestion and islet purification

A favourable ethical opinion was obtained for use of human pancreatic tissue for research prior to the start of this study (LREC Q5/2801/47 – Coventry and Warwickshire Ethics Committee). In all instances the organs were delivered in a temperature controlled box, on ice and double bagged. Prior to processing, the pancreas was examined for an intact capsule (required for adequate perfusion and distension of the gland with Liberase) and, if attached, the duodenum and spleen were removed. An assessment was carried out to determine the degree of fibrosis in the gland (by palpation) and the extent of fat infiltration (by eye) and if these were found to be of an appropriate level a decision was made to proceed with islet isolation.

The pancreas was transferred to a Class II microbiological safety cabinet. Overlying fat was removed along with a small section of the pancreas head in order to clearly expose the pancreatic duct. The sections removed from the head were processed for later histological and microbiological analysis. Once all extraneous tissue was removed the pancreas was weighed and the duct cannulated. Ductal infusion of a collagenase blend (Liberase – 3.5mg/ml – Roche Diagnostics Ltd, West Sussex, UK) was then performed injecting a total of 60ml of enzyme solution into the gland, thus causing distension. The degree of distension

was noted. The gland was then placed whole into a 1L glass beaker containing a large magnetic flea and 200-250ml of Hanks Balanced Salt Solution (HBSS) was added (sufficient to cover the pancreas). The beaker was sealed with parafilm and transferred to a water bath where the temperature of the organ and the enzyme solution was raised to 37^{o}C. Once the required temperature was achieved, the beaker (with contents), was placed in a stirrer oven at 37^{o}C, thus enabling the liberase to commence enzymatic digestion in combination with continuous mechanical agitation. Every 5-10mins a sample of the digesting material was collected using a pastette and examined for evidence of liberated islets by staining with dithizone (Diphenylthiocarbazone – DTZ, Sigma-Aldrich UK.co.Ltd. – 0.75μg/ml – see below for further details). If necessary the pancreas was divided into 3-4 pieces during the digestion phase to assist islet liberation. On evidence of islet cleavage (i.e. observation of islets completely dissociated from surrounding exocrine tissue - usually after 25-30 mins), the beaker containing the digested pancreas was returned to the cabinet, and the enzymatic reaction stopped by addition of 0.5L of ice cold HBSS supplemented with 0.5% bovine serum albumin (BSA, Sigma-Aldrich).

The pancreatic digest was divided equally between 4-6 large conical centrifuge tubes (250ml) and centrifuged at 400*g* for 5 mins and the supernatant discarded. The resulting pellets were re-suspended in HBSS + 0.5% BSA (each pellet in 50 ml), and any remaining large tissue fragments were removed by passing the digest through a 500μm mesh. The digest was re-centrifuged at 400*g* for 5 mins, the supernatant decanted and the pancreatic digest re-suspended in 50ml of University of Wisconsin (UW) solution which is an organ preservation solution used clinically for the preservation of abdominal organs destined for

transplantation, and incubated on ice for 30 mins to allow the islets to de-granulate. The digest was then harvested by centrifugation at 300*g* for 5 mins, the UW solution discarded and the total volume of digested material calculated.

For islet purification, Ficoll gradient columns were prepared using Histopaque-1077 (Sigma-Aldrich) diluted in HBSS+0.5% BSA and the pancreatic digest thoroughly mixed into the gradient, ensuring homogeneity. The gradient:digest mixture was then divided into 50ml conical centrifuge tubes (13mls/tube) and "capped" with 5mls of HBSS prior to centrifugation at 800*g* for 5 mins (without brake). Following centrifugation the heavier exocrine material formed a pellet while the islets formed a compact layer at the interface between the Histopaque and HBSS. A plastic pastette was used to gently aspirate the islets from the interface and transferred to a 50ml centrifuge tube. The islets were washed with HBSS+0.5% BSA and centrifuged at 300*g* for 5 mins. The supernatant was discarded and the islet pellets re-suspended in Medium-199 supplemented with 10% foetal bovine serum (FBS), 100U/ml penicillin, 100μg/ml streptomycin and 10μg/ml of amphotericin B.

2.2.3. Method for assessment of islet purity, viability, counting and standardisation to islet equivalents (IEQ)

Isolated human islets were stained with DTZ to assist identification and counting. Dithizone is traditionally used as a trace for heavy metals as it readily complexes with mercury and lead. In islets, dithizone forms a stable complex with zinc ions resulting in the formation of a red colour reaction within the insulin containing islets (Latif et al., 1988). Contaminating exocrine tissue (non-zinc containing) is therefore readily distinguished from the islets enabling subjective analysis of islet purity.

To quantify β-cell mass a 10-50μl sample of DTZ stained islets was placed in a 24-well tissue culture plate the base of which was etched with gridlines to assist orientation and counting. Using an inverted microscope equipped with a $1cm^2$ counting grid (in the eyepiece) all of the islets in the sample were counted and sized (100-150 islets per 50μl sample). This counting procedure was performed in triplicate and the mean values used to calculate islet equivalents (IEQ), where each IEQ represents a "standard" islet with a diameter size of 150μm (Ricordi et al., 1990). The structural viability of the isolated islets was assessed by Trypan Blue (dye) score, using an adaptation of the technique described by Goto et al (Goto et al., 2009). In brief, 50μl of islet suspension was diluted in an equal volume of Trypan Blue solution (0.4%- Sigma-Aldrich). Following a brief period (3-5 mins) of equilibration the islet suspension was viewed under an inverted microscope and examined for evidence of dye uptake. Those islets with good structural integrity (i.e. intact membranes) successfully excluded the dye whilst damaged cells showed cytoplasmic (blue) staining characteristic of

non-viable cells. As islets are multi-cellular structures it was necessary to score the degree of dye uptake on a scale of 0 to 3; islets showing no evidence of staining were awarded the highest score, heavily stained islets received a score of 0, intermediate scores were assigned to islets with only a few cells (between 1 and 5, mainly on the periphery) staining positive for the dye. Up to 30 islets were thus sampled and scored; the percentage viability was calculated by dividing the aggregate score by the maximum (i.e.90).

2.2.4. Islet culture

Once counted the islet suspension was adjusted to a density of 500-750 IEQ/ml and seeded into 90mm untreated culture plates (NHS Logistics, Alfreton) with Medium -199 supplemented with 10% foetal calf serum (FCS), 100U/ml penicillin, 100μg/ml streptomycin and 10μg/ml amphotericin B. Islets were cultured for 24 hours at 30°C in a humidified atmosphere of 95%O_2/5% CO_2 to allow recovery and acclimatisation prior to functional assessment as described below.

2.2.5. Functional Assessment

Islet functional viability was determined by assessing glucose-stimulated insulin secretion (GSIS) 24 hours post-isolation. Static challenge studies were performed as follows: Approximately 1000 IEQ were aspirated from the culture dish using a sterile pastette, placed into a 15ml conical tube and centrifuged at 400*g* for 3 mins. The supernatant was discarded and the resultant pellet re-suspended in a 2ml volume of 1.67mM glucose solution made up in HBSS+0.2%BSA (basal glucose). Islets were then placed in a water bath at 37^{o}C for 1 hour (pre-incubation period). During this hour, an islet count as described above was performed to ascertain the extent of β-cell loss as a consequence of overnight culture. If necessary the islet suspension was readjusted (by the addition of the 1.67mM solution) to a density of 400IEQ/ml and 50μl aliquots (equivalent to 20 IEQ) and transferred to 12mm x 75mm polypropylene tubes (NHS Logistics, Alfreton, UK). A total of 18 tubes were thus prepared, to which 2ml of the appropriate secretagogue, diluted in HBSS+0.2% BSA, was added as follows: 6 tubes received 1.67mM glucose solution to assess basal insulin secretion. To a

further 6 tubes a 16.7mM solution of glucose was added to determine stimulated insulin secretion. The remaining tubes received 2ml of 16.7mM glucose supplemented with 10mM theophylline; a potentiator of insulin secretion. The racked tubes were sealed with parafilm and placed into the water bath at 37^{o}C for 1 hour to allow insulin secretion (incubation period). Following incubation the tubes were gently vortexed and then centrifuged at 400*g* for 5 mins. The resultant supernatant was harvested for analysis of insulin content using an enzyme-linked immunosorbent assay (ELISA; Mercodia, Diagenics UK), according to the manufacturer's instructions. Assessment of glucose-induced insulin secretion is a standard method for estimating the functional viability of islets in the early post-isolation period and is presented as the stimulation index (S.I; fold increase in insulin release in response to a known secretagogue compared to basal secretion).

2.2.6. Statistical analysis

Statistical differences in response to insulin secretagogues were assessed by one way analysis of variance (ANOVA) using insulin secretion under basal conditions as the control. In all comparisons a p value of <0.05 was considered to be statistically significant. Statistical analysis was performed using SigmaStat software version 3.5 (Systat Software Inc, Chicago, USA).

2.3. Results

A total of seven human pancreata (five female and 2 male donors with a mean age of 47.14±3.35 years) were procured and successfully processed to obtain the islets used in the present study. The mean cold ischemia time was 10.04±1.25 hours. An average of 70g of pancreatic tissue was processed yielding a mean of approx 111,000 IEQ post-purification (Table 2). In all instances sufficient numbers of viable islets were isolated for research purposes.

2.3.1. Morphological characteristics of isolated islet preparation

The human islet isolation protocol employed in the present study resulted in the harvest of structurally intact islets which were well cleaved from the surrounding exocrine tissue, as previously reported (Murray et al., 2009, Murray et al., 2005) (Fig.1). The purity of the islet suspension following Ficoll gradient -assisted separation ranged from 70-85%, with islets mostly sized between 100-500μm. Trypan Blue exclusion served as an indicator of preserved islet structural integrity.

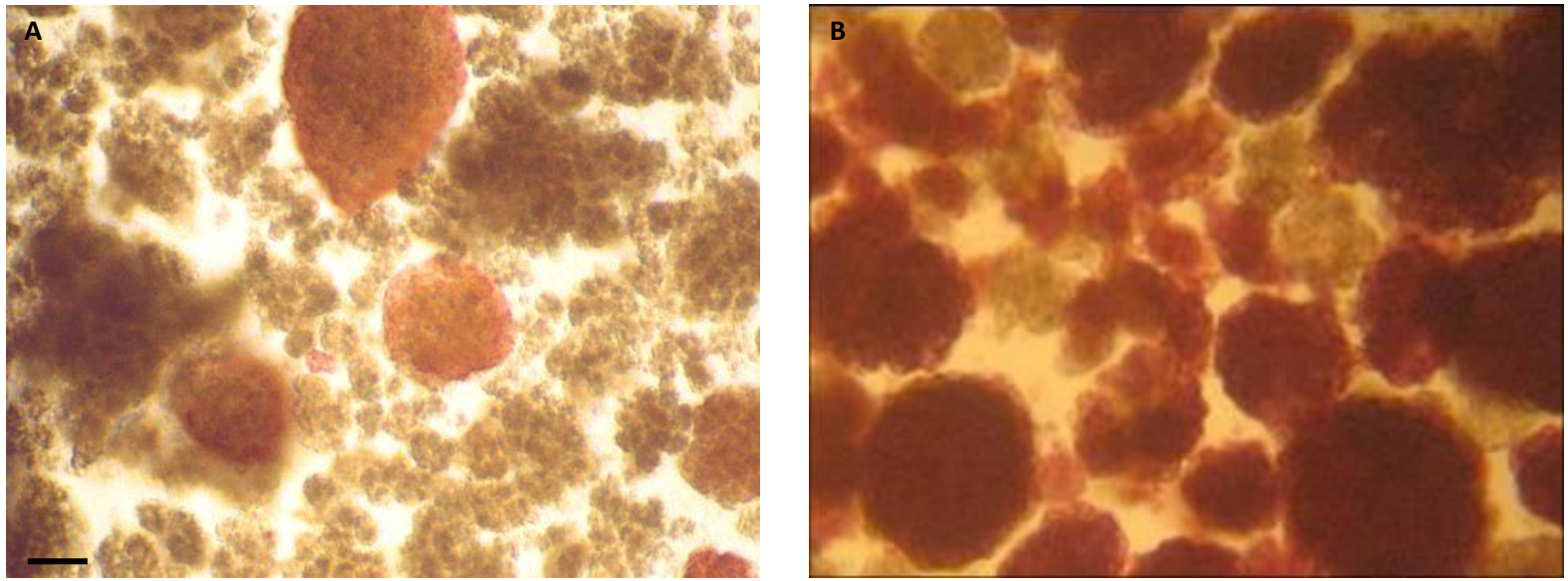


Figure 1. Human pancreatic islets visualised by dithizone staining (red cells) observed following pancreatic digestion (A) and after Ficoll gradient assisted centrifugation (B). Scale bar = 100μm

Table 2 - Donor characteristics and islet isolation outcome for the pancreata used in this study

	Donor code	Age (years)	Gender(M/F)	Organ descrip.	CIT	Weight used (g)	Purity	% Viability	IEQ purified
	H4190607	52.00	F	fatty/fibrous	15.50	70.00	80	71.6	26000.00
	H7241107	43.00	F	damaged capsule/fatty	10.00	62.00	75	73.3	66000.00
	H8141207	31.00	M	damage to head/fatty	6.50	102.00	75	70.0	75000.00
	H1160108	54.00	M	fatty	10.50	84.00	85	73.3	314000.00
	H6160508	42.00	F	slight damage to capsule	13.00	56.00	70	66.7	103000.00
	H7150808	53.00	F	damage to head/fatty	7.75	64.00	80	73.3	70000.00
	H1240109	55.00	F	damage to head	7.00	60.00	65	73.3	125000.00
n		**7.00**			**7.00**	**7.00**	**7.00**	**7.00**	**7.00**
mean		**47.14**			**10.04**	**71.14**	**75.71**	**71.64**	**111285.71**
s.e.m.		**3.35**			**1.25**	**6.19**	**2.54**	**0.95**	**35755.60**

Abbreviations; CIT = cold ischemia time, IEQ = islet equivalents

2.3.2. Functional Assessment

Insulin secretory function 24 hours post-isolation

In the present study isolated islets gave a robust response to high (16.7mM) glucose which was consistently increased (S.I. 2.63±0.21) when compared with basal release. Stimulated insulin release was further enhanced by the presence of 10mM theophylline achieving an S.I. of 3.67±0.34 (Fig.2.).

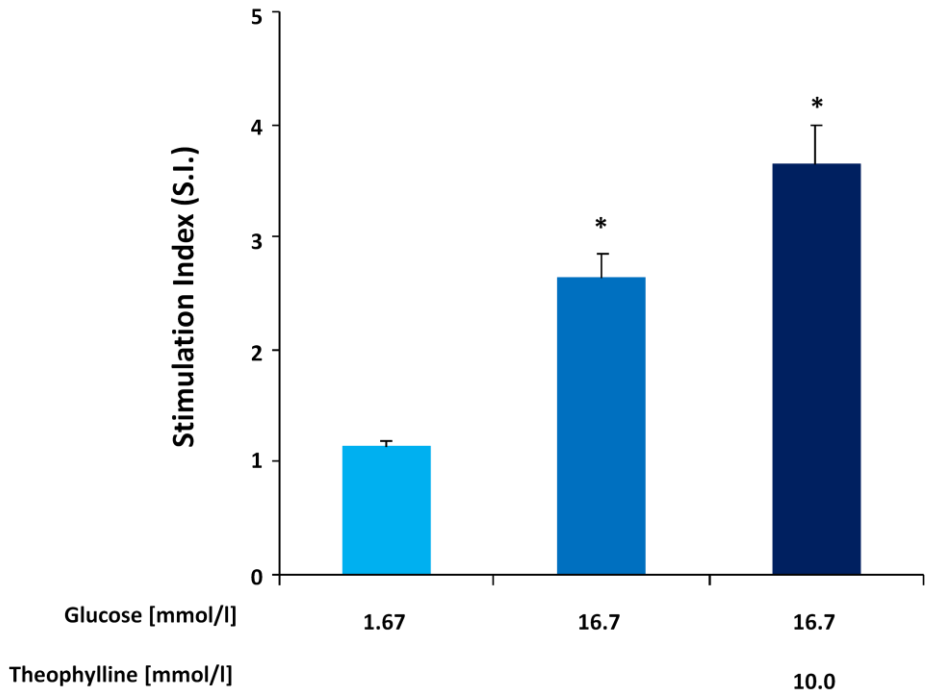


Figure 2. *Insulin secretion from isolated human islets in response to nutrient stimulation during static challenge experiments performed 24 h post isolation.* Islets were maintained under conventional static culture conditions prior to assessment of insulin secretory function. Insulin release was measured in response to 1.67 mmol/l glucose (basal release), 16.7 mmol/l glucose and 16.7 mmol/l glucose plus 10mmol/l theophylline. Results are expressed as the mean ± SEM fold increase in insulin release in response to nutrient stimulation relative to release under basal conditions (stimulation index S.I.). n=7 independent islet preparations. The absolute mean value for insulin secretion under basal conditions was 86.5±17.2 µUml-1 [20 islets]–1 h–1. * p<0.05 vs. basal conditions. *(One way analysis of variance)*

2.4. Discussion

The aim of the present chapter was to outline the process of procuring human pancreatic tissue for research and the subsequent isolation of islets by use of an in-house manual separation method.

Clinical islet transplant programmes require standardisation of islet isolation in order to conform to strict regulatory guidelines for the manufacture of tissue for transplant purposes. The semi-automated method of islet retrieval is amenable to validation with well-defined standard operating procedures which may be readily adopted by multiple transplant centres involved in clinical trials, ensuring that outcomes may be directly compared (Linetsky and Ricordi, 2008). The trade-off arises from the resource-rich and labour-intensive nature of the process, with the costs involved making islet isolation prohibitive to all but a few centres across the UK, and indeed worldwide (Paget et al., 2007).

Whilst human islets used for research purposes are required to be of a similarly high standard the method of islet procurement may differ in terms of the processes employed. In contrast to the semi-automated-method where the procedures must be rigidly adhered to, the manual method of islet retrieval may be subject to adaptation with changes made to the protocol depending on the characteristics of the pancreas being processed. The ability to make modifications during the procedure increases the likelihood of successful islet isolation, despite the frequent use of marginal (sub-optimal) organs. This ensures optimum use of the scarce resource of human pancreatic tissue for islet procurement.

In this study the use of a manual method of islet isolation was demonstrated to be an effective means of obtaining human islets of suitable quality for research. The technique may be readily mastered, with appropriate training, by researchers new to the field. The cost of isolation is affordable, conducted by two personnel with fewer consumable resources expended and no specialised equipment required in comparison to the semi-automated process. Successful isolations were achieved with most organs procured and the yields obtained were of an appropriate level (ranging from 25,000-300,000 IEQ per pancreas) for the planned experiments.

The pancreata obtained through NHS Blood and Transplant had been declined for use in whole organ or islet transplantation. In certain instances this may have been due to anatomical incompatibility with the intended recipient or due to clinical service limitations. However, on most occasions the organs were deemed unsuitable due to the period of cold ischaemia, certain characteristic of the donor (age, BMI, medical history) or due to physical damage to the organ. The manual method of islet isolation can be adapted to address these shortcomings. For instance, islets from a donor with a high BMI may have fat infiltration which would make it unsuitable for islet isolation using the semi-automated method, due to the extended period of enzymatic digestion which is required. In the clinical isolation process, increasing the digestion phase of the process is problematic and often leads to over digestion of the preparation and subsequent islet fragmentation. However, using the manual technique, the process of digestion is placed under greater surveillance with frequent sampling of the digest (as frequently as every 5 mins) ensuring that islets are liberated without risk of excessive digestion. Additionally, use of a discontinuous gradient (as opposed

to the COBE 2991) allows greater control of the purification process and conducting it at room temperature with less β-cell toxic gradients (McCall et al., 2011) provides the opportunity to "rescue" poorly fractionated preparations and attempt further separation. As a result it was possible to achieve purity of up to 70% in the batches of islets used in this study; dithizone staining provided subjective evidence that that there was minimal exocrine contamination and enabling us to exclude any compounding effects arising from the presence of non-islet cellular components.

It has been argued that the use of manual methods of islet isolation increases the risk of bacterial and viral infection (Goto et al., 2004). However, in the present study all islet preparations were subject to microbiological analysis (performed by our Trust microbiologists) and were found to be free of contaminating pathogens. The additional steps involved in isolation are also thought to equate to a loss of morphological or functional viability (Gurol et al., 2004). However, in this study, morphological analysis confirmed the structural integrity of the cells. Furthermore, the results of the glucose challenge test demonstrated the functional viability of the islets, notably their appropriate response to nutrient stimulation as observed 24 hours after isolation. In clinical islet transplantation the stimulation index is used, in part, to determine whether a batch of islets meets the criteria for transplantation (D'Aleo et al., 2010). In most instances islet preparations with an S.I. between 2.5 and 4.0 are transplanted although there is no clear correlation between pre-transplant S.I. and long-term graft performance (Ryan et al., 2004). This, coupled with the purity, suggests that the islets obtained would have been suitable for clinical use (Linetsky

and Ricordi, 2008) and were therefore an appropriate model for use in subsequent studies to design low immunogenic tissue constructs for use in islet transplantation.

CHAPTER 3: HUMAN AMNIOTIC EPITHELIAL CELLS: ISOLATION, MORPHOLOGICAL AND FUNCTIONAL ASSESSMENT

3.1. Introduction-Amnion-derived cells as candidates for transplantation therapies

During gestation the developing foetus is surrounded by amniotic fluid, enclosed in a sac lined by the amniochorionic membrane (Fig.3.). The innermost layer is composed of amniotic epithelial cells (AEC), resting on a basement membrane and underlying avascular stromal cells which collectively form the amniotic membrane (AM) (Hoyes, 1975). The amniotic component of the foetal sac originates from the epiblast of the inner cell mass which contrasts with the origin of the chorion, derived from extra-embryonic tissues (Benirschke and Kaufmann, 2000).

The placenta gives rise to a number of distinct cell populations which have recently become the subject of considerable interest in the fields of cell and tissue transplantation and regenerative medicine (Parolini and Caruso, 2011, Parolini et al., 2008). Of note, the stem- and progenitor-like characteristics of these cells, coupled with their relative immune-inertness makes them strong candidates in the search for surrogates for use in cell replacement therapy in a variety of clinical situations. The critical role played by the placenta during pregnancy is understood to involve maintenance of feto-maternal tolerance, preventing the partially allogeneic foetus from being rejected. As a consequence, the placenta and its associated membranes are endowed with certain characteristics pertinent to the modulation of the surrounding immune micro-environment. Of note, in extensive

studies the following human placental derived cell types have been shown to possess immuno-modulatory potential with importance for the inhibition of inflammatory and/or immune events.

i. chorionic trophoblastic cells (CTC) – isolated from the chorionic trophoblast of term placenta
ii. chorionic mesenchymal stromal cells (CMSC) – obtained from the mesenchymal layer of the chorionic membrane
iii. amniotic mesenchymal stromal cells (AMSC) – derived from the mesenchymal layer of the amniotic membrane
iv. Amniotic epithelial cells (AEC) – sourced in large quantities from the epithelium of the amniotic membrane

For the purpose of the present study this summary will focus on the cells derived from the human amniotic membrane; i.e. amniotic epithelial cells whilst the others are comprehensively reviewed elsewhere (Parolini and Caruso, 2011).

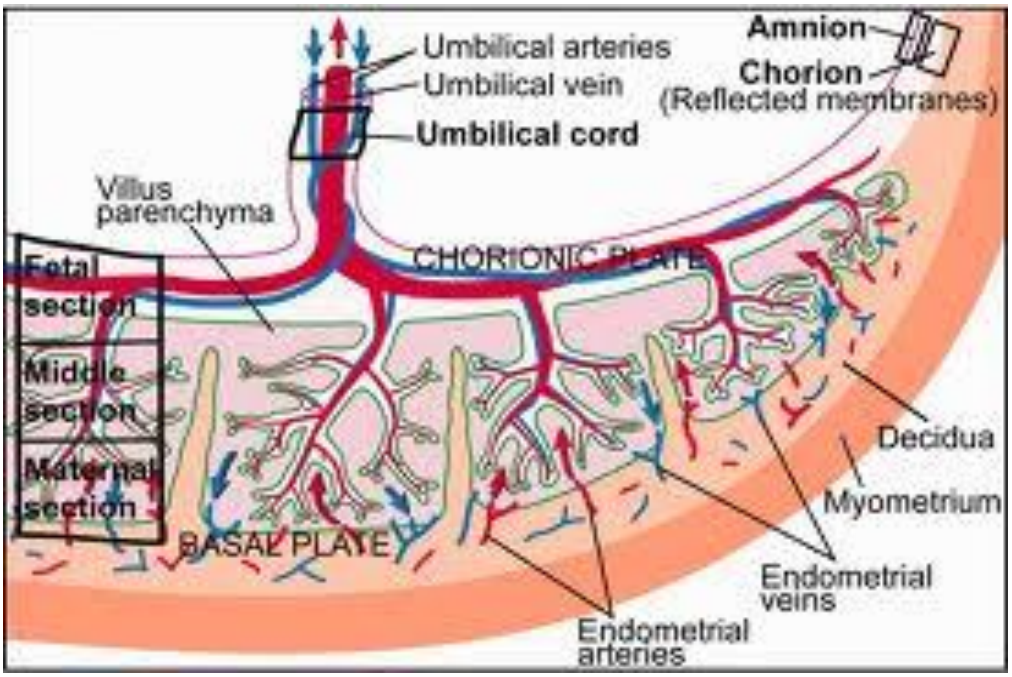


Figure 3. Illustration of the architecture of the human placenta and amniotic membranes

3.1.1. Clinical use of human amniotic membrane (AM)

The low-antigenicity and marked anti-inflammatory properties of human AM underlies its importance in reconstructive and transplant medicine. Its' most acknowledged use is in ophthalmic surgery, where using AM as a basement membrane substitute or as a temporary graft has become commonplace. AM has been shown to reduce inflammation and scarring, prevent angiogenesis and fibrosis and is thought to be the source of certain growth factors which encourage the re-epithelisation of the surface of the eye (Meller et al., 2011). Despite the absence of controlled randomised clinical data directly supporting the role of AM transplantation in ophthalmic surgery, the results of numerous case studies strongly suggest that it serves as an effective approach to corneal and conjunctival reconstruction and has been successfully applied to the treatment of burns (Meller et al., 2000), acute Stevens-Johnson Syndrome (Gregory, 2011), intractable corneal ulcers (Nubile et al., 2011) and persistent epithelial defects (Seitz et al., 2009).

