A role for the human mesenchymal stem cell secretome in attenuation of cytokine-induced apoptosis in pancreatic beta cells

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Abstract

Diabetes is a lifelong condition caused by an inability of the body to break down glucose due to a defect in either insulin synthesis or the target cells becoming resistant to secreted insulin. There are two main types of diabetes, type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus. T2DM mainly occurs due to insulin resistant, inability of cells to respond to normal levels of insulin, the treatment of T2DM usually involves exercise, diet control, drugs and in some cases insulin is needed. In T1DM, the immune system starts to attack the β-cells, the cells responsible for insulin hormone synthesis and secretion from the endocrine pancreas. The aim of T1DM management is to restore carbohydrate metabolism as close to the normal condition as possible. To achieve this goal insulin hormone must be provided daily. Insulin is given in different ways such as injection (which is the most common method), pump and inhalation. Insulin sources can be either recombinant or animal-based. Treatment of T1DM is continuous and even with the best treatment options people with diabetes can develop serious complications such as acute diabetic coma or other long-term complications. These include diabetic cardiovascular disease, diabetic retinopathy, and diabetic nephropathy. Injection of insulin and daily monitoring of glucose levels presents a substantial burden to both the diabetic patient and also to associated dependents or carers i.e paediatric diabetes. So there is a strong need to develop an alternative treatment to reduce the burden, enhance control, and even ultimately cure diabetes mellitus.

A recent medical invention is the use of stem cells in the treatment of many disease conditions. Stem cells have a remarkable ability to develop into many different cell types. Stem cells therapy offers a new method for treating disease such as diabetes. The results of many studies demonstrate the capability of mesenchymal stem cells (MSCs) in the treatment of bone disease, cardiac disorder, and multiple sclerosis. However, much work remains to be done in the clinic and laboratory to optimise the use of these cells.

The main aim of this study was to explore the therapeutic effectiveness of MSCs conditioned media to restore the viability and function of β-cells.

In order to establish an *in vitro* model of cytokine driven β-cells apoptosis, pancreatic β-cells were treated with rising concentrations of pro-inflammatory cytokines TNF-α, IFN-γ and IL-1β and endotoxin LPS for 24 h. The optimal concentration of each cytokine or endotoxin was assessed by MTT assay. Optimal concentrations were deemed to be those that induced an approximate reduction in cell viability of 50%. The cells were treated with the optimal concentration and cell viability was monitored over time in addition to assessment of anti-apoptotic gene induction via qPCR assay. Mesenchymal stem cells (MSCs) secretome was collected as conditioned media and the β-cells cultured in non-conditioned and conditioned media during cytokine-driven apoptotic induction. β-cell viability and anti-apoptotic gene expression was determined to evaluate the therapeutic effectiveness of mesenchymal stem cells conditioned media (MSC-CM) in protecting β-cells from pro-inflammatory cytokines.

We observed a significant increase in the viability of pancreatic β-cell lines cultured in conditioned media when compared to those cultured in non-conditioned media. After that, we sought to identify a possible candidate that is present in the MSC-CM and help the cells to overcome the effect of pro-inflammatory cytokines. We found a high concentration of IL-10 in our conditioned media, in addition to the presence of IL-4, PIGF and VEGF in variable amount. Based on our present findings, cytokine-induced apoptosis is mediated through the TRAIL-dependent pathway. However, the addition of MSC-CM blocked cytokine-induced apoptosis and downregulated the genetic expression of A20 and TRAIL. Also, IL-10 was able to block IFN-γ and TNF-α-induced apoptosis.

Content

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Poster presentation

1- **TCES (Tissue and Cell Engineering Society) 2015:** Buthainah Al-Azzawi[1], Catriona Kelly[2], Nicholas R Forsyth[1]. Stem cell product-based blockage of cytokine-induced apoptosis: implications for diabetes.

2- Mesenchymal Stem Cell Meeting 2015: Buthainah Al-Azzawi[1], Catriona Kelly[2], Nicholas R Forsyth[1]. Mesenchymal Stem cell- derived product attenuate cytokines-induced apoptosis in pancreatic β-cell lines.

3- **Diabetes UK professional conference 2016**: Buthainah Al-Azzawi[1], Marwan M. Merkhan[1] **,**Catriona Kelly[2], Nicholas R Forsyth[1]. Mesenchymal Stem cell- derived product attenuate cytokines-induced apoptosis in pancreatic β-cell lines.

4- **ISTM postgraduate symposium 2016:** Buthainah Al-Azzawi[1], Catriona Kelly[2], Nicholas R Forsyth[1]. Mesenchymal stem cells conditioned media protect β-cells from TRAIL-induced apoptosis.

Abbreviations

AGE	Advanced Glycosylated End Product
ATP	Adenosine tri-phosphate
ADP	Adenosine di-phosphate
APC	Antigen presenting cells
AIF	Apoptosis-inducing factor
AP-1	Activating protein-1
β –cells	Beta –cells
CAD	Caspase activated DNase
CHOP	C/EBP homologous protein
COPD	Chronic obstructive pulmonary disease
CTL	Cytotoxic T-lymphocyte
DISC	Death-inducing signalling complex
DC	Dendritic cells
DM	Diabetes Mellitus
DIDMOAD	Diabetes insipidus, diabetes mellitus, optic atrophy and deafness
DKA	Diabetic ketoacidosis
ER	Endoplasmic reticulum
EDTA	Ethylenediaminetetra acetic acid
EMT	Epithelial Mesenchyme transition
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain
FACE	Flow cytometry
GLUT	Glucose transport protein
GAD	Glutamic acid decarboxylase
HLA	Human leukocyte antigen
HBSS	Hank's Balanced Salt Solution

HSCT	Haematopoietic stem cell transplantation
HIF-1α	Hypoxia –inducible factor alpha
HGF	Hepatocyte growth factor
ICA	Islets cell autoantibodies
ICAD	Inhibitor caspase activated DNase
IFN-γ	Interferon- gamma
IBMX	Isobutyl-1-methylxanthine
IDDM	Insulin dependent diabetes mellitus
IL-1β	Interleukin 1 beta
IL-7	Interleukin- 7
IL-15	Interleukin -15
IL-10	Interleukin-10
IL-1R1	Interleukin 1 receptor 1
IL-1R2	Interleukin 1 receptor 2
IRAK-1	IL-1 receptor-associated kinase 1
IRAK-4	IL-1 receptor-associated kinase 4
IKK	Inhibitor of NF-kB kinase
IKB	Inhibitor of k light chain gene enhancer B-cell
IRF-3	Interferon regulatory factor 3
IMS	Industrial methylated spirits
ISCT	Mesenchymal and Tissue Stem cell Committee of the International Society for Cellular Therapy
JNK	c-Jun-N-terminal kinase
LBP	LPS binding protein
MyD88	Myeloid differentiation primary response gene 88
MAPK	Mitogen-activated protein kinase
MKK	Mitogen kinase kinase

MSCs	Mesenchymal stem cells
MTT assay	(4,5-dimethylthiazol-2-yl)-2-5-diphnyltetrazolium
MSC-CM	Mesenchymal stem cell conditioned media
MD2	Lymphocyte antigen 96
mRNA	Messenger ribose nucleic acid
NK	Natural killer cells
NO	Nitric oxide
NEAA	Non-essential amino acid
NOD	Non-Obese Diabetic
NF-kB	Nuclear factor kappa-B
PKC	Protein kinase C
PKR	Protein kinase R
PCR	Polymerise chain reaction
PDS	Prospective diabetes study
PD-1	Programmed death 1 molecule
PP	Pancreatic polypeptide
RIP-1	Receptor-interacting protein 1
ROS	Reactive oxygen species
STZ	Streptozotocin
STAT	Signal transducer and activator of transcription
SODD	Silencer of death domain
TAK2	Transforming growth factor-activated kinase 2
TAB	TAK1 binding protein
TLR4	Toll-like receptor 4
TRAF-2	TNFR-associated factor 2
TRAF6	TNFR-associated factor 6

TRIF-3	Toll-interleukin receptor domain 3
TRADD	TNFR-associated death domain
TNF-α	Tumour necrosis factor-Alpha
T1DM	Type 1 diabetes mellitus
TGF-β	Transforming growth factor beta
TNFR1	Tumour necrosis factor receptor 1
TNFR2	Tumour necrosis factor receptor 2
Th1	T-helper 1 cells
Th2	T-helper 2 cells
UKPDS	UK prospective diabetes study
VEGF	Vascular endothelial growth factor
VNTR	Variable number of tandem repeats
ZNT8	Zinc transporter 8

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Chapter 1

Introduction

1.1 Endocrine Pancreas

The pancreas is a glandular organ, located in the digestive system with endocrine and exocrine functions. The adult human pancreas is a 6-10 inch elongated organ weighing 65 to 160 grams and lying in the abdominal cavity. It lies posterior to the stomach and anterior to the kidney. The head of the pancreas is on the right side of the abdomen and is connected to the duodenum through a small tube called the pancreatic duct. The narrow end of the pancreas called the tail, extends to the left side of the body.[(1)](Figure 1.1).

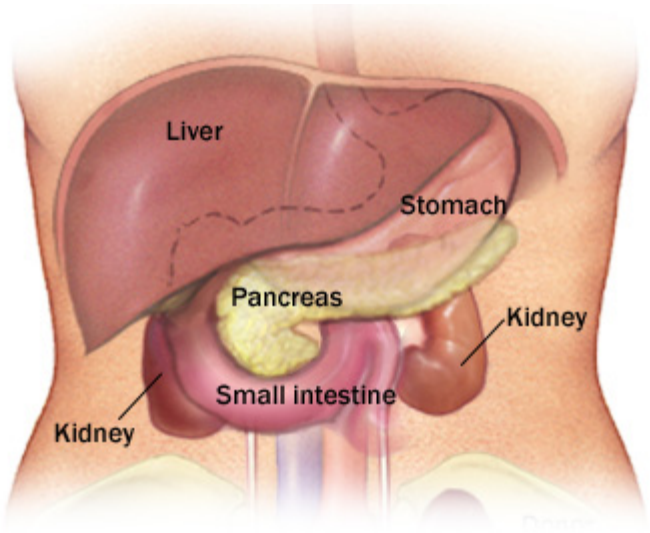

Figure 1.1 Pancreas position. Anatomical demonstration of pancreas position in relation to other organs. The image is reprinted from (http://www.hitachimed.com/self-learning-activity/docs/AbdominalImagingModule/images/figure-77.jpg).

The endocrine portion of the pancreas makes several hormones, which help to regulate the body`s metabolism. These hormones are made by specific cell types, which gather or cluster together to form small islands (islets) within the pancreas[2]. The islets are called islets of Langerhans and there are about one million islets scattered through the adult pancreas. The islets contain five different types of cells (alpha cells, β-cells, delta cells, F or PPcells and Epsilon cells) as shown in (Figure 1.2). The β-cells make up 65-

80% of the total population of islets cells, with alpha cells accounting for 15-20% and delta cells accounting for 3-10% of islets cells. F cells also known as pancreatic polypeptide cells (PP) make up 3-5% of all islets cells. The recently identified Epsilon cells make up less than 1% of all islet cells. The hormones made by these cells include.[(2)]:-

1- Insulin produced by β-cell -, which lowers blood glucose level.
2- Glucagon produced by alpha cell –, which raises blood glucose levels.
3- Somatostatin produced by delta cells –, which inhibits the release of insulin and glucagon.
4- Pancreatic Polypeptide produced by F or PP cells – the functions of pancreatic polypeptide remain largely unknown it has been suggested that it reduces appetite, in response to food intake.[(2)]
5- Ghrelin produced by Epsilon cells - again the function is not completely understood.

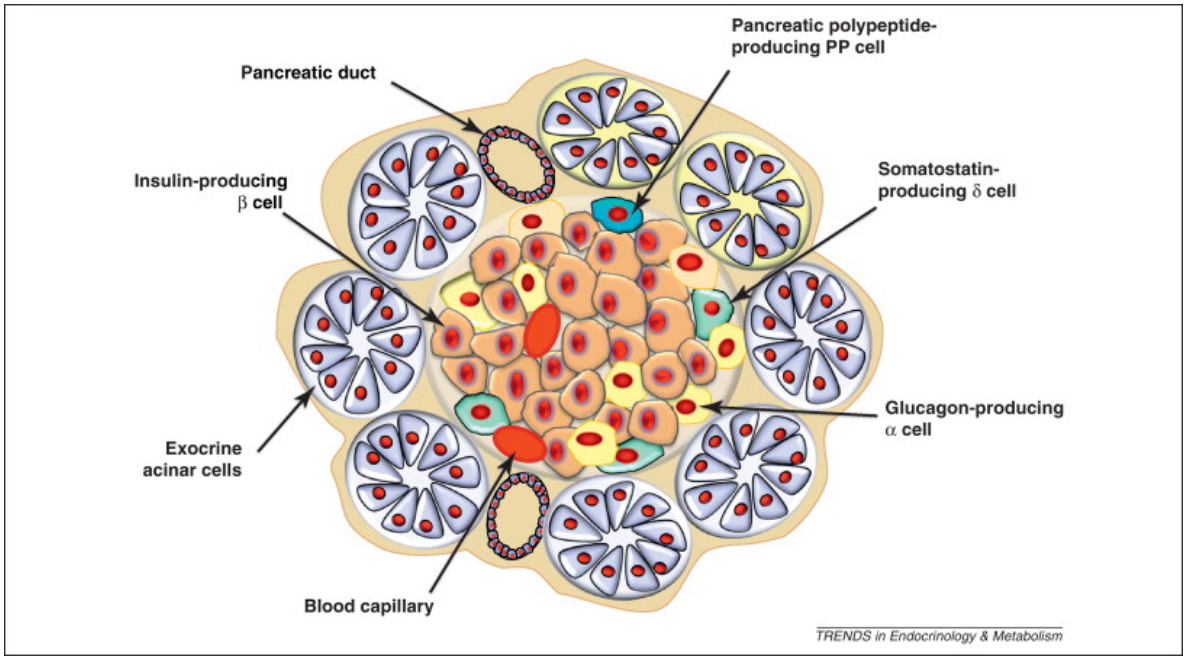

Figure 1.2 Diagram of pancreatic islets and surrounding accini. Diagram reprinted from (http://www.cell.com/trends/endocrinology metabolism/fulltext/S1043-2760(12)00049-5)

1.2 Insulin Hormone

Insulin is a small protein with an approximate molecular weight of 5.8 kilodaltons. It has two chains, which are held together by disulfide bonds. The amino acid sequence is nearly the same in all vertebrates, so the insulin in one mammal is biologically active in another. Insulin derived from animal sources like pigs and cows was widely used until the 1980s, when recombinant varities became commonplace.[3] β-cells in the pancreas are responsible for insulin secretion. Initially, the insulin is synthesized as a single chain precursor called preproinsulin, which is converted to proinsulin in the endoplasmic reticulum.[4] Proinsulin consists of three parts: a carboxy terminal A chain, an amino-terminal B-chain and C-peptide in the middle. Insulin inside the endoplasmic reticulum will undergo post-translational modification by several specific endopeptidases resulting in the removal of the C-peptide and generation of mature insulin. The C-peptide along with the mature insulin will be packed in the Golgi apparatus as secretary granules, which accumulate in the cytoplasm[4] as shown in (Figure 1.3). When the β-cells are stimulated, insulin is exocytosed with an equal volume of C-peptide and diffuses into the surrounding capillary blood network.[5]

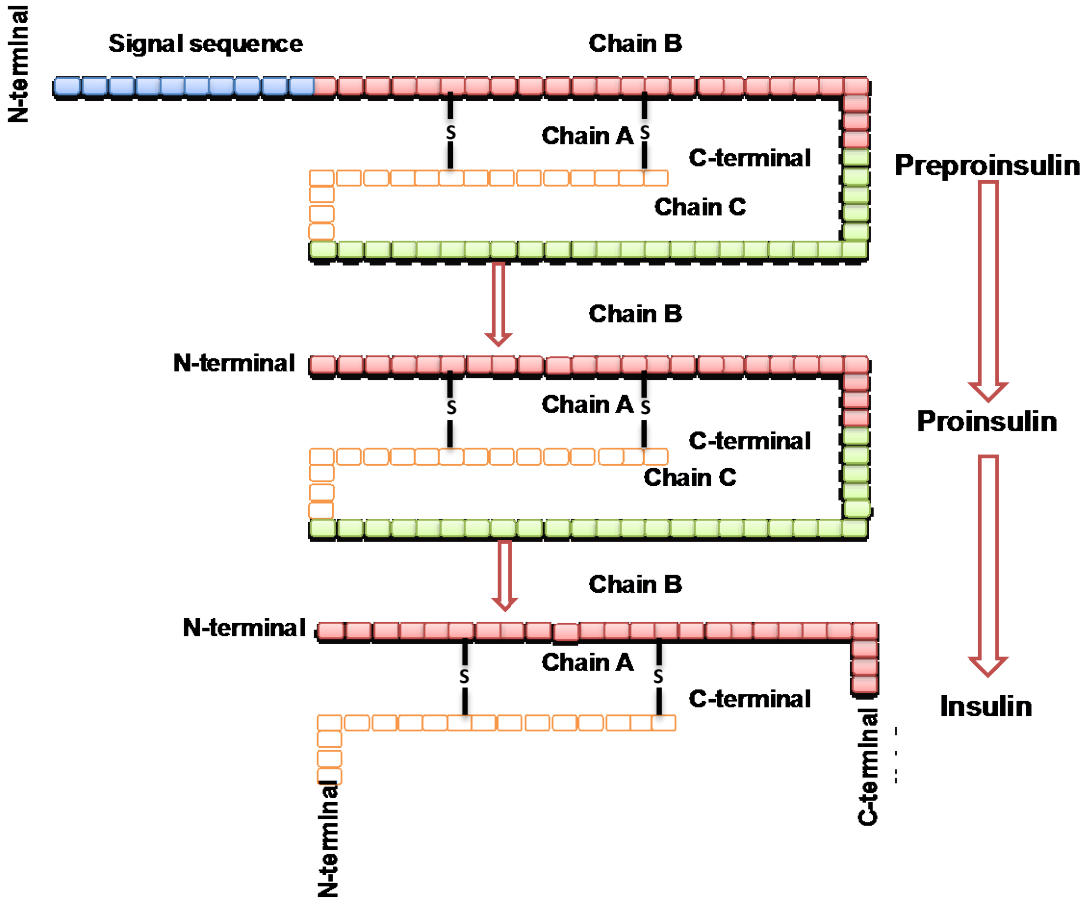

Figure 1.3 Post-translational modifications leading to the production of mature insulin. This diagram demonstrates the steps of insulin hormone synthesis starting from the polypeptide chain preproinsulin, proinsulin is formed by removal of signal peptide and the removal of C-chain lead to the formation of insulin.

1.3 Control of insulin secretion

The primary regulator of insulin secretion is glucose. However, other stimuli that may also cause insulin secretion include neurotransmitters, amino acids, and fatty acids.[6] The mechanisms behind insulin secretion are complex and not fully understood. However, the following process has been established[7]:-

When blood glucose concentration increases, glucose enters the cell by facilitated diffusion. This is mediated by glucose transporter proteins (GLUTs). There are 12 types of this GLUT transporter, with GLUT2 facilitating glucose entry into the β-cell of the pancreas. Glucose is then phosphorylated to glucose-6-phosphate by the action of the enzyme glucokinase, which is considered to be a glucosensor for β-cells. The β-cells then undergo membrane depolarization (mainly due to an alteration of the ATP:ADP ratio along with the closure of the potassium-sensitive K_{ATP} channel) and movement of extracellular calcium into the cell. This intracellular increase of calcium promotes exocytosis of the secretary granules that contain insulin hormone.[7]

1.4 Diabetes Mellitus

Diabetes Mellitus (DM) encompasses a group of autoimmune diseases characterized by hyperglycemia. It is characterized by an inability of the pancreas to produce sufficient quantities of the insulin hormone as a result of either a defect in insulin synthesis (total absence of the insulin hormone, insufficient quantity produced or poor quality of the produced insulin), or the target cells becoming resistant to the secreted insulin.[8] The body compensates for this elevation in glucose concentration by drawing water from intracellular to extracellular compartments in order to dilute the glucose concentration and excrete it in the urine. Thus, polyuria, polydipsia, and polyphagia are the most common clinical features in people with diabetes.[9]

The primary types of DM include type 1 DM (T1DM), which is characterized by β-cell destruction that ultimately leads to an absolute insulin requirement to prevent the development of ketoacidosis, coma, and death. It is also called insulin dependent diabetes mellitus (IDDM). The second type of DM is Type 2 DM, which results from

either a disturbance in insulin action or secretion. It is generally referred to as non-IDDM and can be treated with diet, exercise, and insulinotropic drugs.[10]

There is other less common subset of DM including: -

1- Gestational diabetes: develops in pregnant women that have never had diabetes before developing hyperglycemia during pregnancy. It affects 4% of pregnant women and many proceed to type 2 DM or occasionally T1DM. However, the vast majority of women return to normoglycemia postpartum.[11]
2- Maturity-onset diabetes of the young (MODY): characterized by a monogenic defect of β-cell function that leads to mild hyperglycemia in young people. This is generally genetic disorder inherited in an autosomal dominant manner.[12]
3- A genetic variation of DM: In these instances diabetes is secondary to systemic disorders involving multiple organ systems of the body e.g. Wolfram syndrome or DIDMOAD (diabetes insipidus, DM, optic atrophy and deafness) Frederic's ataxia, dystrophia myotonia and glycogen storage disease.[13]

1.4.1 Type 1 diabetes mellitus

T1DM is an autoimmune disease in which the immune system produces antibodies against β-cells of the pancreas.[14] Causative agents in T1DM include:

1.4.1.1 Genetic

T1DM is a polygenic disease with several genes contributing to its onset. Two *HLA* (human leukocyte antigen) genes located on chromosome 6 accounts for 40 – 50% of the inherited diabetic risk. The first gene that confers risk is called *DR* (cell surface antigen that mediates immune response). It has two forms (*DR3, DR4*), which are

found in about 95% of people with diabetes. The second *HLA* gene that confers risk for diabetes is *DQ* (a cell surface receptor type protein found on antigen presenting cells) and in particular, *DQA* and *DQB* are associated with the development of T1DM. Most people with diabetes tend to inherit one or more forms of both genes.[15]

Mutation in the insulin gene itself seems to affect the liability of the person to develop diabetes. The insulin gene has a short nucleotide sequence of DNA that is organized as a tandem repeat called the Variable Number of Tandem Repeat (VNTR) which varies in length.[15] The long VNTR regions comprise 140-200 DNA repeats while the smaller region has only 26-63 repeats. The probability of developing T1DM is fivefold higher in people that inherit two short VNTR compared in those inherit one long VNTR.[16]

1.4.1.2 Diet and environment

Several lines of evidence support the importance of exogenous factors in the pathogenesis of T1DM. Studies in identical twins indicate a 13-33% pairwise concordance for T1DM suggesting that there is either an inconsonant in the genetic information or the exposure to different environmental factors.[17] The geographic variations in the occurrence of T1DM in children are obvious even among Caucasians living in areas of Europe. In children under 15 years of age, the lowest incidence is reported in Macedonia and the highest incidence has been observed in Finland.[17] The differing rates are unlikely to be explained by genetics alone.[17]

A link between diet and the development of T1DM where cow's milk triggers an autoimmune response has been suggested but no connection has been confirmed between the antibodies against proteins in cow's milk and T1DM.[18] Researchers in

Northern Finland believe that there is an inverse relationship between vitamin D supplementation and the risk of developing T1DM later in life. They propose that the ingestion of 2000 IU of vitamin D every day in the first year of life help to prevent T1DM.[19] On the other hand, neglecting breastfeeding was associated with a risk of T1DM increase in Czech children.[20] Diet plays an important role in the development of type 2 DM with obese people at high risk of insulin resistance. Controlling of diet and exercise helps in the treatment of type 2 DM. However, the role of diet and exercise in T1DM is less well understood.

1.4.1.3 Virus

A theory proposed by Fairweather and Rose,[21] suggest that T1DM is caused by antibodies reacting against a viral infection, predominantly against Coxsackie and Rubella viruses. The theory depends on the fact that when there is an autoimmune response to viral invasion the antibody will attach to the infected cells along with β-cells. This cannot be applied to everybody since not everyone infected with these viruses will go on to develop T1DM.[21]

1.4.1.4 Chemical and drugs

In 1976, a rodenticide was introduced in the United States known as Pyrinuron (Vacor N-3-pyridylmethyl-N-p nitrophenyl urea). It was found later to be one of the causes of β-cell death when ingested either accidently or intentionally.[22] Zanosar (streptozotocin) is another example of a chemical that leads to β-cell destruction. It is an antineoplastic used in the treatment of pancreatic cancer but will also destroy the β-cells and lead to T1DM and is currently used to induce T1DM in animal models.[23]

1.4.2 Autoimmune destruction of β-cells

The pathophysiological mechanism for each risk factor can differ amongst individuals. However, common to all risk factors of T1DM is the resultant autoimmune response against β-cells, which involves β-cell autoantigens, B lymphocytes, T lymphocytes, macrophage and dendritic cells.[24]

1.4.2.1 Islet autoantibodies

The immune system has two essential parts, the innate and adaptive immune response based on the specificity and speed of the reaction. The innate response is the first line of defense of the human body against foreign antigens like bacteria or viruses and consists of monocytes, macrophages, neutrophils and positive acute phase proteins, which could help in destroying the microbes like c-reactive protein which binds to the surface of the bacteria and promote phagocytosis via macrophages.[25] Adaptive immunity is a secondary and more complicated immune response that consists of antigen-specific reaction via B and T lymphocytes. Adaptive immunity is a highly sophisticated process that starts within days or weeks of infection, resulting in immunological memory that allows the body to act rapidly in case of subsequent exposure.[26]

Despite being a highly organized system, the immune system occasionally fails to differentiate between self and non-self-resulting in an exaggerated antibody-mediated reaction. Under normal circumstances, the immune system has the ability to distinguish the body's own cells from foreign substances that pose a threat. Unfortunately, this regulated and efficient system can also turn on self-antigens causing destruction to a target tissue and development of autoimmune disorders such as T1DM. Autoantibodies against islets cell (ICAs) were first described over thirty years ago.[27]

A method used to identify autoantibodies specified for each autoantigen is the immunoprecipitation test, which is based on antigen-antibody reaction. For example, in children newly diagnosed with diabetes, the use of biochemical analysis of the purified immune complexes after standard immunoprecipitation tests was found to be helpful in the diagnosis of autoantigens recognized by immunoglobulin.[28] The first autoantigens discovered the presence of 64K protein, which was found later to have glutamic acid decarboxylase (GAD) activity and also to represent a previously unknown isoform, GAD65.[29]

Continued research has subsequently revealed the presence of the phosphatase related IA-2 molecule. It is co-precipitated with GAD65 in the sera of patients that contain 64K protein.[30] The most recent major islet autoantigens, zinc transporter 8(ZnT8) was discovered in 2007 by screening for highly expressed, islet β-cell specific molecules.[27] The three autoantigens can be labelled by iodination or polymerase chain reaction (PCR) making it easy for autoantibodies assays to identify biochemical antibodies.[31]

The ability to measure autoantibodies in T1DM using recombinant autoantigens opened the door for further identification of several different autoantigens detected by autoantibodies in other genetic disorders that involve the immune system.[32] This can help in the diagnosis of many autoimmune diseases before the appearance of clinical features. Further studies on T1DM revealed that not all autoantigens involved had been discovered; ICA reactivity does not always correlate with defined autoantigens suggesting the existence of other, unknown, forms of autoantigens.[32] Therefore, ICAs, autoantibodies against GAD65, insulin autoantibodies and autoantibodies against IA-2 can predict the appearance of T1DM even with normal blood glucose

concentrations.[33] However, it should be emphasized that despite increased risk, not everyone positive for ICAs progresses to T1DM. The time interval between the detection of autoantibodies and the development of frank clinical symptoms varies among individuals and can range from a few months to years.[32]

1.5 Management of T1DM

1.5.1 Insulin therapy

The management of T1DM requires replenishment of the absolute insulin requirement and restoration of glucose homeostasis. This is generally achieved through exogenous insulin therapy. For years the first line of treatment for T1DM has been an exogenous injection of porcine or recombinant insulin, and more recently, insulin pumps or inhalers have been developed. However, the clinical preparations of porcine or recombinant insulin differ from endogenous human insulin. Notably, C-peptide is absent in exogenously formed insulin.[34] Most of the insulin that are used today are produced from non-human sources although the clinical evidence conflicts as to whether this insulin is any less likely to produce allergic reactions. Adverse reactions have been reported including loss of warning signs, which may result in sufferers slipping into a coma through hypoglycaemia, convulsions, memory lapse and loss of concentration.[3]

A high degree of compliance and monitoring is required in order to achieve glycemic control and prevent many of the long-term complications associated with T1DM. Complications of diabetes can support the theory that inflammation is important in T1DM because it is usually associated with increases in inflammatory and oxidative

stress markers like C-reactive protein, nitrotyrosine and vascular cell adhesion molecule 1, which are increased in microvascular and cardiovascular complications.[35] The shortcomings of current diabetes therapies have promoted research into alternative therapies. Whole pancreas or islet transplantations are performed presently, but only for patients who meet strict guidelines and whose disease is poorly controlled and life-threatening.

1.5.2 Pancreas transplantation

The purpose of pancreas transplantation is to ameliorate T1DM and produce complete insulin independence. The first successful pancreas transplantation in conjunction with kidney transplantation was performed by W.D. Kelly and Richard Lillehei from the University of Minnesota in 1966. Until about 1990, the procedure was considered experimental now it is a widely accepted therapeutic modality. However, pancreas transplantation is recommended when diabetes is advanced and complications of the disease are having a serious impact on the patient's quality of life.[36] The pancreas transplant is usually performed along with a kidney transplant in patients with kidney failure. However, it is also possible to perform independent pancreas transplantation. It is a very complicated operation that carries many risks of complications. One in four patients needs further surgery in the first few days after transplantation to deal with problems such as infection and bleeding. In addition to the risk of rejection due to the immune response against the new organ the long-term use of immunosuppressant carries it is own risk of complications, such as increased vulnerability to infection and cancer.[37] One of the problems associated with pancreas transplantation is the liability of developing an increase in the blood glucose level especially if the patient is on prednisone treatment that results in the return of diabetes symptoms and the need of glucose management.[38]

1.5.3 Islet transplantation

Transplantation of isolated islets is an alternative to whole pancreas transplantation.[39] It is generally considered a safer and less invasive procedure and allows the exocrine portion of the recipient's pancreas to remain intact and functioning. The newly transplanted cells will produce insulin sufficient to maintain a normal level of blood glucose.[40]

The operation involves the implantation of islets into the liver of the patients. Immunosuppressant drugs are administered to prevent graft rejection. Follow-up of patients that undergo islet transplantation reveals that 75% of patients are insulin independent one year after the operation.[41] However, the long-term need for immunosuppressive therapy and shortage of donor islets hampered the widespread application of islet transplantations. In addition, a five year follow-up of patients revealed that only 10% remain insulin independent.[42]

The Edmonton Protocol is the most recent method that is used for islet transplantation. Shapiro and Lakey (University of Alberta, Edmonton) developed this protocol. Their procedure has some points of difference from its predecessors where the main difference is in the use of immunosuppressant drugs (glucocorticoid-free immunosuppressant regime) and the infusion of an adequate islet mass, obtained from multiple donors into each recipient.[43] There are risks associated with the use of immunosuppressive drugs like the development of lymphopenia, which is usually associated with an increase in serum levels of the homeostatic cytokines interleukin (IL-7) and IL-15, which cause *in vivo* expansion of CD8+T cells. Ultimately this will activate the immune response causing graft failure. Following the Edmonton

Protocol[44], nearly 80% of islet transplant recipients remained insulin independent for one year only and 10% of the recipients were insulin independent for five years post-transplant.[44]

1.6 Diabetic complications

1.6.1 Acute complications

Nearly all acute complications of diabetes are considered dangerous and represent a medical emergency. They are related directly to the glucose concentration in the blood and include diabetic ketoacidosis (DKA), which is caused by the breakdown of fatty acid into ketone bodies.[45] Under normal conditions this happens periodically when there is a need for urgent energy and the body cannot provide it by aerobic means. However, if this is sustained it will become a serious problem. Hypoglycemia is also an acute condition that is caused by several factors such as too much insulin, or incorrectly timed administrations of insulin, or poor management of the type and amount of food taken.[46] If these acute conditions are not treated immediately and properly it may result in diabetic coma and even death. Furthermore, several acute complications including respiratory infection can arise because of an altered immune response.[47]

1.6.2 Long-Term Complications

Long-term complications of T1DM develop gradually, over years. The development depends on earlier onset and poor glycemic control. Eventually, long-term complications may be disabling or even life-threatening. According to World Health Organization, about 80% of diabetic deaths occur due to complications especially in low and middle-income countries.[48]

1.6.2.1Cardiovascular disease

Diabetes dramatically increases the risk of various cardiovascular problems, including coronary artery disease with angina, heart attack, stroke, atherosclerosis and high blood pressure.[49] The central pathological mechanism in macrovascular disease is the process of atherosclerosis. Hyperglycaemia induces a large number of alterations at the cellular level, which leads to narrowing of arterial walls throughout the body. The mechanisms behind atherosclerotic processes involve the following:

a) Glycosylation of proteins and lipids by a nonenzymatic pathway (glucose reacts directly with proteins and lipoproteins in arterial walls) results in changes in molecular conformation, which can cause an alteration in enzymatic activity, reduction in degradative capacity and interfere with receptor recognition. Glycosylated proteins will react with receptors present in all cells specific for the atherosclerotic process like endothelial cells, smooth muscle cells, and monocyte-derived macrophages. This reaction results in oxidative stress and pro-inflammatory responses.[50]

b) Oxidative stress plays a major role in the development of diabetic complications. Cytokines enhance the production of free radicals, which form bonds with other compounds resulting in structural and functional changes in the tissues and cellular destruction. Therefore, the development of oxidative stress is an important step in the pathophysiology of cardiovascular complication.

c) High glucose concentrations activate Protein Kinase C (PKC) by increasing the formation of diacylglycerol, which is derived from the hydrolysis of phosphatidylinositides or from the metabolism of phosphatidylcholine. The elevation of diacylglycerol and subsequent activation of PKC in the vessels can persist for a long time. This subsequently causes an alteration in growth factor

expression, which is thought to lead to thickening of the capillary basement membrane.[51]

These mechanisms are interrelated. For example, hyperglycemia-induced oxidative stress promotes both the formation of advanced glycosylation end product and PKC activation[51].

1.6.2.2 Diabetic neuropathy

The changes in the capillary walls such as the thickening of basement membranes and endothelial hyperplasia will result in neural dysfunction.[52] Diabetic neuropathy is characterized by neural ischemia especially in the limbs where numbness and tingling start at the tips of the toes or fingers and radiates centrally. Diabetic neuropathy can be classified into peripheral neuropathy (characterized by loss of feeling in the upper and lower extremities); proximal neuropathy (causing pain in the thighs, hips or buttocks regions); and focal neuropathy (which occurs suddenly causing weakness in any nerve in the body).[53] The nerves of the gastrointestinal tract can also be affected by neuropathy and the patient will suffer from nausea, vomiting, and diarrhoea or constipation.[54]

1.6.2.3 Diabetic retinopathy

Diabetic retinopathy is the most common microvascular complication of diabetes. The risk of developing diabetic retinopathy or other microvascular complications of diabetes depends on both the duration and the severity of hyperglycaemia. The UK Prospective Diabetes Study (UKPDS) showed that most patients with T1DM develop evidence of retinopathy within 20 years of diagnosis.[55]

The development of diabetic retinopathy is multifactorial and involves several pathways including the polyol pathway, where glucose is converted to sorbitol via the action of Aldose reductase. Hyperglycaemia increases the flux of glucose molecules through the polyol pathway. Therefore, sorbitol will accumulate causing osmotic stress, which has been proposed as an underlying mechanism in the development of diabetic retinopathy.[56]

Another well-characterized pathway is damage resulting from the accumulation of advanced glycosylated end product (AGEs). Hyperglycaemia promotes the non-enzymatic formation of AGEs. Studies in animal models reveal the relationship between AGEs and the formation of microaneurysms and pericyte loss. After 26 weeks of induced hyperglycaemia, the retinal capillaries of diabetic rats have marked accumulation of AGEs. Furthermore diabetic rats treated with aminoguanidine (AGE formation inhibitor) have reduced AGE accumulation and reduced histological changes, including microaneurysm and pericyte loss.[57]

Free radical and reactive oxygen species are also involved in the development of diabetic retinopathy. The use of antioxidants like vitamin E may attenuate some vascular dysfunction as have been shown in animal studies but, the treatment with anti-oxidants has not yet been shown to alter the development or progression of retinopathy.[58]

1.6.2.4 Diabetic nephropathy

People with diabetes are at increased risk of developing renal disorders like urinary tract infection, papillary necrosis, and glomerular lesions. The early stages of diabetic nephropathy cause an elevated glomerular filtration rate with an enlarged kidney.

These changes can be caused by increasing mesangial cell matrix production and mesangial cell apoptosis in addition to increased glomerular basement membrane thickness and microaneurysm formation.[59] Chronic renal failure could be the end stage of diabetic nephropathy. Many of the mechanisms that are involved in the development of diabetic retinopathy are also involved in nephropathy.[60]

1.7 Cytokine-driven β-cell apoptosis

Cytokines are small signalling molecules that can be classified into peptides, proteins, and glycoproteins. They also have immunomodulatory properties. They differ significantly from classical hormones in that they circulate in much lower concentrations (picomolar range) and can be increased in response to trauma and infection.[61] In addition, cytokines are produced by specific tissues or cell types and not by specialized glands. Their mechanism of action is dependent on paracrine or autocrine signalling but not endocrine signalling.[62]

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Cytokines act by releasing a signal at the site of the infected cell (caused by different factors like pathogens and injury) to attract other immune molecules to the site. The effect of cytokines depends on the availability of the complementary receptor at the cell surface and the intracellular signalling produced by this complex, which leads to changes in cell functions either by upregulation of selected genes and their transcription factors or down-regulation of other genes.[63] Immune-mediated pancreatic β-cells loss is a hallmark of T1DM. T-cells produce pro-inflammatory cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), which are predominant in the early stages of the disease and, which are toxic to β-cells.[34]

1.7.1 IFN-γ

IFN-γ or type II interferon activates macrophages and induces major histocompatibility complex class II (MHC) expression.[64] IFN-γ is the only member of type II interferon family and is unrelated structurally, binds to a different receptor and is encoded by a separate chromosomal locus when compared with type I interferon. The source of IFN-γ was first thought to be exclusively from CD4+ T-helper cells (Th1), CD8+ cytotoxic lymphocyte, and natural killer cells (NK). However, it is now believed that other cells like B-cells and antigen presenting cells (APCs) are also able to produce IFN-γ.[65]

IFN-γ secretion is regulated by cytokines produced by APCs, most notably IL-18 and IL-12 as they act as a bridge to link infection with IFN-γ secretion in the innate immune response. During the inflammatory process, macrophage recognition of many pathogens results in the induction of IL-12 and chemokine production, which in turn attracts NK cells to the site of infection, and promotes IFN-γ secretion. In NK cells, macrophages and T-cells, the combination of IL-18 and IL-12 stimulate a further increase in IFN-γ production. IFN-γ secretion is reduced by interleukin-4 (IL-4), interleukin-10 (IL-10) and growth factors like TGF-β.[66]

The IFN-γ receptor is composed of two ligand-binding IFNGR1 and IFNGR2 chains, which belong to the class II cytokine receptor family. The binding of IFN-γ to its receptor results in the activation of Janus tyrosine kinase (JAK) 1 and signal transducer and activator of transcription (STAT)1. STAT1 translocates to the nucleus and controls the transcription of the target gene.[67] (Figure 1.4). The gene products activated by IFN-γ (also known as interferon-stimulated genes (ISGs)) are responsible for IFN-γ's antiviral, anti- proliferative and immunomodulatory properties.[68] The host defence gene protein kinase R (PKR) is one of these genes that is stimulated by IFN-γ and it is

thought that PKR is required for the activation of NF-κB. The latter is known to stimulate the transcription of genes that play important roles in apoptosis, immune modulation, and induction of inflammatory cytokines. Many IFN-γ-inducible genes are also TNF-α-inducible, and these genes are usually superinduced by a combination of these factors.[69]

1.7.2 TNF-α

Tumour necrosis factor alpha was first known as an endotoxin-induced glycoprotein, which caused hemorrhagic necrosis of sarcomas that had been inoculated into mice.[70] TNF-α has since been implicated in a wide range of infectious, inflammatory and malignant conditions.[71] TNF-α is produced by activated T-lymphocytes and macrophages and is usually not detected in healthy individuals it is only elevated in serum and tissues during an inflammatory or infectious diseases.[72] Although, the main source of TNF-α is macrophages/monocytes there are other cells that can also secrete TNF-α like mast cells, T, and B lymphocytes, NK cells and neutrophils.[73]

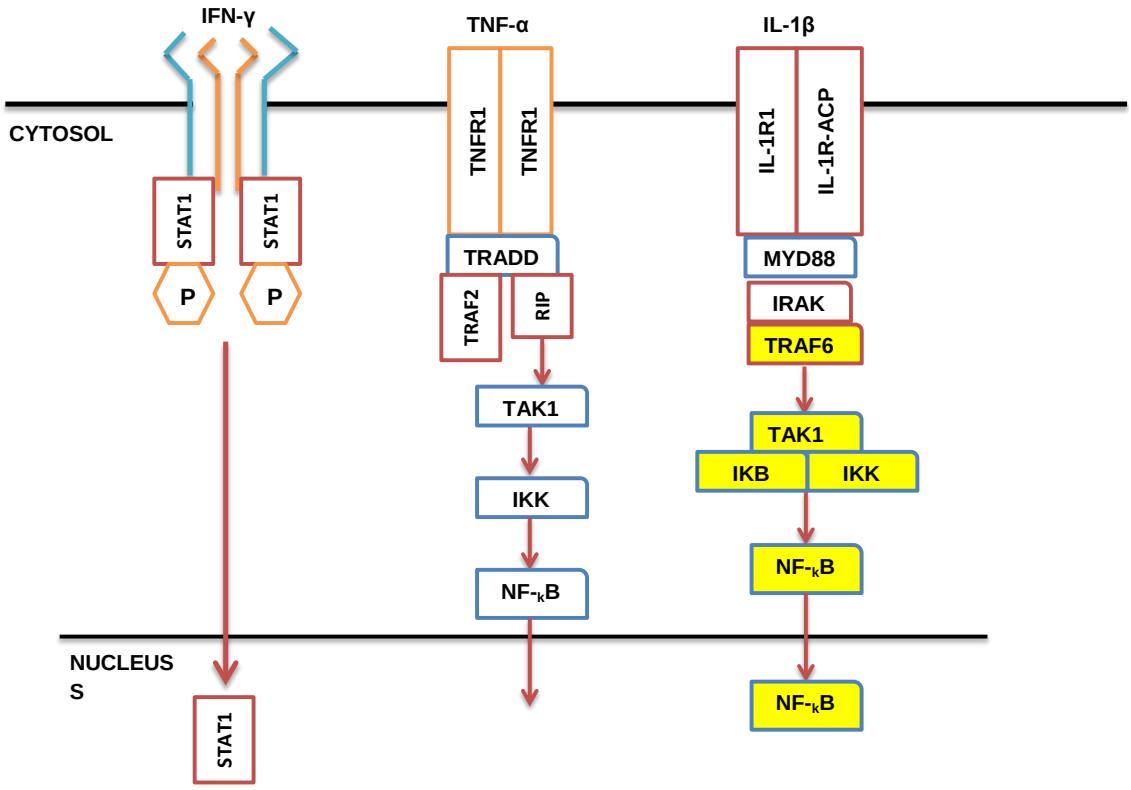

Figure 1.4 ***Pro-inflammatory signalling pathways activated by cytokines.*** This figure demonstrates the signal transduction pathways for IFN-γ, TNF-α, and IL-1β. IFN-γ phosphorylates and activates the STAT1, which will be translocated to the nucleus leading to transcription of several genes. TRADD and MYD88 are adapter proteins that mediate the action of TNF-α and IL-1β.