In addition to its role in ophthalmic surgery AM has also been used in the management of ulcers refractory to other treatments, and in venous leg ulcers AM grafts were shown to encourage re-epithelisation from the edge of the wound inwards with a concomitant reduction in fibrosis and associated pain (Mermet et al., 2007). The treatment of paediatric burns using human AM as a temporary graft or for skin graft fixation has also been explored clinically with promising results (Sheridan and Moreno, 2001, Mohammadi and Johari, 2010).

3.1.2. Clinical potential of human amniotic epithelial cells

The innate anti-inflammatory characteristics of AM have been attributed, at least in part, to the cells lining the membrane surface, the amniotic epithelial cells (AEC) which, when isolated, have been shown to exhibit immunomodulatory potential. *In vitro* studies demonstrate the ability of human AEC to suppress T-cell activation in both mixed lymphocyte and mitogen-induced proliferation assays (Wolbank et al., 2007, Li et al., 2005) and AEC are amenable to both allogeneic and xenogeneic engraftment in immune-competent recipients (Akle et al., 1981, Kong et al., 2008, Kubo et al., 2001, Sankar and Muthusamy, 2003). Furthermore, the expression of several mediators of localised immune suppression including HLA-G, Fas ligand and TGFβ have been characterised in isolated AEC or culture supernatant (Li et al., 2005, Harirah et al., 2002, Hammer et al., 1997, Kubo et al., 2001, Lefebvre et al., 2000). Such immuno-mediators have the capacity to counteract the potentially harmful actions of immune cells; evidence suggests that AEC may be capable of creating a microenvironment conducive to sustained allogeneic graft survival (Wolbank et al., 2007, Li et al., 2005, Bailo et al., 2004, Kong et al., 2008, Sankar and Muthusamy, 2003).

Coupled with their relative immune-inertness, AEC also express a variety of stem cell markers indicating multi-lineage differentiation potential. Indeed, under defined culture conditions human AEC have the capacity to differentiate into cells from all three germ layers, giving rise to bone, fat, liver, pancreas and neural cells (Murphy et al., 2010). As such, in experimental models, human AEC have been shown to be beneficial in the treatment of Parkinson's disease, stroke, spinal cord injury, DM, peripheral vascular disease, pulmonary and liver fibrosis (Parolini and Caruso, 2011). There is limited evidence that differentiated

AEC are able to directly participate in tissue regeneration in vivo (Okawa et al., 2001). However, the beneficial AEC-mediated effects observed are largely considered to be due to the secretion of bioactive molecules that act on other cells and promote endogenous tissue repair through paracrine effects (Parolini and Caruso, 2011).

A substantial body of evidence therefore exists in favour of a role for human AEC in regenerative medicine. When compared with stem cells derived from an embryonic source, the wide availability and relative lack of ethical constraints associated with procurement of this tissue make AEC an ideal candidate for further exploration.

Aims of the Chapter

This study aims to provide evidence that AEC may be used as an adjunct to islet cell transplantation, offering vital trophic support, whilst simultaneously protecting the islet graft from immune assault. In the following sections we describe the methods of isolation and subsequent characterisation of the AEC used in the present investigation, including their immunomodulatory potential.

3.2. Materials and Methods: I: AEC Isolation and morphological characterisation

3.2.1. Donor recruitment and consent

All studies using human amniotic tissue were performed according to ethically approved protocols (LREC: Q5/2801/70- Coventry and Warwickshire Ethics Committee) and with the informed consent of the tissue donor. For amniotic membrane procurement, potential participants were identified from an elective Caesarean section list. Women undergoing normal vaginal delivery were excluded from the study to reduce the risk of microbial contamination of the amnion sample. The prospective tissue donors were seen in the pre-operative clerking clinic 24 hours beforehand and given information about the research project. Following discussion of the proposed work, women were invited to take part in the study and if appropriate gave informed, written consent prior to delivery.

3.2.2. AM harvest and dissociation

The amnion was harvested under aseptic conditions in the operating theatres. After the placenta had been delivered and inspected by the midwife, the AM was mechanically dissociated from the chorion layer. 2-3.5 cm^2 pieces were stripped off and washed twice in 150ml of filter sterilised PBS containing 100U penicillin, 100μg streptomycin and 10μg amphotericin B. The tissue was placed in a sterile pot containing 100ml of filter sterile PBS supplemented with 200U penicillin, 200μg streptomycin and 20μg amphotericin B for transport to the laboratory.

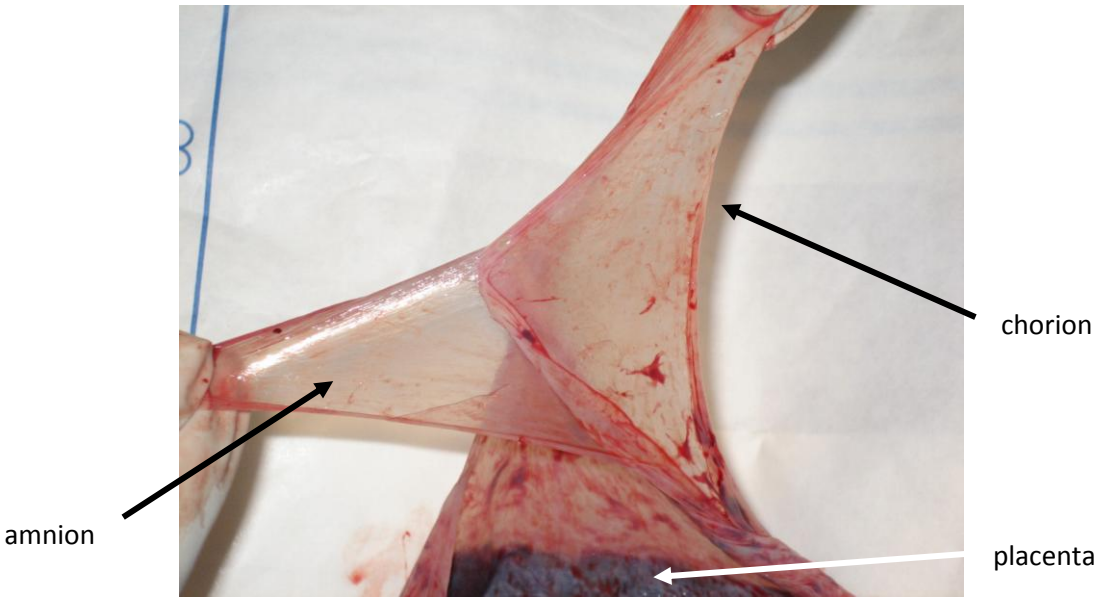


Figure 4. Human amniotic membrane mechanically separated from the chorion. Samples not in direct contact with the placenta were collected for processing.

The tissue was transferred to a Class II microbiological safety cabinet and the amnion was cut into small pieces to increase its surface area. The AM fragments were placed in a conical tube containing HBSS before being centrifuged at 400*g* for 5 mins (brake set to 5). The supernatant was decanted leaving the pelleted amniotic tissue. A 0.25% solution of porcine Trypsin (Sigma-Aldrich) was made up in HBSS in a sterile container and placed in a water bath to reach a temperature of 37^{o}C. Once achieved, 100ml of the trypsin solution was used to re-suspend the amnion tissue and both were transferred to a glass beaker equipped with a magnetic flea. The beaker was tightly sealed with a layer of parafilm and placed in a stirrer oven at 37^{o}C for 30 mins.

Following the first digestion period the dissociating tissue was passed through a 500μm mesh to harvest any detached cells; the tissue retained by the mesh was collected and returned to the glass beaker, re-suspended in a fresh 100ml volume of 0.25% trypsin solution and placed in the stirrer oven for a further 30 mins incubation period. The collected filtrate (Fraction 1) was placed into 50ml conical tubes and centrifuged at 700*g* for 5 mins (brake set on 5). The supernatant was decanted and the cell pellet re-suspended in 2mls RPMI 1640 containing 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin and 10μg/ml of amphotericin B.

The above process was repeated until the amniotic epithelium was fully dissociated and the resulting, dispersed AEC were collected in a total of 4 separate cell fractions (Fractions 1-4), each of which was suspended in 2mls RPMI 1640 medium containing additives as described above. In most instances fraction 1 was discarded due to the high number of contaminating

red blood cells. Fractions 2-4 were subsequently pooled and plated into T-75 flasks. Each flask received 1ml of cell suspension (containing between 1-3 million AEC) and was made up to 20ml total volume with RPMI 1640 + additives. The flasks were transferred to an incubator for 48 hours at 37 °C in a humidified atmosphere of 95%O_2/5% CO_2 to allow cell attachment. At the first medium change (48-72 hours) 15mls of medium was removed from each flask and replaced with 10mls of fresh medium. Thereafter a full medium change occurred at 2-3 day intervals. Flasks of dispersed amniotic epithelial cells reached confluence at between 7-10 days post isolation. In this study AEC were routinely used at passage 1 and at this time the cells were counted and assessed for viability.

3.2.3. Passaging of AEC – assessment of yield and viability

At confluence AEC cultures were passaged as follows:

The flasks containing the AEC monolayers were transferred to the hood and the medium was gently aspirated using a sterile 10ml pipette. This was immediately replaced with 10mls of filter sterilised PBS (Sigma-Aldrich) which was used to rinse the cells. Rinsing was performed 3 times to ensure all of the FBS-containing culture medium was removed (as this would inhibit the subsequent trypsinisation process). 1ml of 0.025% Trypsin-EDTA in PBS (Sigma-Aldrich) was added to each flask, gently swirling the flask to ensure that the entire monolayer was covered by the enzyme. The flasks were then returned to the incubator for 20mins to assist mild dissociation of the monolayer. The AEC monolayers were viewed under an inverted microscope to assess the level of disruption; AEC rolled up and become detached from the base of the flask as the trypsin took effect. If necessary, cell detachment

was further assisted by gently tapping the flask against the palm of the hand. Once all the AEC were free-floating the trypsin reaction was quenched by addition of 10ml of culture medium contain 10% FBS. The AEC suspension was harvested into 50ml conical tubes for centrifugation at 500*g* for 3 mins. Each AEC pellet was re-suspended in 1ml of culture RPMI 1640+ additives, (i.e. 1 ml of culture medium per flask of AEC) and all the pellets were pooled for counting and viability assessment.

Samples of AEC (50-100μl) were placed in an Eppendorf tube to which an equal volume of 0.4% Trypan Blue solution was added. Following mixing, a portion of the cell suspension was used to fill the lower counting chamber of a haemocytometer – by capillary action. Cells occupying the 4 quadrants (composed of 16 squares) of the chamber were counted and the mean value used to determine the total cell number in one ml of suspension. From this total AEC numbers were calculated. In addition, the non-viable cells (i.e. blue-stained) in each grid were counted to give the percentage cell viability. The AEC suspension was adjusted to a final density of $1x10^6$/cells per ml for subsequent use or for cryopreservation.

3.2.4. AEC Cryopreservation

As the ultimate aim of this study was to determine whether human AEC could be applied to clinical cell transplantation we sought to determine whether these cells were amenable to cryopreservation thus ensuring their ready availability. To this end studies were performed to assess AEC function following a period in ultra-low temperature storage. AEC at passage 1 (P1) were counted as described above and adjusted to a final density of $1x10^6$ cells per ml in

RPMI 1640 + additives and 10% dimethyl sulfoxide (DMSO – Sigma-Aldrich, cell culture tested). After allowing 5 mins of equilibration the cells were transferred to 1ml cryo-tubes™ (Appleton Woods, Birmingham, UK) and placed in a Nalgene® "Mr Frosty" (Fisher Scientific, Loughborough, UK). This solvent-assisted cooling chamber permitted controlled, step-wise freezing of the AEC cryo-solution to a temperature of - 45°C over a 24 hour period. After this the tubes were racked and stored at - 80°C for up to 3 months.

3.2.5. AEC Histological Characterisation

P1 AEC were counted and adjusted to a final density of 1 x 10^6cells/ml in supplemented RPMI 1640, as previously described. A 24-well culture plate was prepared by placing a pre-washed and autoclaved 13mm glass coverslip in the base of each well and adding 950µl of culture medium. 50µl of cell suspension was then added, giving a total of 50,000 cells per well and the plate was placed at 37 °C in a humidified atmosphere of 95%O_2/5% CO_2 to allow cell attachment. After approximately 72 hours the AEC attached to the glass coverslips at which time they were processed for histology as follows:

Sub -confluent AEC monolayers were fixed with freshly made 4% paraformaldehyde (Sigma – Aldrich) in PBS for 30 mins at room temperature (RT). Three 10 mins washes in PBS were followed by antigen-retrieval (0.3% Triton-X-100, Sigma- Aldrich) and blocking using either 10% normal goat serum (NGS) or 10% normal rabbit serum (NRS – both from Vector Laboratories Ltd, Peterborough, UK) depending on the species in which the secondary antibody was raised. The AEC monolayers were then incubated with the following primary antibodies: anti-human cytokeratin 19 (CK19), anti-human vimentin (Dako UK Ltd,

Cambridgeshire, UK– 1:100), anti-human insulin (AbD Serotec, Oxford, UK 1:10) and anti-human Fas Ligand (FasL, Sigma-Aldrich, 1:50), for 1 hour at RT and at 4°C overnight. Secondary antibody (goat anti-mouse IgG-FITC for CK19, vimentin, FasL, goat anti-rabbit IgG-TRITC for insulin – Cambridge Biosciences, Cambridge, UK, 1:100) was applied for 3 hours at RT. The coverslips were rinsed and mounted in fluorescence mounting medium (Dako UK Ltd) before cell imaging using a Zeiss Axioskop 40 fluorescence microscope equipped with an AxioCam MRC colour camera and incorporating Axiovision imaging software (Carl Zeiss, Hertfordshire, UK). Controls involved omission of the relevant primary antibody.

3.2.6. Analysis of cytokine Profile of AEC supernatant and lysate

Cell supernatants collected from cultured AEC (plated at 50,000cells/ml for 72 hours) and cell lysates prepared by mild sonication of cultured AEC were processed by Randox Laboratories Ltd. Using BioChip Array Analysis (BAT) a comprehensive panel of 12 cytokines relevant to immune modulation were investigated in the AEC samples. In this study the Cytokine Array I High Sensitivity assay was used which detects Interleukin (IL)-1 α,IL-1β, IL-2,IL-4,IL-6, IL-8, IL-10, Epidermal Growth Factor (EGF), Interferon-γ (IFN-γ), Monocyte Chemotactic Protein-1 (MCP-1), Tumour Necrosis Factor-α (TNF-α), and Vascular Endothelial Growth Factor (VEGF). Due to the combination of highly specific antibodies and advanced chemistries the array enables all 12 cytokines and growth factors to be detected simultaneously in a single sample. The limit of sensitivity for each analyte is presented in table 3. Each sample was measured in duplicate and 2 samples from each group (supernatant or lysate) were provided for analysis.

3.3. Material and Methods II: Assessment of Immunomodulatory potential

3.3.1. Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats obtained from healthy volunteers through the National Blood Service (NBS, Birmingham, UK) with local research ethics approval and with the consent of the donor. In all instances the buffy coats came from regular blood donors who were screened for pathogens and who were free of any illness which might adversely affect lymphocyte reactivity. To isolate the PMBC, buffy coats were collected into 150 ml sterile pots and diluted in an equal volume of HBSS. This was carefully layered onto 12ml of density gradient adjusted to 1077g/ml (Histopaque®-1077 - Sigma-Aldrich), centrifuged at 700*g* for 30 mins (with no brake), and the resulting leucocyte layer harvested from the interface using a sterile pastette. The isolated PBMC were washed three times in HBSS, centrifuged at 500*g* for 10 mins, re-suspended in RPMI 1640 (supplemented as described above). The separated PBMC were counted using haemocytometer and viability was assessed using trypan blue exclusion. The cells were then cultured in uncoated plastic petri dishes at 37°C, 5%CO_2, 95%O_2 overnight. The PBMC cultures were incubated overnight, half of them in the presence of the plant mitogen phytohaemagglutinin (PHA, Sigma-Aldrich, 5μg/ml), for 24 hours prior to use in proliferation assays.

3.3.2. AEC vs. PBMC – Proliferation Assay

AEC at P1 were prepared as described in section 3.2.3 above. Once adjusted to a final volume of $1x10^6$cells/ml in supplemented RPMI 1640, aliquots of the AEC suspension were added to the appropriate wells of a 24-well plate. In preliminary studies a dose response curve was performed plating AEC at 5,000, 50,000 and 500,000 AEC per well. Each well was supplemented with medium up to a total volume of 1ml and the plates placed at 37°C, 5%CO_2, 95%O_2 for 72 hours to permit cell anchorage. Additionally, in selected experiments cryopreserved AEC were rapidly thawed, rinsed in PBS and seeded as described above for the fresh AEC prior to their use in PBMC assays as follows: AEC seeded plates were processed by repeated washing in filter sterile PBS to ensure all unattached cells/cellular debris was removed from the wells. Resting or PHA-activated PBMC were added at a density of 50,000 cells/well either alone which served as a control or to wells pre-seeded with 50,000 firmly anchored AEC prior to co-incubation at 37°C, 5%CO_2, 95%O_2. Activated PBMC continued to be cultured in the presence of 5μg/ml PHA throughout the assay period. After 72 hours the PBMC were harvested, washed and assayed for intracellular ATP content as described in section 3.3.4 (*below*).

3.3.3. AEC conditioned medium (CM) vs. PBMC

In additional selected experiments P1 AEC were re-plated into T-75 flasks at a density of $2x10^5$cells/ml (equivalent to $3x10^6$cells per flask) in 15mls of supplemented RPMI 1640 medium. The flasks were left for 72 hours without a medium change to allow concentration of putative soluble factors released by the AEC. The resulting AEC-conditioned medium (CM)

was harvested and centrifuged at 1300*g* to ensure removal of all cells/cellular debris prior to use in PBMC proliferation assays. 0.5ml of CM was dispensed to the appropriate wells of a 24-well plate and 5 x 10^4 resting or PHA activated PBMC were added; adjusting the total volume to 1.0ml using standard RPMI medium. Plates were incubated at 37°C, 5%CO_2, 95%O_2. After 72 hours the PBMC were harvested, rinsed and processed as described in section 3.3.4 (below).

3.3.4. Quantification of PBMC proliferation - luminescent detection of intracellular ATP

After 72 hours the harvested PBMC were solubilised using cell lysis reagent (Vialight – Lonza Ltd, Wokingham, UK) and analysed for ATP content using a commercial chemiluminescence assay (Lonza Ltd) according to the manufacturer's instructions. Concentration of ATP per well, measured as relative light units (RLU) is directly proportional to cell number and thus indicative of the proliferative activity of PBMC in culture (Sottong et al., 2000). Results were expressed as the percentage increase in relative cell number compared to the control *viz.* resting PBMC incubated in the absence of AEC.

3.3.5. Statistical analysis

Significant differences in PBMC proliferation in response to co-culture with AECs was determined using Mann-Whitney U (by Rank) and Tukey's multiple comparison tests, with the response of resting PBMC serving as the control. In all comparisons a p value of <0.05 was considered to be statistically significant. Statistical analysis was performed using SigmaStat software version 3.5 (Systat Software Inc, Chicago, USA).

3.4. Results I

3.4.1. Morphological characteristics of isolated AEC

Human amniotic epithelial cells when isolated from the membrane and held in suspension culture readily adopted a spherical morphology forming an apparently homogeneous population (Fig.5.A). Once plated at high density in T75 flasks AEC readily attached and flattened to form a monolayer (Fig.5.B), the vast majority of these cells staining positive for the epithelial cell marker cytokeratin 19 (Fig.5.C). Additionally, a discreet sub-population of cells stained positive for the intermediate filament marker vimentin (Fig.5.D).

3.4.2. Cytokine Analysis –multiplex immunoassay

AEC supernatants collected after 72 hours of culture as described in section 3.2.6., and cell lysates prepared from AEC monocultures were processed by Randox Laboratories Ltd using a cytokine array multiplex immunoassay.

Several cytokines relevant to immune-modulation were detected either in AEC cell supernatant, lysate or both, as presented in Table 3. Despite using AEC at the same density as that employed in the proliferation assays the concentration of cytokines detected were, in many cases, below the level of sensitivity for the immunoassay.

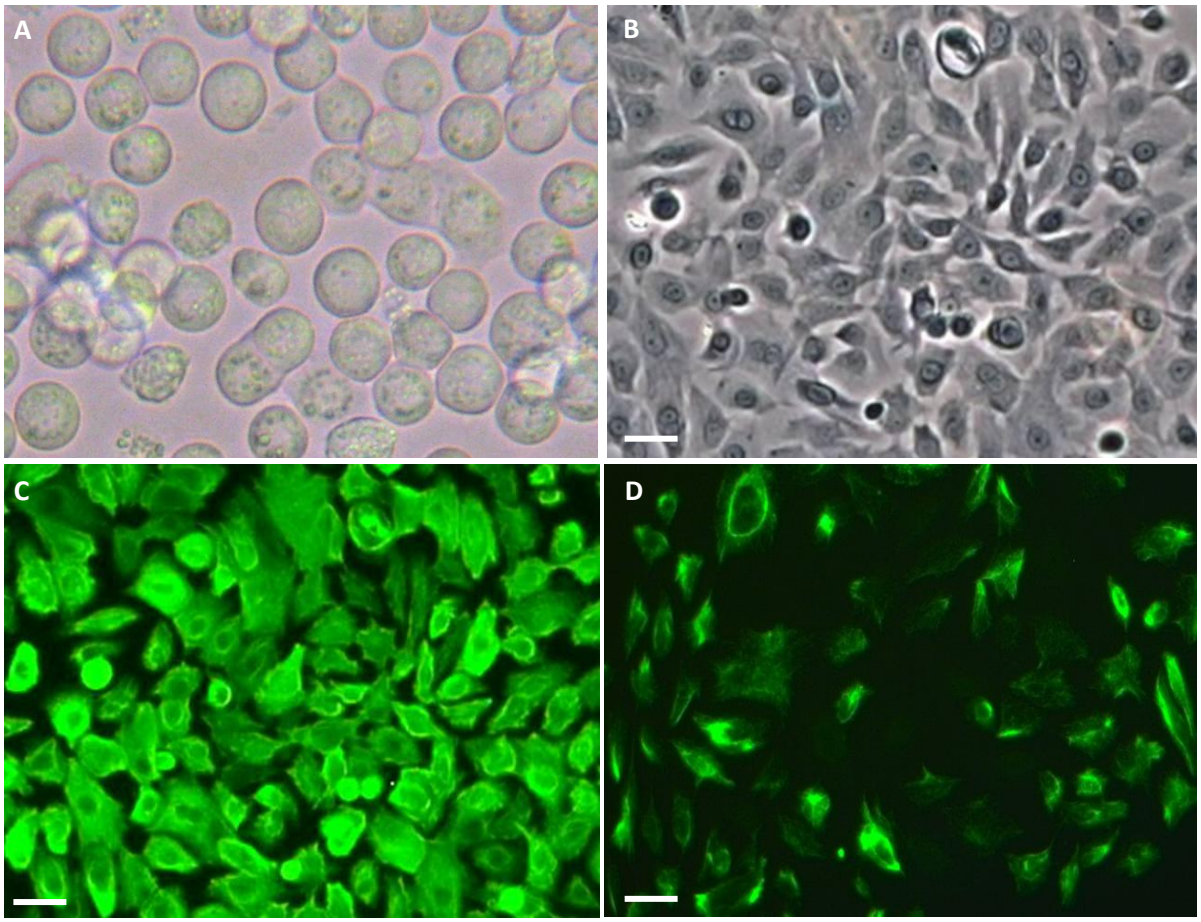


Figure 5. Human amniotic epithelial cells (AEC) viewed by light microscopy immediately following isolation (A), viewed first under phase contrast (B) and using fluorescence immunocytochemistry for the localisation of the epithelial cell marker cytokeratin 19 (CK19) (C) and the intermediate filament protein vimentin (D). A FITC-conjugated secondary antibody was used for visualisation. Vimentin expression by AEC maintained in monolayer culture differs from that of CK19 which is a cytoskeletal protein marker and is distributed throughout the cytoplasm. Most epithelial cell types co-express vimentin. For A original magnification x40, B,C,D Scale bar = 100μm.