TNF-α signal transduction is complicated and not fully defined. The regulation of transcription factor NF-κB is a key component of TNF-α signal transduction. Responses to TNF-α are triggered by its binding to one of it is two receptors TNFR1 and TNFR2. These are tightly regulated on various cell types under normal and disease conditions.[74] Based on studies with receptor knockout mice and cell culture work, both the pro-inflammatory and apoptotic pathways that are stimulated by TNF-α are largely mediated via TNFR1. Signal transduction following activation of TNFR2 is less well characterized. However, TNFR2 has been shown to mediate signals that promote angiogenesis and tissue repair.[75]

TNFR1 is type I transmembrane receptor protein. In a resting state, TNFR1 is associated with a silencer of death domain (SODD), which is a cytoplasmic protein that is thought to prevent TNFR1 from signaling. However, when TNF-α binds to TNFR1 SODD is released. The TNFR1 will then activate TNFR-associated death domain protein (TRADD), which in turn will activate two additional proteins, the receptor interacting protein 1 (RIP-1) and TNFR-associated factor 2 (TRAF-2).[76] This complex (TRADD-RIP-1-TRAF-2) is released from TNFR1 inducing subsequent signaling events that involve the activation of different mitogen-activated protein kinase; e.g. MEKK-3 and TAK1, which activate the transcriptional factor NF-κB that results in NF-κB translocation to the nucleus and the initiation of gene transcription.[77] The complex can also activate the apoptotic signaling kinase-1 through activation of Janus kinase, which can phosphorylate and activate transcription factor activating protein-1 (AP-1).[78]

In addition to mediating apoptosis and pro-inflammatory responses via NF-κB and AP-1, TNFR1 can also stimulate cell death via binding to Fas-associated death domain protein (FADD). This involves the binding of TRADD and FADD and the subsequent activation of pro-caspase 8, which initiates apoptosis via activation and cleavage of pro-caspase 3 (Figure 1.4). The signaling pathways initiated by TNFR2 are less clearly defined, but it is thought that TNFR2 can signal by sharing and opposing effects of TNFR1.[79]

1.7.3 IL-1β

IL-1β is another cytokine that has an important role in the immune and inflammatory response. Cells activated by IL-1β display an inflamed phenotype and an upregulation of genes that encode proteins that directly participate in the immune response.[80]

These genes include cytokines, cytokines receptors, acute-phase reactants, growth factors extracellular matrix components and adhesion molecules.[81]. The IL-1β receptor system and associated signal transduction have been under intense investigation in the past 10 years, providing a remarkable improvement in our understanding of how IL-1β produces its effect. There are two types of IL-1 receptor (IL-1R1 and IL-1R2). IL-1R2 is a decoy cytokine receptor that inhibits the activity of it is ligands.[82] The binding of IL-1β to IL-1R1, with the help of co-receptor IL-1 receptor accessory protein (IL-1RAP), forms a trimeric complex. This complex will stimulate the myeloid differentiation primary response gene 88 (MyD88), which will trigger the phosphorylation of IL-1 receptor-associated kinase 4 (IRAK 4), which then phosphorylates IRAK 1. This in turn will activate TGF-β kinase 1 (TAK1) and TAK1 binding protein (TAB).[83] From this point, the signal can propagate through two main pathways: NF-κB and/or c-Jun N-terminal kinase (JNK). To mediate IL-1β action through the NF-κB pathway, the phosphorylated TAK1 activates the inhibitor of NF-κB kinase subunit (IKK), which in turn will phosphorylate the NF-κB inhibitor (IKB), which gets degraded so that NF-κB kinase is released and transferred to the nucleus.[84] TAK1 can also activate MAPK p38, JNK and extracellular signal-regulated kinase (ERK) through interacting with MAP kinase (MKK) proteins, resulting in the activation of several transcription factors including c-Jun, c-Fos, c-Myc and ATF2, which leads to cellular events such as gene expression or cell cycle.[85](Figure 1.4).

1.7.4 Bacterial Lipopolysaccharide (LPS)

Many studies describe a role for the endotoxin lipopolysaccharide (LPS) in the development of autoimmune disease such as T1DM.[86] The outer membrane of the gram-negative bacteria contains LPS, which makes them easily recognized by the immune cells, which will produce pro-inflammatory cytokines as a defence mechanism against this pathogen.[87] It is believed that LPS binding protein (LBP), which is a protein produced by the liver and, which circulates in the blood will be the first to recognize and bind to LPS and then form a temporary complex with CD14 mediating the transfer of LPS to the LPS receptor complex is composed of Toll-like receptor-4 (TLR4) and lymphocyte antigen 96 (MD2).[88] This is followed by the activation of several adaptor proteins such as myeloid differentiation primary response gene 88 (MyD88).[89] TLR4 signal transduction has been divided into MyD88-dependent and MyD88-independent pathways.[90] Several studies on MyD88-deficient macrophages proved the importance of this pathway in stimulation the expression of pro-inflammatory cytokines while the MyD88-independent pathway stimulates type I interferon and interferon-inducible genes.[91] The activation of MyD88 results in the activation of IL-1 receptor-associated kinase-4 (IRAK-4) and the knock-out of IRAK-4 in mice resulted in severely impaired production of pro-inflammatory cytokines after stimulation with LPS. [91]

TNF receptor-associated factor 6 (TRAF6) is another adaptor protein that is important to the IRAK-4 and IRAK-1 signalling pathway, TRAF6 will activate TAK1(transforming growth factor activated kinase-1), which will activate IKK and MAPK (mitogen-activate protein kinase) pathways.[92] The activation of IKK will phosphorylate and degraded IkB (inhibitor of k light chain gene enhancer in B-cell) and promote the translocation of NF-κB, which controls the expression of many pro-inflammatory cytokines.[93]

Toll-interleukin receptor domain-containing adaptor inducing IFN (TRIF) is an important adaptor protein that mediates the MyD88-independent pathway. Studies using TRIF-deficient macrophages have revealed the importance of this protein in the activation of transcription factor IRF3 and the late phase activation of NF-κB and MAPK.[94]

These effects of LPS are not fully understood in people with T1DM. LPS could mediate a reduction in insulin secretion and insulin gene expression through stimulation of inflammatory responses involving TLR4 and NF-κB which may contribute to β-cell dysfunction and death. This would lead to T1DM and is supported by the identification of the TLR4 expression in human and mouse islets.[95] In addition a study confirmed that toll-like receptors play an essential role in islet allograft rejection.[96] Unfortunately, our knowledge regarding the early development of the disease process are far from complete there is still a big question of how and why the circulating immune cells targeted the β-cells only and not the other cells present in islets (Figure 1.5).

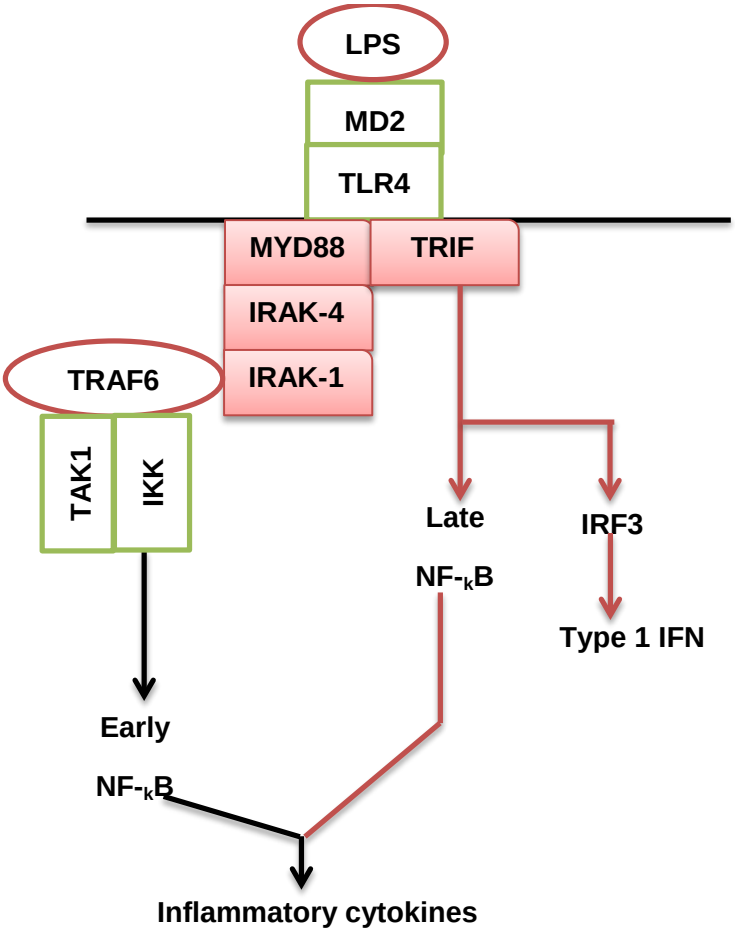

Figure 1.5 ***LPS signalling pathway.*** This figure outlines the MyD88-dependent and independent signal transduction pathways for LPS.

1.7.5 The role of pro-inflammatory cytokines in β-cell apoptosis

The exposure of β-cells to secreted pro-inflammatory cytokines results in an alteration of β-cell function and may lead to cell death. Although the reason is unclear, it is well established that T1DM is associated with dysregulation of cellular and humoral immunity.[97] Th1 cells and their cytokine products (IL-2, IFN-γ, TNF-α, and TNF-β) are the mediators of cellular immunity. These cytokines will activate vascular endothelial cells to attract circulating leukocytes to the site of infection, which then leads to the elimination of antigen-bearing cells.[98] Th1 cytokines induce β-cell destruction directly through accelerating activation-induced cell death (apoptosis). Th2 cytokines are

effective in stimulating humoral immune responses, which are responsible for immunoglobulin production by B-cells. It is also believed that the responses of Th1 and Th2 are reciprocally inhibitory, thus, Th2 cytokines (IL-4, IL-10) can inhibit the production of Th1 cytokines and these, in turn, can inhibit Th2 cytokines.[99]

Most of our available information regarding the role that T-cells and macrophage-derived cytokines play in the pathogenesis of T1DM came from studies on genetically IDDM animals like nonobese diabetic mice.[100] Based on these results, cytokines were shown to induce/and or exacerbate T1DM through direct and indirect mechanisms. The possible mechanisms through, which cytokines produce their effect could be listed as;
1) activation of stress-activated protein kinases such as c-Jun NH_2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK),
2) triggering endoplasmic reticulum (ER) stress mechanisms, and
3) The release of death signals from mitochondria.[101]

As mentioned previously, cytokines are able to activate JNK, ERK, and MAPK and some researchers suggested that using an inhibitor of these kinases will result in blocking cytokine-induced apoptosis in insulin-producing cells. However, these findings have not been confirmed in primary β-cells.[102]

ER stress responses are a group of specific cellular mechanisms that result from disruption of ER homeostasis. This disruption is induced via changes in Ca^{+2} concentrations that trigger the accumulation of unfolded protein and activation of stress responses, which aim to restore ER homeostasis and function. These cellular responses could involve translational attenuation, up-regulation of ER chaperones, and

degradation of misfolded proteins.[103] Prolonged activation of ER stress will end in apoptosis, which is driven via the activation of the transcription factor CHOP, MAPK, JNK, and caspase-12. β-cells are particularly vulnerable to ER stress due to their high rate of protein synthesis.[104] In addition to the ER, mitochondria are an important organelle for β-cell function and survival and disruption of mitochondrial homeostasis can trigger β-cell apoptosis. Members of the Bcl-2 protein family control the mitochondrial response to pro-apoptotic signals by preventing the release of cytochrome C, which will prevent the activation of caspase 9 and 3.[105] A study on RINm5F cells suggested that cytokines have the ability to disrupt the mitochondrial membrane, but that overexpression of Bcl-2 partially protected both mouse[106] and human[107] islets against cytokines-induced apoptosis. However, Bcl-2 overexpression failed to prevent adenovirus-induced cell death[108] or spontaneous diabetes in NOD mice.[109] This indicates the presence of other pathways that could trigger apoptosis and/or that Bcl-2-linked mitochondrial events and activation of caspases are late stage events in the apoptotic process when the fate of cells has already been decided.[110] Other pro-apoptotic genes that could also be activated by cytokines include Bid, Bak, and caspase-3[111] with suggestions of cross talk between the mitochondria and ER to decide whether β-cells will undergo apoptosis or not.[112]

Autoimmune β-cell destruction begins when autoantigens (i.e. GAD65) are released during spontaneous β-cell turnover. The antigens are processed by dendritic cells and/or macrophages and presented to helper T cells (CD4+ TH1 cells). Activated macrophages secrete IL-12, which activates CD4+ TH1-type T cells. CD4+ T cells secrete cytokines such as IFN-α, TNF-α, TNF-β, and IL-2, which cause macrophages to become cytotoxic and release β-cell–cytotoxic cytokines (including IL-1 β, TNF-α, and IFN- γ). The pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-1β) along with the

endotoxin (LPS) are believed to activate NF-κB through their signal transduction pathways. The activation and translocation of the NF-κB into the nucleus will stimulate the formation of different pro-inflammatory genes like iNOS (inducible nitric oxide synthase gene), this could be one of the possible pathways through which pro-inflammatory cytokines and endotoxin are producing apoptosis in β-cells.

1.8 Apoptosis

The term apoptosis was first used in 1971 to describe a form of cell death. Apoptosis occurs normally in aging and development and as a homeostatic process to keep cell populations constant in tissues. It also occurs as a defense mechanism when cells are damaged due to diseases and noxious agents, or in response to an immune reaction.[113] There are many types of stimuli that can trigger apoptosis, however, not all cells die in response to the same stimulus e.g. corticosteroids, which can result in apoptosis in some cells while other cells are unaffected or stimulated.[114]

The morphological changes that are associated with apoptotic processes include cell shrinkage, chromatin condensation followed by chromatin fragmentation, extensive plasma membrane blebbing, followed by nuclear rupture, separation of cell fragments into apoptotic bodies (cytoplasm containing tightly packed organelles, with or without a nuclear fragment. The apoptotic bodies will then be phagocytosed by macrophages or parenchymal cells and degraded within phagolysosomes.[115]

Apoptosis is a safe process as apoptotic cells do not release their content into the surrounding interstitial tissue and are quickly eliminated by macrophages. They do not therefore stimulate an inflammatory reaction themselves in contrast to necrosis, which

is considered to be a toxic process due to the rupture of the cell membrane and release of cellular contents into adjacent tissues resulting in initiation of inflammatory reactions.[116]

There are multiple signal pathways that can trigger apoptosis and these are regulated by multiple complicated extrinsic and intrinsic ligands. To date, research indicates that there are two main apoptotic pathways: 1) the intrinsic or mitochondrial pathway, and 2) the extrinsic or death receptor pathway. However, there is evidence that connects the two pathways together.[117] The extrinsic pathway involves transmembrane receptor-mediated interactions involving receptors that are members of TNF receptor gene superfamily such as TNF-α/TNFR1, FasL/FasR, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5.[118] The signal is mediated through a cytoplasmic death domain which will transmit the death signal from cell surface to the intracellular signaling that starts with activation of adapter proteins like FADD, TRADD, and RIP (based mainly on the type of receptors stimulated). This complex will, in turn, activate the pro-caspase 8 and once the caspase 8 is activated, the execution phase of apoptosis is triggered.[119] Caspases are a family of cysteine proteases that are aspartate-specific, which serve as a mediator of apoptosis. Caspase activation occurs following receipt of an intrinsic or extrinsic death signal.[120] The intrinsic pathway is initiated by an intracellular signal like DNA damage, growth factor withdrawal, or loss of contact with the extracellular matrix. These stimuli cause changes in the inner mitochondrial membrane, which lead to the opening of the mitochondrial permeability transition pore. This, in turn, will result in the release of pro-apoptotic proteins from intermembrane space to the cytosol.[121] The first group includes cytochrome C, serine protease HtrA2/Omi, and Smac/DIABLO proteins that activate the caspase dependent-mitochondrial pathway. Cytochrome C will bind and activate the caspase 9 and Apaf-1forming apoptosome. HtrA2/Omi and

Smac/DIABLO proteins promote apoptosis through suppressing the IAP (inhibitors of apoptosis protein).[121] The second group of pro-apoptotic proteins consists of apoptosis-inducing factor (AIF), endonuclease A, and caspase activated DNase which are released in later stages when the cell is committed to death. AIF will transfer to the nucleus causing DNA fragmentation. Endonuclease G will also translocate to the nucleus where it cleaves nuclear chromatin to produce oligonucleosomal DNA fragments. CAD action will also lead to oligonucleosomal DNA fragments and a more advanced chromatin condensation.[122] Finally, in the perforin/granzyme pathway, cytotoxic T-lymphocyte (CTL) are able to eliminate target cells through the extrinsic pathway and FasL/FasR interaction.[123] Perforin and Granzyme are also able to exert their cytotoxic effects on tumour cells and virus-infected cells through a novel pathway that involves the production of the transmembrane pore-forming molecule perforin with subsequent release of granules via the pore and into the target cell.[124] These granules have serine protease granzyme A and B within them.[125] Granzyme B has the ability to cleave proteins at aspartate residues, which results in activation of caspase 10 and can also cleave factors like ICAD.[126] It can also utilize the mitochondrial pathway for amplification of the death signal via cleaving the Bid, which is a pro-apoptotic protein belonging to the Bcl-2 family. The protease cleaves Bid and it will transfer to the mitochondria where it increases mitochondrial permeabilization and the release of cytochrome C.[127] Granzyme B can also activate caspase 3 directly. All these stimuli will end in apoptosis and cell death.[128] Granzyme A-induced cell death is mainly characterized by the formation of single strand DNA nicks. It induces loss of mitochondrial inner membrane potential and release of reactive oxygen species (ROS). The mitochondrial outer membrane remains intact so there will be no release of cytochrome C, serine protease HtrA2/Omi, and Smac/DIABLO proteins.[129] ROS will result in the translocation of the ER-associated SET complex, which includes pp32,

NM23-H1, Ape1, and TREX1. Granzyme A will cleave the SET complex thus releasing inhibition of NM23-H1resulting in apoptotic DNA degradation.[129](Figure 1.6).

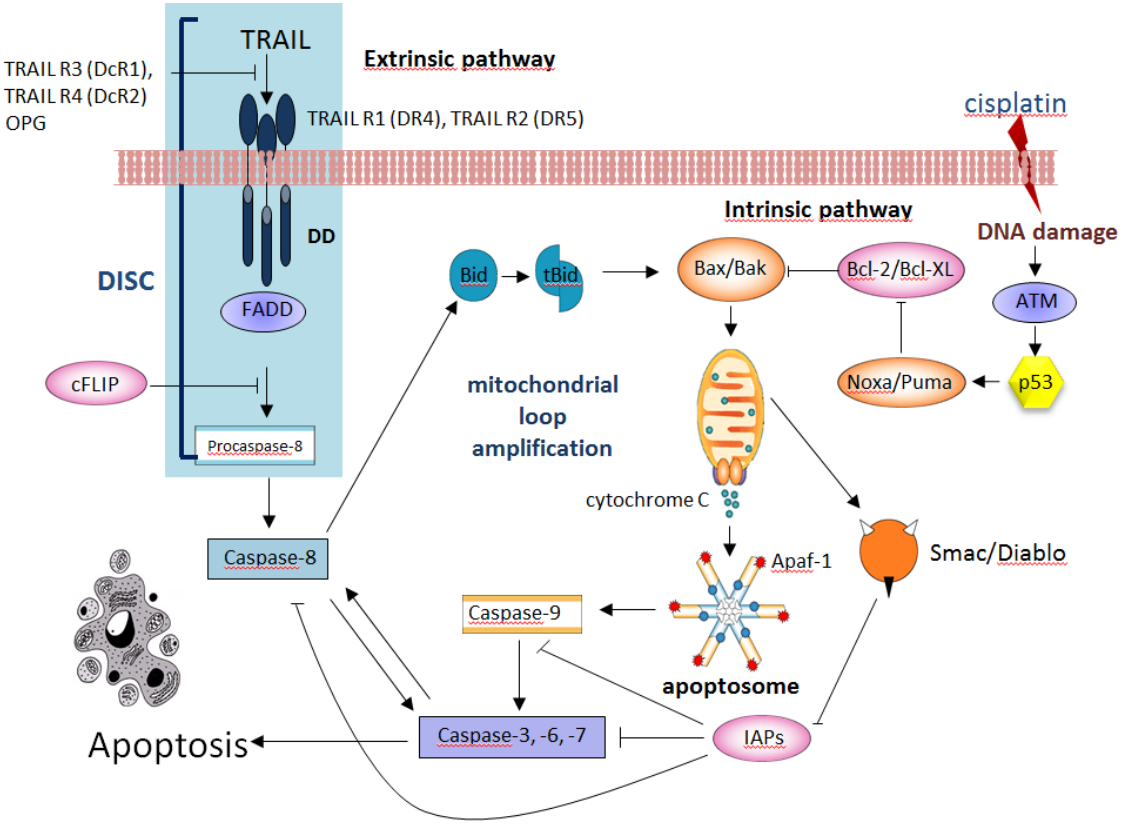

Figure 1.6 ***Extrinsic and intrinsic activation of apoptosis (this image reprinted from Chapter 11of biological significant of apoptosis in ovarian cancer http://www.intechopen.com/books/ovarian-cancer-a-clinical-and-translational-update).*** Two types of apoptosis are known: (1) an extrinsic pathway, which depends on the presence of death receptor (like TRAIL) that will form death-inducing signalling complex (DISC) that will initiate series of caspase activation, which will end in apoptosis; and (2) an intrinsic pathway initiated by an intracellular signal that causes changes in the mitochondrial membrane resulting in the release of cytochrome c that eventually lead to apoptosis.