Table 3: Cytokine analysis

	IL2 (pg/ml)	IL4 (pg/ml)	IL6 (pg/ml)	IL8 (pg/ml)	IL10 (pg/ml)	INFγ (pg/ml)	TNFα (pg/ml)	IL1α (pg/ml)	IL1β (pg/ml)	MCP1 (pg/ml)	VEGF (pg/ml)
AEC Supernatant	5.69	3.21	6.43	2.45	ND	ND	ND	ND	0.76	24.76	60.34
AEC Lysate	5.24	4.24	ND	ND	1.2	ND	ND	1.07	0.67	26.81	ND
Limits of Detection	4.8	6.6	1.2	4.9	1.8	3.5	4.4	0.8	1.6	13.2	14.6

ND = not detected

3.4.3. Results II – Immunomodulatory Potential of isolated AEC and AEC-conditioned medium

PBMC proliferation was evident following exposure to the plant-derived mitogen – phytohaemagglutinin - 5μg/ml (PHA) as demonstrated by a robust (20-fold) increase in intracellular ATP concentration as measured by chemiluminescence detection. By contrast, despite the fact that the two cell populations are derived from different donors and are therefore allogeneic, PBMC grown in the presence of varying numbers of human AEC failed to proliferate to a significant degree. At the highest concentrations of AEC (i.e. 50,000 and 500,000) there was a slight increase in PBMC numbers but this did not reach statistical significance and was small in comparison to the magnitude of response seen to the non-specific mitogen. PHA-mediated PBMC stimulation was significantly reduced by their co-culture with AEC. In the dose response experiment AEC-induced inhibition of PBMC proliferation was evident at an AEC:PBMC ratio of 1:10 (Fig.6) demonstrating a 60% inhibition in cell numbers compared to the control viz. PBMC expansion in the absence of AEC. Increasing numbers of AEC inhibited PBMC proliferation by a similar magnitude.

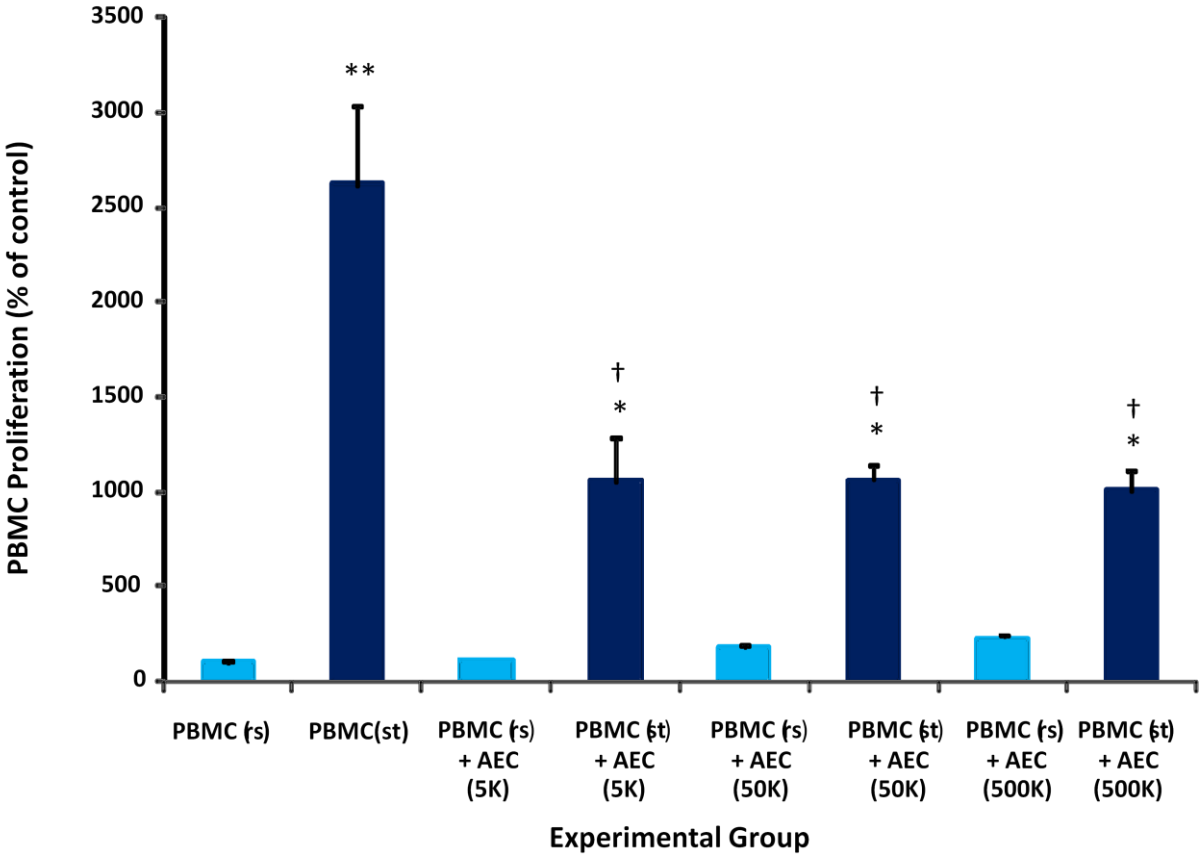


Figure 6. *Modulation of peripheral blood mononuclear cell (PBMC) proliferation by co-incubation with varying numbers of human amniotic epithelial cells (AEC):* AEC were plated at 5,000 (5K), 50,000 (50K) or 500,000 (500K) cells/ml into 24-well plates and allowed to firmly attach for up to 72 hours. PBMC were then added and incubated either in the absence (■) of presence (■) of the plant mitogen phytohaemagglutinin (5μg/ml) for a further 72 hours. The rate of PBMC proliferation following this period was measured using an ATP chemiluminescence assay. Data shows the percentage increase above control (resting PBMC) from 3 individual AEC preps and represents the typical observation. * $p < 0.05$, ** $p < 0.01$ PHA induced increase in PBMC numbers as compared to resting control. † $p < 0.01$ inhibition of PHA-activated PBMC proliferation in the presence vs. absence of AEC. *(Mann-Whitney U (by Rank) and Tukey's multiple comparison tests)*

As this study seeks to provide evidence to justify clinical application of AEC in cell transplantation therapy it was important to ascertain whether these cells were amenable to cryopreservation. To this end in a sub-set of experiments AEC which had been frozen for between 1 and 3 months were used to determine their immunomodulatory capacity in relation to PBMC. When compared to fresh AEC, cryopreserved AEC elicited a mild stimulation of PBMC on co-culture (Fig.7). However this response was small in comparison to their response to PHA. A similar magnitude of inhibition (55%) of PHA-mediated proliferation was observed when PBMC were co-incubated with either fresh or cryopreserved AEC.

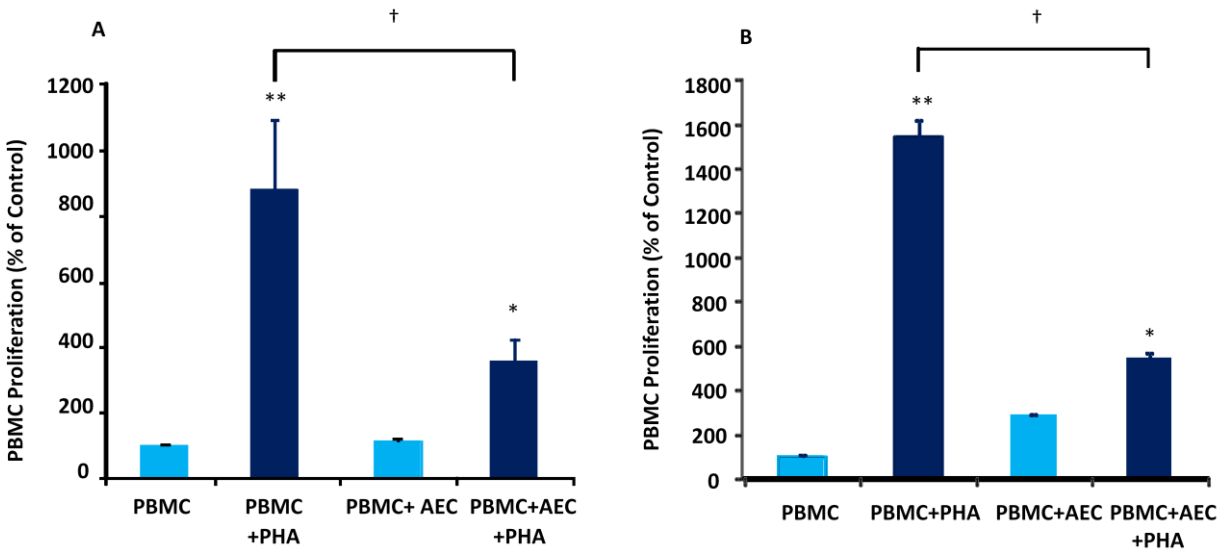


Figure 7. *Modulation of peripheral blood mononuclear cell (PBMC) proliferation by fresh (A) and cryopreserved human amniotic epithelial cells (AEC) (B).* Resting (■) or PHA-activated (■) human PBMC were maintained in 24-well plates either alone, or in the presence of an equal number of human amniotic epithelial cells for a period of 72 hours. The rate of PBMC proliferation following this period was measured using an ATP chemiluminescence assay. Data shows the percentage increase above control (resting PBMC) from 6 individual AEC preps and represents the typical observation in fresh and cryopreserved AEC. * $p < 0.05$, ** $p < 0.01$ compared to control. † $p < 0.01$ for PHA-activated PBMC proliferation in the presence or absence of AEC. *(Mann-Whitney U (by Rank) and Tukey's multiple comparison tests)*

In a sub-set of experiments the effect of AEC- conditioned medium on PBMC proliferation was also determined. In our study AEC- conditioned medium had comparable immunosuppressive activity on PHA-activated PBMC as the AEC inhibiting proliferation by up to 60% (Fig.8).

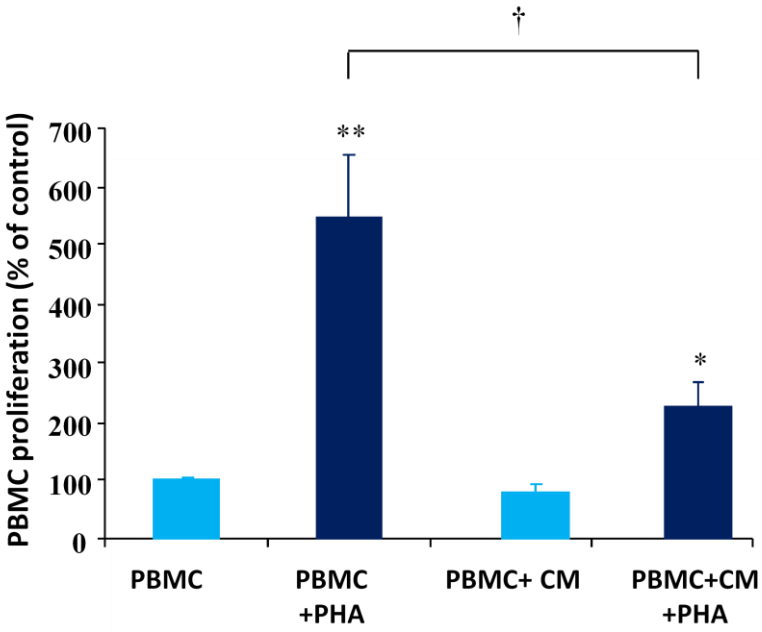


Figure 8. *Modulation of peripheral blood mononuclear cell (PBMC) proliferation during exposure to AEC-conditioned medium (CM).* Resting ( ) or PHA-activated ( ) human PBMC were maintained in 24-well plates either alone or in the presence of 0.5mls of AEC-conditioned medium for a period of 72 hours. The rate of PBMC proliferation following this period was measured using an ATP chemiluminescence assay. Data shows the percentage increase above control (resting PBMC) from 4 individual AEC-CM preps and represents the typical observation. * $p < 0.05$, ** $p < 0.01$ compared to control. † $p < 0.01$ for PHA-activated PBMC proliferation in the presence or absence of AEC-conditioned medium. *(Mann-Whitney U (by Rank) and Tukey's multiple comparison tests)*

3.4.4. Immunocytochemical analysis of immune mediators

In an attempt to identify other potential immune modulators underlying the inhibitory actions of human AEC, we sought to identify Fas Ligand expressing cells within P1 AEC monocultures. The Fas/FasL pathway has been implicated in feto-maternal tolerance and has previously been shown to be expressed in foetal membranes (Koenig and Chegini, 2000) and amniotic cells (Li et al., 2005). The results of the immunocytochemical investigation confirm that a small sub-population of AEC express FasL in culture. Whilst counterstaining for cell nuclei was not performed in this study a comparison of the cell populations using phase contrast and fluorescence microscopy suggests that approximately 30% of the AEC population exhibited cytoplasmic localisation of FasL protein (Fig.9).

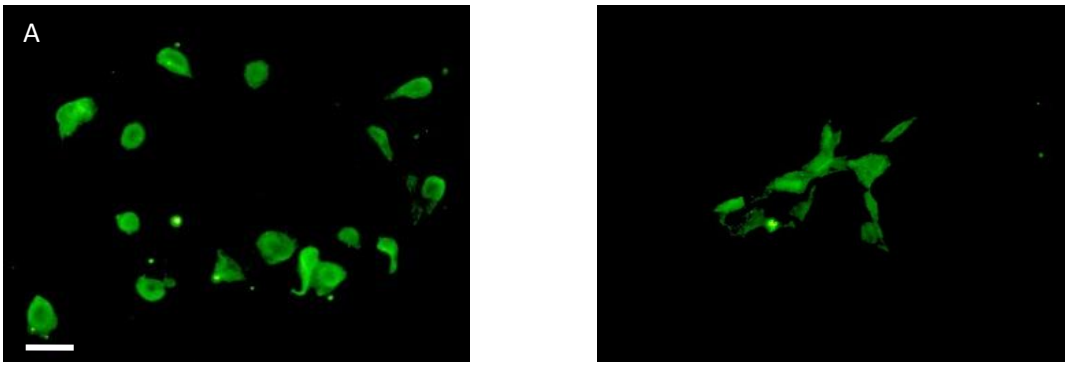


Figure 9. The images (*right*) show FasL expressed by AEC .FasL is a soluble membrane bound protein expressed by approx. 30% of isolated AEC. Scale Bar = 100μm

3.5. Discussion

The results of this series of studies suggest that AEC are capable of eliciting a suppressive effect on peripheral mononuclear cell proliferation, inducing effective inhibition even at a ratio of 1:10 for AEC:PBMC. The immunomodulatory capabilities of human amniotic membrane have been studied extensively (Hori et al., 2006, Kubo et al., 2001, Trelford et al., 1975). Our findings that isolated AEC abrogate mitogen-induced PBMC proliferation confirm the results of previously published studies using comparable amnion-derived epithelial cell populations (Li et al., 2005, Wolbank et al., 2007). Furthermore, the findings of the conditioned medium studies support those of others (Ueta et al., 2002, Hori et al., 2006, Li et al., 2005) and, indicate that AEC secrete immunomodulatory factors at concentrations sufficient to create a microenvironment of localised immunosuppression.

PBMC numbers did not increase during co-culture with AEC suggesting a lack of allo-antigenicity. This may relate to the absence of HLA-A, -B, -C or -DR antigens, reported for AEC in culture and the lack of MHC Class I and low level expression of MHC Class II in primary AEC at low passage (Akle et al., 1981, Adinolfi et al., 1982). In addition, cultured AEC show a lack of surface expression of co-stimulatory molecules (CD80, CD86 and CD40) (Pratama et al., 2011). Their expression and that of HLA is required for appropriate T-cell activation and progression of a full allogeneic response. As such, their absence confers a degree of immune-privilege on AEC, as found in the present study, and may have relevance to their wider use in clinical transplantation.

In addition to being immune inert, AEC elicit a marked decrease in PHA-mediated PBMC proliferation during co-culture, suggesting these cells have an immunosuppressive capability. This has been reported in other studies which indicate that AEC secrete immunomodulatory factors pertinent to the suppression of the innate and adaptive immune responses. Characterisation of the mediators involved have been conducted resulting in the identification of several soluble factors including TGFβ, HLA-G, IL-6 and IL-10 (Lefebvre et al., 2000, Li et al., 2005, Pratama et al., 2011) all of which have the potential to affect localised immunosuppression. The limited cytokine analysis performed in the present study confirms the localisation of certain anti-inflammatory cytokines either in the AEC-conditioned medium or in AEC cell lysate. However, very low concentrations of cytokines including certain pro-inflammatory cytokines, notably IL-2 and IL-8 were also detected. It has been suggested that the immunomodulatory properties of certain stem cells including mesenchymal stem cells are activated in the presence of pro-inflammatory mediators (e.g. TNF1β, INFγ) (Bassi et al., 2011) which act through Toll-like receptors to increase the production of immune regulatory factors. Thus, it is possible that a more relevant AEC cytokine profile may be determined by measuring the AEC supernatant following exposure of the cells to pro-inflammatory molecules. Nevertheless, the anti-inflammatory mediators we have detected and which have also been reported by others are known to cause T-cell arrest through a number of pathways. TGF-β inhibits Th1 proliferation and as a result rebalances the Th1/Th2 ratio toward Th2 – anti-inflammatory. In addition TGF-β and IL-6 prevent dendritic cell (DC) maturation and induces T-reg expression, whilst HLA-G-mediates inhibition of T-cell proliferation and the suppression of the cytotoxic activity of Natural Killer (NK) cells (Bassi et al., 2011). AEC may also secrete or enhance DC secretion of IL-4 and IL-10

thus reducing the prominence of Th1 in favour of Th2 cells and creating a more anti-inflammatory, tolerogenic microenvironment.

Finally, from the results of the limited immunocytochemical analysis it is evident that AEC express immune-modulators. Localisation of FasL in a sub-population of AEC indicates the potential involvement of the Fas/FasL pathway in the underlying mechanism of T-cell arrest. FasL is an immunomodulatory factor associated with naturally occurring T-cell evasion in the testis, eye, and brain (Green and Ferguson, 2001). Localisation of FasL within the placenta and amnio-chorionic membranes is implicated in maternal tolerance developed to the foetus during pregnancy (Kauma et al., 1999, Harirah et al., 2002). In the present study expression of FasL in the AEC monocultures raises the possibility that FasL- mediated apoptosis of Fas bearing T-cells may also play a role in the absence/loss of T-cell proliferation during PBMC/AEC co-culture. Thus, in the context of cell transplantation, AEC are associated with a number of factors which have the capacity to directly or indirectly modulate the immune system. A number of these mediators could potentially operate in concert to produce a microenvironment capable of sustaining allogeneic tissue by diminution of the T-cell response.

In terms of their clinical application, the findings of the present study indicate that human amniotic membrane represent a source of a readily expandable population of immunomodulatory cells which are amenable to cryopreservation without loss of function. The tissue is readily available, through elective Caesarean section, and as it is generally viewed as clinical waste, it is not subject to significant ethical constraints. The ability of AEC

to create localized immunosuppression is supportive of their potential role in creating immune-tolerance in allogeneic tissue and cell transplantation with significance for the further development of regenerative therapy for the treatment of diabetes and other chronic conditions.

In Chapter 4 we detail studies which extrapolate this hypothesis to determine whether the inherent immunosuppressive properties of isolated AEC could be manipulated to confer a state of immune-privilege onto otherwise immunogenic cell populations.

CHAPTER 4: BIO-ENGINEERING OF ISLET:AEC CONSTRUCTS; MORPHOLOGICAL AND FUNCTIONAL CHARACTERISATION

4.1. Introduction

The separation and purification of the endocrine component of the pancreas is imperative to successful clinical islet transplantation yet the isolation process, involving both enzymatic and mechanical disruption of the human pancreas, negatively influences long-term islet viability. The intact pancreas has a microenvironment which is conducive to islet function; appropriate β-cell activity being dependent not only on interaction with other endocrine cells, (α, δ, pp) but also with the non-endocrine compartment of pancreas and the extracellular matrix (ECM) in which islets are embedded (Hopcroft et al., 1985, Ilieva et al., 1999). Cell-to-cell and cell-to-ECM communication is disrupted during islet isolation leading to the elimination of trophic/paracrine support provided by the non-endocrine pancreatic cells and also loss of structural viability, resulting in a decline in β-cell numbers. This is considered to be responsible, at least in part, for the eventual diminution of islet graft function in the post-transplantation period.

It has been suggested that the pre-transplant culture of islets represents a "window of opportunity" to perform interventions aimed at rehabilitating "isolation- impaired" islets and also to endow them with attributes to enhance their survival in the post-transplant setting. Although not incorporated in the original Edmonton Protocol (Shapiro et al., 2000) other participants in the Islet Transplantation Network (ITN) have successfully cultured islets prior to implantation allowing time to confirm islet cell viability and sterility and enabling the

preparation and pre-treatment of the potential graft recipient with novel immunosuppression protocols (Kin et al., 2008, Froud et al., 2005).

A number of approaches have been used to enhance islet function in culture. These include alteration of temperature and culture media composition (Clayton et al., 2001, Kim et al., 2005, Brandhorst et al., 2005), culturing on surfaces coated with relevant ECM components (Xiaohui et al., 2006), islet encapsulation (Mikos et al., 1994, Beck et al., 2007, Kizilel et al., 2005), the use of bioreactors (Stepkowski et al., 2006, Rutzky et al., 2002, Murray et al., 2009, Murray et al., 2005, Tobin et al., 2001) and co-culture models (Murray et al., 2009, Johansson et al., 2008, Johansson et al., 2005, Tian et al., 2005, Park et al., 2010). In the present chapter we will outline the findings of studies where the latter two approaches have been combined in an attempt to improve β-cell function whilst simultaneously altering the immune profile of isolated islets.

Previous research in this laboratory has demonstrated the advantage of culturing islets in a novel rotational cell culture system (RCCS) based on original bioreactor technology developed as part of the National Aeronautics and Space Administration (NASA) space program (Paget et al., 2011, Murray et al., 2009, Murray et al., 2005). In an attempt to study the effect of microgravity on human cells NASA developed a rotary cell culture chamber, known as a Rotating Wall Vessel (RWV) which enables cells and tissue constructs to experience low gravity conditions; held by the continuous circular motion of the fluid medium. The RWV promotes re- assembling of cells into anatomically appropriate three-dimensional structures over extended periods of time, forming tissue constructs with

relevant functionality (Unsworth and Lelkes, 1998). The technology was made commercially available and researchers, including those of this laboratory, have further adapted microgravity cell culture to study a wide variety of human cells and tissues under conditions appropriate for their long term survival (Navran, 2008).

The Rotational Cell Culture System (RCCS) used in the present investigation incorporates use of High Aspect Ratio Vessels (HARV) to simulate low gravity microenvironment where continuous cell movement allows optimal oxygenation and nutrient transport. The human islets are kept in a semi-buoyant state avoiding contact with the vessel walls thus limiting physical damage, central necrosis and fibroblast overgrowth (Murray et al., 2005). Furthermore, the minimal shear forces and enhanced cellular contact prevents the cells from being exposed to the detrimental effects of mechanical stirring supporting propagation of physiologically appropriate cell aggregates (Murray et al., 2005, Murray et al., 2009). Studies performed in this laboratory suggest that when maintained within the RCCS human islets exhibit preservation of structural integrity and glucose stimulated insulin release over a 10-day culture period; superior to the results obtained by culturing islets in the conventional static culture conditions (CSC) (Murray et al., 2005, Daoud et al., 2010). This time frame allows pre-transplant manipulation of islets to enhance function and improve long-term graft survival. Further studies indicate significant remodelling of islets under rotational cell culture conditions which promotes the transport of nutrients to the islet core, as well as the depletion of dendritic cells which serves to reduce islet immunogenicity (Rutzky et al., 2002, Stepkowski et al., 2006).

Our own and other studies report a positive impact on islet function resulting from the experimental co-culture of islets with other relevant cell types. Notably, trophic support to islets has been demonstrated by their co-culture with pancreatic ductal epithelial cells (Murray et al., 2009), dermal fibroblasts (Jalili et al., 2011) bone marrow-derived mesenchymal cells (Duprez et al., 2011), and Sertoli cells (Li et al., 2011, Teng et al., 2005) with enhancement of islet function and/or graft survival being reported. It is considered that these cell populations interact with islets in a paracrine fashion, releasing trophic factors with relevance to β-cell survival, proliferation and differentiation including insulin-like growth factor (IGF) - I and -II, epidermal growth factor (EGF), hepatocyte growth factor (HGF), transforming growth factor (TGF)-beta and keratinocyte growth factor (KGF) (Adams et al., 1994, Dheen et al., 1997, Robertson et al., 2008, Jiang and Harrison, 2005). Additionally, components of the extracellular matrix are vital for appropriate pancreatic development and several integrin receptors and their associated ligands including laminin, fibronectin and collagen I are expressed by these cell types, notably epithelial cells (Jiang et al., 1999, Cirulli et al., 2000, Jiang and Harrison, 2005).

We therefore propose that combining the use of bioreactor technology with tissue-engineering to modify the function of transplantable therapeutic cells represents a novel approach to improving clinical outcomes in islet replacement therapy. It is our hypothesis that islet cells may be modified in vitro and adapted under defined culture conditions to enhance survival in the post-transplant environment.

Aims of the Chapter

The specific aim of this section of the project was to construct heterotypic cell composite grafts with the capacity to:

- provide important paracrine regulatory and trophic support to native beta-cells by the synthesis and release of appropriate growth factors
- counteract islet-induced allo-immune responses by mediating localised suppression of the innate and adaptive immune system

To this end, the work as outlined in Chapters 2 and 3 was extended to exploit the observed immunomodulatory potential of human amniotic epithelial cells (AEC), employing a rotational cell co-culture model to provide these beneficial characteristics to populations of isolated, and purified human islets. As AEC are also reported to synthesise and secrete a range of growth factors which may have relevance for the sustained functional viability of islets (Fiaschi-Taesch et al., 2008, Movassat et al., 2003, Hanley and Rosenberg, 2007, Koizumi et al., 2000, Kakishita et al., 2003, Scharfmann and Czernichow, 1996) we also explored the impact of AEC co-culture on islet viability and functionality. The effectiveness of this intervention was assessed using in vitro models of insulin-secretory function and immunomodulation as detailed below.

4.2. Materials and Methods

4.2.1. Bioengineering of islet:AEC constructs

For co-culture studies islet suspensions obtained as described in section 2.2 were adjusted to a density of 500-1000 IEQ per ml and placed under either conventional static culture (CSC) conditions in 90mm culture plates (NHS Logistics, Alfreton, UK) or in a rotational cell culture system (RCCS) in high aspect ratio vessels (HARVs, Cellon Ltd, Bereldange, Luxembourg). The growth medium for both culture models was composed of Medium-199 (M199) supplemented with 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin and 10μg/ml of amphotericin B – no additional trophic factors were added. The cultures were maintained at 30^{o}C in a humidified atmosphere of 95%O2/5%CO2. Confluent AEC monolayers at passage 1 were disrupted by mild enzymatic digestion (0.025% trypsin-EDTA in PBS, Sigma-Aldrich Ltd) and the resulting cell suspension was washed in PBS and introduced to the islet cultures (both CSC and RCCS) at a final density ranging from 1 x 10^4-1x10^5 cells per ml. The islet:AEC co-cultures were maintained under conditions as described above for 72 hours. The speed of rotation of the HARV's was initially set to 8 rpm and increased to a maximum of 15rpm as the size of the islet:AEC aggregates increased. Control cultures consisted of islets seeded at equal density (CSC and RCCS) in the absence of AEC.

4.2.2. Immunocytochemical analysis of the islet:AEC constructs

For immunocytochemistry islet:AEC co-cultures maintained for 72 hours either under CSC conditions or within the RCCS were collected into separate centrifuge tubes and centrifuged at 400*g* for 3 mins, the resulting cell pellets being re-suspended in 1ml of supplemented RPMI-1640 as previously described for AEC culture. Autoclaved 13mm glass coverslips were placed into each well of a 24-well plate and 0.5ml of supplemented RPMI-1640 was added followed by an aliquot of cell suspension containing approximately 20 IEQ or islet:AEC aggregates. The well volume was made up to 1 ml by addition of Medium-199 supplemented as described above. The islet:AEC constructs were thus allowed to anchor to the glass coverslips during a culture period of 48 hours at 37^{o}C, 5%CO_2, 95%O_2. Adhered cell aggregates were fixed in freshly prepared 4% paraformaldehyde during a 30 mins incubation at RT (Sigma –Aldrich). Three 10 mins washes in PBS were followed by antigen-retrieval (0.3% Triton-X-100, Sigma-Aldrich) and blocking using either 10% normal goat serum (NGS) or 10% normal rabbit serum (NRS – both from Vector Laboratories Ltd, Peterborough, UK) depending on the species in which the secondary antibody was raised. The constructs were then incubated with the following primary antibodies: anti-human cytokeratin 19 (CK19), anti-human vimentin (Dako UK Ltd, Cambridgeshire, UK– 1:100) or anti-human insulin (AbD Serotec, Oxford, UK 1:10) for 1 hour at RT and at 4^{o}C overnight. Secondary antibody (goat anti-mouse IgG-FITC for CK19 and vimentin, goat anti-rabbit IgG-TRITC for insulin – Cambridge Biosciences, Cambridge, UK, 1:100) was applied for 3 hours at RT. The coverslips were rinsed and mounted in fluorescence mounting medium (Dako UK Ltd) before cell imaging using a Zeiss Axioskop 40 fluorescence microscope equipped with an AxioCam MRC

colour camera and incorporating Axiovision imaging software (Carl Zeiss, Hertfordshire, UK). Controls involved omission of the relevant primary antibody.

4.2.3. Functional Assessment of the islet:AEC Constructs

4.2.3.1. Estimation of glucose sensitivity - Static Challenge

Functional viability of islet:AEC constructs was determined by assessing glucose-stimulated insulin secretion (GSIS). Static challenge studies were performed as described in Chapter 2 with the following modifications: Constructs (or unmodified islets - controls) were harvested from the relevant culture systems by aspiration using a wide bore sterile pastette. The tissue suspensions were placed into 15ml conical tubes and centrifuged at 400*g* for 3 mins, the supernatants discarded and the resultant pellets re-suspended in a 2ml volume of 1.67mM glucose solution made up in HBSS+0.2%BSA (basal glucose). The tubes containing constructs (or unmodified islets) were then placed in a water bath at 37^{o}C for 1 hour (pre-incubation period). During this hour, a count was performed to determine the number of structurally robust islet:AEC aggregates formed during the 72 hour co-culture period. If necessary the islet/islet:AEC suspensions were re-adjusted (by the further addition of the 1.67mM glucose solution) to achieve a density of 400 constructs/ml. After the pre-incubation period 50µl aliquots (equivalent to 20 IEQ/aggregates) of the cell suspension were transferred to 12mm x 75mm polypropylene tubes (NHS Logistics, Alfreton, UK) using a pipette fitted with a "cell-saver" tip to avoid damaging larger constructs. For each culture condition (i.e. CSC vs. RCCS islets vs. islet:AEC) a total of 18 tubes were thus prepared, to which 2ml of the appropriate secretagogue, diluted in HBSS+0.2% BSA, was added as follows: 6 tubes received 1.67mM

glucose solution to assess basal insulin secretion. To a further 6 tubes a 16.7mM solution of glucose was added to determine stimulated insulin secretion. The remaining tubes received 2ml of 16.7mM glucose supplemented with 10mM theophylline. The racked tubes were sealed with parafilm and placed into a water bath at 37^{o}C for 1 hour to allow insulin secretion (incubation period). Following incubation the tubes were gently vortexed and then centrifuged at 400*g* for 5 mins. The resultant supernatant was harvested for analysis of insulin content using an enzyme-linked immunosorbent assay (ELISA; Mercodia, Diagenics UK), according to the manufacturer's instructions.

4.2.3.2. Estimation of Immunomodulatory function – Mixed islet-lymphocyte reaction (MILR)

Islet:AEC constructs were also assessed for immunomodulatory potential in comparison to unmodified islets cultured in isolation for the same period. Once adjusted to a final volume of approx. 1000 aggregates/ml in supplemented RPMI 1640, 20μl of the islet or islet:AEC suspension was added to the appropriate wells of a 24-well plate and made up to 1ml total volume using the same medium. Thus, approximately 50 IEQ or 50 islet:AEC constructs were added to each well and the plate placed at 37°C, 5%CO_2, 95%O_2 for 72 hours to permit stable cell attachment. Islet: AEC seeded plates were processed by repeated washing in filter sterile PBS to ensure all unattached cells/cellular debris was removed from the wells. Thereafter, resting or PHA-activated PBMC were added at a density of 50,000 cells/well either alone or to wells pre-seeded with firmly anchored islet:AEC constructs prior to co-incubation at 37°C, 5%CO_2, 95%O_2. Activated PBMC continued to be cultured in the

presence of 5μg/ml PHA throughout the assay period. After 72 hours the PBMC were harvested, washed and assayed for intracellular ATP content as detailed in Chapter 3.

4.2.4. Statistical Analysis

Statistical differences in response to insulin secretagogues were assessed by one way analysis of variance (ANOVA) using insulin secretion from control islets (islet alone in maintained under CSC conditions). Significant differences in PBMC proliferation in response to co-culture with islet:AEC constructs was determined using Mann-Whitney U (by Rank) and Tukey's multiple comparison tests, with the response of resting PBMC serving as the control. In all comparisons a p value of <0.05 was considered to be statistically significant. Statistical analysis was performed using SigmaStat software version 3.5 (Systat Software Inc, Chicago, USA).

4.3. Results I

4.3.1. Morphological analysis of islet:AEC constructs

Constructs formed using sub-optimal numbers of AECs (CSC and RCCS)

In preliminary studies human islets were co-cultured under both CSC conditions and within the RCCS employing AEC at low density (less that $5x10^4$/ml). Under these conditions the AEC failed to adequately integrate with the islets with limited numbers of AEC attaching to the islet surface (Figs. 10 and 11). This was observed in both CSC and RCCS cultures and mirrored our observations in Chapter 3 suggesting that AEC require plating at high density to achieve adequate attachment to the growing surface. In subsequent studies islet:AEC co-cultures were initiated with a minimum of $1x10^5$ cells per ml to encourage optimal cellular aggregation of the two cell types.

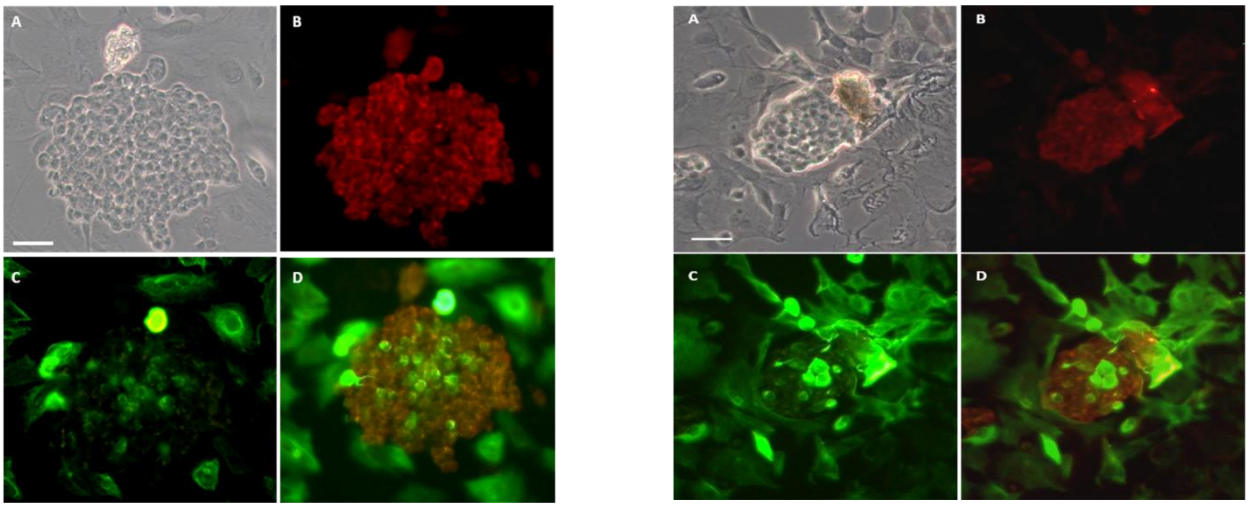
Islet:AEC constructs formed using optimal AEC density: CSC vs RCCS.

When a sufficient density of AEC was used in the co-culture system islets and AEC under both CSC conditions and within the RCCS demonstrated a degree of cell association: However, the extent of cellular integration differed between the two culture conditions. When the constructs were formed using static cultures loose aggregates formed with AEC overlying the surface of the islet; seemingly using the islet as a matrix (Figs. 12 and 13).

Robust, tightly formed cellular constructs exhibiting good integration of the two cell types was achieved when islets and AEC were co-cultured for 72 hours within the RCCS. The vast

majority of islets within the RCCS became associated with AEC although, in most instances, the AEC did not form a complete layer (Figs.14 and 15).

Constructs formed by islet co-culture with sub-optimal numbers of human AEC under conventional static culture and within the rotational cell culture system.



Figures 10. Islet and AEC constructs with a sub-optimal number of AECs cultured under conventional static culture (CSC) conditions. Visualisation was achieved using TRITC (red, insulin) or FITC (green, CK19) conjugated secondary antibodies (A) phase contrast image of the constructs (B) insulin expression (C) CK19 expression (D) overlay image showing very poor cellular interaction between islets and AECs
Scale bar=20μm

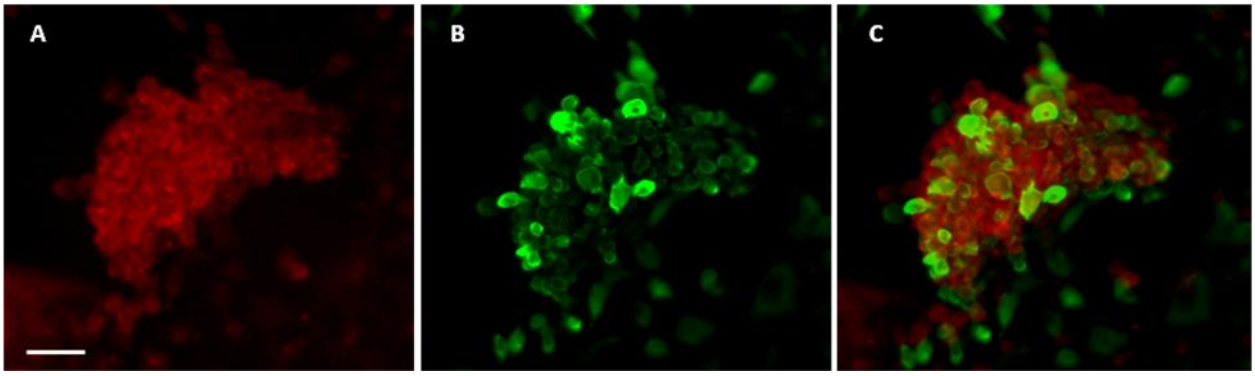


Figure 11. Islet and AEC constructs with a sub-optimal number of AECs maintained in a rotational cell culture system (RCCS). Visualisation was achieved using TRITC (insulin) or FITC (CK19) conjugated secondary antibodies (A) insulin expression (B) CK19 expression (C) overlay image showing very poor cellular interaction between islets and AECs
Scale bar= 50μm

Constructs formed by islet co-culture with optimal numbers of human AEC under conventional static culture.

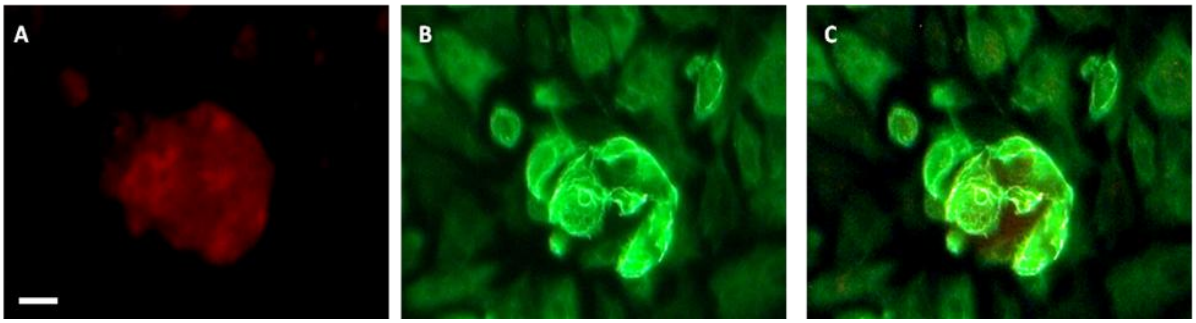


Figure 12. Islet and AEC constructs in static culture conditions. Visualisation was achieved using TRITC (insulin) or FITC (CK19) conjugated secondary antibodies (A) insulin expression (B) CK19 expression (C) overlay image showing some cellular interaction between islets and AEC. Scale bar= 50μm

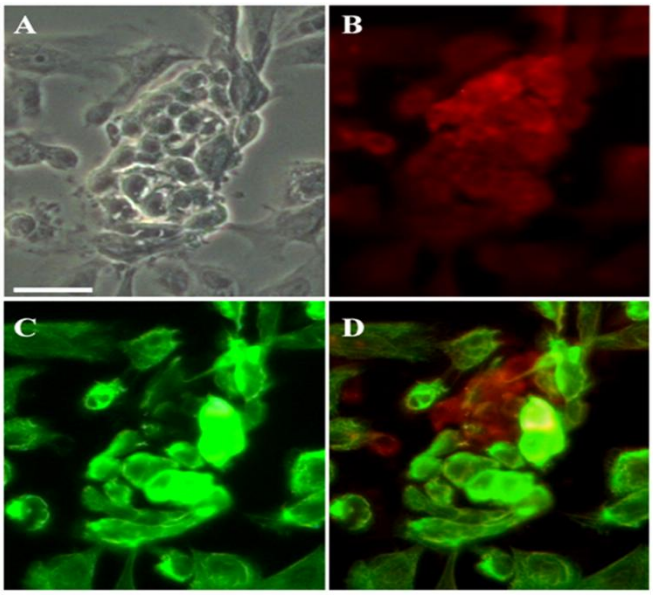


Figure 13. Islet and AEC constructs in static culture conditions. Visualisation was achieved using TRITC (insulin) or FITC (CK19) conjugated secondary antibodies (A) phase contrast image of the constructs (B) insulin expression (C) CK19 expression (D) overlay image showing some cellular interaction between islets and AECs
Scale bar=50μm

Constructs formed by islet co-culture with optimal numbers of human AEC within the rotational cell culture system.

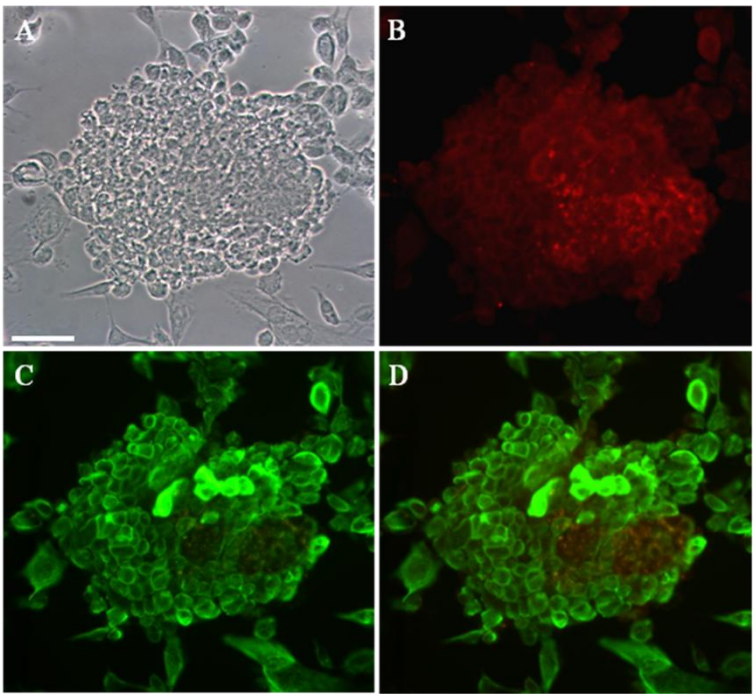


Figure 14. Islet and AEC constructs in rotational culture conditions. Visualisation was achieved using TRITC (insulin) or FITC (CK19) conjugated secondary antibodies (A) phase contrast image of the constructs (B) insulin expression (C) CK19 expression (D) overlay image showing good cellular interaction between islets and AECs and tight robust constructs. Scale bar=50μm

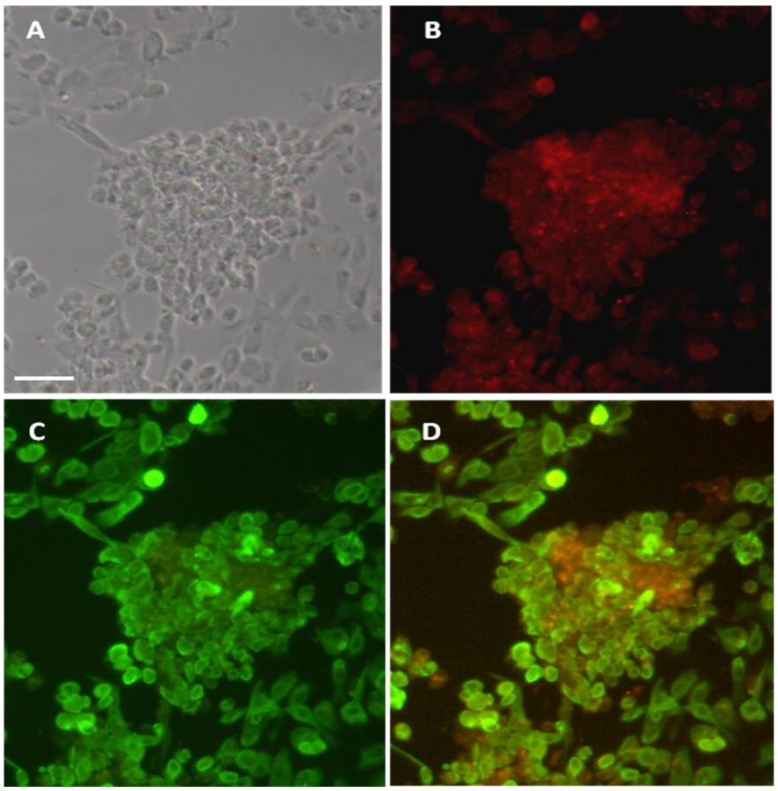


Figure 15. Islet and AEC constructs in rotational culture conditions. Visualisation was achieved using TRITC (insulin) or FITC (CK19) conjugated secondary antibodies (A) phase contrast image of the constructs (B) insulin expression (C) CK19 expression (D) overlay image showing good cellular interaction between islets and AECs and tight robust constructs. Scale bar=50μm

4.4. Results II

4.4.1. Analysis of insulin secretory capacity of islet:AEC constructs

Following a 72 hour period of culture islet and islet:AEC constructs were subjected to further glucose challenge studies. Preliminary experiments indicated that isolated AEC do not secrete insulin when maintained in either static or rotational culture (data not shown) and were therefore not assessed alone during this investigation. In the presence of elevated (16.7mM) glucose, control islets held under CSC conditions throughout the period of the investigation responded minimally in terms of insulin secretion (S.I. 1.24±0.07), as previously noted (Murray et al., 2009, Murray et al., 2005), although a combination of 16.7mM glucose and 10mM theophylline elicited more marked ($p< 0.05$) insulin secretion (S.I. 1.53±0.1: Fig.16). By contrast, maintenance of islets within the RCCS preserved glucose responsiveness with significant insulin secretion occurring in response to 16.7mM glucose both in the absence (S.I. 1.59±0.08; $p < 0.05$) and the presence (S.I. 2.49±0.28; $p < 0.01$) of theophylline. Co-culture of islets with AEC under both CSC conditions or within the RCCS had an apparently beneficial effect on beta cell function, with islets continuing to respond to glucose stimulation (S.I. 1.65±0.12 and 2.89±0.34 for islets under CSC condition in response to 16.7mM glucose alone and 16.7mM glucose plus 10mM theophylline respectively; S.I. 1.83±0.11 and 3.15±0.32 for islets maintained in the RCCS in response to 16.7mM glucose alone and 16.7mM glucose plus 10mM theophylline respectively: Fig.16).