1.9 Cell therapy

Given the limitations of current insulin replacement therapies for those with T1DM and the potentially devastating effect of diabetic complications, attention has focused on

cell-based therapies for the treatment of T1DM. Two hundred years ago James Blundell (Guy's Hospital, London, UK) was the first to record human-human blood transfusion. This knowledge developed over the years to the advanced cellular therapies of our days. Initially, these were based on clinical trial and error and then developed a dependency on laboratory science that cares about the necessary critical mass and unique challenges in order to justify being a distinct industry in its own right. Cell therapy is considered the fourth and most modern therapeutic pillar of global healthcare.[130]

Cell therapy has two main approaches to treating patients. Cells are either harvested from a patient and expanded or treated and returned back to the same patient (known as the autologous method), or the cells are taken from one or a few universal donors followed by large-scale expansion and banking of multiple doses (known as allogeneic cell therapy).[131]

Cells are prepared in a manufacturing facility and stored under controlled conditions for subsequent manipulation and use. Stem cell therapy has the potential to treat a wide range of diseases and disorders. The most commonly used stem cell therapies at present rely on mesenchymal stem cells due to their plasticity, established isolation procedure and capacity for *ex vivo* expansion.[132]

1.9.1 Stem cells

Stem cells are a class of undifferentiated cells that are able to differentiate into different cell types. Stem cells have two main properties: self-renewal (the ability to go through numerous cycle of cell division while maintaining the undifferentiated state), and potency, which is the ability to differentiate into specialized cells e.g. cells of the blood,

heart, bones, skin, muscles and many other types of cells.[133]. Stem cells come from two main sources: embryonic, which are found in the inner cell mass of blastocysts, and adult stem cells, found throughout the body and, which act as a repair system for the body.[134] In the inner cell mass of the pre-implantation blastocyst, embryonic stem cells have the potency to differentiate into all the specialized cells and are known as pluripotent cells. Other types of stem cells are classified as being totipotent cells (can differentiate into embryonic and extraembryonic cell types, these cells are produced from the fusion of an egg and sperm cell. Cells produced by the first few divisions of the fertilized egg are also totipotent) , multipotent cells (stem cells that can differentiate into number of cells, but only those of a closely related family of cells), oligopotent cells (can differentiate into only a few cells such as lymphoid or myeloid stem cells) and unipotent stem cells (cells that can produce only one cell type, their own, but have the property of self-renewal, which distinguishes them from non-stem cells (examples include progenitor cells and muscle stem cells).[135]as shown in (Figure 1.7).

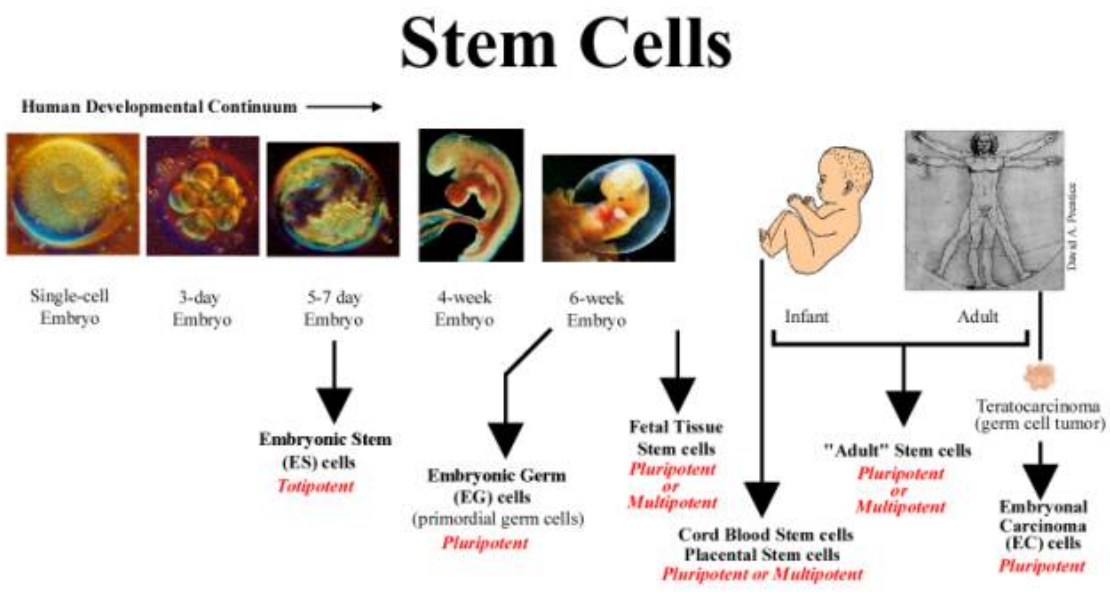

Figure 1.7 Types of stem cells. This figure demonstrates the normal development of stem cells and types of stem cells in relation to the normal development. Reprinted from.https://smediacacheak0.pinimg.com/736x/a2/84/6b/a2846b4886265fc1d1cc81ae5334ed8a.jpg

1.9.2 Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are multipotent stem cells that have the potential to differentiate into many cell types like osteoblasts (bone cells), chondrocytes (cartilage cells) and adipocytes (fat cells). MSCs are predominantly isolated from bone marrow, but may also be found in other tissues including peripheral blood, cord blood and the fetal liver and lung.[136]

In addition to being multipotent, MSCs can achieve limited expansion during *in vitro* culture. It is thought that MSCs response to stress or injury is somewhat similar to the innate and adaptive immune response in that MSCs travel to the site of infection when supplied exogenously.[124] Also, MSCs have the ability to undergo *ex vivo* differentiation into many cell types.[137] The International Society for cellular therapy proposes minimal

criteria to identify human MSCs like MSCs must be plastic-adherent when maintained in culture condition also the flow cytometric analysis demonstrated the expression of distinguishing MSCs antigens (CD105, CD73, and CD90), and the absence of hematopoietic and endothelial antigens (CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR) and also MSCs must differentiate into adipocyte, chondrocyte and osteocyte.[138]

1.9.2.1 Development and function of MSCs

The origin of MSCs is not fully known, it is believed that MSCs are specialized connective tissue cells, which are undifferentiated and found in the early stages of fetal life.[139] MSCs can easily migrate, unlike epithelial cells, which migrate slowly.[140] MSCs have been isolated from different types of tissues like bone marrow.[141] adipose tissue,[142] lung tissue[143]and umbilical cord blood[144] suggesting that MSCs may reside in all postnatal organs.[145]

MSCs have an immunomodulatory capacity, in addition to an expansion capacity and multipotent differentiation capacity. The secretion of soluble factors by the MSCs can alter the production of dendritic cells (DCs) causing an increase in the production of anti-inflammatory cytokines including IL-10, whilst inhibiting the production of IFN-γ and IL-12.[146] MSCs are able to prevent the proliferation of T cells by binding the programmed death 1 molecule (PD-1) to its ligands PD-L1 and PD-L2. This binding will produce soluble factors (e.g. transforming growth factor beta (TGF-β) or IL-10) that inhibit T-cell proliferation through interaction with DCs. MSCs have the ability to increase the number of T regulatory cells that suppress the immune response.[147] The production of soluble factors by MSCs can suppress the proliferation and IgG secretion

of B cells. Therefore, the therapeutic properties of MSCs can be related to their ability to release trophic and immunomodulatory factors.[148]

1.9.2.2 Clinical utility of MSCs

MSCs have generated a great amount of excitement over the past decade as a novel therapeutic strategy for different disease conditions such as bone disease,[149] cardiac disorder,[150] multiple sclerosis[151] and T1DM[152] as shown by *in vivo* studies in animal models. In experimental animal models of rheumatoid arthritis, a single injection of MSCs prevented severe damage of bone and cartilage by inducing T cell hypo-responsiveness and modulation of inflammatory cytokines like TNF-α.[153] Furthermore, experiments on rat models with glomerulonephritis show that the use of MSCs accelerates glomerular healing.[154] The use of MSCs and bone marrow cell co-transplantation in mice with T1DM supported the regeneration of recipient-derived pancreatic insulin secreting cells and prevented the immune response against the newly formed β-cells by suppression of T cell responses.[155]

The ability of MSCs to differentiate into multiple lineages, secrete soluble factors that have immunosuppressive and anti-inflammatory effects, in addition to their tendency to migrate to the site of injury made them an attractive therapeutic candidate. MSCs have been used in various clinical trials and have shown promising results. Children with osteogenesis imperfecta (clinical condition characterized by a defect in type 1 collagen) underwent allogeneic haematopoietic stem cell transplantation (HSCT) and showed an improvement in bone structure and function.[156] Also for cardiovascular repair; a pilot study involved 69 patients with acute myocardial infarction who displayed a significant improvement in cardiac function following MSC administration.[157] There is also clinical

trials that test the beneficial effects of MSCs in Crohn's disease and the initial results are promising.[158]

1.9.2.3 MSC and tissue repair

The extraordinary regenerative ability of MSCs along with their immunomodulatory properties have opened the door for many studies based on the use of MSCs in tissue repair. Studies are performed on animal models with a different type of tissue injuries such as skeletal defect, lung injuries, kidney disease, graft versus host disease and myocardial infarction.[159] The results showed the ability of MSCs to serve as effective therapeutic agents but the mechanism by, which the MSCs work is still unclear. Some studies suggest that the repair ability is secondary not to transdifferentiation of MSCs into the appropriate cell phenotype or to cell fusion, but rather to the production of soluble factors that change the microenvironment of the tissue.[159]

Transforming growth factor, hepatic growth factor, prostaglandins E2 and IL-10 are all soluble factors that are secreted by MSCs and, which have been found to suppress T-cell mediated antigen response *in vitro*. Burchfield *et al* stated that secretion of the anti-inflammatory cytokine IL-10 by MSCs plays a major role in their therapeutic effectiveness in mice with myocardial infarction.[160] IL-10 is critical for regulation of the immune system, and is involved in the differentiation of T-regulatory cells, which is essential for inhibition of autoimmune reactivity and in termination of the inflammatory response.[160] IL-10 is one of the anti-inflammatory cytokines that has been confirmed to be present in MSCs secretome.[161] Another study suggests a role for TGF-β and IL-4 in the treatment of mouse model with induced asthma.[162] Furthermore, according to Meisel *et al* MSC inhibition of T-cell proliferation could also be due to a depletion of tryptophan (using the indolamine2, 3-dioxygenase enzyme (which induced by IFN-γ

and other proinflammatory cytokines) to catalyzes the conversion of tryptophan to kynurenine, this conversion has been identified as a major immunosuppressive effectors pathway that inhibits T-cell response to autoantigens *in vivo*) and subsequent inhibition of T-cell proliferation.[163] Inducible nitric oxide synthase heme oxygenase-1 expressed by MSCs has also been implicated for their immunosuppressive properties. It is likely that these mechanisms are not exclusive and that the relative contribution of each mechanism to modulating immune response varies in different experimental models. It is also interesting to hypothesize that immunomodulation of MSCs in different tissues may be mediated by different factors.[164]

1.9.2.4 Immunomodulatory effect of MSC and diabetes

The immunomodulatory capacity of MSCs makes the researcher consider the ability of MSCs in the treatment of T1DM. In Non-Obese Diabetic mice (NOD), it appears that a dysregulation in the immune system leads to the development of autoimmune diabetes, a combination of immune cell dysfunction (including T-cells, B-cells, and dendritic cells), as well as the presence of inflammatory cytokines, leads to β-cell failure.[165] The immunomodulatory properties of MSCs may interrupt autoimmunity. MSCs may have the capacity to regulate T1DM by presenting differential levels of negative co-stimulatory molecules and secreting regulatory cytokines such as transforming growth factor and IL-10 that control autoreactive T-cells. MSCs have the ability to suppress B-cell proliferation and terminal differentiation resulting in the suppression of antigen-specific antibodies.[166] In addition, it has been observed that MSCs are able to interfere in the differentiation, maturation and function of dendritic cells (DCs), which represent the most important antigen presenting cells (APC) playing a cardinal role in humoral and cellular immune response against foreign and self-antigen they possess a number of receptors that increase the uptake of antigen and convert them to major

histocompatibility complex that could be recognized by lymphocytes.[167] DCs have the capacity to secrete IL-12 and activate T-lymphocyte. Furthermore, MSCs were found to inhibit T-cell proliferation and differentiation by interfering in the conversion of naïve $CD4^+$ T-cells into T-helper 1 (Th1) effector cells.[168] MSCs have been used to induce apoptosis of infiltrated leukocytes in pancreatic islets of non-obese diabetic mouse (NOD).[169] MSCs also demonstrate a similar effect in a study that involves human islets transplanted into mice in which they increased T-regulatory cell production leading to immune tolerance of transplanted human islets.[170] However, the mechanisms underlying the immunomodulatory abilities of MSCs are still unclear and need further research and clinical trials to prove the effectiveness of MSCs in the treatment of T1DM.

1.10 Aims of this study

Owing to their immunomodulatory and self-renewal properties, MSCs have emerged as a new therapeutic tool for the treatment of several chronic conditions. Given the complex autoimmune pathways involved in the destruction of β-cells in T1DM, we hypothesised that MSCs and specifically, MSC-derived products may protect against β-cell death and theoretically, halt the progression of T1DM. Therefore, the aims of this project were:

- To establish cellular models of cytokine-driven β-cell death.
- To determine if culturing conditions (oxygen concentration and the presence of serum in the culture media) sensitized cells to the effects of cytokines.
- To explore the therapeutic effectiveness of MSC-CM (MSC conditioned medium) in the prevention of β-cell death.

- To identify candidate anti-inflammatory proteins from MSC-CM that may protect against β-cell death
- To investigate potential mechanisms by which candidate proteins confer protection against β-cell death

Chapter 2

Materials & Methods

2.1 Materials

Table 2.1 list of materials, catalogue number and supplier

Name	Catalogue number	Supplier
(3-(4,5-dimethylthiazol-2yl)-2,5diphenyltetrazolium bromide	M2128	Sigma-Aldrich
3-isobutyl-1-methylxanthine (IBMX)	I7018	Sigma-Aldrich
4′,6-Diamidino-2-phenylindole (DAPI)	D9542	Sigma-Aldrich
ABTS (2.2-azino-bis(3-ethylbenzothiazolin-6-sulphonic acid)	A3219	Sigma-Aldrich
ALPCO Elisa kit	80-INSRTH-E01	ALPCO-USA
Agarose	BP1356-500	Fisher Scientific
Alcian blue	A3157	Sigma-Aldrich
Alizarin red S	A5533	Sigma-Aldrich
Ascorbic acid phosphate	A8960	Sigma-Aldrich
β-glycerophosphate	G9422	Sigma-Aldrich
βTC1.6 cells	CRL-11506™	ATCC
Bovine serum albumin (BSA)	BP9703-100	Fisher Scientific
Collagenase P solution	17018-029	Fisher Scientific
Cryopreserved human bone marrow mononuclear cells (MNCs)	2M-125C	Lonza

Calcium chloride	499609	Sigma-Aldrich
Dexamethasone	D2915	Sigma-Aldrich
Direct Load Wide Range DNA Marker	D7058	Sigma-Aldrich
Dimethyl sulfoxide	D2650	Sigma-Aldrich
Donkey anti goat polyclonal antibody	SC-45102	Santa Cruz biotechnology
Donkey anti rabbit polyclonal antibody	SC-2089	Santa Cruz biotechnology
Dulbecco's Modified Eagle Medium (DMEM)	BE12-707F	Lonza
Ethidium bromide	E1510	Sigma-Aldrich
Ethanol (absolute)	E0650/17	Fisher Scientific
FBS (foetal bovine serum)	DE14-801F	Lonza
Fibronectin	F0895	Sigma-Aldrich
Flow cytometry staining buffer	FC001	R&D system biotechne
Gel Loading Buffer	10816-015	Thermo fisher
Glucose	G7021	Sigma-Aldrich
Goat anti mouse monoclonal antibody	SC-16516	Santa Cruz biotechnology
Goat polyclonal anti-GLUT2	SC-7580	Santa Cruz biotechnology
Goat polyclonal anti-IRS-1	SC-559	Santa Cruz biotechnology

Hydrogen peroxide (H_2O_2)	216763	Sigma-Aldrich
Human IL-10 mini ELISA kit	900-M21	PeproTech
Human IL-4 mini ELISA kit	900-M14	PeproTech
Human PIGF-1 ELISA kit	900-K307	PeproTech
Human VEGF mini ELISA kit	900-M10	PeproTech
Hepes	H-3375	Sigma-Aldrich
Indomethacin	I7378	Sigma-Aldrich
Insulin	I9278	Sigma-Aldrich
Insulin, Transferrin, Selenium (ITS)	I3146	Sigma-Aldrich
In situ direct DNA fragmentation kit	Ab66108	Abcam
Industrial methylated spirits	I99050	Genta Medical
Isopropanol	P/7500/17	Fisher Scientific
L-Glutamine	BE17-605E	Lonza
L-Proline	P5607	Sigma-Aldrich
Methanol	M/3900/17	Fisher Scientific
Mouse monoclonal anti-HNF-1α	SC-135939	Santa Cruz biotechnology
Magnesium sulphate	M-5921	Sigma-Aldrich

Non-essential amino acids	BE13-114E	Lonza
DNA-Dye NonTox	A9555.1000	VWR
Magnesium chloride	M-8266	Sigma-Aldrich
Oil Red O	O0625	Sigma-Aldrich
Paraformaldehyde	P/0840/53	Fisher Scientific
Penicillin, streptomycin, amphotericin B	BE17-745E	Lonza
Penicillin-Streptomycin (P.S)	17-602F	Lonza
Phosphate buffered saline	BE17-516F	Lonza
Phycoerythrin conjugated antibodies CD105	130-098-845	Miltenyi Biotec
Phycoerythrin conjugated antibodies CD14	130-098-167	Miltenyi Biotec
Phycoerythrin conjugated antibodies CD19	130-098-168	Miltenyi Biotec
Phycoerythrin conjugated antibodies CD34	130-098-140	Miltenyi Biotec
Phycoerythrin conjugated antibodies CD45	130-098-141	Miltenyi Biotec
Phycoerythrin conjugated antibodies CD73	130-097-932	Miltenyi Biotec
Phycoerythrin conjugated antibodies CD90	130-098-906	Miltenyi Biotec
Phycoerythrin conjugated antibodies HLA-DR	130-098-177	Miltenyi Biotec
Phycoerythrin conjugated antibodies IgG1	130-098-849	Miltenyi Biotec

Potassium chloride	P9333	Sigma-Aldrich
Potassium phosphate	PHR 1330	Sigma-Aldrich
Phenol red	P0290	Sigma-Aldrich
Pierce BCA protein assay kit	23221	Thermo fisher
Quantifast SYBR green RT-PCR kit	204154	Qiagen
Radio immune precipitate assay buffer (RIPA)	R0278	Sigma-Aldrich
Rabbit anti-murine IL-10	500-p60	PeproTech
Rabbit Anti-murine TRAIL	500-P303	PeproTech
Rabbit polyclonal anti-insulin	SC-9168	Santa Cruz biotechnology
Rabbit polyclonal anti-KCNQ1	SC-20816	Santa Cruz biotechnology
Rabbit polyclonal anti-KIR6.2	SC-20809	Santa Cruz biotechnology
Rabbit polyclonal anti-SUR-1	SC-25683	Santa Cruz biotechnology
Recombinant human IFN-γ	300-02	PeproTech
Recombinant human IL-1β	200-01B	PeproTech
Recombinant human VEGF	100-20B	PeproTech
Recombinant human PIGF	200-04	PeproTech
Recombinant human IL-4	100-20B	PeproTech

Recombinant human TNF-α	300-01A	PeproTech
Recombinant murine IL-10	210-10	PeproTech
Recombinant murine TRAIL	315-19	PeproTech
RNase Zap	R2020	Sigma-Aldrich
RNeasy mini kit	74104	Qiagen
Rosewell Park Memorial Institute (RPMI1640)	12-918F	Lonza
SuperScript III One-Step RT-PCR System with Platinum *Taq* High Fidelity DNA Polymerase kit	12574030	Life Technologies/ Fisher Scientific
Sodium Chloride	S3014	Sigma-Aldrich
Sodium Phosphate	S-0876	Sigma-Aldrich
Sodium bicarbonate	S-7277	Sigma-Aldrich
Tris-Acetate-EDTA (TAE) buffer (50X) (2 M Tris Acetate, 100 mM Na_2EDTA)	EC-872	National Diagnostics
Triton X-100	9002-93-1	Sigma-Aldrich
Tween 20	66368	Analar

2.2 Methods

2.2.1 Cell Models

All cell culture was performed in an aseptic environment using a laminar flow hood, the hood was sterilized by UV light for at least half an hour prior to use and all equipment was sterilized with 70% Industrial Methylated Spirit (IMS) (Genta Medical UK) prior to use.

2.2.1.1 Pancreatic β-cell lines

BRIN-BD11 cells were a kind gift from Ulster University. The insulin-secreting cell line was established after electro-fusion of RINm5F cells with New England Deaconess Hospital rat pancreatic islet cells. Morphological studies established that these cells grow as cell monolayers with epithelioid characteristics and maintain stability in tissue culture for more than 50 passages.[171] βTC1.6 cells were purchased from ATCC (LGC Standards, UK) and cultured according to the supplier's instructions. βTC1.6 cells are an insulinoma cell line derived by expression of the SV40 T antigen (Tag) incorporated under control of the insulin promoter in transgenic mice.[171]

BRIN-BD11 cells were maintained in a T75 flask with 10-15 ml of warm Rosewell Park Memorial Institute (RPMI1640) media (Lonza, UK) supplemented with 10% fetal bovine serum (FBS) (Lonza, UK) and 1% Penicillin-Streptomycin (P.S) (Lonza, UK). βTC1.6 cells were cultured in a T25 flask with 5-7 ml of warm Dulbecco's modified Eagle's (DMEM) media (Lonza,UK) supplemented with 15% heat inactivated FBS (Lonza, UK) and 1% P.S (Lonza, UK). All cells were maintained in an incubator at an atmosphere of 37 °C and 5% CO_2. Cells were checked microscopically daily to ensure they were healthy and growing as expected. Both cell lines grow as adherent monolayers as shown in Figure 2.1, are round or elongated in shape and refract light around their membrane. Cells were discarded if they detached in large numbers and/or look shrivelled and grainy/dark in colour, or displayed evidence of quiescence.