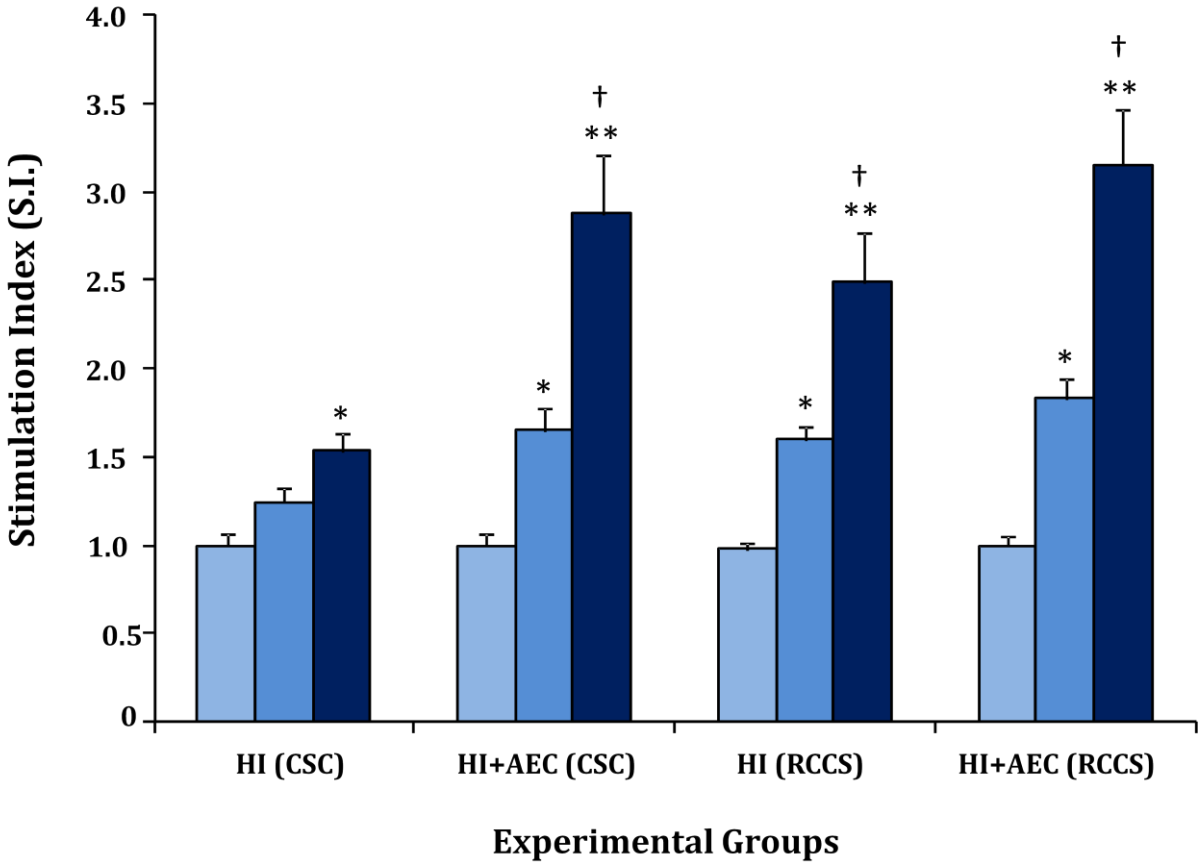
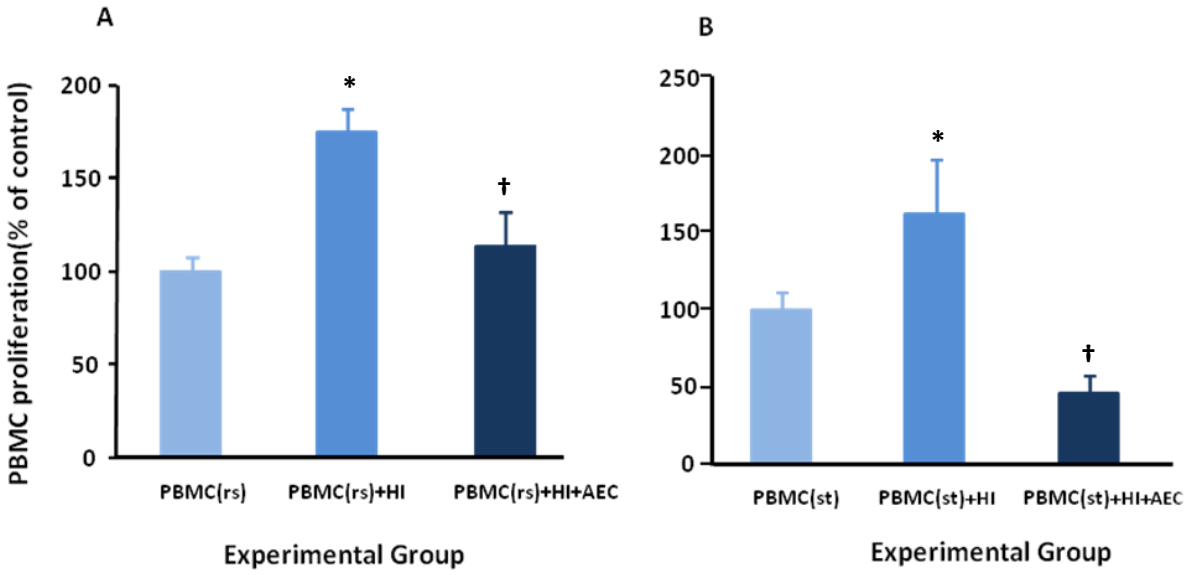


Figure 16. *Glucose stimulated insulin release from human islets (HI) maintained under conventional static culture (CSC) conditions or within the rotational cell culture system (RCCS) either in the presence or absence of human amniotic epithelial cells (AEC) for 72 hours.* Insulin release was measured in response to 1.67 mmol/l glucose ( ), 16.7 mmol/l glucose ( ), and 16.7 mmol/l glucose plus 10 mmol/l theophylline ( ). Results are expressed as the ratio of stimulated insulin release compared to basal, mean ± S.E.M. n=4. * $p < 0.05$, ** $p < 0.01$ stimulated insulin secretion compared to basal release. † $p < 0.01$ for stimulated release in treatment groups compared to the control (*ANOVA)*

4.4.2. Analysis of immunomodulatory potential of the islet:AEC constructs

Exposure of resting PBMC to unmodified human islets which were maintained within the RCCS elicited a marked ($p < 0.05$) proliferative response (Fig.17.A). The presence of AEC attenuated the resting PBMC proliferation elicited by human islets. PHA-stimulated PBMC proliferation was increased on contact with isolated islets, but was significantly ($p<0.01$) suppressed when islets were in co-culture with AEC (Fig.17.B).



Figures 17.A and 17.B. *Modulation of peripheral blood mononuclear cell (PBMC) proliferation by exposure to human islets and human islet:AEC constructs:* Resting (A) or PHA-activated (B) human PBMC were maintained in 24-well plates either alone or in the presence of human islets (HI) or islet:AEC constructs for a period of 72 hours. The rate of PBMC proliferation following this period was measured using an ATP chemiluminescence assay. Data depicts the response from 4 individual human islet and AEC preps and represents the typical observation. * $p < 0.01$ compared to PBMC alone (resting or activated). † $p < 0.01$ for PBMC proliferation in response to islet:AEC constructs compared to unmodified islets. (*Mann-Whitney U)*

4.5. Discussion

In previous chapters (2&3) the isolation, culture and morphological and functional characterisation of human islets and AEC has been outlined in detail. The experiments detailed in the present chapter sought to demonstrate that islet cells may be modified through in vitro, pre-transplant interventions designed to enhance post-implant survival; specifically, to provide evidence for our hypothesis that the co-culture of human islets with a purified population of human amniotic epithelial cells modulates the immunogenic potential of transplantable islet cells without impairment of β-cell function. The results obtained suggest that it is possible to bring these two cell types into close proximity whilst preserving their respective insulin secretory function and immunomodulatory capabilities.

This chapter deals therefore, with the impact of co-culturing these two cell types and to evaluate their combined function. Notwithstanding their disparate origins, the co-culture of human islets and AEC under either conventional static or rotational cell culture conditions resulted in successful physical interaction between the two cell types. The degree of association was dependent on the density of AEC seeded with a minimum of $5x10^4$cells/ml required before significant aggregation was observed. The observation correlates with our earlier findings that AEC require plating at relatively high density in monolayer culture in order to achieve good cell attachment and proliferation, which has been reported elsewhere (Parolini et al., 2008).

The RCCS provided a more conducive environment for cellular aggregation, with the formation of robust constructs exhibiting frequent spatial association of the insulin and CK19

expressing cells and a preserved islet-like morphology. The high aspect ratio vessels (HARVs) are designed to create a microgravity environment with low shear forces permitting a greater degree of cell-cell interaction (Unsworth and Lelkes, 1998) which may underlie the efficient formation of stable islet:AEC constructs observed in the present study.

The close proximity of AEC to the human islets had no adverse effect on beta-cell function. Indeed, the insulin-secretion data indicate preservation of glucose-sensitivity in human islets maintained in co-culture with AEC. This may be compared with islets held alone under CSC conditions which showed a diminution of glucose responsiveness. Previous studies conducted in this laboratory indicate a beneficial impact of pancreatic ductal-epithelial cell co-culture in preserving islet function (Murray et al., 2009), most likely due to their ability to provide trophic support to neighbouring beta-cells (Rosenberg and Vinik, 1992). Similarly, AEC are reported to synthesise and secrete a range of growth factors which may have relevance for the sustained functional viability of islets seen in this novel co-culture model. Of note, mRNA expression of TGFβ, EGF and KGF, known mediators of beta cell replication (Fiaschi-Taesch et al., 2008, Movassat et al., 2003, Hanley and Rosenberg, 2007) have been reported in intact human amniotic membrane and isolated amniotic epithelial cells (Koizumi et al., 2000). Furthermore, dissociated AEC secrete biologically active neurotrophins including brain derived neurotrophic factor (BDNF) (Kakishita et al., 2003) which have been linked to β-cell development and survival (Scharfmann and Czernichow, 1996). Other studies suggest the trophic actions of AEC mediate repair processes in experimental models of Parkinson's Disease, stroke, spinal cord injury and liver fibrosis by encouraging

regeneration of host tissue or supporting the growth and engraftment of transplanted cells (Parolini and Caruso, 2011) .

Isolated islets are known to release inflammatory cytokines (including IL-1β, IL-6 and TNFα) and pro-inflammatory molecules (including tissue factor and monocyte chemoattractant protein-1) which have deleterious effects on β-cell function, with subsequent impairment of islet graft function (Marzorati et al., 2006, Matsuda et al., 2005). Recent studies suggest that AEC exert anti-inflammatory properties as demonstrated in animal models of lung and liver fibrosis (Manuelpillai et al., 2011, Manuelpillai et al., 2010a, Murphy et al., 2011), reducing the tissue levels of pro-inflammatory cytokines with concomitant release of IL-10. It is possible that in our co-culture model these anti-inflammatory factors serve to subdue cytokine mediated β-cell damage thus sustaining islet function. Overall, it is likely that the close association of AEC to islets as provided by their co-culture within the RCCS permits the paracrine release of soluble mediators able to support insulin secretory capacity in the post isolation period with beneficial consequences in terms of sustained islet graft function.

The proposition that the immunosuppressive properties of isolated AEC may be manipulated to confer a state of immune-privilege on other cells capable of provoking an immune response is confirmed by the mixed islet-lymphocyte reaction (MILR) study. Sustained proliferation of resting PBMC was demonstrated in the presence of unmodified islets, yet those which were closely associated (co-cultured) with AEC failed to elicit an allogeneic response. This effect was not dependent on complete encapsulation of the islets by the AEC; further indicative of a role for soluble immunoregulatory factors. Also, the

immunomodulatory response to activated (PHA-stimulated) T-cells was as robust in the islet:AEC co-cultures as in AEC monocultures. Combined, these data suggest that AEC exhibit a potent and generalised immunosuppressive capability, inducing an anti-proliferative response in T-cells subjected both to mitogen and allo-antigen challenge.

This is the first study to demonstrate that the immunomodulatory capabilities of human AEC, as observed in vitro, may be conferred on another, otherwise, immunogenic cell population, provided that they are held in close proximity. The finding has relevance for the wider use of islet cell replacement therapy as a treatment for Type 1 diabetes. The results are analogous to contemporary studies where alternative immune-suppressing cell types have been co-cultured/co-transplanted with islets, albeit in animal models. Notably, in the context of islet transplantation the use of Sertoli cells (SC) to create a local milieu conducive to long-term allograft and xenograft survival has been demonstrated experimentally and clinically (Isaac et al., 2005, Kin et al., 2002, Valdes-Gonzalez et al., 2007) and more recently the use of bone marrow-derived mesenchymal stem cells (MSC) to regulate the immunogenicity of islet allografts has also been reported (Ding et al., 2009). We propose that immuno-protection could be achieved by the use of AEC, effectively bio-engineering a state of immune-privilege within the graft tissue promoting the localised release of soluble immunoregulatory mediators. While the widespread clinical use of human SC and MSC would pose certain technical challenges associated with accessibility and standardisation, human amnion is readily available and not subject to the same ethical constraints. Additionally, the present studies suggest that amnion provides an expandable pool of immunomodulatory cells which are amenable to cryopreservation, readily integrate with

isolated human islets and do so without causing adverse alterations to beta cell viability, cellular transplant volumes or nutrient exchange. Furthermore, the experimental and clinical use of amniotic membrane is well established (Gomes et al., 2005, Hasegawa et al., 2007, Sheridan and Moreno, 2001, Cargnoni et al., 2009) and successful engraftment of human AEC without evidence of tumorigenesis has been reported (Bailo et al., 2004). Direct application of this approach awaits "proof-of-concept" studies evaluating the function of implanted islet:AEC constructs in immune-competent, diabetic animal models.

These findings raise the possibility of engineering insulin-secreting tissue constructs applicable to cell-based therapies for diabetes, which are capable of restoring endogenous insulin production without the need for adjuvant chronic systemic immunosuppression.

CHAPTER 5: AEC-MEDIATED IMMUNOMODULATION. SPECIFIC T-CELL TARGETS AND RELEVANCE TO ISLET TRANSPLANTATION

5.1. Introduction

The previous chapters detailed a series of investigations to demonstrate the potential of human AEC to modulate the actions of the immune system. The results suggest that AEC are capable of subduing the proliferative activity of human PBMC in response to a known mitogen as has been previously reported (Wolbank et al., 2007). Additionally, and for the first time, this study provides *in vitro* evidence that AEC are able to confer their immunomodulatory properties to other adjacent cell types with the overall effect of reducing the immunogenic profile of, in this instance, human islet cells, and do so without impairing function *viz.* physiological release of insulin. Such a property may have beneficial implications for tissue/cell replacement therapy where localised immune-privilege mediated by AEC may serve to shield co-transplanted therapeutic cells from immune rejection. At present the precise mechanism(s) by which AEC restrict lymphocyte proliferation require further elucidation; clinical application of the immunomodulatory actions of AEC would benefit from a clearer understanding of the individual T-cell sub-populations targeted by AEC and the role that such T-cells play in islet graft rejection.

Our understanding of the mechanism(s) underlying allo and auto-reactivity in islet-cell replacement therapy is based on experimental and limited clinical transplantation data. The presence of islet-specific autoreactive CD4+ T- cells at time of transplantation coupled with

increased frequencies of circulating CD8+ T- cells (insulin B10–18 reactive) is considered to significantly influence clinical outcome of islet transplant recipients (Huurman et al., 2008, Pinkse et al., 2005). The insulin-specific CD8+ T- cells show potential cytolytic activity, producing granzyme B and IFN-γ and are therefore potentially able to destroy insulin-producing β-cells. Equally the absence of these markers, and therefore presumably of auto-reactivity, correlates with good clinical outcome (Huurman et al., 2008, Pinkse et al., 2005).

Allo-rejection of transplanted islets is considered to be mediated by both CD4+ and CD8+ T-cells and both populations are required to accomplish β-cell death. The actions of a number of other immune cells including macrophages, dendritic cells (DC) and B lymphocytes are co-ordinated to induce and sustain the immune assault. Macrophages and DC act as antigen-presenting cells and stimulate the migration and infiltration of grafted cells by peripheral CD4+ and CD8+ T-cells. The islets are also targeted by natural killer cells (NK) and B lymphocytes. Infiltrating macrophages serve to activate cytotoxic CD8+ cells which, in the same manner as auto-reactive CD8+ T-cells cause β-cell destruction by the release of cytolytic agents. In allograft rejection CD4+ T-cells may also act indirectly through B-cell activation and the generation of complement fixing antibodies. Pro-inflammatory cytokines including interleukin (IL)-12 released by macrophages activate Th1-type CD4+ T-cells which subsequently secrete IL-2, interferon-γ, and TNF-α to further augment the CD8+ response.

Aims of the Chapter

As a means of gaining further understanding of the relevance of AEC- mediated immunomodulation to islet graft protection the next series of studies sought to more closely examine the specific immune cell targets involved. As populations of CD4+ and CD8+ T-cells play a major role in both auto and allo-graft rejection this study sought to determine the modulatory potential of AEC in regard to these two cell types.

5.2. Materials and Methods

5.2.1. CD4+ T-cell isolation

A Dynabead®-mediated negative selection system was employed to isolate CD4+ T-cell populations (Dynabeads® Untouched™ Human CD4 T-cells, Life Technologies, Paisley, UK). The isolation kit is designed to deplete B cells, NK cells, monocytes, platelets, dendritic cells, CD8+ T-cells, granulocytes and erythrocytes from platelet-poor PBMC samples, leaving isolated CD4+ T- cells free of bead and antibody, thus making them appropriate for use in subsequent proliferation assays. In the present study PBMC were isolated from CD leucocyte cones (leucocyte concentrates) obtained from healthy donors (NHS Blood and Transplant, Birmingham). The cells were processed within 18 hours of blood collection and PBMC isolation was performed as described in section 3.3.1. The PBMC preparation was subjected to 3 washes in isolation buffer; Phosphate Buffered Saline (PBS) without Mg_2^+ and Ca_2^+ supplemented with 0.6% sodium citrate and 0.1% BSA. PBMC were counted and adjusted to a density of $1x10^8$cells/ml in isolation buffer. A 200µl aliquot of the PMBC suspension was transferred to a 15ml conical tube to which was added 40µl of heat inactivated foetal calf serum (FCS, Sigma-Aldrich Ltd). This was followed by addition of 40µl of antibody mix containing mouse IgG antibodies for CD8, CD14, CD16 (specific for CD16a and CD16b), CD19, CD36, CD56, CDw123 and CD235a (GlycophorinA), being sure that the suspensions were thoroughly mixed. The cell/antibody suspension was incubated for 20mins at 4°C prior to thorough washing in 4mls of isolation buffer. Cells were harvested by centrifugation at 300*g* for 8 mins at 4°C and re-suspended in 200µl of isolation buffer. To this was added 200µl of pre-washed Depletion MyOne® Dynabeads at the same density as the cells, followed by

incubation for 15 mins at RT with gentle tilting and rotation using a Hulamixer®. The bead-bound cells were re-suspended by vigorously triturating the sample through a 1000μl pipette tip (approx. 10 times) before addition of 2mls of isolation buffer. The tube containing the cells was then placed into a magnet for 2 mins before transferring the supernatant to a new tube. The original tube was washed with another 2mls of isolation buffer and returned to the magnet. The supernatant was again collected into a fresh tube. Finally the supernatants were pooled and placed in the magnet for a further 2 mins to remove any remaining beads. The supernatants containing the free CD4+ cells were then washed in isolation buffer and cells harvested by centrifugation at 300*g* for 5 mins at 4°C prior to counting and assessment of viability as described in section 3.3.1.

5.2.2. CD8+ T-cell isolation

CD8+ T-cells were isolated using a Dynabead®-mediated negative selection system (Dynabeads® Untouched™ Human CD8 T-cells, Life Technologies, Paisley, UK) as described above for CD4+ T- cells but with the following modifications. 500μl of PBMC suspension (at a density of 1 x 10^8cells/ml) were transferred to a 15ml conical tube. The cells were supplemented first with 100μl of FCS and then with 100μl of antibody mix consisting of biotinylated mouse IgG antibodies for CD4, CD14, CD16 (specific for CD16a and CD16b), CD19, CD36, CD56, CDw123 and CD235a (Glycophorin A) ensuring that cell and antibody solutions were well mixed. The cells were incubated for 20 mins at 4°C prior to thorough washing in 10mls of isolation buffer. Cells were harvested by centrifugation at 350*g* for 8 mins at 4°C and re-suspended in 500μl of isolation buffer. To this was added 500μl of pre-

washed Depletion MyOne® Dynabeads followed by incubation for 15 mins at RT with gentle tilting and rotation using a Hulamixer®. The bead-bound cells were re-suspended by vigorously triturating the sample before addition of 5mls of isolation buffer. Magnetic assisted separation of the bead-bound cells and the resulting purified CD8+ cells were harvested, counted and assessed for viability.

5.2.3. Confirmation of T-cell purity by Flow Cytometry

Purity of the CD4+ T-cell subset was confirmed by cell surface marker labeling and flow cytometry. Cell surface staining was achieved by use of a Brilliant Violet 421™conjugated anti-human CD4+ antibody or the Isotype control (BioLegend, Supplied by Cambridge Bioscience, Cambridge, UK). The antibodies were diluted 1:100 in Fluorescence activated cell sorting (FACS) buffer and added to samples of the CD4+ T-cells prior to incubation on ice in the dark (covered with foil) for 15 mins. 300µl of FACS buffer was then added before centrifugation at 400*g*, 4°C, for 5 mins. The supernatant was removed and replaced with 100µl of Fix Buffer (made as per manufacturer's instructions) to each tube, which were incubated on ice for 30 mins. CD4+ were harvested by centrifugation at 4°C, 400*g* for 6 mins and washed again with 300µl of FACS Buffer. Following centrifugation at 400*g,* 4°C for 6 mins, the supernatant was removed and the cells were washed 2 times with 200µl of Perm Buffer (prepared according to manufacturer's instructions) pelleting between washes by centrifugation 400*g*, 4°C for 6 mins.

A Cyan 3 laser 9-colour flow cytometer equipped with Summit™ data software (Beckman Coulter, High Wycombe, UK) was used to analyse the CD4+ T-cell population on the basis of the forward scatter/side scatter profile of cells labelled with BV –anti-CD4+ as detailed above using an isotype-matched negative control antibody to quantify the degree of positive staining.

5.2.4. Proliferation Studies

5.2.4.1. CD4+ T-cell proliferation: modulation with phytohaemagglutinin (PHA), human amniotic epithelial cells (AEC)

In preliminary studies we used our existing protocol to determine whether CD4+ T-cell proliferation was influenced by exposure to human amniotic epithelial cells (AEC). To this end P1 AEC were plated at a density of $5x10^4$/ml into the wells of a 24-well plate and incubated at 37°C for 72 hours to enable firm anchorage. CD4+ T-cells, processed as described above, were plated at equal density either in the presence or absence of the attached AEC. Additionally, further groups of CD4+ T-cells were plated in the presence of the plant mitogen phytohaemagglutinin at a concentration of 5μg/ml. Incubation was carried out at 37°C for 72 hours before the CD4+ T-cells were harvested prior to intracellular ATP analysis using chemiluminescence (see section 3.3.4).

5.2.4.2. PBMC, CD4+ and CD8+ T-cell proliferation: modulation with anti CD3/CD28, human amniotic epithelial cells (AEC) and human islets

In light of the findings of the preliminary study (detailed in section 5.3.1. below) the next series of experiments were performed using a more specific T-cell activation method. Dynabeads® Human T-Activator CD3/CD28 beads (Invitrogen, Life Technologies, Paisley, UK) were employed as an alternative to PHA. CD3/CD28 activator beads are reported to provide physiological activation and expansion of human T-cells including CD4+ and CD8+ cells. The cells once activated can be analysed or subjected to further differentiation protocols (e.g. differentiation to T-helper cells). Proliferation assays were initiated using AEC at P1 plated at a density of $5x10^4$cells/well, as described above. Unfractionated PBMC, purified CD4+ or CD8+ T-cells were plated at the same density either in the presence or absence of AEC and a further group of cells were plated in the presence of $5x10^4$ pre-washed CD3/CD28 activation beads. Incubation was carried out at 37°C for 96 hours before the cells were harvested for intracellular ATP analysis using chemiluminescence (see section 3.3.4). In a small series of experiments mixed islet:lymphocyte proliferation studies were performed. For these studies islets were cultured in the presence or absence of AEC in RCCS for 72 hours to allow aggregates to form. Islets and islet:AEC constructs were seeded into 24-well plates (50IEQ/aggregates per well) and allowed to anchor for up 72 hours. Proliferation assays were then established as for the AEC monocultures as described above.

5.3. Results

5.3.1. Expansion of CD4+ T-cell populations using PHA and CD3/CD28 activation beads

Flow cytometry confirmed the purity of the CD4+ T-cells used in the present studies. In preliminary investigations it was observed that isolated CD4+ T-cell populations were largely unresponsive to the routinely used stimulator, phytohaemagglutinin (PHA). Whereas unfractionated PBMC numbers increased in the presence of 5μg/ml PHA, CD4+ T-cell numbers did not change significantly. By contrast incubation of both unfractionated PBMC and isolated CD4+ T-cells with CD3/CD28 stimulator beads produced a robust increase in numbers of both cell populations (Fig 18). In subsequent proliferation studies CD3/CD28 beads were adopted as the stimulator of choice.

These results were used as the basis for subsequent proliferation assays using fractionated CD4+ and CD8+ T-cells. In these studies it was observed that activation of both cell types are modulated on co-culture with an equal number of allogeneic human AEC (Fig.19). Resting CD4+ and CD8+ T-cells were largely unresponsive to co-culture with allogeneic AEC but responded robustly to the presence of an equal number of CD3/CD28 activator beads. The degree of T-cell expansion induced by the beads was significantly ($p < 0.01$) abrogated in the presence of allogeneic AEC with a greater than 50% inhibition for both T-cell populations (Fig.19).

5.3.2. Fractionated CD4+ and CD8+ T-cell proliferation assays. Impact of co-culture with human AEC

This series of studies first sought to determine whether human AEC are able to modulate the proliferative capacity of fractionated CD4+ and CD8+ T-cells. As observed with PBMC, allogeneic AEC failed to provoke significant CD4+ T-cell proliferation when co-cultured at varying densities ranging from $2.5x10^3$-$5x10^4$. Employing CD3/CD28 activator beads as the stimulus, AEC-mediated inhibition of CD4+ T-cell proliferation was observed at all ratios tested from 1:20 – 1:1, exhibiting a degree of dose-dependency (Fig.20).

5.3.3. Response of CD4+ and CD8+ T-cell populations on co-culture with allogeneic human islets and islet:AEC constructs

In a limited study human islets prepared as outlined in Section 2.2. were used in a mixed islet:lymphocyte reaction. In vitro, allogeneic human islets provoked a moderate activation of both CD4+ and CD8+ T-cells (Fig 21). Expansion of both T-cell populations was observed during a 96 hour co-culture period with human islets. By contrast, when human islets were pre-cultured with human AEC to form constructs as detailed section 4.2.1, the response of CD4+ of CD8+ cells was significantly attenuated (Fig.21).

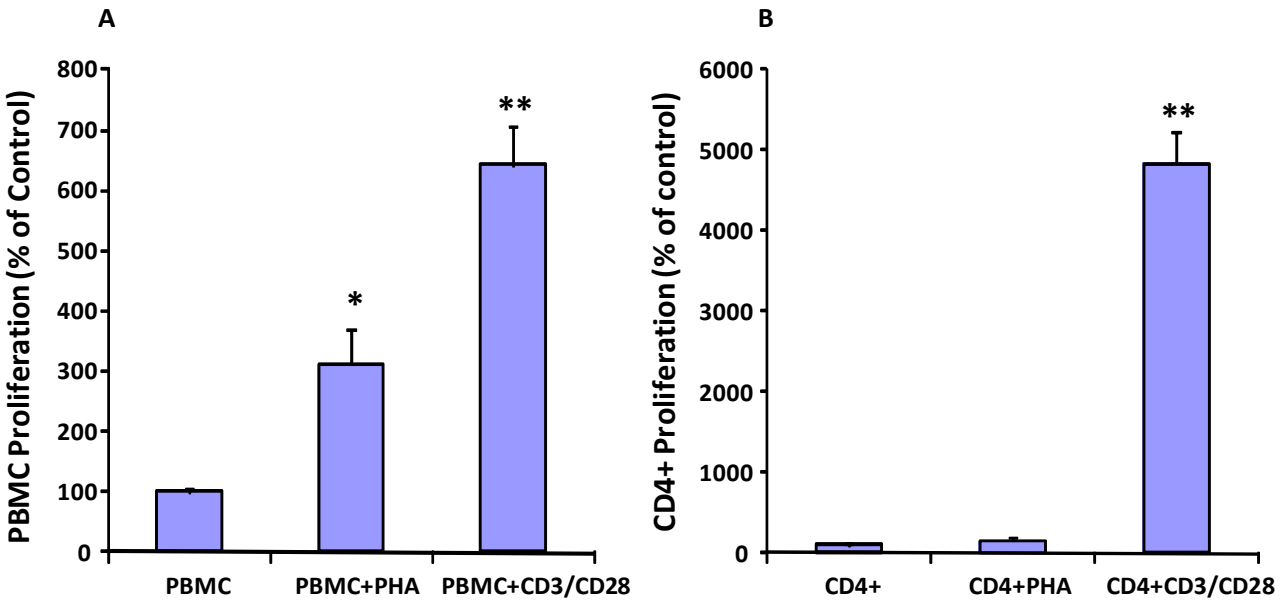


Figure 18. *Activation of PBMC (A) and fractionated CD4+ T-cells (B) on exposure to either phytohaemagglutinin (PHA - 5μg/ml) or an equal number (5x10^4) of CD3+/CD28+ activation beads.* PHA produced a robust increase in PBMC numbers but a greater response was observed in both cell populations when the activator beads where employed as the stimulus. n = 4. * $p < 0.05$, **$p < 0.01$ compared with control (resting cells). *One way ANOVA*

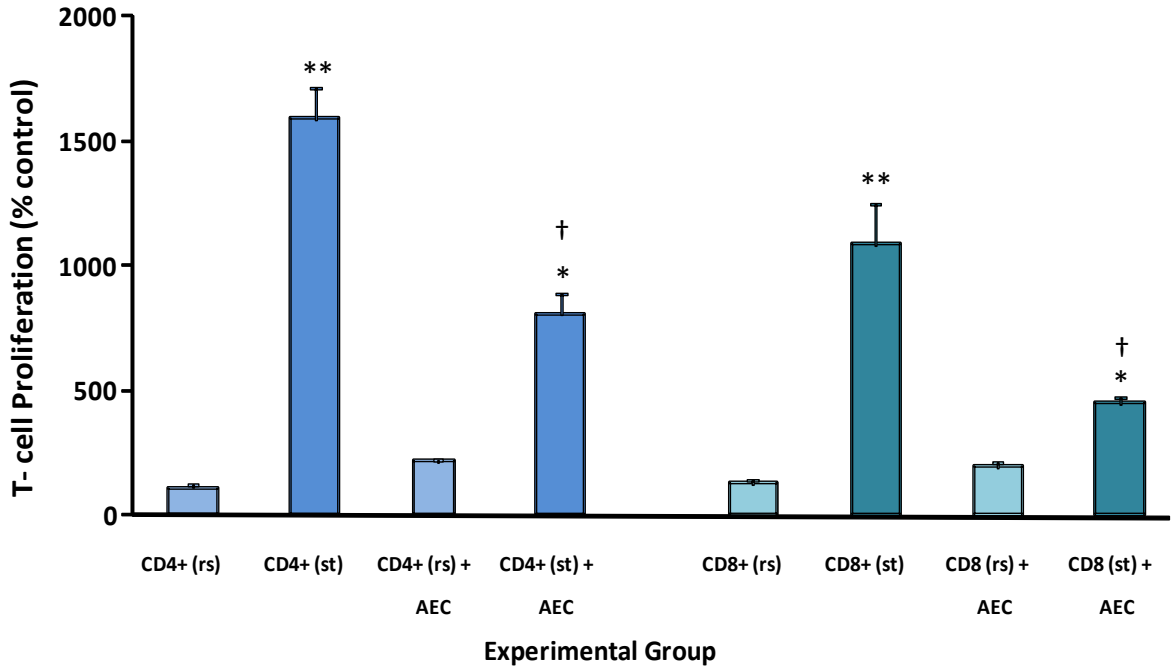


Figure 19. *Modulation of CD4+ and CD8+ T-cell proliferation by allogeneic human amniotic epithelial cells (AEC):* Resting (rs) or CD3/CD28-activated (st) CD4+(blue shaded bars), and resting (rs)or CD3/CD28-activated (st) CD8+ (green shaded bars)T-cells were maintained in 24-well plates either alone, or in the presence of an equal number of human amniotic epithelial cells for a period of 96 hours. The rate of T-cell proliferation following this period was measured using an ATP chemiluminescence assay. Data shows the percentage increase above control (resting T-cell). n = 3. * $p < 0.05$, ** $p < 0.01$ compared to control. † $p < 0.01$ for CD3/CD28-activated T-cell proliferation in the presence or absence of AEC. (Mann-Whitney U and Tukey's multiple comparison tests)

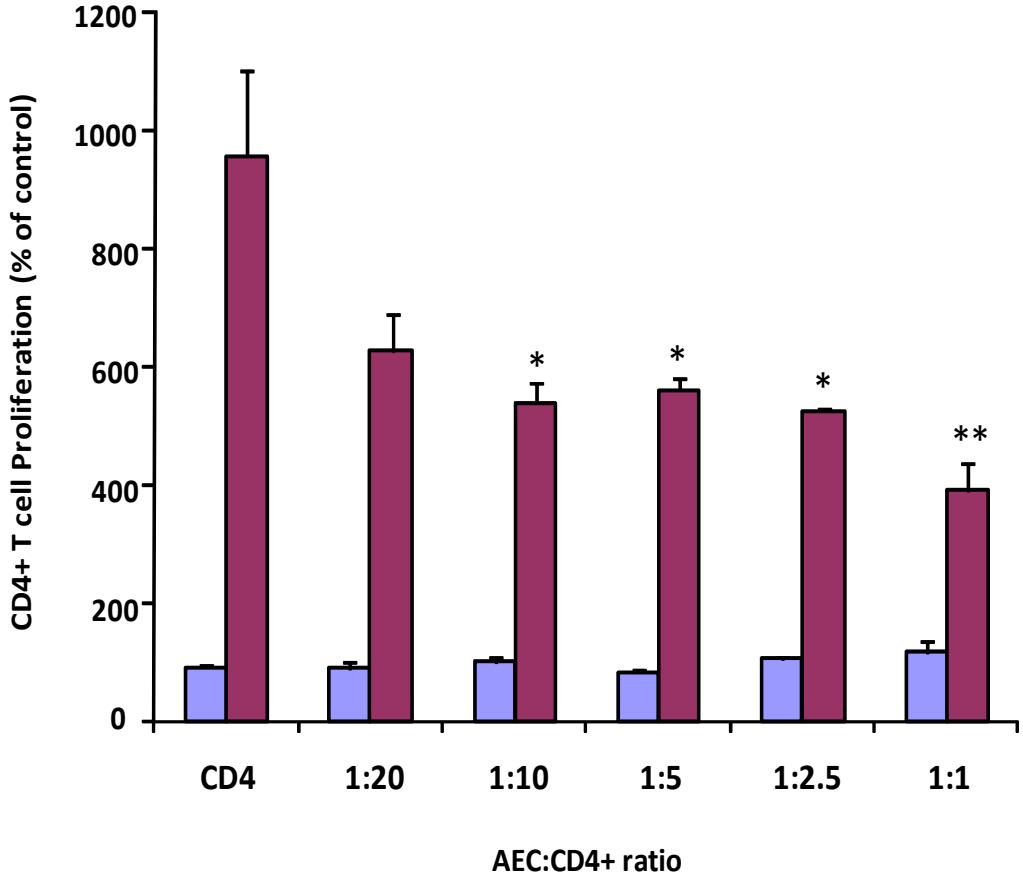


Figure 20. *Dose-dependent modulation of CD4+ T-cells on co-culture with human amniotic epithelial cells (AEC):* AEC were pre-plated at densities ranging from $2.5x10^3$-$5x10^4$ cells/well and co-cultured with $5x10^4$ CD4+ T-cells for 96 hours. Cell proliferation was measured by chemiluminescence assay. n = 3. * $p < 0.05$, ** $p< 0.01$ for CD4+ T-cell proliferation in the presence vs. absence of AEC. *One way ANOVA*

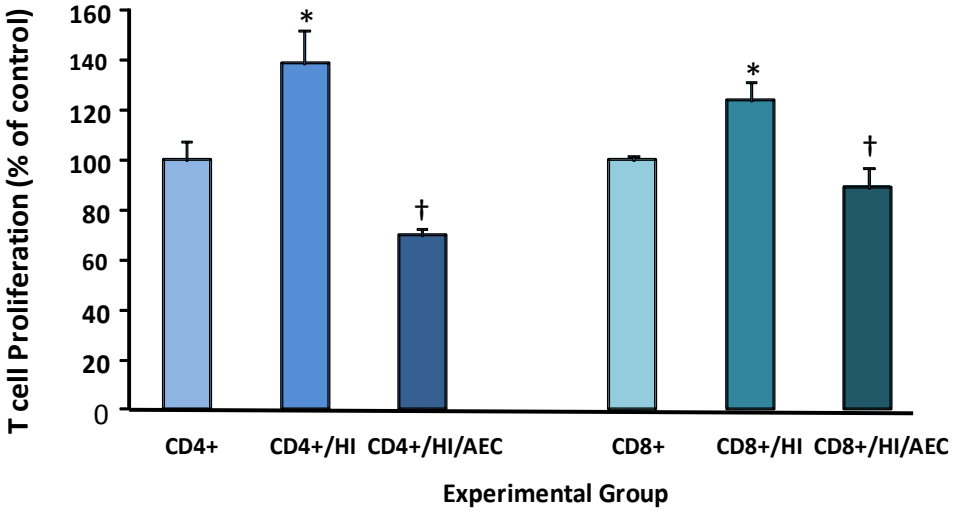


Figure 21. *Modulation of CD4+ and CD8+ T-cell proliferation by exposure to human islets and human islet:AEC constructs:* Resting CD4+ T-cells (blue bars) and CD8+ T-cells (green bars) were maintained in 24-well plates either alone or in the presence of human islets (HI) or islet:AEC constructs (HI/AEC) for a period of 96 hours. The rate of T-cell proliferation following this period was measured using an ATP chemiluminescence assay. Data depicts the response from 2 individual human islet and AEC preps and represents the typical observation. * $p < 0.01$ compared to resting levels. † $p < 0.01$ for T-cell expansion in the presence of HI/AEC constructs compared to HI alone. *(Mann-Whitney U and Tukey's multiple comparison tests)*

5.4. Discussion

This investigation provides further evidence that the immuno-modulatory potential of human AEC may have relevance to the protection of islet allografts from immune assault. The findings suggest that several of the targets of AEC-induced immunomodulation have relevance to islet allograft rejection and as such indicate the potential use of AEC populations as mediators of “bystander” immunosuppression in islet transplantation. The data indicate that both CD4+ and CD8+ T-cell activation is responsive to AEC and that bringing these immune cells into close proximity of AEC prevents their in vitro expansion in response to a physiological stimulus *viz.* CD3/CD28 complex. As the activation and proliferation of CD4+ and CD8+ T-cells is crucial to the appropriate and successful activation of the innate and adaptive immune response, culminating in allograft rejection, suppression of their function by AEC may have relevance for providing a localised means of providing immune protection to transplanted islet cells and circumvent the need for long-term, non-specific systemic immunosuppression.

The lack of responsiveness of fractionated CD4+ T-cells to the routinely used mitogen PHA prompted modification to the protocol for assessing AEC-mediated modulation in the present chapter. The failure of PHA to cause significant CD4+ T-cell expansion at a concentration which effectively increased PBMC numbers indicates that accessory cell populations (i.e. macrophages, dendritic cells) may be required for appropriate CD4+ stimulation in the presence of this mitogen. Indeed it has been reported that CD4+ T-cells fail to express IL-2 receptor (CD25) or respond appropriately to IL-2 in the absence of cells involved in antigen presentation and as a result, expansion of T-cell population is blunted

(Halvorsen et al., 1988, Leivestad et al., 1988). Hence it was necessary when conducting studies with purified CD4+ and CD8+ populations to use a stimulus which would provide the required antigen presentation and which also has relevance in terms of clinical application. Immuno-magnetic anti-CD3 anti-CD28 coated beads are employed for *in vitro* T-cell expansion prior to infusion in immunotherapy (Thompson et al., 2003). Commercially available expansion beads seek to mimic physiological activation of CD4+ and CD8+ T-cells by simulating in vivo T-cell proliferation in response to antigen-presenting cells. The system utilizes the two activation signals CD3 and CD28, bound to a three-dimensional bead similar in size to endogenous antigen-presenting cells. Thus this method provides appropriate T – cell activation without the need to re-introduce macrophages/dendritic cells which may themselves be directly or indirectly modulated by contact with human AEC.

Having made changes to the protocol it was possible to clearly demonstrate that CD4+ and CD8+ T-cell proliferation, similarly to unfractionated PBMC, was abrogated by co-culture with AEC. In contrast to the results with unsorted PBMC, we observed a relatively small increase in CD4+ and CD8+ T-cell numbers under basal conditions which could be indicative of a mild allogeneic response. However, as AEC are also a source of many trophic factors (Parolini and Caruso, 2011) it is also possible that the increase in cell numbers under resting conditions reflects the generalised cell-supportive characteristics of AEC/AEC-conditioned medium which may serve to promote survival of purified resting T-cells and reduce the basal rate of apoptosis.

The results of the initial mixed islet:lymphocyte study suggests that CD4+ and CD8+ cells are activated by islet allo-antigens. The subdued nature of their response to islet co-culture may be due to the decline of immunogenicity and depletion of carrier (host-derived) dendritic cells which occurs in human islets during prolonged culture, especially within the RCCS (Rutzky et al., 2002). Yet, elevated CD4+ and CD8+ T-cell numbers were observed during T-cell:islet co-culture but this increase was abrogated in the islet:AEC constructs as demonstrated in Chapter 4.

There is a general consensus, based on studies in experimental islet transplantation that both CD4+ and CD8+ T-cells are involved islet allograft rejection with initial infiltration of antigen-presenting cells, such as macrophages and DC mediating the response. The release of cytokines notably IL-2 and IL-6 trigger lymphocyte activation and the migration of CD4+ and CD8+ T-cells to the site of the graft. Additional pro-inflammatory cytokines secreted by CD4+ T-cells prompt the invasion of NK cells and B lymphocytes which on activation generate complement fixing antibodies. CD8+ cytotoxic cells cause further β-cell damage by the release of chemicals granzymes and perforins which induce cell lysis. The allogeneic response is exacerbated by pro-inflammatory cytokines released by activated Th1 type CD4+ T-cells which secrete IL-2, interferon-γ, and TNF-α which maximise CD8+ activation.

To summarise, in combination the immunoregulatory properties of AEC have the capacity to influence several aspects of immune-mediated destruction of the islet graft. Firstly they may directly induce apoptosis of CD4+ and CD8+ T-cells by the actions of soluble FasL and HLA-G. Whilst the Fas/FasL pathway is normally associated with CD8+ mediated cell destruction,

and has the propensity to activate neutrophil-mediated inflammation (Turvey et al., 2000) the presence of FasL bearing AEC may become dominant in a microenvironment in which the Th1/Th2 ratio is in favour of Th2 cells and therefore anti-inflammatory mediators predominate (Pearl-Yafe et al., 2006). The secretion of TGF-β and IL-10 by AEC may also serve to prevent the activation and proliferation of T effector cells and inhibit the production of pro-inflammatory cytokines. Additionally, AEC also secrete macrophage inhibitory factor, reducing the magnitude of immune cell migration and therefore graft infiltration (Li et al., 2005)

CHAPTER 6: FUTURE WORK, GENERAL DISCUSSION AND CONCLUSIONS

Diabetes mellitus is a metabolic disorder resulting in hyperglycaemia and the onset of secondary complications, which are in turn associated with significant morbidity and mortality. The pharmacological approach to its treatment (insulin, oral hypoglycaemics) serves to manage the condition, but is not a cure and does not provide full protection from the potentially life-threatening consequences of the disease. The definitive therapeutic strategy for T1DM is the re-instatement of endogenous insulin production to restore physiological glucose control. Both whole organ *viz.* pancreas and islet cell transplantation offer the recipient the possibility to achieve and maintain euglycaemia and since the early 2000s and the introduction of the Edmonton protocol, islet transplantation outcomes have improved significantly where 1- year insulin-independence rates have reached 70% to 90% in experienced transplant centres (Shapiro et al., 2006). Yet whilst early results are good, current protocols are still associated with a progressive and steady decrease in graft function; up to 90% of recipients return to insulin therapy within 5 years of their first infusion (Ryan et al., 2005).

As discussed in Chapter 1, the underlying cause of islet graft attrition is likely to be multifactorial, involving recurrence of autoimmunity, allogeneic rejection, toxicity of the immunosuppressive drug regimen, poor graft re-vascularisation and “metabolic fatigue” of the islet graft. Determining the precise cause of graft failure in each clinical case is restricted by the limitations in technology for monitoring transplanted islets although much research is

now concerned with graft imaging and surveillance for signs of immunologic events in the peripheral blood (Berney and Toso, 2006). The impact of immune-mediated β-cell loss is, however, likely to be significant, with studies indicating an association between pre-transplant cellular auto-reactivity and poor clinical outcome (Huurman et al., 2008). Furthermore, in case studies graft failure is demonstrated to be preceded by the detection of antibodies to HLA class II antigens (Kessler et al., 2009).

Immunosuppressive drugs fall short of producing long term graft protection and additionally, expose the recipient to toxicity, increased vulnerability to infection and heightened susceptibility to malignancy. These risks are factored into the pre-operative evaluation/assessment of potential islet transplant recipients, being balanced against the potential benefits of the procedure in terms of reducing insulin requirement, providing good glucose control and limiting the frequency of hypoglycaemic events by restoring hypoglycaemic awareness. Such considerations invariably result in only the most poorly controlled diabetic individuals being selected for transplant, and then only after intensive insulin therapies have been explored, including the use of insulin pumps. If islet transplantation is to be made more readily available, becoming a routine treatment option for diabetes, it is of paramount importance to develop more long term, effective and safe methods of islet graft immuno-protection.

This thesis seeks to provide preliminary evidence in support of the clinical exploitation of immune-privileged tissue to locally control the recipient immune response in cellular transplantation. The unique features of the islet graft *viz.* discrete cell clusters transplanted

in a relatively small volume means that it may be possible to avoid chronic, systemic immunosuppression by pre-transplant modification of the graft combined with manipulation of the implantation site. Human amniotic epithelial cells (AEC) are chosen as a potential candidate for this role due to certain key characteristics, notably their immune inertness on transplantation and ability to markedly reduce T-cell proliferation as demonstrated in this study (Chapter 3) and by others (Parolini et al., 2008). The present study is the first to seek to capitalise on these features of AEC in the context of islet transplantation and to provide direct (*in vitro*) evidence that AEC are capable of modulating the immunogenicity of human islets to the T-cells directly implicated in graft rejection (Chapter 4&5).

Complete characterisation of the mechanisms underlying the immunosuppressive potential of AEC will form the basis of future work. Our current understanding of the properties of AEC will determine which potential mechanisms merit closer investigation. Special attention should be paid to the possibility that the unique repertoire of soluble mediators secreted by AEC create graft tolerance either by a direct cytotoxic effect on CD4+ and CD8+ T-cells (potentially via soluble HLA-G) (Pratama et al., 2011, Hammer et al., 1997) or more interestingly, by the induction of regulatory T-cells (T-regs). Increasingly, regulatory T-cells ($CD4^+/CD25^+$/Foxp3 regulatory T-cells) are being implicated in the suppression of allogeneic graft rejection and the development of immune-tolerance (Wood, 2011, Wood et al., 2003). In the periphery T-regs are derived from naïve $CD4^+$ cells and are thought to arise as a consequence of appropriate exposure to antigen and co-stimulation in the presence of cytokines which induce expression of Foxp3, a gene which, in turn, orchestrates T-reg expansion. AEC secrete two cytokines considered to be implicated in the induction of Foxp3

namely transforming growth factor beta (TGF-β) and IL-10 (Manuelpillai et al., 2010b, Pothoven et al., 2010, Chung et al., 2009, Li et al., 2005) and are therefore capable, at least in theory, of creating a local environment conducive to the expansion of T-reg populations. In the context of islet transplantation AEC could induce the expression of alloantigen-specific T-regs able to counteract the deleterious effects of CD4+ and CD8+ T-cells and therefore prevent graft rejection and mediate link unresponsiveness. To test this theory, future studies using T-cell proliferation assays would be conducted to detect changes in the levels of Foxp3 expression, by flow cytometry, in CD4+ T-cells cultured in the presence or absence of dispersed AEC and AEC supernatant, and also with islet:AEC constructs. Subsequent studies would attempt to correlate changes in Foxp3 expression to the prevailing levels of TGF-β and IL-10 in the assay supernatant, and further validate their role by addition of TGF-β and IL-10 antibodies to block Foxp3 induction.

Additional potential targets for AEC- mediated immunosuppression include restriction of dendritic cell maturation (Li et al., 2006b) and inhibition of T-cell proliferation by indoleamine 2,3 dioxygenase (IDO) via tryptophan depletion (Jones et al., 2007) which may also be explored as part of ongoing studies.

The islet:AEC constructs bioengineered in the present study exhibit both insulin-secretory and immunomodulatory capabilities (Chapters 4&5). These two characteristics make islet:AEC constructs ideal for use in cell replacement therapy for the treatment of diabetes. In future work an assessment of their ability to survive and perform under experimental, physiological conditions must be undertaken using animal models, thus providing “proof-of-

concept". A positive outcome would provide the necessary rationale for studies in higher non-human primates prior to transferring the technology to a clinical setting.

The series of in vivo studies would include assessment of construct survival in both immune-deficient and immune-competent murine models using the renal sub-capsular space as the implant site and detection of circulating human C-peptide and insulin as indicators of graft function. Additional studies in diabetic murine models (streptozotocin-treated and non obese diabetic – NOD) will confirm the ability of the islet:AEC constructs to restore and maintain normoglycaemia in the absence of chronic immunosuppression. Appropriate histological examination of explanted tissue i.e. haematoxylin and eosinophil staining, immunocytochemical localisation of human insulin, glucagon, E-cadherin, and infiltration of murine CD4+, CD8+ and Foxp3 positive cells will further define the degree of graft survival and increase our understanding of the mechanism(s) underlying graft immuno-protection.