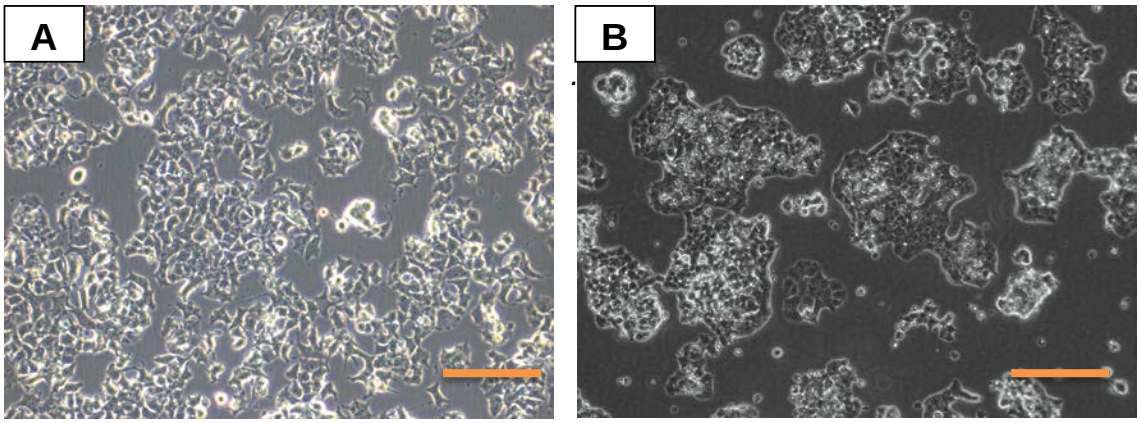

Figure 2.1 Light micrograph images of pancreatic β-cell lines grown in culture as monolayers.(A) BRIN-BD11 cells monolayers, (B) βTC1.6 cells monolayer. X20 magnification, the scale bar is 100 µm.

Cells were passaged at (80-90)% confluency. Briefly, cells were washed with 4 ml phosphate buffer saline (PBS) (Lonza, UK) twice. Pre-heated 1x trypsin/EDTA (2.5ml) (Lonza, UK) was added to the flask, which was returned to the incubator for 3-5 mins. The flask was gently tapped to aid cellular detachment, which was confirmed microscopically. Once detached, an additional 4 ml of media was added to the flask and the resultant suspension transferred to a 15 ml centrifuge tube, and the tube centrifuged at 1000 rpm (152 g)

for 2 mins. After centrifugation the solution was aspirated, taking care not to dislodge the pellet, which was resuspended in 1 ml of media. The cells were then counted using a disposable haemocytometer (Hycor Kova Glasstic slide 10, US) and returned to culture or utilized for experimentation.

On occasion cells were also cryopreserved in liquid nitrogen until required. Freezing media was made up of 80% FBS (Lonza, UK), 10% dimethyl sulfoxide (DMSO) (Sigma, UK) and 10% complete media (RPMI or DMEM, Lonza, UK). Briefly cells were detached from the flask with 1x trypsin/EDTA (Lonza, UK) resuspended in the freezing media and immediately transferred to cryovials and placed in isopropanol filled Mr Frosty freezing container for controlled cooling to -80 ^{O}C before being transferred to liquid nitrogen storage dewars.

2.2.1.2 Isolation and characterization of primary pancreatic islets of Langerhans

2.2.1.2.1 Islets isolation and culture

Pancreatic islets were isolated from CD1 mice aged 12-16 weeks. All procedures were conducted in accordance with the Animals Scientific Procedures Act 1986. Animals were euthanized under Schedule 1 methods and the pancreas was excised and transferred to Hank's Balanced Salt Solution (HBSS) transport buffer comprising 8 g/l NaCl, 0.4 g/l KCl, 0.14 g/l CaCl2, 0.1 g/l MgSo4, 0.1 g/l MgCl2, 0.06 g/l Na2HPO4, 0.06 g/l KH2PO4, 1 g/l Glucose, 0.02 g/l phenol red, 0.35 g/l NaHCO3 and 10 mM Hepes The pancreas was subsequently chopped into small pieces and placed in collagenase P solution (0.5 mg/ml collagenase clostridium histolyticum, (Fisher, UK) in HBSS) to allow enzymatic digestion of the exocrine pancreatic tissue. Enzymatic digestion was aided by mechanical disturbance as the pancreas solution was shaken in a water bath at 37 ^{O}C for 10 minutes. Following this, HBSS supplemented with 0.1% Bovine Serum Albumin (BSA; Sigma, UK) was added to stop the action of collagenase. Pancreatic tissue was then centrifuged for 5 mins at 1000 rpm (152 g) The supernatant was carefully removed and the tissue pellet resuspended in 5 ml wash buffer (HBSS + 5% FBS). This step was repeated twice and the homogenised pancreas was passed through a fine mesh filter into a 50 ml Falcon tube. The filtrate was centrifuged for 5 mins at 1000 rpm during which time the isolated islets formed a pellet at the bottom of the Falcon tube. The isolated islets were cultured in a petri dish using RPMI media supplemented with 5% FBS and 1% P.S. The islets were maintained in an incubator at 37 ^{O}C and 5% CO_2 for a minimum of 24 hours before experimentation.

2.2.1.2.2 Characterization of pancreatic islets

Freshly isolated islets were fixed with 4% paraformaldehyde (PFA) in 1x PBS (Lonza, UK) for 30 mins at room temperature. The islets were subsequently washed three times with 1x PBS for 10 minutes. This was followed by permeabilization using 0.3% Triton X-100 (Sigma, UK) in 1x PBS for three hours at room temperature. Then a blocking solution (5% BSA,

0.15% Triton X-100 in 1x PBS) was applied overnight at 4 ºC. The following morning, the islets were centrifuged for 5 mins at 900 rpm (123 g) and the blocking solution removed and replaced with a buffer solution of 1% BSA (Sigma, UK), 0.2% Triton X-100 in 1x PBS for a further 40 minutes. The primary and secondary antibodies were diluted in buffer solution as shown in Table 2.2 and incubated for 24 hours respectively at 4 ºC. The islets were examined using laser scanning confocal microscope (Olympus, Japan) sequential narrow band filter accounts for spectral bleed-through (FITC excitation set as 473 nm).

Immunogen	Primary anti body	Secondary	Dilution	
			primary	secondary
Insulin	Rabbit polyclonal anti-insulin	Donkey anti rabbit polyclonal anti-insulin	1:50	1:100
GLUT2	Goat polyclonal anti-GLUT2	Donkey anti goat polyclonal anti-GLUT2	1:50	1:200
IRS-1	Goat polyclonal anti-IRS-1	Donkey anti goat polyclonal anti-IRS-1	1:50	1:200
KCNQ1	Rabbit polyclonal anti-KCNQ1	Donkey anti rabbit polyclonal anti-KCNQ1	1:50	1:100
HNF-1α	Mouse monoclonal anti-HNF-1α	Goat anti mouse monoclonal anti-HNF-1α	1:50	1:100
KIR6.2	Rabbit polyclonal anti-KIR6.2	Donkey anti rabbit polyclonal anti-KIR6.2	1:50	1:100
SUR-1	Rabbit polyclonal anti-SUR-1	Donkey anti rabbit polyclonal anti-SUR-1	1:50	1:100

Table 2.2 Antibody dilutions for immunohistochemistry of pancreatic islets.

2.2.1.3 Culture and characterization of bone marrow-derived mesenchymal cells (MSCs)

Human Bone Marrow Mononuclear cells were purchased from (Lonza, UK). Cells were cultured at a density of 1 x 10^5 mononuclear cells (MNC)/cm^2 in T75 flasks. Flasks were pre-coated with 10 ng /ml of fibronectin (Sigma,UK) in PBS for one hour at room temperature. MNC cells were cultured in DMEM media (Lonza,UK) supplemented with 5% FBS (Lonza,UK), 1% L-Glutamine (Lonza, UK), 1% Non-essential amino acid (Lonza, UK) and 1% Penicillin Streptomycin Amphotericin-B (PSA) (Lonza, UK). After one week a 50% media change was performed and cells incubated for a further week after which a 100% media

change was performed. Following this a routine media changes were carried out twice a week. Cells were passaged when needed as described in Section 2.2.1.1.

2.2.1.3.1 Trilineage Differentiation

In order to evaluate the multipotency of bone marrow mesenchymal stem cells, the cells were differentiated into osteogenic, adipogenic and chondrogenic lineages using differentiation media.

Cells were seeded into twelve well plates at $1X10^5$ cell/well and grown to 70-80% confluence in complete culture media. Once at the required confluency cells were then cultured with differentiation media specific for osteogensis, adipogensis and chondrogensis with media changes every three/four days. For control purposes bone marrow mesenchymal stem cells were cultured in complete grow media for the same duration with identical timing of media change.

2.2.1.3.1.1 Osteogenic differentiation

Osteogenic differentiated media consisted of low glucose DMEM supplemented with 10% FBS, 1% NEAA, 1% L-glutamine, 50mM ascorbic acid (Sigma, UK), 10mM betaglycerophosphate (Sigma, UK) and 0.1mM dexamethasone (Sigma, UK).

2.2.1.3.1.2 Adipogenic differentiation

Adipogenic differentiated media consisted of low glucose DMEM supplemented with 10% FBS, 1% NEAA, 1% L-glutamine, 0.5mM IBMX(Sigma, UK), 10µg/ml insulin (Sigma, UK), 100mM indomethacin (Sigma, UK) and 0.5mM dexamethasone.

2.2.1.3.1.3 Chondrogenic differentiation

Chondrogenic differentiation media comprised low glucose DMEM supplemented with 1% FBS, 1%NEAA, 1% L-glutamine, 1% ITS (Sigma, UK), 0.1mM dexamethasone, 50mM ascorbic acid (Sigma, UK), 40µg/ml L-proline (Sigma, UK), 1% sodium pyruvate (Sigma, UK) and 10ng/ml TGF-β3 (Sigma, UK).

2.2.1.3.2 Histological staining

After 21 days of culture in differentiation media the bone marrow mesenchymal stem cells had media removed followed by a wash with PBS and fixation with 4% paraformaldehyde (Sigma, UK) in PBS for Adipogenic differentiated cells and 95% methanol (Sigma, UK) for Osteogenic and Chondrogenic differentiated cells for 10 minutes before being washed twice with PBS. Samples were then covered with the stain according to the anticipated differentiation lineage.

2.2.1.3.2.1 Alizarin red for osteogensis

Osteogenic lineage differentiation was identified with a 2% alizarin red S solution. An aqueous solution of 2% (w/v) of alizarin red S (Sigma, UK) was prepared in distal water (dH_2O) and paper filtered. The cells were covered with the stain for 15 minutes at room temperature. The stain was then removed and the cells washed with ddH_2O twice followed by microscopic observation (Olympus CKX41, Japan), and image capture (1X 2-SLP, Micropublisher S-ORTV, Japan).

2.2.1.3.2.2 Oil red O for adipogensis

Samples were stained with oil red O prepared as 0.5% solution (Sigma, UK) in isopropanol (Fisher scientific, UK), to prepare a working solution, a small volume of stock solution diluted with ddH_2O (3:2). The cells were immersed in oil red O solution for 15 minute followed by two ddH_2O washes and microscopic observation and image capture as above.

2.2.1.3.2.3 Alcian blue for chondrogensis

To detect glycosaminoglycans secreted by chondrocytes, the cells were stained with 0.1% alcian blue solution (Sigma, UK). The alcian blue was prepared as 1% (w/v) alcian blue in 0.1M aqueous HCl and paper filtered. Then the stain was applied on the cells for 15 minutes followed by its removal, ddH_2O washes (x2) and microscopic observation and image capture as described above.

2.2.1.3.2 Flow cytometry (FACS)

To test that our MSCs follows the minimum criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy which define human MSC, the expression of (CD73, CD90, CD105) which must be positive along with the negative (CD14, CD19, CD34, CD45, HLA-DR) was explored using antibodies from (Miltenyi biotec, UK). Isotype control IgG1 and IgG2 antibodies were included (Miltenyi biotec, UK)**.** To perform FACS MSC cells were first grown to 80-90% confluence in a T75 flask. The cells were then washed with PBS (4-5 ml) twice before exposure to trypsin EDTA (3-5 ml) (Lonza, UK) for 3-5 minute. Cells were examined under the microscope to ensure detachment and then transferred to a 15ml tube and centrifugation at 900 rpm (14 g) for 5 minutes. The supernatant was removed and 1ml of buffer solution was added (R & D staining buffer). Cell counts were performed and $1X10^5$ cells were placed into 10 X 1.5 microtubes to accommodate the 10 different antibodies. Cells were then centrifuged at 300 RPM for 10 minutes and the supernatant aspirated completely. The cells were resuspended with 100µl of R&D buffer solution and for each microtube 10µl of the specific antibodies were added. Solutions were mixed well and incubated for 10 minutes in the dark at 4 °C. After that the cells were washed by adding 1-2 ml of buffer and centrifuged at 300 RPM for 10 mins. The supernatants were aspirated and the cell pellet resuspended in a suitable amount of buffer for analysis by flow cytometry (Beckman Coulter Cytomics FC 500). At least $1\text{-}3X10^4$ events were acquired and the data analysis was carried out using flowing software version 2. The

isotopes controls IgG1 and IgG2, were used to establish positive and negative events for each antibody measured. The percentage of positive events was determined by using gates to exclude 99% of the appropriate control events.

2.2.2 Preparation of conditioned media

For preparation of MSC conditioned media, the MSCs were allowed to become 80-90% confluent in T75 tissue culture flasks. Cells were washed once with 10 ml PBS and twice with 10 ml DMEM (serum-free) media. Then the MSCs were conditioned with 15 ml RPMI-1640 or DMEM media (Lonza, UK) with and without serum. After 24 hour the media were collected, centrifuged to remove any cell debris, filtered through 0.2 µm filter and stored at -80 °C until required for experimental use.

2.2.3 Treatment of pancreatic β-cell lines and primary islets with recombinant cytokines and endotoxin

The pro-inflammatory cytokines; Tumor Necrosis Factor-α (TNF-α), Interferon Gamma (IFN-γ), and Interleukin-1β (IL-1β) along with bacterial lipopolysaccharide (LPS) isolated from *E. coli* were purchased from PeproTech and Sigma-Aldrich, respectively. These cytokines were chosen as they have a well-documented role in triggering β-cell death[34]. To explore the effect of the above cytokines and endotoxin on cell viability and the induction of apoptosis, cell lines were seeded at a density of 5 x 10^4 cells/well/96-well plate and allowed to attach overnight under culture conditions of 5% CO_2 and 37 °C. Islets were isolated as described in Section 2.2.1.2 and seeded at a density of 25 islets/well/96-well plate and maintained in culture overnight. Cell lines and islets were subsequently exposed to cytokines or endotoxin as described in the following paragraph and the resultant effect of cell viability and the induction of apoptosis assessed as outlined in Sections 2.2.4 and 2.2.5 below.

Briefly, cell lines and islets were treated with rising concentrations of TNF-α, IFN-γ, and IL-1β (0.1 ng/ml - 1000 ng/ml) for 24 h. Similarly, cell lines and islets were exposed to rising concentrations of LPS (0.1 µg/ml – 1000 µg/ml) for 24 h. The optimal concentration of each cytokine or endotoxin was assessed by MTT (Section 2.2.4) in the first instance. Optimal concentrations were deemed to be those that induced an approximate reduction in cell viability of 50%. Finally, cell lines were treated with optimal concentrations of cytokines or endotoxin for 0 h, 15 mins, 1 h, 2 h, 4 h and 24 h to monitor cell viability over time and to allow assessment of anti-apoptotic gene induction as outlined in Section 2.2.6.

2.2.4 Assessment of cell viability by calorimetric MTT Assay

To evaluate the effect of cytokines on BRIN-BD11, βTC1.6 and islet cell viability and to optimise cytokine concentration for subsequent experiments, the MTT (3-(4,5-dimethylthiazol-2yl)-2,5diphenyltetrazolium bromide) cell viability assay was performed to estimate cell metabolic activity using the MTT reagent thiazolyl blue tetrazolium bromide (Sigma, UK), which reduces to insoluble formazan by the action of NAD(P) H dependent oxidoreductase enzyme. The cells were seeded in 96-well plates at a density of 5×10^4 cells/well for cell lines, and 25 islets per well for primary cultures and remained in culture overnight. The cell lines and islets were exposed to cytokines as described in section 2.2.1 and 2.2.2.

Following the basic protocol 500µl of MTT reagent (5 mg/ml) (Sigma, UK) was mixed with 5ml of RPMI1640 media. Media was removed from well plates and 220 µl added to each well followed by incubation for 2 hours at 37 °C. MTT solution (195 µl) was then removed and 100 µl DMSO added to each well and mixed thoroughly by pipetting before incubating again at 37°C for a further 45 minutes. The absorption was measured with a micro-plate reader (Dynatech, MR5000 version 3.7) at a wavelength of 570 nm with a reference wavelength reading at 650 nm.

2.2.5 TUNEL assay for assessment of apoptosis

Following optimisation of cytokine concentration by MTT, the TUNEL (terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labelling) assay (TUNEL in situ direct DNA fragmentation kit (Abcam, UK)) was employed to ensure that any reduction in viability observed resulted from apoptotic processes and not necrosis. The principle of the kit depends on the use of terminal deoxynucleotidyl transferase (TdT) enzyme catalysis of fluorecein-12-dUTP incorporation into the free 3´-hydroxyl ends of fragmented DNA. The fluorescence labelled D terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end labelling DNA can then be detected.

Cell lines were seeded at a density of 5 x 10^4 cells/well/96-well plates and allowed to attached overnight under culture conditions of 5% CO_2 and 37 °C. Islets were seeded at a density of 25 islets per well and placed in culture overnight. Cell lines and islets were treated with the optimal concentration of cytokines identified by MTT assay. The TUNEL assay was performed following a modified manufacturer's protocol as follows; media was first removed from cells and islets (islets were gently centrifuged at 1000 rpm prior to each step in the following protocol), which were washed once with PBS, and cell lines were fixed with 95% methanol for 10 mins (for the islets fixation we followed the steps mentioned in Section 2.2.1.2.2). The methanol was removed and the cells washed twice with washing buffer followed by resuspension in 51 µl of staining solution (10 µl reaction buffer, TDT enzyme 0.75 µl, 8 µl FITC_dUTP and 32.25 µl ddH2O per well) -. The cells were incubated at 37 ^{O}C for one hour and the staining solution was then removed. The cells were washed with rinse buffer twice after which 50 µl of dapi (4,6-Diamidino-2-phenylinodle) (Sigma, UK) was added for 30 mins at room temperature. Images of cell lines were acquired by fluorescent microscope (Olympus Fluoview, Nikon Eclipse, Japan) while islets were visualised using a laser scanning confocal microscope (Olympus, Japan).

2.2.6 Measurement of anti-apoptotic gene induction following cytokine or endotoxin exposure

The ability of cytokines or endotoxin to induce highly-regulated early anti-apoptotic genes in pancreatic β-cell lines was assessed by reverse transcription polymerase chain reaction (RT-PCR). In the first instance, optimization of primer annealing temperature and confirmation of gene induction was established using end-point semi-quantitative RT-PCR. Subsequently, gene induction was quantified using real-time RT-PCR.

2.2.6.1Extraction and quantification of total RNA

Total RNA was isolated using the RNeasy Mini kit (Qiagen, UK) following exposure of BRIN-BD11 and βTC1.6 cells to cytokines as outlined in Section 2.2.3. Cells were lysed *in situ* with 600 µl/well RLT lysate buffer. Vigorous pipetting was carried out to ensure cells were completely detached and disrepute, the resulting lysate was transferred to QIAshredder minispin column centrifuged at 1700 rpm (439 g) for 2 min.

Following lysis and homogenization the lysate was then transferred to a 1.5 ml microtube to which 300 µl of 70% molecular grade ethanol was added. After mixing thoroughly the resulting solution was transferred to an RNeasy Mini spin column, which was centrifuged for 15 sec at 8000 RPM (9731 g) and the flow through discarded leaving total RNA bound to the column membrane. To wash the column membrane, 700 µl of RW1 washing buffer was added and the column centrifuged at 800 rpm (97 g) for 15 sec. The flow through was discarded and 500 µl of RPE washing buffer was added. The column was again centrifuged at 800 rpm for 15 sec. The flow through was discarded and this step repeated with an extended centrifugation step of 2 mins. The spin column was placed into a fresh, clean, collection tube and centrifuged for 1 min at 1000 rpm to ensure the membrane was completely dried of washing buffers. The collection tube was discarded and replaced by 1.5 ml eppendorf tube. RNase-free water (30 µl) was carefully added to the centre of the spin column membrane and allowed to soak for 2 mins. The column was then centrifuged for 1

min at 1000 rpm and the RNA was eluted from the column into the eppendorf tube below, which was immediately transferred to ice. RNA concentration was quantified using a Nanodrop 2000 (Thermo Scientific, UK) and stored at -80°C until required.