It is envisaged that the clinical use of modified islets whether by co-aggregation with human AEC, bone marrow-derived mesenchymal stem cells or other immunomodulatory cell populations would ultimately operate in conjunction with other novel strategies of graft immuno-protection. It is feasible to suggest that during the initial transplant period additional methods of immune protection would be required, giving the AEC time to establish and exert influence at the site of implantation. The putative mechanism(s) underlying AEC-mediated immunosuppression may well be compatible with some of the newer methods of graft protection currently being explored, including co-stimulation blockade (notably the CD28/CD80/86 and CD40/CD154 pathways) and the use of anti-CD3 or

T-cell depleting antibodies (Bellin et al., 2012). Their use as induction therapy in conjunction with islet:AEC co-grafting may circumvent the need for the use of calcineurin inhibitors (tacrolimus) and other more conventional immunosuppressive agents (sirolimus) and therefore eliminate their attendant toxic actions both on the graft and the recipient.

Whilst islet transplantation continues to be defined as a research or experimental treatment it delivers real improvements both to the health status and quality of life of graft recipients. Unquestionably, there is a need to refine the technique, reducing the risk:benefit ratio and improving long term clinical outcome if islet transplantation is to become a serious rival to exogenous insulin therapy. The work detailed in the present thesis provides a rationale to examine novel cell-based strategies for islet graft immune protection, which may in time, contribute to a more effective means of ensuring sustained islet graft survival.

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APPENDIX

Publications arising from this work

Cell Transplantation, Vol. 20, pp. 523–534, 2011

0963-6897/11 $90.00 + .00
DOI: 10.3727/096368910X528111
E-ISSN 1555-3892
www.cognizantcommunication.com

Human Amniotic Epithelial Cells Induce Localized Cell-Mediated Immune Privilege In Vitro: Implications for Pancreatic Islet Transplantation

Khalid M. Qureshi,* Robert J. Oliver,* Michelle B. Paget,*
Hilary E. Murray,* Clifford J. Bailey,† and Richard Downing*

*The Islet Research Laboratory, Worcester Clinical Research Unit, Worcestershire Acute Hospitals NHS Trust, Worcester, UK
†School of Life and Health Sciences, Aston University, Birmingham, UK

Chronic systemic immunosuppression in cell replacement therapy restricts its clinical application. This study sought to explore the potential of cell-based immune modulation as an alternative to immunosuppressive drug therapy in the context of pancreatic islet transplantation. Human amniotic epithelial cells (AEC) possess innate anti-inflammatory and immunosuppressive properties that were utilized to create localized immune privilege in an in vitro islet cell culture system. Cellular constructs composed of human islets and AEC (islet/AEC) were bioengineered under defined rotational cell culture conditions. Insulin secretory capacity was validated by glucose challenge and immunomodulatory potential characterized using a peripheral blood lymphocyte (PBL) proliferation assay. Results were compared to control constructs composed of islets or AEC cultured alone. Studies employing AEC-conditioned medium examined the role of soluble factors, and fluorescence immunocytochemistry was used to identify putative mediators of the immunosuppressive response in isolated AEC monocultures. Sustained, physiologically appropriate insulin secretion was observed in both islets and islet/AEC constructs. Activation of resting PBL proliferation occurred on exposure to human islets alone but this response was significantly ($p < 0.05$) attenuated by the presence of AEC and AEC-conditioned medium. Mitogen (phytohaemagglutinin, 5 μg/ml)-induced PBL proliferation was sustained on contact with isolated islets but abrogated by AEC, conditioned medium, and the islet/AEC constructs. Immunocytochemical analysis of AEC monocultures identified a subpopulation of cells that expressed the proapoptosis protein Fas ligand. This study demonstrates that human islet/AEC constructs exhibit localized immunosuppressive properties with no impairment of β-cell function. The data suggest that transplanted islets may benefit from the immune privilege status conferred on them as a consequence of their close proximity to human AEC. Such an approach may reduce the need for chronic systemic immunosuppression, thus making islet transplantation a more attractive treatment option for the management of insulin-dependent diabetes.

Key words: Human islets; Human amniotic epithelial cells; Immune privilege; Rotational cell culture system; Peripheral blood lymphocytes; Immunosuppression; Insulin; Fas ligand

INTRODUCTION

Islet transplantation offers a more physiological approach to the restoration of glucose homeostasis than exogenous insulin therapy (8,30), but its use is restricted to a discrete population of individuals with type 1 diabetes who experience frequent and unpredictable episodes of hypoglycemia. More widespread application of islet transplantation awaits solution of technical limitations, in particular the requirement for chronic systemic immunosuppression, which poses risks both to the islet graft and its recipient (4,11,17,32).

Development of a safe, biocompatible method of islet immune isolation that circumvents the need for systemic immunosuppression has been the subject of extensive research. Macro- and microencapsulation devices have been the preferred option, resulting in limited clinical application (6,39), but loss of capsule integrity and impaired gaseous and nutrient exchange undermine long-term β-cell function. Nanocapsule devices, formed from layers of biocompatible polymer applied to the islet surface, address some of these limitations, but the process is technically involved and incurs significant loss of β-cell mass (35,40). Hence, a clinical role for encapsulated islet transplants must await improvements in capsule composition and biocompatibility.

Received November 17, 2009; final acceptance August 31, 2010. Online prepub date: September 30, 2010.
Address correspondence to Khalid M. Qureshi, The Islet Research Laboratory, Worcestershire Acute Hospitals NHS Trust, Worcester, WR5 1HN, UK. Tel: 01905 760 251; Fax: 01905 760 262; E-mail: khalidqureshi@doctors.org.uk

A more physiological approach to cellular immune evasion exploits the properties of cells with innate immunomodulatory capabilities involved in creating anatomical sites of immune privilege. Sertoli cells (SC) induce a state of immune neutrality in the testis to support resident germ cells and prevent rejection of allogeneic and xenogeneic intratesticular islet grafts. Furthermore, SCs may also confer immune privilege at anatomical sites that would otherwise be unable to sustain graft survival without systemic immunosuppression. When preengrafted to the renal capsule of chemically induced diabetic mice, SCs enhance subsequent islet allograft survival (20); examples of xenograft protection at ectopic sites in large mammals have also been documented (16). Intriguingly, such studies suggest that complete encapsulation of islets by SC is not a prerequisite to prevent rejection; nonetheless, obtaining sufficient numbers of SC for use in human transplantation would pose logistical challenges.

Human fetal membranes may provide an alternative source of immunoregulatory cells, readily obtainable in large numbers without ethical constraints. Amniotic membrane possesses anti-inflammatory and immunesuppressing properties that underlie its clinical use in the treatment of wounds, burns, and in ophthalmic surgery where it is grafted without rejection (2,9,14,33). Human amniotic epithelial cells (AEC) isolated from the membrane suppress T-cell activation in both mixed and mitogen-induced lymphocyte proliferation assays (24,41): AEC are amenable to both allogeneic and xenogeneic engraftment in immune-competent recipients (1,22). The expression of potential mediators of immune suppression, including HLA-G, Fas ligand and TGF-β have been identified in human AEC (13,23,24), which may serve to inhibit immune cell functions to create a microenvironment conducive to allogeneic graft survival.

In the present study we examined the potential of human AEC to modify the immune response to isolated human islets. Specifically, we sought to test the hypothesis that the presence of AEC in close proximity to human islets alters the immediate microenvironment sufficiently to induce a localized immunosuppressive response on invading peripheral blood lymphocytes. A rotational cell culture system (RCCS) (26,27) was employed to bioengineer novel cellular constructs composed of islets and AEC (islet/AEC), the functional and immunological characteristics of which were then investigated under in vitro conditions.

MATERIALS AND METHODS

Human Islet Isolation

Pancreases from multiorgan donors were supplied by the UK Human Tissue Bank (De Montfort University, Leicester, UK) with the appropriate consent and local Research Ethics Committee approval. A total of seven pancreases (five female, two male; mean age 47.1 ± 3.3 years) were used, with an average cold ischemic time of 10.04 ± 1.25 h. Organs were dissociated by a combination of enzymatic digestion (Liberase HI, Roche Diagnostics, West Sussex, UK) and mechanical agitation, and islets separated from the resulting pancreatic digest using density gradient centrifugation on Ficoll columns as previously described (26,27). Staining with dithizone (500 μg/ml, Sigma Aldrich Ltd, Dorset, UK) was used to assist islet counting and conversion to islet equivalents (IEQ) (28), while trypan blue (0.4% v/v) exclusion confirmed islet cell viability. The islet preparations were seeded at a density of 750–1000 IEQ/ml in Medium 199 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml amphotericin B (Sigma Aldrich Ltd) supplemented with 10% fetal calf serum (First Link Ltd, Birmingham, UK), and maintained in nonadherent culture (27) for a period of 24 h to allow acclimatization.

Human Amniotic Epithelial Cell (AEC) Isolation

Human amniotic membrane was obtained according to ethically approved protocol and with informed consent from 17 women (mean age, 32.5 ± 1.6 years) undergoing elective Caesarean section. Samples (10 × 10 cm) of amniotic tissue were separated from the chorion layer by blunt dissection (avoiding areas overlying the placenta). The tissue was rinsed three times in phosphate-buffered saline (PBS, Sigma Aldrich Ltd) containing 200 U/ml penicillin, 200 μg/ml streptomycin, and 20 μg/ml amphotericin B, and reduced to small pieces for digestion in 0.25% (w/v) trypsin in Hanks balanced salt solution (HBSS, Sigma-Aldrich Ltd) for 20 min at 37°C. The resulting tissue suspension was passed through a 500-μm mesh to retain larger pieces of amnion, which were subjected to three further incubation cycles with trypsin to liberate all available epithelial cells. Pooled fractions of cell suspension thus obtained were centrifuged at 400 × *g* for 5 min and the pellets resuspended in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich Ltd), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg amphotericin B. AEC were seeded at high density in T-75 flasks and cultured at 37°C, 5% CO_2, 95% O_2 in a humidified atmosphere for 48–72 h, to form a flattened confluent monolayer. In some instances cultures of AEC harvested at confluence by mild trypsinization (0.025% trypsin-EDTA in PBS, Sigma Aldrich Ltd) were resuspended in supplemented RPMI containing 10% DMSO and cryopreserved at −80°C for later analysis of immunomodulatory potential.

Peripheral Blood Lymphocyte (PBL) Isolation

Peripheral blood lymphocytes (PBL) were isolated from buffy coats obtained from nine healthy volunteers

through the National Blood Service (NBS, Birmingham, UK) with local research ethics approval. Briefly, buffy coat fractions were resuspended in an equal volume of HBSS and layered onto 12 ml of Histopaque-177 (Sigma-Aldrich Ltd), centrifuged at $700 \times g$ for 30 min (with no brake), and the resulting leucocyte layer harvested using a sterile pastette. The isolated PBLs were washed three times in HBSS, centrifuged at $500 \times g$ for 10 min, resuspended in RPMI-1640 (supplemented as described above), and cultured in uncoated plastic petri dishes at 37°C, 5% CO_2, 95% O_2 overnight. A portion of the isolated PBLs was incubated with the mitogen phytohemagglutinin (PHA, 5 μg/ml, Sigma Aldrich Ltd) for 24 h prior to use in proliferation assays.

Islet/AEC Coculture: Conventional Static Culture Versus a Rotational Cell Culture System (RCCS)

For coculture studies islet suspensions were adjusted to a density of 500–1000 IEQ/ml and placed under either conventional static culture (CSC) conditions in 90-mm culture plates (NHS Logistics, Alfreton, UK) or in a rotational cell culture system (RCCS) in high aspect ratio vessels (HARVs, Cellon Ltd, Bereldange, Luxembourg) as previously described (27). The cultures were maintained at 30°C in a humidified atmosphere of 95% O_2/5% CO_2. Once confluent the AEC monolayers were disrupted by mild enzymatic digestion (0.025% trypsin-EDTA in PBS, Sigma Aldrich Ltd) and the resulting cell suspension washed in PBS and introduced to the islet cultures (both CSC and RCCS) at a final density of 1×10^5 cells/ml. The islet/AEC cocultures were maintained under conditions as described above for 72 h. Control cultures consisted of islets seeded at equal density (CSC and RCCS) in the absence of AEC.

Morphological Analysis of AEC Monocultures and Islet/AEC Cocultures Using Fluorescence Immunocytochemistry

For immunocytochemistry isolated AEC and islet/AEC cocultures maintained for 72 h either under CSC conditions or within the RCCS were anchored to glass coverslips and fixed with 4% paraformaldehyde for 30 min at room temperature (RT). Three 10-min washes in PBS were followed by antigen retrieval (0.3% Triton X-100, Sigma Aldrich Ltd) and blocking (10% normal goat serum in PBS for CK19, vimentin, FasL; 10% normal rabbit serum in PBS for insulin, Vector Laboratories Ltd, Peterborough, UK). The AEC or islet/AEC constructs were then incubated with primary antibodies, anti-human cytokeratin 19 (CK19), anti-human vimentin (Dako UK Ltd, Cambridgeshire, UK; 1:100), anti-human insulin (AbD Serotec, Oxford, UK; 1:10), or anti-human Fas Ligand (FasL, CD95L, Sigma Aldrich Ltd, 1:10) for 1 h at RT and at 4°C overnight. Secondary antibody (goat anti-mouse IgG-FITC for CK19, vimentin, and FasL, goat anti-rabbit IgG-TRITC for insulin; Cambridge Biosciences, Cambridge, UK, 1:100) was applied for 3 h at RT. The coverslips were rinsed and mounted in fluorescence mounting medium (Dako UK Ltd) before cell imaging using a Zeiss Axioskop 40 fluorescence microscope equipped with an AxioCam MRc color camera and incorporating Axiovision imaging software (Carl Zeiss, Hertfordshire, UK). Controls involved omission of the relevant primary antibody.

Insulin Secretory Capacity: Static Glucose Challenge

Cultures of islets or cocultures consisting of islets and AEC maintained either under CSC conditions or within the RCCS as described above were assessed for preserved glucose responsiveness. The impact of culture condition on islet function was determined by measuring insulin release in response to glucose under basal conditions viz. in the presence of 1.67 mmol/L glucose in modified HEPES-buffered HBSS comprised of (mmol/L): HEPES (9.9); NaCl (113.2); $NaHCO_3$ (4.1); Na_2HPO_4 (0.33); KCl (5.36); $CaCl_2$ (0.95); $MgSO_4.7H_2O$ (0.8); KH_2PO_4 (0.44), containing 0.2% BSA, pH 7.4, at 37°C, and subsequent to stimulation with high glucose (16.7mmol/L) or a combination of 16.7 mmol/L glucose and 10 mmol/L theophylline according to methods previously described (26,27). The secretory capacity demonstrated by islets maintained under CSC conditions was compared with that seen in islets held within the RCCS and to islets in coculture with AEC under both culture conditions. Response to glucose stimulation was quantified by measurement of insulin in the incubation medium using a commercial ELISA (Diagenics Ltd, Milton Keynes, UK) and expressed as a ratio of insulin secretion under basal conditions (stimulation index, SI). The islets were assessed for insulin secretory capacity at 24 h postisolation and at 72 h after the initiation of the islet/AEC cocultures (viz. 5–7 days postisolation).

Immunomodulation: PBL Proliferation Assay

AEC Monocultures. Confluent monolayers of AEC were dispersed and transferred to 24-well plates at a final density of 5×10^4 cells/well in supplemented RPMI as described above. The cells were allowed to attach and flatten prior to the initiation of PBL proliferation assays. Resting or PHA-activated PBLs were added to each well at equal density (5×10^4/well) for coincubation at 37°C, 5% CO_2, 95% O_2. Activated PBLs in contact with AEC continued to be cultured in the presence of 5 μg/ml PHA. After 72 h the PBLs were harvested, solubilized (VialightPlus, cell lysis reagent, Lonza Ltd, Wokingham, UK), and analyzed for ATP content using a commercial chemiluminescence assay (Lonza Ltd) according to the manufacturer's instructions. Concentration of

ATP per well, measured as relative light units (RLU), is directly proportional to cell number and thus indicative of the proliferative activity of PBLs in culture (34). Results were expressed as a percentage of control (i.e., resting PBLs incubated in the absence of AEC). In selected experiments cryopreserved AEC were rapidly thawed, rinsed in PBS, and seeded as described for the fresh AEC prior to their use in PBL assays as detailed above.

AEC-Conditioned Medium. In a separate set of experiments confluent monolayers of AEC were dispersed and replated in T75 flasks in supplemented RPMI medium as described above. The flasks were left for 72 h without a medium change to allow concentration of putative soluble factors released by the AEC. The resulting AEC-conditioned medium was harvested and centrifuged at $1300 \times g$ to ensure removal of all cells and cellular debris prior to use in PBL proliferation assays. Conditioned medium (0.5 ml) was dispensed to the appropriate wells of a 24-well plate and 5×10^4 resting or PHA-activated PBLs were added, adjusting the total volume to 1.0 ml using standard RPMI medium. Plates were incubated at 37°C, 5% CO_2, 95% O_2. After 72 h the PBLs were harvested and processed as described above. Results were expressed as a percentage of control (i.e., resting PBLs incubated in the absence of AEC-conditioned medium).

Islet/AEC Cocultures. As the islets and islet/AEC cultures maintained within the RCCS demonstrated superior viability both with regard to morphology (islet/AEC integration) and insulin secretory capacity compared to those held under CSC conditions, these cultures were subjected to PBL proliferation studies. Cells were transferred from the HARVs to 24-well plates (50–100 IEQ or 50–100 islet/AEC aggregates/well). Following a 48-h period to allow attachment the islet or islet/AEC cultures were exposed to either resting or PHA-activated PBLs (5×10^4 cell per well) for a period of 72 h, after which time the PBLs were harvested and analyzed for ATP content as described above.

Statistical Analysis

Statistical differences between the culture conditions in response to insulin secretagogues were assessed by one-way analysis of variance (ANOVA) using islet monocultures maintained under CSC conditions as the control group. Significant differences in PBL proliferation in response to AEC, conditioned medium, islets or islet/AECs were determined using Mann-Whitney *U* and Tukey's multiple comparison tests (by Rank), with the response of resting PBLs serving as the control. In all comparisons a value of $p < 0.05$ was considered to be statistically significant. Statistical analysis was performed using SigmaStat software version 3.5 (Systat Software Inc, Chicago, IL, USA).

RESULTS

Morphological and Immunocytochemical Assessment of Human Islet and Amniotic Epithelial Cells Postisolation

The human islet isolation protocol employed in the present study resulted in the harvest of structurally intact islets, which were well cleaved from the surrounding exocrine tissue, as previously reported (26,27). The purity of the islet suspension following Ficoll-assisted separation ranged from 70% to 85%, with islets mostly sized between 100 and 500 μm. Trypan blue exclusion served as an indicator of preserved islet structural integrity.

AEC plated at high density in T75 flasks readily attached and flattened to form a monolayer (Fig. 1A); the vast majority of these cells stained positive for the epithelial cell marker cytokeratin 19 (Fig. 1B). A discreet subpopulation of cells also stained positive for the intermediate filament marker vimentin (Fig. 1C) and a significant number (~30%) expressed Fas ligand (Fig. 1D). Islets held in coculture with AEC under CSC conditions demonstrated a degree of cell association: AEC were overlying islets in some instances (Fig. 2) and more robust, tightly formed cellular constructs exhibiting good integration of the two cell types were achieved by 72-h coculture of islets and AEC within the RCCS (Fig. 3). The vast majority of islets within the RCCS became associated with AEC although, in most instances, the AEC did not form a complete layer.

Islet Secretory Function at 24 h Postisolation

Islets maintained under CSC conditions for a period of 24 h postisolation demonstrated functional viability as indicated by their response to a glucose challenge. Insulin secretion was consistently increased by 16.7 mM glucose (SI 2.63 ± 0.21) compared with basal release. This was further enhanced by the presence of 10 mM theophylline (SI 3.67 ± 0.34) (Fig. 4).

Impact of islet/AEC Coculture Condition on β-Cell Function

Following a 72-h period of culture islet and islet/AEC constructs were subjected to further glucose challenge studies. Preliminary experiments indicated that isolated AEC do not secrete insulin when maintained in either static or rotational culture (data not shown) and were therefore not assessed during this investigation. In the presence of elevated (16.7 mM) glucose, control islets held under CSC conditions throughout the period of

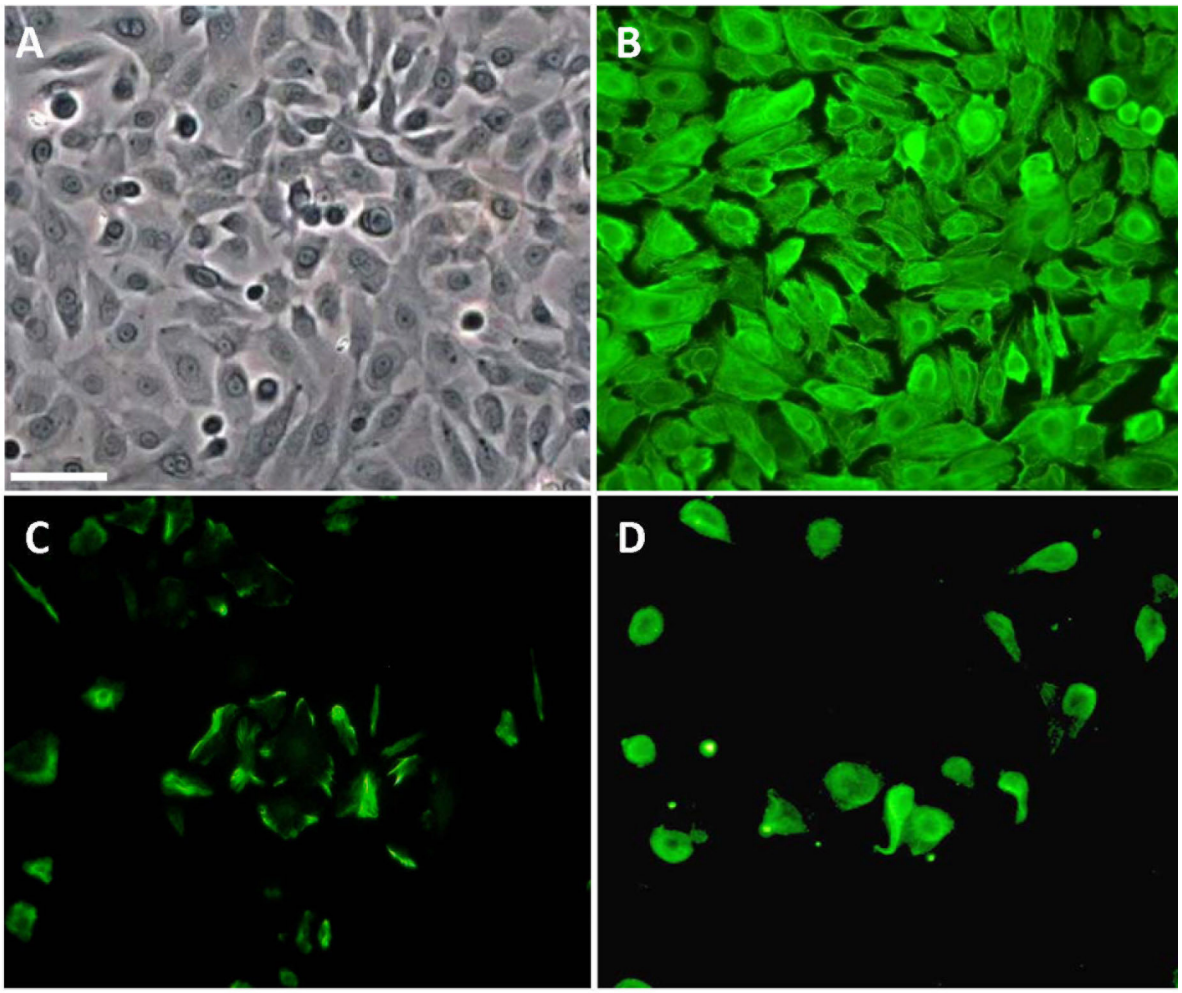

Figure 1. Morphological characteristics of human amniotic epithelial cells (AEC) in confluent monolayer culture. Phase contrast visualization of isolated human AEC (A). Immunocytochemical localisation of cytokeratin 19 (CK-19) (B), vimentin (C), and Fas Ligand (FasL) (D). Scale bar: 100 μm.

the investigation responded minimally in terms of insulin secretion (SI 1.24 ± 0.07), as previously noted (26, 27), although a combination of 16.7 mM glucose and 10 mM theophylline elicited more marked ($p < 0.05$) insulin secretion (SI 1.53 ± 0.1) (Fig. 5). By contrast, maintenance of islets within the RCCS preserved glucose responsiveness with significant insulin secretion occurring in response to 16.7 mM glucose both in the absence (SI 1.59 ± 0.08; $p < 0.05$) and the presence (SI 2.49 ± 0.28; $p < 0.01$) of the potentiator. Coculture of islets with AEC under both CSC conditions or within the RCCS had an apparently beneficial effect on β-cell function, with islets continuing to respond to glucose stimulation (SI 1.65 ± 0.12 and 2.89 ± 0.34 for islets under CSC condition in response to 16.7 mM glucose alone and 16.7 mM glucose plus 10 mM theophylline, respectively; SI 1.83 ± 0.11 and 3.15 ± 0.32 for islets maintained in the RCCS in response to 16.7 mM glucose alone and 16.7 mM glucose plus 10 mM theophylline, respectively) (Fig. 5).

PBL Proliferation: Influence of AEC and AEC-Conditioned Medium

PBLs taken from healthy volunteers demonstrated a six- to ninefold stimulation in the presence of 5 μg/ml PHA for a period of 72 h (Fig. 6A, B). Resting PBLs failed to respond on contact with an equal number of AEC or on exposure to AEC-conditioned medium over the same time period. The proliferation of PHA-activated lymphocytes was abrogated by coculture with AEC (Fig. 6A). A similar inhibition to PHA-mediated PBL proliferation was seen in AEC subjected to a period

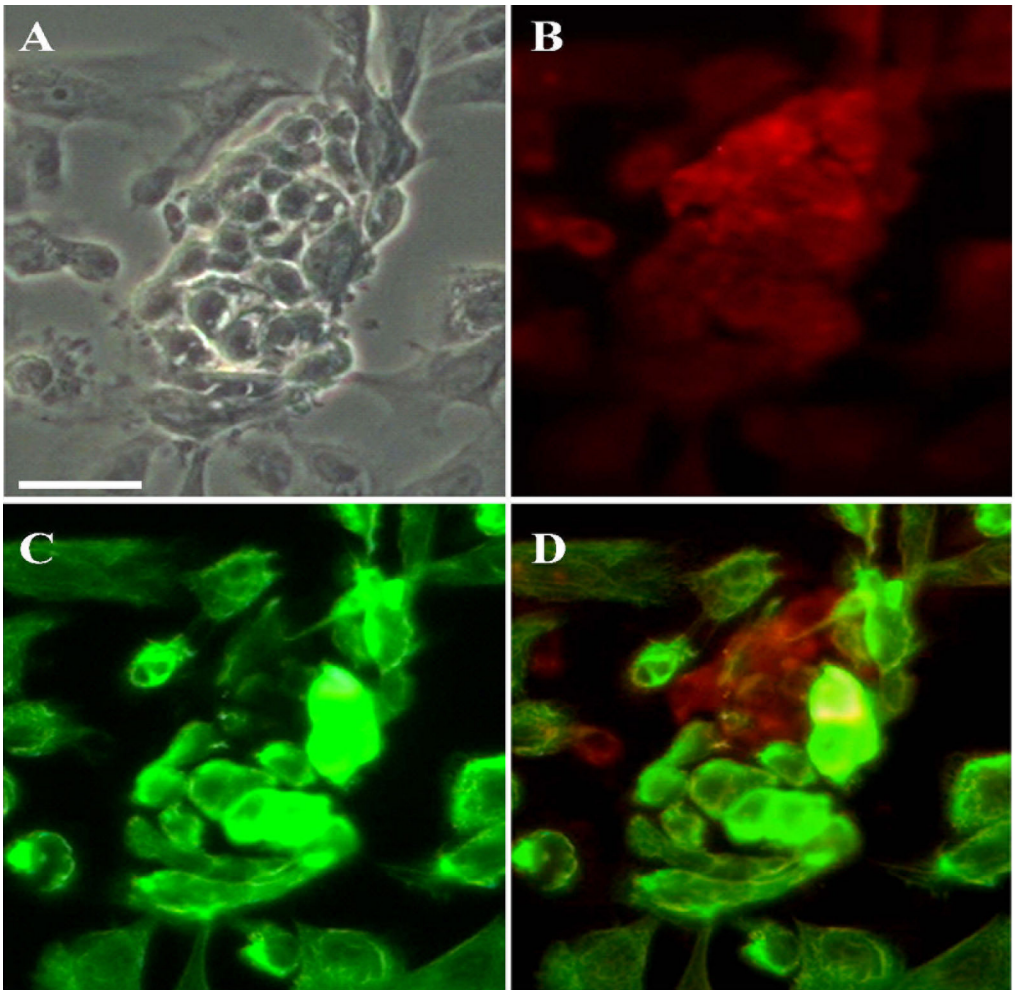

Figure 2. Morphological characteristics of human islet/AEC constructs formed by coculture under conventional static culture (CSC) conditions for 72 h. Phase contrast image of typical cell construct (A). Immunocytochemical localization of insulin (TRITC) (B) and CK19 (FITC) (C). Overlay image showing the spatial interaction of the two cell types (D). Scale bar: 50 μm.

of cryopreservation. Furthermore, AEC-conditioned medium had comparable immunosuppressive activity on PHA-activated PBLs (Fig. 6B).

PBL Proliferation: Islets Versus Islet/AEC

Exposure of resting PBL to unmodified human islets that were maintained within the RCCS elicited a marked ($p < 0.05$) proliferative response (Fig. 7A). By contrast, the presence of AEC attenuated resting PBL proliferation. PHA-stimulated PBL proliferation was sustained on contact with isolated islets, but was significantly ($p < 0.01$) suppressed when islets were in coculture with AEC (Fig. 7B).

DISCUSSION

This investigation has demonstrated that human amniotic epithelial cells possess innate immunoregulatory properties in vitro as indicated by their ability to suppress mitogen-induced lymphocyte proliferation, thus confirming previous studies (1,24,41). In addition, the outcome of the coculture studies suggests, for the first time, that the immunosuppressive properties of AEC may confer a state of immune privilege in otherwise immunogenic cells. These novel observations are relevant to the potential use of human AEC as an adjunct to cell replacement therapies, such as islet transplantation. Conceivably, the creation of a localized region of immunosuppression might reduce or obviate the obligatory requirement for chronic immunosuppressive therapy.

Notwithstanding their disparate origins, the coculture of human islets and AEC under either conventional static or rotational cell culture conditions resulted in successful physical interaction between the two cell types. As previously reported (27), the RCCS provided a more conducive environment for cellular aggregation, with the

formation of robust constructs exhibiting frequent spatial association of the insulin- and CK19-expressing cells and a preserved islet-like morphology. The high aspect ratio vessels (HARVs) are designed to create a microgravity environment with low shear forces permitting a greater degree of cell–cell interaction (38), which may underlie the efficient formation of stable islet/AEC constructs observed in the present study.

The close proximity of AEC to the human islets had no adverse effect on β-cell function. Indeed, the insulin secretion data indicate preservation of glucose sensitivity in human islets maintained in coculture with AEC. This may be compared with islets held alone under CSC conditions, which showed a diminution of glucose responsiveness. In previous studies we have demonstrated a beneficial impact of ductal epithelial cell coculture in preserving islet function (26), apparently due to their ability to provide trophic support to neighboring β-cells (29). AEC are also reported to synthesize and secrete a range of growth factors that may have relevance for the sustained functional viability of islets seen in the coculture model. Of note, mRNA expression of TGF-β, EGF, and KGF, known mediators of β-cell replication (7,12, 25) has been reported in intact human amniotic membrane and isolated amniotic epithelial cells (21). Furthermore, dissociated AEC secrete biologically active neurotrophins including BDNF (18), which have been linked to β-cell development and survival (31). It is thus likely that the close association of AEC to islets in this coculture model permits the paracrine release of soluble me-

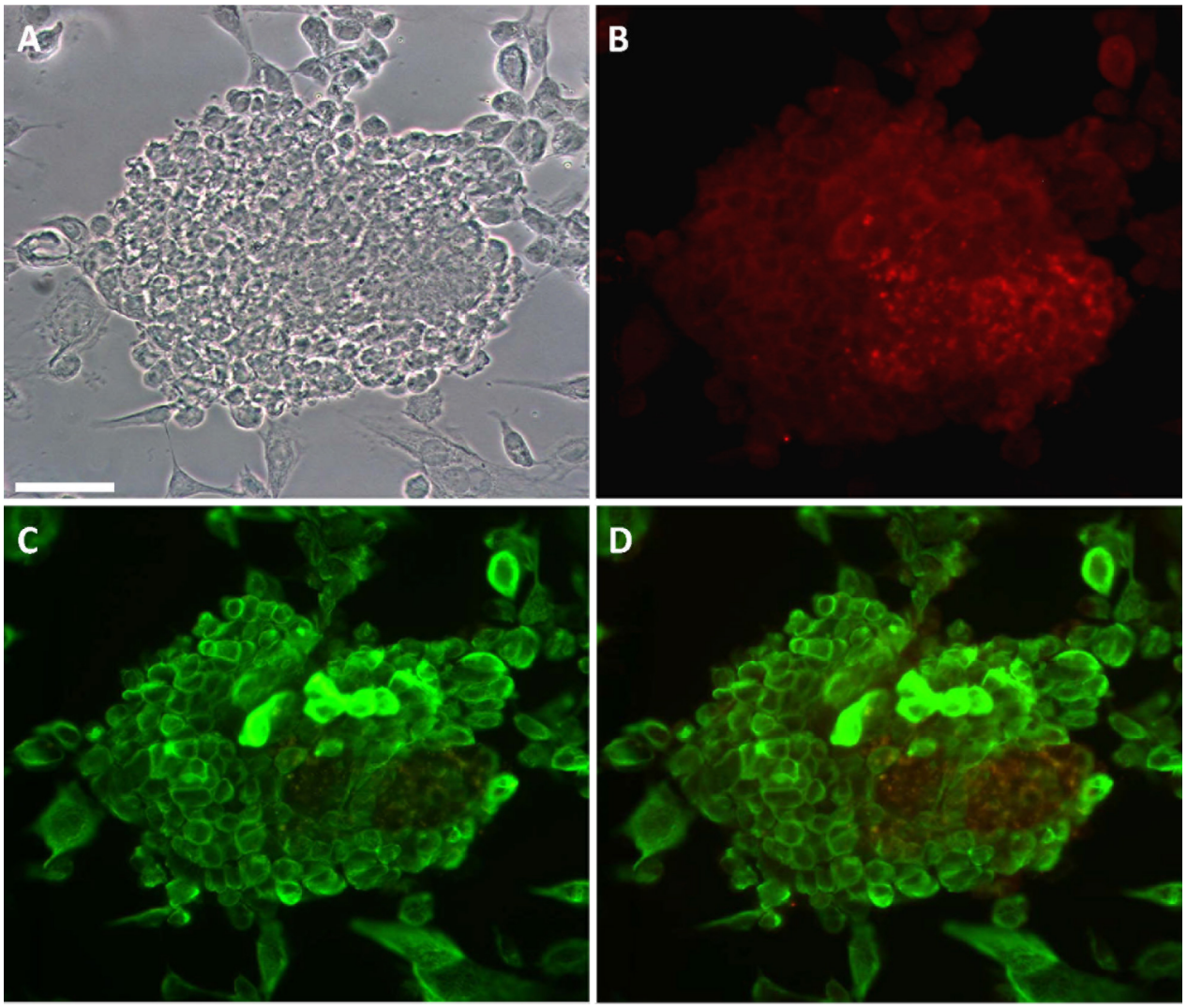

Figure 3. Morphological characteristics of human islet/AEC constructs formed in rotational cell culture (RCCS) over 72 h. Phase contrast image of typical cell construct (A). Immunocytochemical localisation of insulin (TRITC) (B) and CK19 (FITC) (C). Overlay image showing the spatial interaction of the two cell types (D). Scale bar: 100 μm.

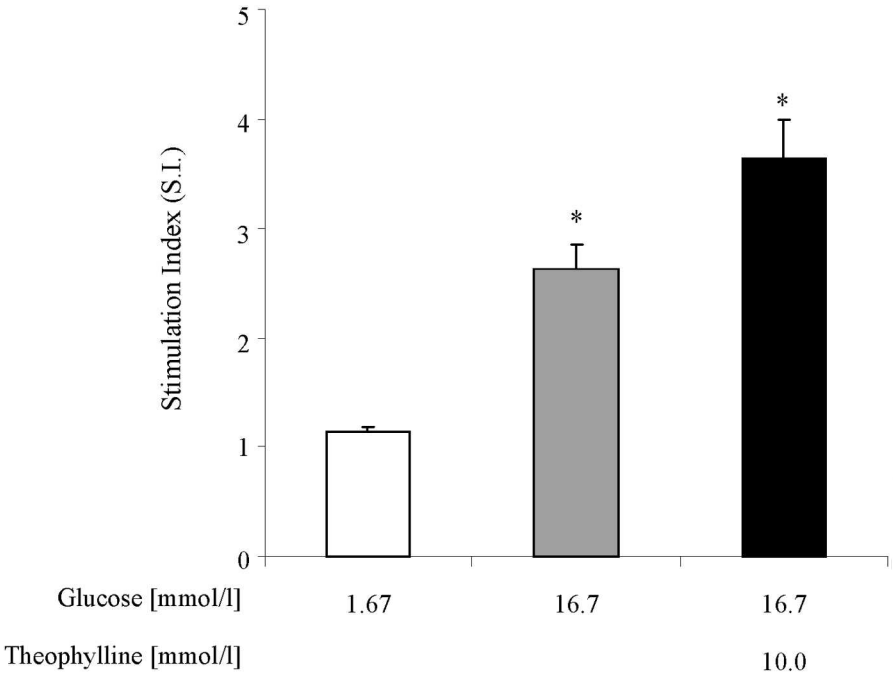

Figure 4. Insulin secretion from isolated human islets in response to nutrient stimulation during static challenge experiments performed 24 h postisolation. Islets were maintained under CSC conditions prior to assessment of secretory function. Insulin release was measured in response to 1.67 mmol/L glucose (basal release), 16.7 mmol/L glucose, and 16.7 mmol/L glucose plus 10 mmol/L theophylline. Results are expressed as the mean ± SEM fold increase in insulin release in response to nutrient stimulation relative to release under basal conditions. $n = 7$ independent islet preparations. The absolute mean value for insulin secretion under basal conditions was 86.5 ± 17.2 μUml^{-1} [20 islets]$^{-1}$ h^{-1}. *$p < 0.05$ versus basal conditions.

diators able to support insulin secretory capacity in the postisolation period with beneficial consequences for long-term β-cell function.

The immunomodulatory capabilities of human amniotic membrane have been studied extensively (15,22, 36). Our findings that isolated AEC abrogate mitogen-induced PBL proliferation confirm the results of previously published studies using comparable amnion-derived epithelial cell populations (24,41). Hence, our extrapolation that the immunosuppressive properties of isolated AEC could be manipulated to confer a state of immune privilege on other cells capable of provoking an immune response. The findings of the conditioned medium studies support those of others (15,24,37), and further suggest that AEC secrete immunomodulatory factors at concentrations sufficient to create a region of localized immunosuppression, with the potential to alter the immunogenicity of other cells in their immediate vicinity. Thus, in our mixed islet/lymphocyte reaction sustained proliferation of resting PBL was demonstrated in the presence of unmodified islets, yet those that were closely associated (cocultured) with AEC failed to elicit an allogeneic response. This effect was not dependent on complete encapsulation of the islets by the AEC, further indicative of a role for soluble immunoregulatory factors. Also, the immunomodulatory response to activated (PHA-stimulated) T cells was as robust in the islet/AEC cocultures as in AEC monocultures. Combined, these data suggest that AEC exhibit a potent and generalized immunosuppressive capability, inducing an antiproliferative response in T cells subjected both to specific and nonspecific antigen challenge.

Studies to identify the soluble factors involved in AEC-mediated immunosuppression and to characterize their T-cell targets are ongoing, yet initial immunocytochemical evidence indicates the potential involvement of Fas ligand (FasL), an immunomodulatory factor associated with naturally occurring T-cell evasion in the testis, eye, and brain (10). Localization of FasL within the placenta and amnio-chorionic membranes is implicated in maternal tolerance developed to the fetus during pregnancy (13,19). Thus, FasL in the AEC cultures raises the possibility of activated, FasL-mediated T-cell apoptosis. It is unlikely that a single mediator is responsible for immune adaptation and, indeed, other soluble factors have been identified within the AEC population, includ-

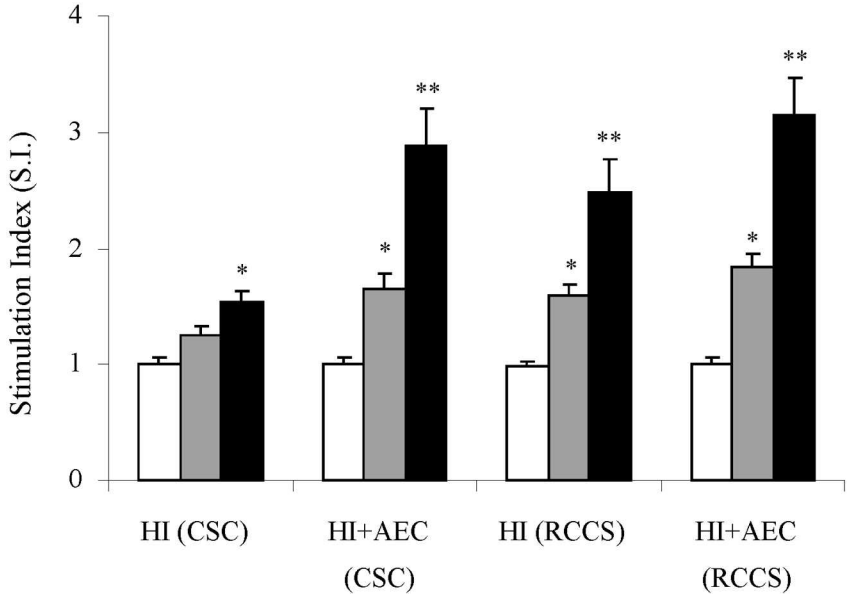

Figure 5. Glucose-stimulated insulin release from human islets (HI) maintained under conventional static culture (CSC) conditions or within the rotational cell culture system (RCCS) either in the presence or absence of human amniotic epithelial cells (AEC) for 72 h. Insulin release was measured in response to 1.67 mmol/L glucose (open bars), 16.7 mmol/L glucose (gray bars), and 16.7 mmol/L glucose plus 10 mmol/L theophylline (filled bars). Results are expressed as the ratio of stimulated insulin release compared to basal, mean ± SEM. $n = 4$. *$p < 0.05$, **$p < 0.01$ stimulated insulin secretion compared to basal release.

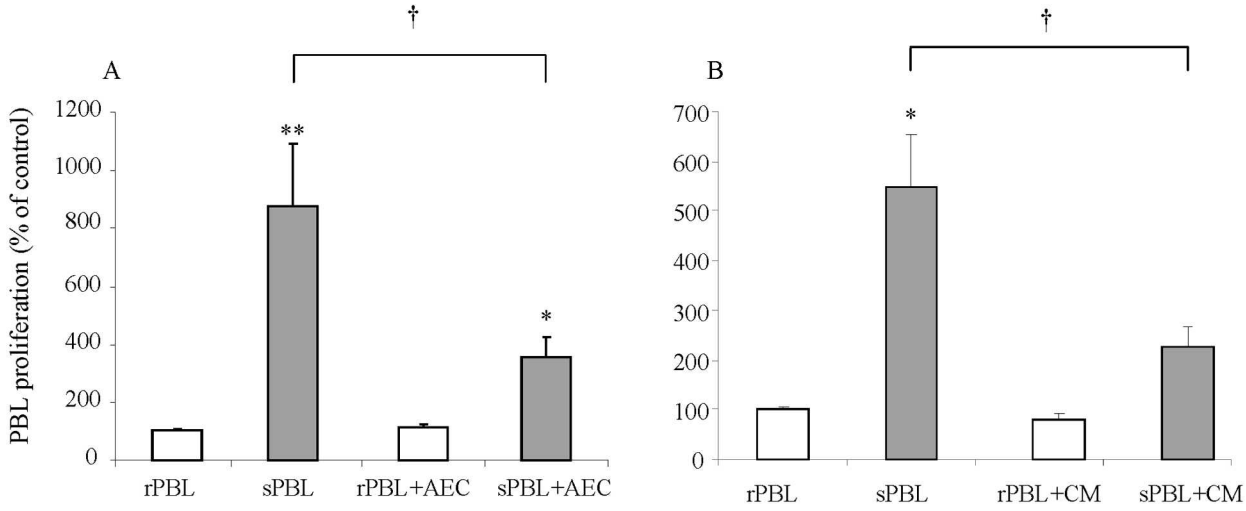

Figure 6. Modulation of peripheral blood lymphocyte (PBL) proliferation by (A) the presence of human amniotic epithelial cells (AEC), and (B) exposure to AEC-conditioned medium (CM). Resting (r; open bars) or PHA-activated (s, filled bars) human PBLs were maintained in 24-well plates either alone, in the presence of an equal number of human amniotic epithelial cells, or 0.5 ml of AEC-conditioned medium for a period of 72 h. The rate of PBL proliferation following this period was measured using an ATP chemiluminescence assay. Data show the percentage increase above control (resting PBLs) from six individual AEC preps and represents the typical observation in fresh and cryopreserved AEC. *$p < 0.05$, **$p < 0.01$ compared to control. †$p < 0.01$ for PHA-activated PBL proliferation in the presence or absence of AEC/AEC-conditioned medium.

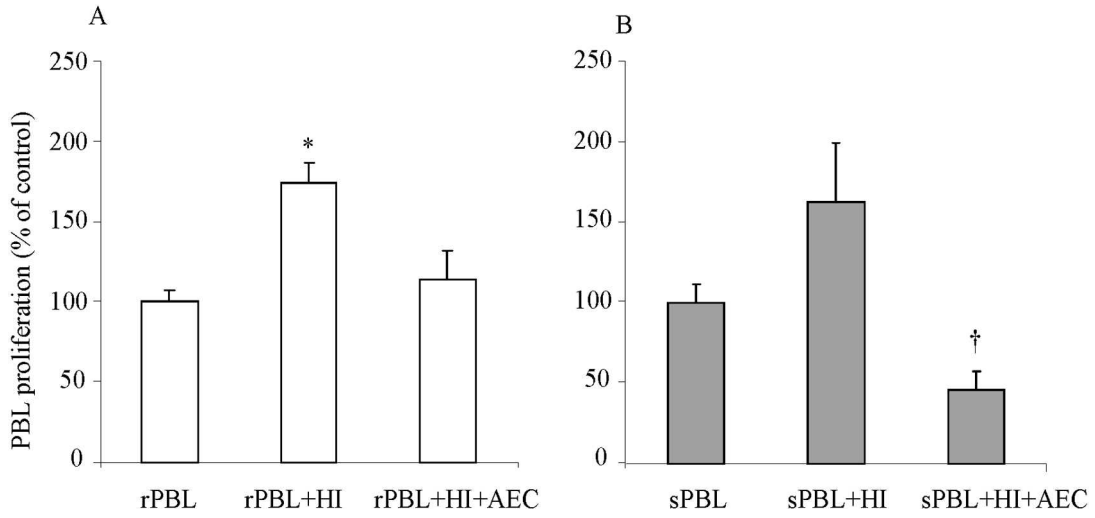

Figure 7. Modulation of peripheral blood lymphocyte (PBL) proliferation by exposure to human islets and human islet/AEC constructs. Resting (A) or PHA-activated (B) human PBLs were maintained in 24-well plates either alone or in the presence of human islets (HI) or islet/AEC constructs for a period of 72 h. The rate of PBL proliferation following this period was measured using an ATP chemiluminescence assay. Data depict the response from four individual human islet and AEC preps and represents the typical observation. $*p < 0.01$ compared to resting levels. $\dagger p < 0.01$ for PHA-activated PBL proliferation in the presence or absence of human islet/AEC constructs.

ing TGF-β, HLA-G, and IL-10 (23,24), all of which have the potential to affect localized immunosuppression. A number of these mediators could potentially operate in concert to produce a microenvironment capable of sustaining allogeneic tissue by diminution of the T-cell response.

The extrapolation of the immune-neutralizing properties of one cell type to modify the immunogenicity of a cotransplanted cell population has been demonstrated elsewhere. Notably, in the context of islet transplantation the use of Sertoli cells (SC) to create a local milieu conducive to long-term allograft and xenograft survival has been demonstrated experimentally and clinically (16,20,39) and more recently the use of mesenchymal stem cells (MSC) to regulate the immunogenicity of islet allografts has also been reported (5). We propose that immunoprotection could be achieved by the use of AEC, effectively bioengineering a state of immune privilege within the graft tissue promoting the localized release of soluble immunoregulatory mediators. While the widespread clinical use of human SC and MSC would pose certain technical challenges, human amnion is readily available and not subject to the same ethical constraints. Additionally, the present studies suggest that amnion provides an expandable pool of immunomodulatory cells, which are amenable to cryopreservation, readily integrate with isolated human islets, and do so without causing adverse alterations to β-cell viability, cellular transplant volumes, or nutrient exchange. Furthermore, the experimental and clinical use of amniotic membrane is well established (3,9,14,33) and successful engraftment of human AEC without evidence of tumorigenesis has been reported (2). Future work will seek to demonstrate that the immune-evasive properties of islet/AEC constructs are sustained in vivo and define how such a bioengineered approach to immune suppression could be adapted for clinical use.

ACKNOWLEDGMENTS: We gratefully acknowledge the financial support of The Sir Halley Stewart Trust, The James Tudor Foundation, The Rowlands Trust, The Eveson Charitable Trust, The South Warwickshire Diabetes UK Voluntary Group, and Worcestershire Acute Hospitals NHS Trust R&D.

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