2.2.6.2 Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was diluted with nuclease-free distilled water to a final concentration of 25 ng/µl. One step RT-PCR was performed with Superscript III RT-PCR platinum Taq High Fidelity Kit (Invitrogen). Following manufacturer's instructions a Master Mix was first prepared which comprised 6.25 µl reaction mix, 0.25 µl of each relevant primer (10 µmol), and 4.50 µl free nuclease water, per sample. Then 1 µl of diluted RNA was aliquoted into a 0.2 ml PCR tube on ice. Finally 0.25 µl of Superscript III RT platinum Taq enzyme was added to the Master Mix and 11.5 µl from the Master Mix was added to each sample to give a final reaction volume of 12.5 µl. Wells with no nucleic acid product were used as a negative control for all experiments.

PCR primers were designed using rat gene sequences from NCBI map viewer and designed using Primer3 open-source PCR primer design software and obtained from Invitrogen Ltd. (Paisley, UK). Designed primers were evaluated in NCBI Primer-BLAST to check specificity before being purchased from Invitrogen. Primer sequences and product sizes are listed in Table 2.3. PCR cycling conditions are given in Table 2.4.

Primers	Sequence		Product size
β-Actin	F	“ATGCAGAAGGAGATTACTGC”	218
	R	“TAAAACGCAGCTCAGTAACA”	
A20	F	“CAGATATCCCATCGTCCTTG”	262
	R	“ATCTAACTTGGCAGCATTGA”	
Trail	F	“AAGAGGTGACTTTGAGAACC	193
	R	“GTTTCTATCTTCTGGCCCAA”	
IL-10RB	F	“CTCCCCAGTATGACTTTGAG”	259
	R	“AAGGCGTACTTTGTCTTCTT”	
IL-10RA	F	“TAGACCACATCCCCTTGTTA”	175
	R	“TAGACCACATCCCCTTGTTA”	
TNFRSF10A (TRAIL-R1)	F	“CAAAGAATCAGGCAATGG”	196
	R	“GTGAGCATTGTCCTCAGG”	
TNFRSF10B (TRAIL-R2)	F	“CAGGTGTGATTCAGGCAC”	220
	R	“CCCACTGTGCTTTGTACC”	

Table 2.3 Primer sequences for RT-PCR

Step	Temperature (°C)	Time	Number of cycle
1-Conversion to cDNA	50	30 mins	1
2-Initial denaturation	94	2 mins	1
3-Denaturation	94	15 sec	40
4-Primer annealing	55	30 sec	40
5-Primer extension	68	1 min	40
6-Final extension	15	∞	

Table 2.4 RT-PCR cycling conditions

2.2.6.3 Agarose Gel Electrophoresis

Following RT-PCR amplification, samples were fractionated by agarose gel electrophoresis on a 2% gel (2 g of agarose powder (Fisher scientific, USA) was dissolved in 100 ml 1x tris-acetate EDTA (TAE) buffer (Sigma, UK)). The agarose solution was boiled in a microwave oven until the solution was clear and agarose powder was no longer visible. The hot, dissolved, agarose solution was immediately poured into an agarose gel case containing a comb. After 1 hr at room temperature, the gel had set and was ready to use. The gel was placed into the gel tank (Bio-Rad DNA Sub cell) and 5 µl of each PCR sample was mixed with 5 µl of DNA NonTox Dye (VWR, UK) and then pipetted into the correct well. For comparison of fragment size, direct load wide range DNA marker (Sigma, UK) was also run. All gels were run for one hour at 100 V and were viewed using an Ultraviolet Transilluminator using Syngene gel documentation system (Cambridge, UK).

2.2.6.4 Quantitative real time-polymerase chain reaction (qRT-PCR)

Relative gene expression in cell lines treated with cytokines or endotoxin at different time points (as described in section (2.2.2)) were assessed using the QuantiFast SYBR Green RT-PCR kit (Qiagen, UK). SYBR green is a commonly used fluorescent DNA binding dye, it binds to all double stranded DNA and detection is monitored by measuring the increase in

fluorescence throughout the cycle at the end of either the annealing or the extension step when the greatest amount of double stranded DNA is present. To generate a specific gene amplicon a combination of primers and master mix were used. Replicate reactions for each gene of interest and for housekeeping genes were performed for each sample. To compare the gene expression difference among our samples the ΔΔCt were calculated. Threshold cycle (Ct) value is the cycle number at which the fluorescence generated within the PCR reactions, were a detectable amount. ΔCt value was calculated for each sample as the difference between the Ct for the gene of interest and the house keeping gene. ΔΔCt was measured as the difference between ΔCt values of an experimental sample and the control sample, the fold-change in gene expression was measured as $2^{-(\Delta\Delta Ct)}$.

Samples were prepared by adjusting the concentration of RNA to 100 ng/ml in a final volume of 3.5 µl of RNA with free nuclease disttilled water and this volume placed into each experimental well of a chilled 96 PCR plate (Sigma, UK) and kept on ice till use. A master mix of 6.25 µl SYBR green, 1.25 µl of each relevant primer (10 µM) was prepared. Immediately before the transfer to the PCR machine 0.125 µl/sample of enzyme was added to the master mix thoroughly mixed and 8.8 µl of the master mix was added to each sample well, an optical adhesive cover (Thermo fisher, UK) was used to seal the plate and then transferred to a Stratagene MX3005P real time thermal cycler. The thermal cycler was programmed as shown in Table 2.5.

Step	Temperature (°C)	Time	Number of cycles
1-Conversion to cDNA	50	10 mins	1
2-Initial denaturation	95	5 mins	1
3-Denaturation	95	10 sec	40
4-Primer annealing	55	30 sec	40
5-Primer extension and Fluorescent determination	60	30 sec	40
6-Pre-programmed melt curve and Fluorescent determination as temperature increased from 60°C to 95°C	95	1 mins	1
	60	30 sec	1
	95	30 sec	1

Table 2.5 qRT-PCR cycling conditions

2.2.7 Stimulation and measurement of insulin secretion from β-cell lines

To determine the effect of conditioned media on insulin secretion from pancreatic β-cells, cell lines were seeded at a density of $1x10^5$ cells/well/24-well plate and allowed to attach overnight. Following this step the cells were treated with pro-inflammatory cytokines (IFN-γ. TNF-α, IL-1β and endotoxin LPS) with and without conditioned media as described above (Section 2.2.3). Glucose solutions were prepared in 1x Hepes buffered saline (HBS comprising 10 mM Hepes, 145 mM NaCl, 5 mM Kcl and 1 mM MgSO4) at three different concentrations (1.1 mM, 5.6 mM and 16.7 mM D-Glucose). After exposing the cells to cytokines for 24 hours the media was removed and the cells were washed twice with 1 ml HBS followed by addition of 1.1 mM glucose solution for 40 mins to prime the cells. This step was followed by removal of the glucose solution and addition of 1.1, 5.6 or 16.7 mM glucose solution for a further 20 mins after which the supernatant was removed and kept at -20 °C for further analysis.

Cells were lysed using 200 µl/well/24-well plate RIPA buffer (Sigma, UK) and transferred to fresh eppendorf tubes, which were maintained on ice with regular vortexing for 20 mins.

Lysates were centrifuged at full speed and 4 °C for 20 mins. The total protein present in the resulting supernatants was quantified using the Pierce BCA Protein Assay Kit (Thermo scientific, UK). Following the kit instruction BCA working reagent was prepared by adding 50 parts of BCA reagent A to one part of BCA reagent B, 25 µl of standard, blank and samples were added to each well of 96-well plate along with 200µl of working reagent. The plate was covered and incubated at 37 ^{O}C for 30 mins. The plate was cooled to room temperature and read at 562 nm on a microplate reader (Dynatech, MR5000 version 3.7).

2.2.7.1 Insulin Enzyme linked immunosorbent assay (ELISA)

Insulin secretion into the supernatants was quantified using ALPCO ELISA kits (ALPCO, USA) according to the manufacturer's instructions. Briefly, 10 µl of control, standard and samples were added to the provided insulin-coated 96-well microplate. This was followed by the addition of 75 µl of working strength conjugate solution to each well. The microplate was sealed and incubated for 2 hours on a shaker (700-900 rpm) at room temperature. The contents of the wells were then discarded and the plate washed 6 times by filling the wells with the provided wash buffer using a wash bottle. The plate was inverted to discard the wash buffer and firmly tapped on absorbent paper towel between each washing step. Then 100 µl of TMB (tetramethyl benzidine) substrate was added to each well and the microplate was sealed and incubated for 15 mins at room temperature on a plate shaker (700 – 900 rpm). Following this step, 100 µl of the provided stop solution was added to each well and the plate gently agitated to mix the contents. Absorbance was read using a microplate reader (Dynatech, MR5000 version 3.7) at a wavelength of 450 nm. A standard curve was plotted using linear regression as shown in Figure 2.2 and insulin values interpolated from the x-axis.

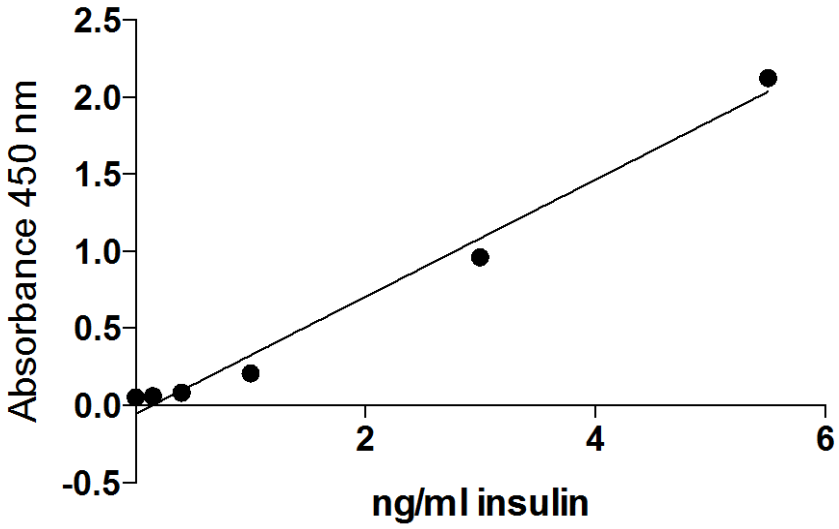

Figure 2.2 A typical standard curve from insulin ELISA assay. Insulin standards were prepared at concentrations of 5.5, 3.0, 1.5, 0.75, 0.25 ng/ml. Values are mean ± SD (n = 3). Interpolation of x-axis was used for determination of insulin concentrations of unknown samples

2.2.8 Measurement of cytokine and growth factor concentration by Enzyme linked immunosorbent assay (ELISA)

Following identification of candidates from a secretome screen of MSC-CM, ELISA was used to quantify the concentration of interleukin-4 (IL-4), interleukin-10 (IL-10), vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) in our MSC-CM. ELISA development kits were purchased from PeproTech (UK) and assay were developed for each cytokine or growth factor according to the manufacturer's instructions as briefly outlined below.

Capture antibody was diluted in PBS to a concentration of 1.0 µg/ml for IL-10, VEGF, PIGF and 0.5 µg/ml for IL-4. Capture antibody solution (100 µl/well/96 well plate) was added to a 96-well Polystyrene Immulon Microtiter plate (Nunc). The plate was sealed with an adhesive film and incubated overnight at room temperature. The next day, the capture antibody was aspirated from the wells and the plate washed 4 times using 300 µl/well of washing buffer

(0.05% Tween-20 in PBS). Following this, 300 µl of blocking buffer (1% BSA in PBS) was added to each well and the plates were sealed and left to incubate at room temperature for an hour. The plates were then washed again four times using 300 µl/well washing buffer. Standards were prepared for IL-4, IL-10, VEGF and PIGF to a concentration of 1ng/ml, 2.5ng/ml, 1ng/ml and 1 ng/ml respectively. Serial dilutions were conducted for each standard to generate standard curves as shown in Figure 2.3. Standards and samples (100 µl/well) were added to the plates in duplicate. The plates were sealed and incubated at room temperature for two hours. The plates were then washed four times with 300 µl/well washing buffer and detection antibody was prepared in PBS as shown in Table 2.5 to a concentration of 0.5 µg/ml for IL-10, IL-4, PIGF and to a concentration of 0.25ug/ml for VEGF. Detection antibody solution (100 µl/well) was added to each well and the plates were allowed to incubate at room temperature for 2 hours. The plates were then washed four times with 300 µl/well washing buffer and 5.5 µl of avidin-HRP conjugate was diluted in 11 ml diluent (0.05% Tween-20, 0.1% BSA in PBS) and 100 µl was added to each well. The plates were incubated for 30 mins at room temperature. The plates were once more washed four times with 300 µl/well washing buffer and 100 µl of ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) (Thermo Fisher, UK) liquid substrate was added. The colour development was monitored with a microplate reader (Dynatech, MR5000 version 3.7) plate reader at 405 nm with a wavelength correction set at 650 nm.

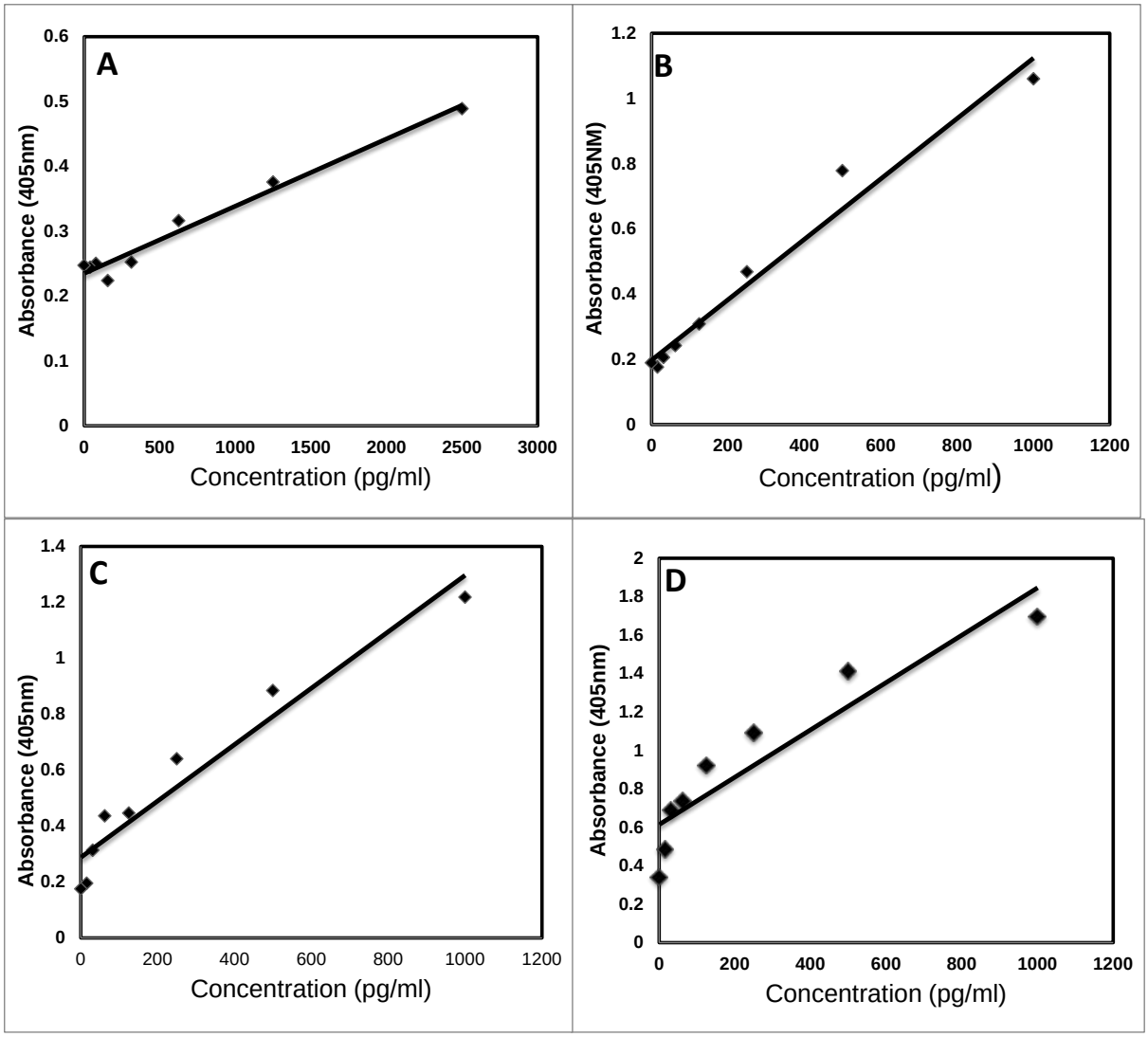

Figure 2.3 Typical standard curves from ELISA assays (A) IL-10 standards were prepared at concentrations of 2500,2000, 1500,1000, 500, 250, 125, 75, 37.5 pg/ml. (B) VEGF, (C) PIGF, (D) IL-4 standards were prepared at concentrations of 1000, 500, 250, 125, 75, 37.5, 18.5 pg/ml/ Values are mean ± SD (n = 3). Interpolation of x-axis was used for determination of cytokine concentrations of unknown samples. IL-10, Interleukin 10; VEGF, vascular endothelial growth factor; PIGF, placental growth factor; IL-4, interleukin 4.

2.2.9 Statistical analysis

Data is presented as mean plus or minus standard deviation (SD) for a given number of observations. Groups of data were compared using one way ANOVA test and two-tailed unpaired Student *t*-tests (Graphpad, PRISM software, USA), with significance being accepted if $P<0.05$.

Chapter 3

Establishment and characterization of *in vitro* model of cytokine-driven β-cell apoptosis

3.1 Introduction

T1DM is a chronic metabolic disorder that is characterized by an inability to release insulin, which results from autoimmune destruction of β-cells in the pancreas.[172] β-cells sense increasing levels of blood glucose and respond by secreting the insulin hormone through a pathway that involves the coupling of glucose uptake and glycolysis with quantitative ATP production via the mitochondrial oxidative pathway.[172] Therefore, oxidative phosphorylation is essential for normal β-cell function. β-cell dysfunction and reduction in their mass are the decisive events in the progression of T1DM. An increase in the concentration of pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-1β was detected both in blood and in pancreatic islets of people with diabetes. Pro-inflammatory cytokines are secreted from activated macrophages that invade the pancreas. These cytokines mediate inflammation through upregulating transcription of pro-inflammatory genes in response to the presence of a foreign body. However, a lack of regulation of pro-inflammatory cytokine production will result in the development of an autoimmune disease like T1DM.[173] There is increasing evidence that these cytokines are responsible for β-cell destruction in T1DM. However, the mechanisms by which cytokines mediate their effects are still unclear.

Recent studies suggest that β-cells become hypoxic under high glucose conditions due to increased oxygen consumption and that pancreatic islets of diabetic mice are moderately hypoxic.[174] However, the impact of moderate hypoxia on β-cell number and function is unclear.[174] Hypoxia could be defined as the lower level of oxygen than that normally experienced by a specific type of cells.[175] Changes in oxygenation could happen as part of normal physiological or developmental process and include hypoxia gradients established during embryonic development. Alternatively, hypoxia may arise as a result of a pathological process like myocardial infarction.[176] Different levels of oxygen will have an impact on mitochondrial activity and ATP production and it is also believed that cells undergo very large changes in gene expression in response to alterations in oxygenation.[177] However,

there is some uncertainty about the precise oxygen concentration experienced by cells in normal tissue.

Some studies suggest that many cells have the ability to adapt to hypoxia through a molecular mechanism regulated by hypoxia-inducible factor (HIF-1α).[172] It is believed now that HIF-1α has a role in the regulation of systemic metabolism and glucose haemostasis in tissue like muscle, liver, and β-cells. There is accumulating evidence that pro-inflammatory cytokines can increase mitochondrial reactive oxygen species (ROS) generation resulting in their accumulation in the intracellular compartment.[178] These mediators exert their effect on transcription factors including HIF-1α by ROS-dependent mechanisms.[179] The activation of HIF-1α is believed to have a negative effect on β-cells[174]. However, it is still unclear how hypoxia affects mitochondrial function in β-cells. In fact, little research has been done on the effect of hypoxia on respiratory capacities and mitochondrial coupling states in β-cells. The primary aim of this study was to assess the *in vitro* effect of pro-inflammatory cytokines on β-cells viability under normoxic (21% O_2) and hypoxic (10% O_2) culturing conditions.

The advance in technical and analytical methods has identified several genes that are upregulated during the apoptosis process like the zinc finger TNF Alpha-Induced Protein 3 (A20). A20 has been identified as the most highly regulated anti-apoptotic gene in the pancreatic beta cell. [180]A20 was originally characterized as a protein that protects cells from TNF-induced cytotoxicity. However, it is now believed that A20 has a dual function as an inhibitor of nuclear factor-kB (NF-κB) activation and apoptosis in TNF receptor 1 signalling pathway (TNFR1) (Figure 3.1).[180] However, the molecular mechanisms by which A20 controls its multiple activities are not fully understood. Some studies demonstrate that the effect of A20 on cell death, mainly depends on the type of cells and there is some sort of balance between, it is anti-apoptotic properties and it impacts on NF-κB inhibition with consequence for the expression of the anti-apoptotic gene.[180]

Another gene that is also found to be upregulated is TNF-related apoptosis-inducing ligand (TRAIL), it is a cytokine functioning as a ligand that induces cell death by binding to certain death receptors like DR4 and DR5 initiating apoptosis which is caspase 8 dependent which will activate a series of effector caspase including caspase 3, 6, and 7 that will lead to cell degradation.[181] Experimental data suggests that TNF-related apoptosis-inducing ligand (TRAIL), an immune system modulator protein, is important in the pathogenesis of type 1 diabetes. Recent evidence from glioblastoma cells now suggests that A20 regulates TRAIL-mediated apoptosis through inhibition of caspase-8.[181] Prior work has shown that addition of recombinant TRAIL protects against beta cell.[182] The TRAIL and A20 genes were upregulated in this study in both BRIN-BD11 and βTC1.6 cell lines in normoxia and hypoxia after exposing them to cytokines.

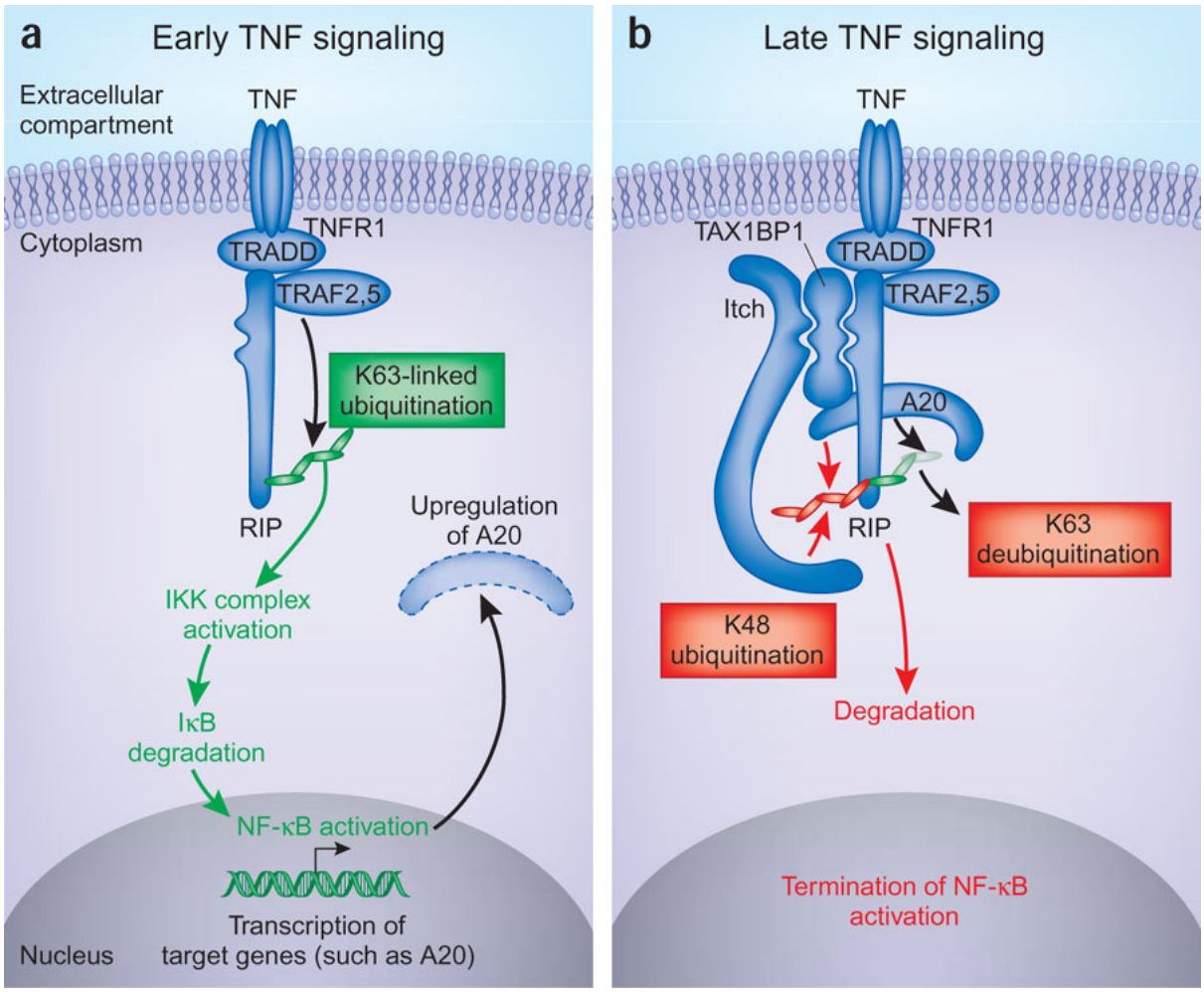

Figure 3.1TNFR1 signal transduction. This figure elucidate the TNFR1 signal transduction and termination by ubiquitin (a) show the early TNF signal transduction that involves activation of RIP leading to activation of NF-kB and A20 upregulation. (b) The late TNF signal involves the formation of a quaternary complex from A20 and RIP leading to the termination of NF-kB activity. This figure reprinted from the following article (Itching to end NF-kB Vigo heissmeyer and Anjana Rao Nature immunology 9, 227-229(2008)doi:10.1038/ni308-227).

3.2 Methods

3.2.1 Materials

Analytical grade reagents and deionized water (Sigma, UK) were used. All chemicals employed are listed in Chapter 2, Section 2.1.

3.2.2 Cell models

The pancreatic β-cell lines BRIN-BD11 and βTC1.6 were cultured, maintained and passaged as outlined in Chapter 2, Section 2.2.1.1. Primary murine islets were isolated after collagenase digestion of the pancreas as described in Section 2.2.1.2. All cell models were maintained in an incubator at an atmosphere of 37 °C and 5% CO_2 under normoxic (21% O_2) and hypoxic (10% O_2) conditions.

3.2.3 Induction of apoptosis by cytokines and endotoxin

Cell lines and primary islets were exposed to recombinant IFN-γ, TNF-α, IL-1B and bacterial LPS as outlined in Chapter 2, Section 2.2.3.

3.2.4 Measurement of cellular viability and apoptosis

Changes in cellular viability following cytokine or endotoxin treatment were assessed by colorimetric MTT assay as outlined in Chapter 2, Section 2.2.4, whilst induction of apoptosis was measured by TUNEL assay as described in Section 2.2.5.

3.2.5 Assessment of early response anti-apoptotic gene induction

Following treatment of cells with cytokines or endotoxin as outlined in Chapter 2, Section 2.2.3, the induction of early response anti-apoptotic genes known to be important in β- cell survival was assessed by PCR as outlined in Section 2.2.6.

3.3 Results

3.3.1 Islets characterization

Primary pancreatic islets isolated from CD-1 mice were characterized by expression of β-cell markers by immunohistochemistry. As shown in Figure 3.2, islets stained positive for the glucose transporter GLUT2, transcription factor HNF1α, potassium channel subunits (Kir6.2, KCNQ11), insulin, and Insulin Receptor Substrate 1 (IRS-1).

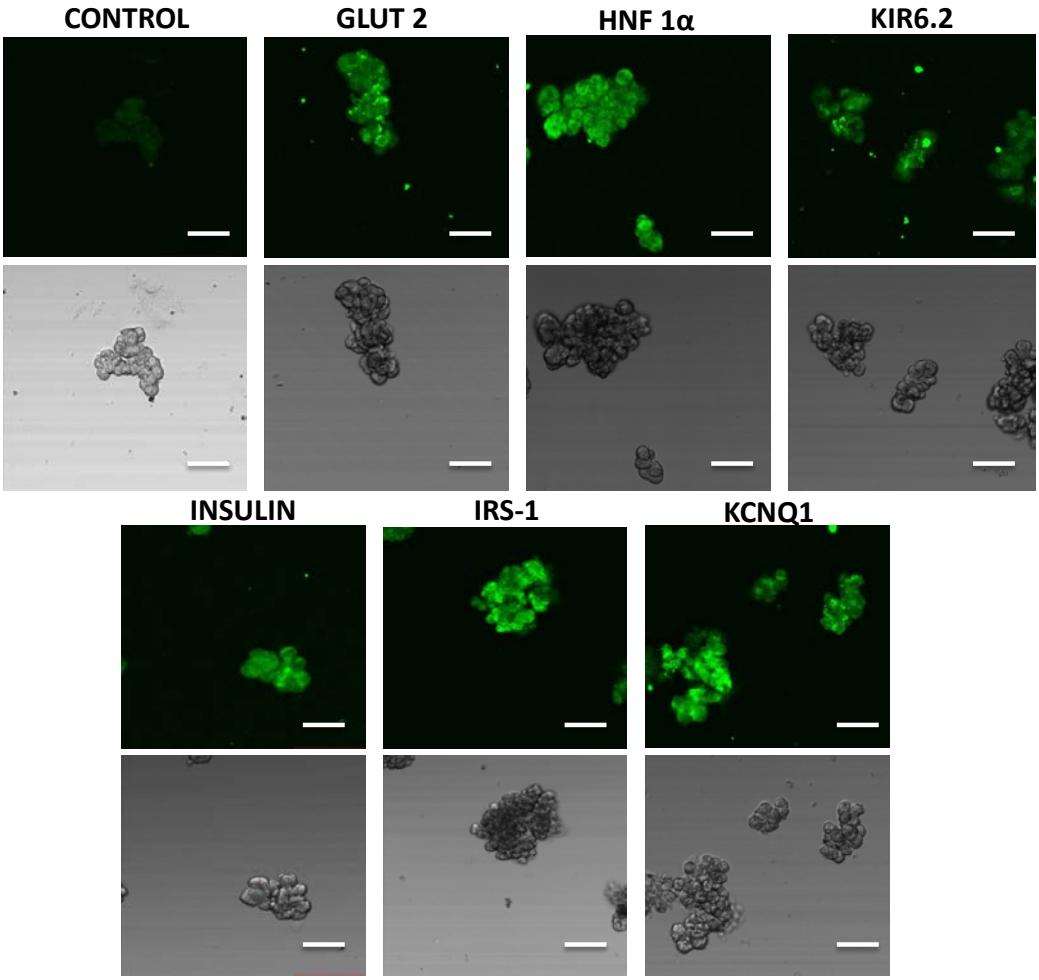

Figure 3.2 Immunostaining images of pancreatic islets isolated from CD-1 mice. Confocal laser scanning microscope images showing a positive staining (green) for β-cells markers (GLUT2, HNF-1α, KIR6.2, INSULIN, IRS-1, and KCNQ1). Microscope magnification used is 20X and the scale bar in all images is 100 µm

3.3.2 Exposure to pro-inflammatory cytokines and LPS resulted in a reduction in β-cell viability

The effect of rising concentrations of IFN-γ, TNF-α, IL-1β and LPS on the metabolic activity of BRIN-BD11, βTC1.6 and primary islets was first explored via the MTT assay. In this instance the MTT assay was being used as a surrogate marker of viability. The aim of these experiments was to optimize the concentrations at which cell viability was reduced by approximately 50% for use in subsequent experiments. MTT data is presented as normalised to the corresponding experimental control in Figures 3.2 – 3.9. Cells were grown under normoxic and hypoxic conditions and in the presence or absence of serum to determine if oxygen concentration or the inclusion of serum sensitized the cells to any of the cytokines or LPS.

3.3.2.1 Determination of pancreatic cell sensitivity to IFN-γ

Following exposure to 0 - 1 µg/ml IFN-γ, cell viability was assessed (Figures 3.3 and 3.4). In all instances, primary islets were more sensitive to the effects of IFN-γ than the β-cell lines. As shown in Figure 3.2, exposure to 1 µg/ml IFN-γ resulted in respective reductions of (-48%) (P<0.0001) and (-47%) (P<0.0001) viability in BRIN-BD11 and βTC1.6 cells grown under normoxic conditions in the presence of serum. Under the same growth conditions, primary islets displayed a (-47%) (P<0.001) reduction in viability in response to 100 ng/ml IFN-γ (Figure 3.2). Similar trends were observed in BRIN-BD11, βTC1.6 and primary islets grown under hypoxic conditions in the presence of serum (Figure 3.2). The culture of cells in the absence of serum did not significantly affect the results (Figure 3.3). As such, 1 µg/ml IFN-γ was chosen for future experiments involving BRIN-BD11 and βTC1.6 cells while 100 ng/ml IFN-γ was chosen for subsequent experiments involving primary islets.

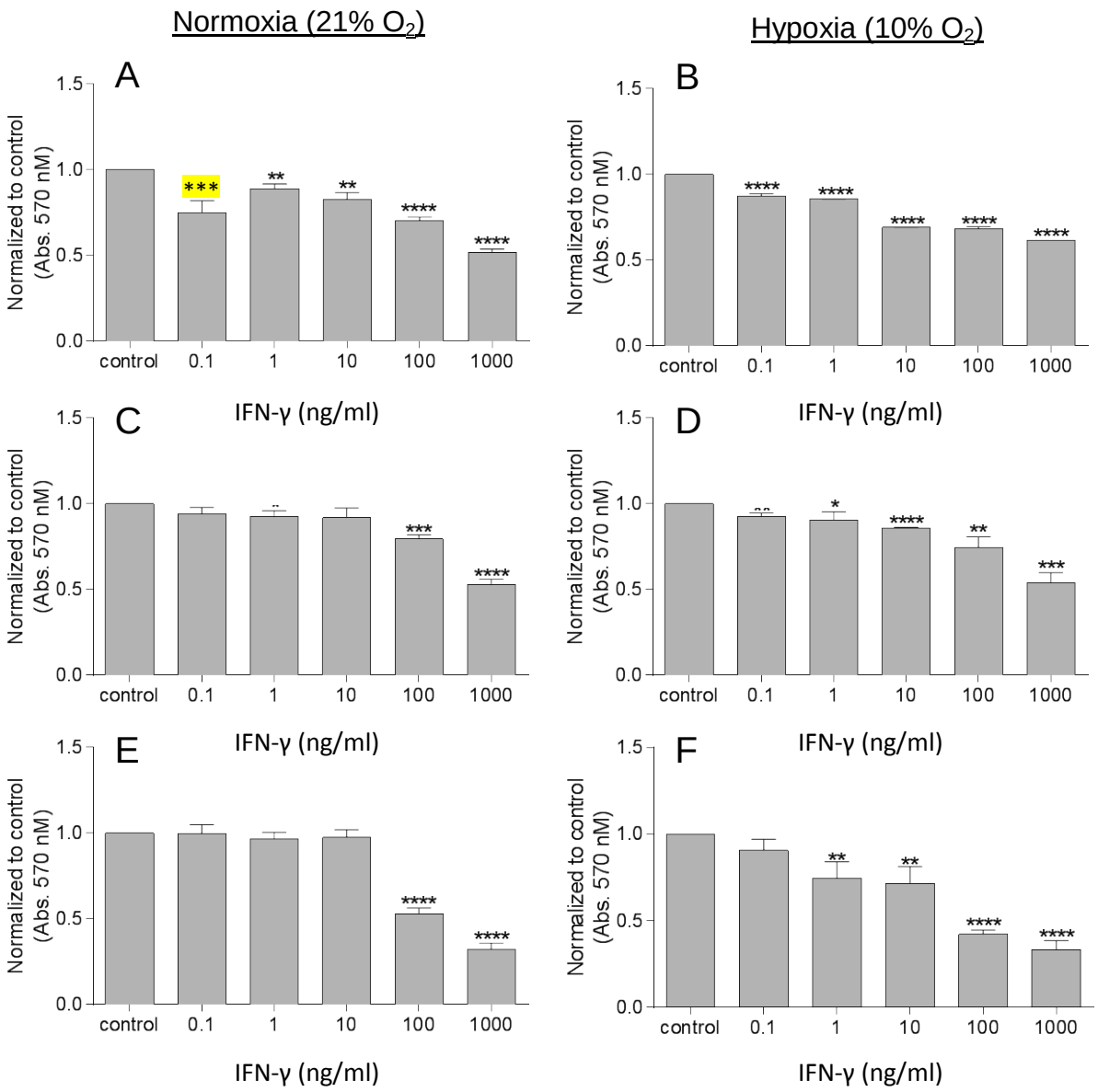

Figure 3.3 ***Effect of IFN-γ on the viability of pancreatic cells cultured in the presence of serum***. Following exposure to rising concentration of IFN-γ for 24 h, the cellular viability of BRIN-BD11 cells (A, B), βTC1.6 cells (C, D) and primary islets (E, F) grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. IFN-γ, Interferon gamma.

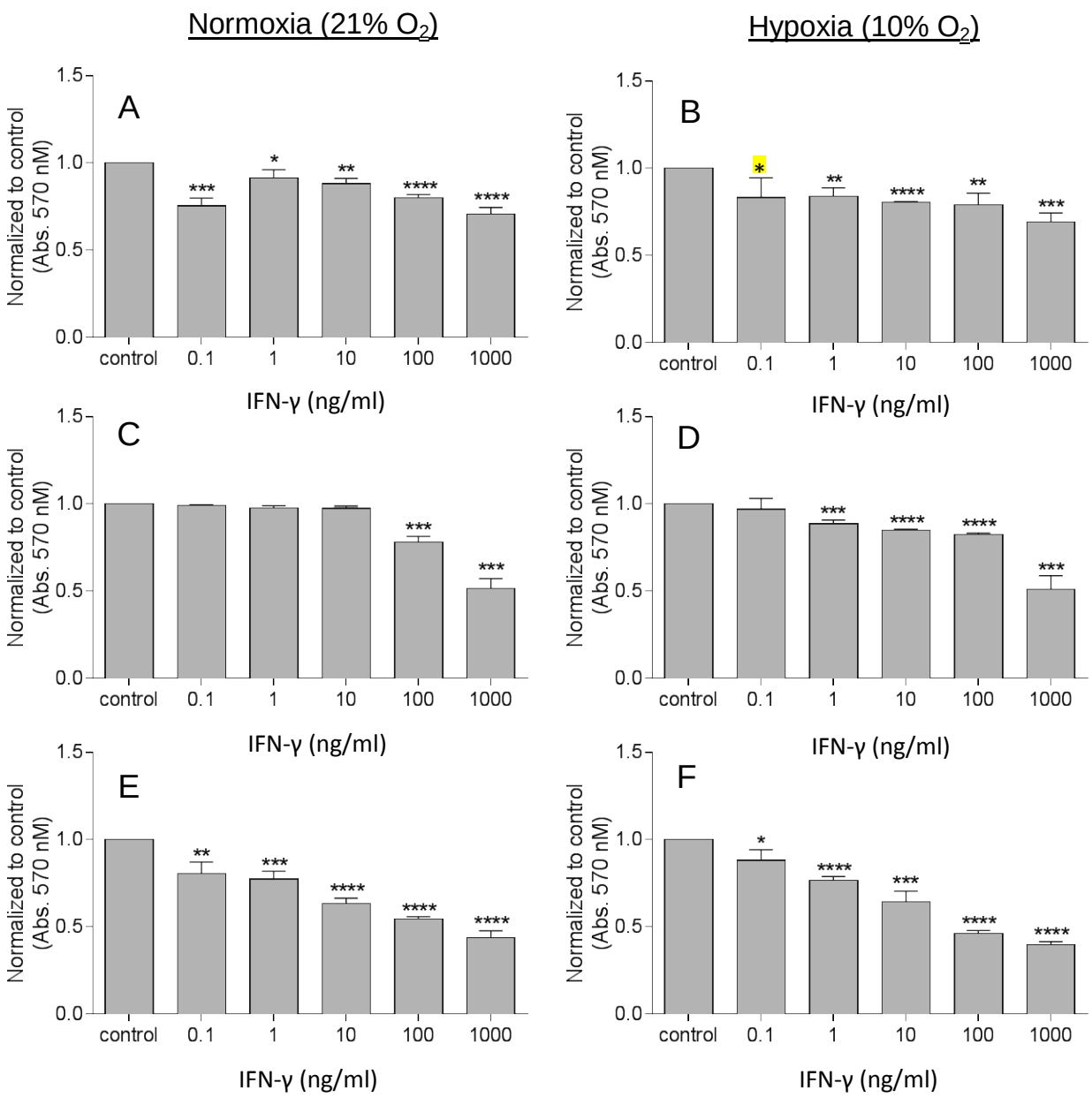

Figure 3.4 ***Effect of IFN-γ on the viability of pancreatic cells cultured in the absence of serum***. Following exposure to rising concentration of IFN-γ for 24 h, the cellular viability of BRIN-BD11 cells (A, B), βTC1.6 cells (C, D) and primary islets (E, F) grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. IFN-γ, Interferon gamma.

3.3.2.2 Determination of pancreatic cell sensitivity to TNF-α

Figure 3.4 demonstrates the ability of 100 ng/ml TNF-α to significantly reduce (P<0.001) the viability of BRIN-BD11 (-46%) and primary islets (-47%) as measured by MTT. Under the same growth conditions βTC1.6 cells displayed a reduction (-43%) (P<0.001) in viability in response to 1 µg/ml of TNF-α. Similar trends were observed in BRIN-BD11, βTC1.6 and primary islets grown under hypoxic conditions in the presence of serum (Figure 3.5).

The absence of serum did not significantly impact on βTC1.6 and primary islet viability. As such, 1 µg/ml TNF-α was chosen for future experiments involving βTC1.6 cells and 100 ng/ml TNF-α was chosen for subsequent experiments involving primary islets (Figure 3.6). For the BRIN-BD11 cell line, 1 µg/ml was chosen for subsequent experiments in the absence of serum (rather than the 100 ng/ml required in the presence of serum) as it showed the highest reduction (-29%; P<0.001) under normoxic conditions. However, under hypoxic conditions, BRIN-BD11 cells grown without serum showed the highest degree of sensitivity in response to 100 ng/ml (-35%; P<0.01) (Figure 3.6).

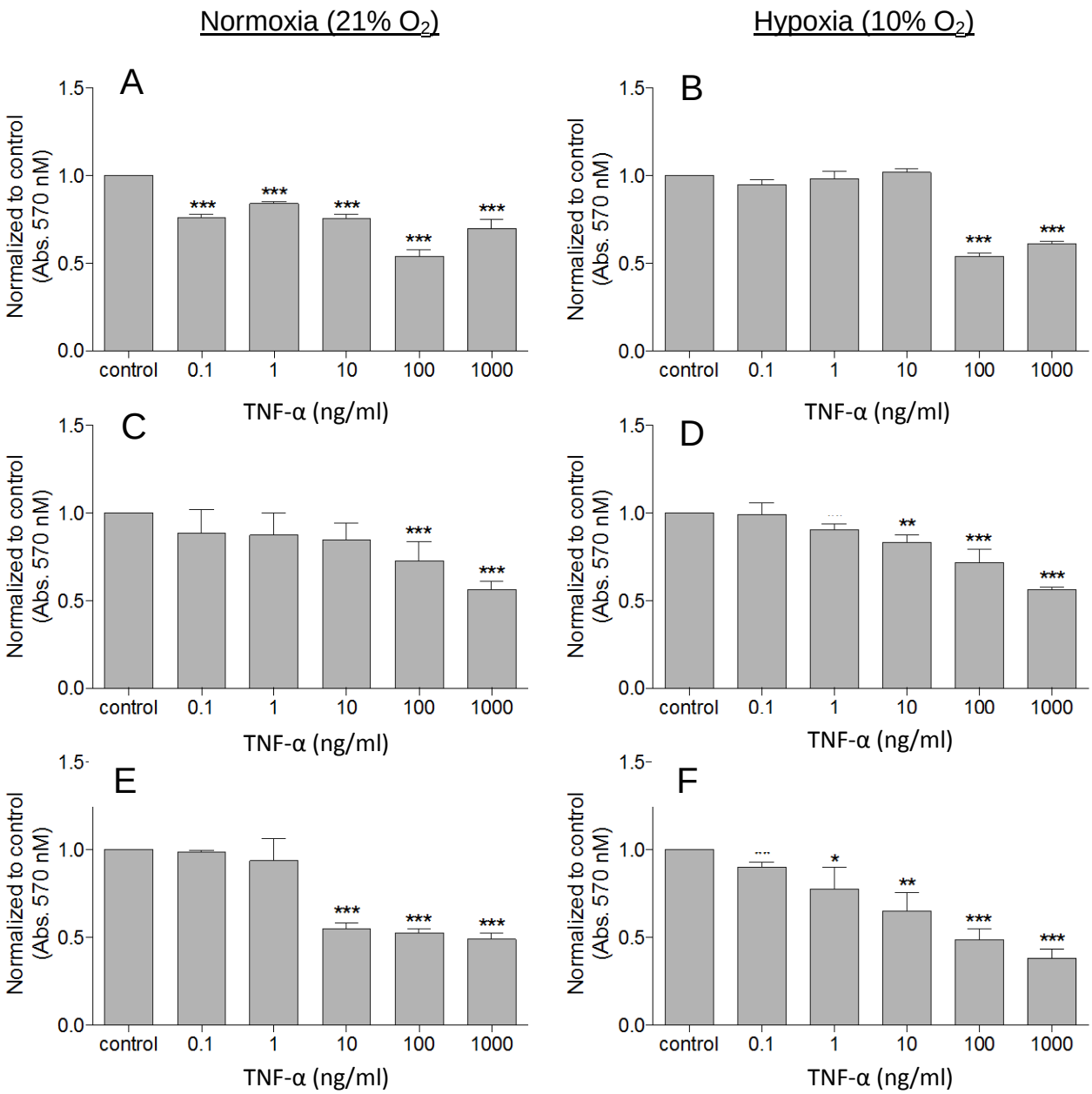

Figure 3.5 Effect of TNF-α on the viability of pancreatic cells cultured in the presence of serum. Following exposure to rising concentration of TNF-α for 24 h, the cellular viability of BRIN-BD11 cells (A, B), βTC1.6 cells (C, D) and primary islets (E, F) grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 compared with untreated controls. TNF-α, Tumour Necrosis Factor alpha.

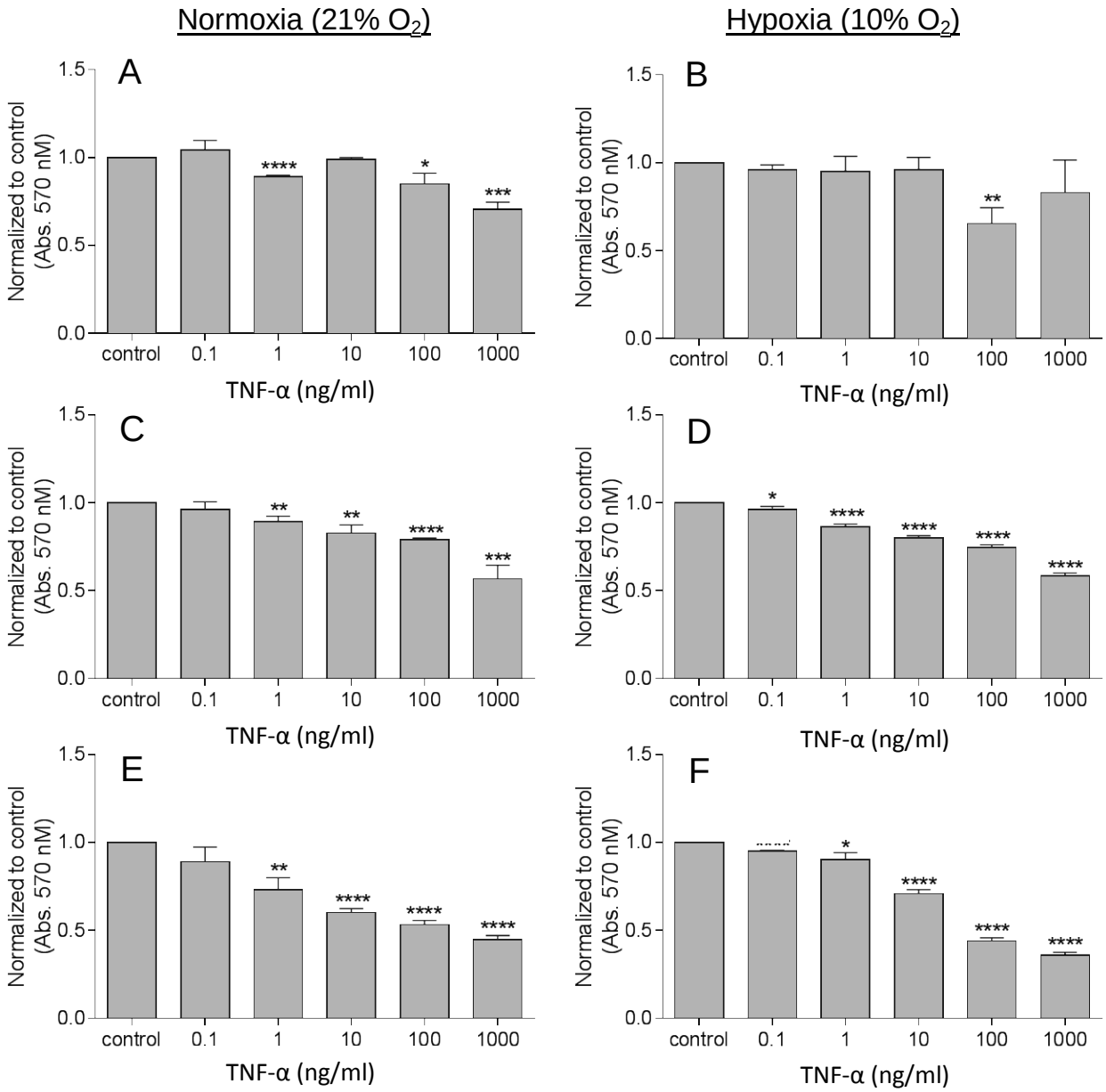

Figure 3.6. Effect of TNF-α on the viability of pancreatic cells cultured in the absence of serum. Following exposure to rising concentration of TNF-α for 24 h, the cellular viability of BRIN-BD11 cells (A, B), βTC1.6 cells (C, D) and primary islets (E, F) grown under normoxic or hypoxia conditions was assessed by colorimetric MTT assay. **P*<0.05, ***P*<0.01, ****P*<0.001 and ****P*<0.0001 compared with untreated controls. TNF-α, Tumor Necrosis Factor alpha

3.3.2.3 Determination of pancreatic cell sensitivity to IL-1β

Normalized MTT assays demonstrated significant reductions in the metabolic activity as an indication of viability of cells under normoxic and hypoxic conditions following exposure to IL-1β (Figures

3.7 and 3.8). Figure 3.6 demonstrates the ability of 100 ng/ml of IL-1β to significantly reduce the viability of BRIN-BD11 cells (-51%; P<0.0001), βTC1.6 cells (-43%; P<0.0001), and primary islets (-32%; P<0.01) in the presence of serum. BRIN-BD11 and βTC1.6 cells cultured under hypoxic conditions showed a similar trend (Figure 3.7). However, primary islets grown under hypoxic conditions were more sensitive to the effects of IL-1β. In this instance, 10 ng/ml was able to significantly reduce (-50%; P<0.0001) the viability of the cells.

The culture of cells in the absence of serum did not significantly impact the viability (Figure 3.7). As such, 100 ng/ml of IL-1β was chosen for future experiments involving BRIN-BD11 cells, βTC1.6 cells, and primary islets. Consistent trends were observed under hypoxic conditions for all cell types (Figure 3.8).

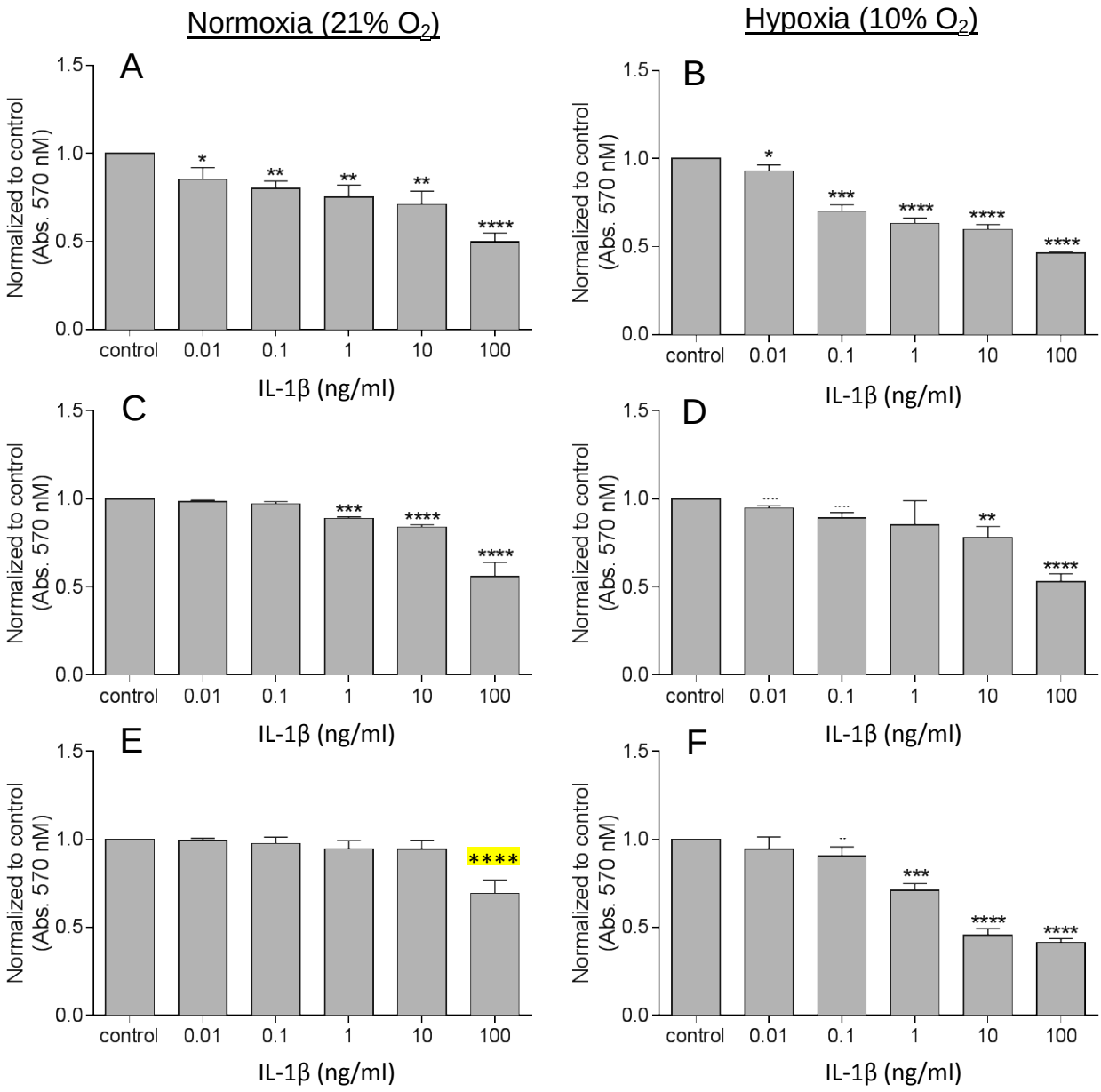

Figure 3.7. Effect of IL-1β on the viability of pancreatic cells cultured in the presence of serum. Following exposure to rising concentration of IL-1β for 24 h, the cellular viability of BRIN-BD11 cells (A, B), βTC1.6 cells (C, D) and primary islets (E, F) grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. IL-1β, Interleukin-1 beta.

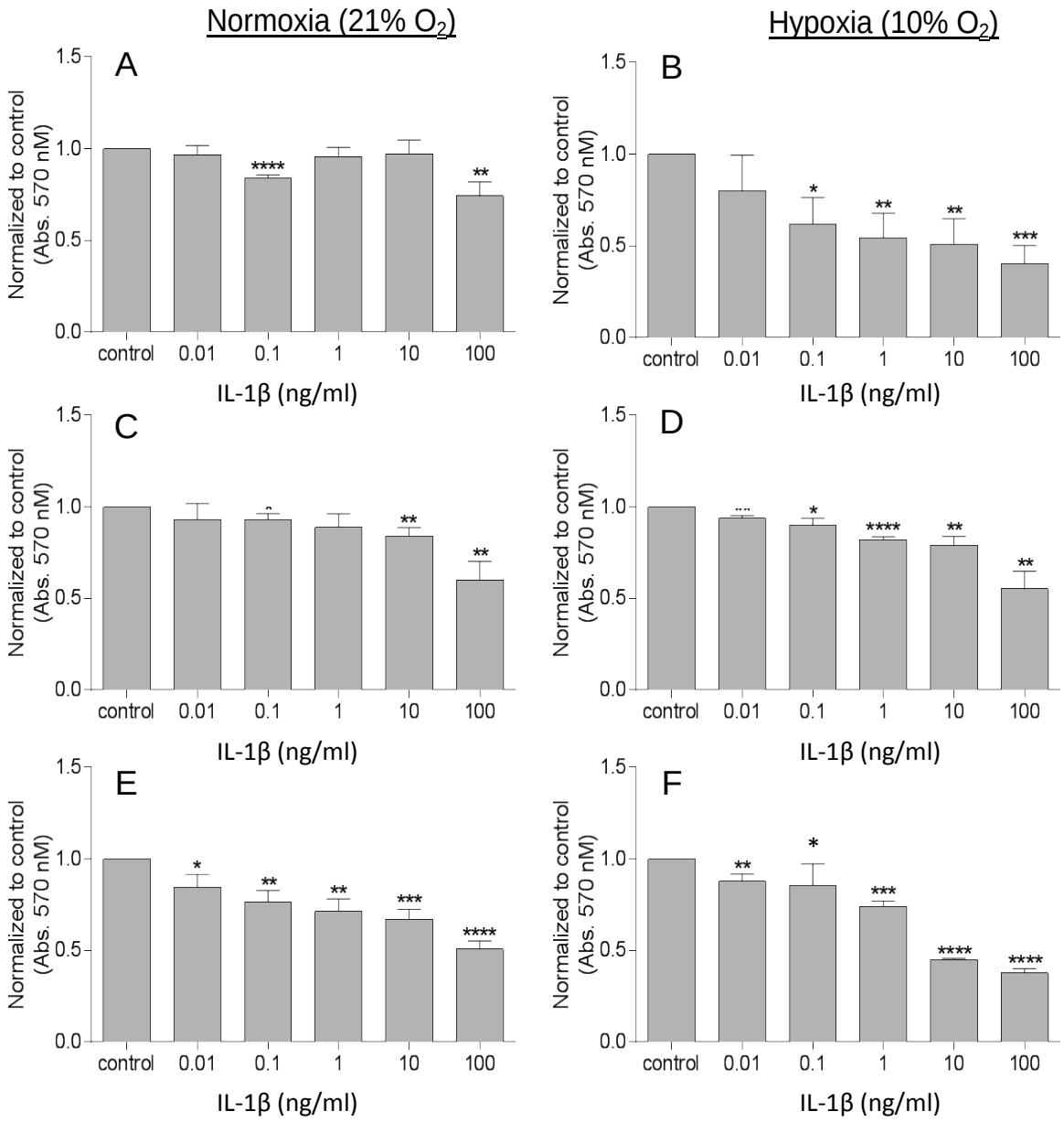

Figure 3.8. Effect of IL-1β on the viability of pancreatic cells cultured in the absence of serum. Following exposure to rising concentration of IL-1β for 24 h, the cellular viability of BRIN-BD11 cells (A, B), βTC1.6 cells (C, D) and primary islets (E, F) grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. IL-1β, Interleukin-1 beta.

3.3.2.4 Determination of pancreatic cell sensitivity to LPS

Normalized MTT assays demonstrated significant reductions in the viability of cells grown under normoxic and hypoxic conditions following exposure to LPS. Figure 3.8 shows that 500 µg/ml of LPS significantly reduced the viability of BRIN-BD11 (-49%; P<0.0001) and βTC1.6 (-44%; P<0.0001) cells. Under the same growth conditions, primary islets displayed a (-48%; P<0.0001) reduction in viability in response to 10 µg/ml. Similar trends were observed in BRIN-BD11, βTC1.6 and primary islets grown under hypoxic conditions in the presence of serum (Figure 3.9). In the absence of serum the concentration chosen for subsequent experiments involving BRIN-BD11, βTC1.6 and primary islets was also 500 µg/ml (-30%; P<0.0001), (-51%; P<0.0001), and (-44%; P<0.0001) respectively (Figure 3.10). For cells grown under hypoxia the dose chosen was 10 µg/ml (-49%; P<0.0001) for BRIN-BD11 cells, 500 µg/ml (-41%; P<0.001) for βTC1.6 cells, and 100 µg/ml (-36%; P<0.0001) for primary islets.

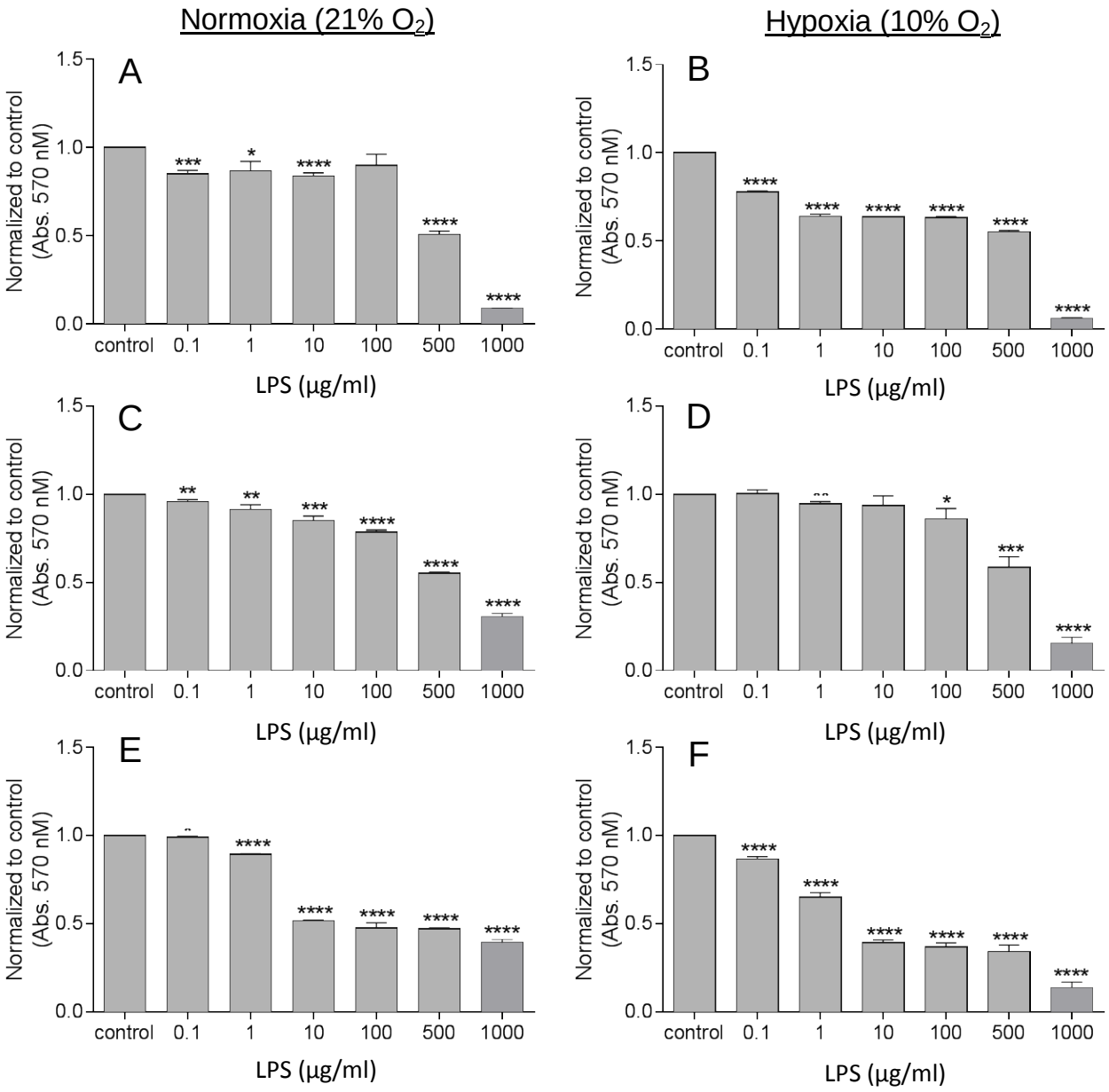

Figure 3.9. Effect of LPS on the viability of pancreatic cells cultured in the presence of serum. Following exposure to rising concentration of LPS for 24 h, the cellular viability of BRIN-BD11 cells (A, B), βTC1.6 cells (C, D) and primary islets (E, F) grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. LPS lipopolysaccharide.

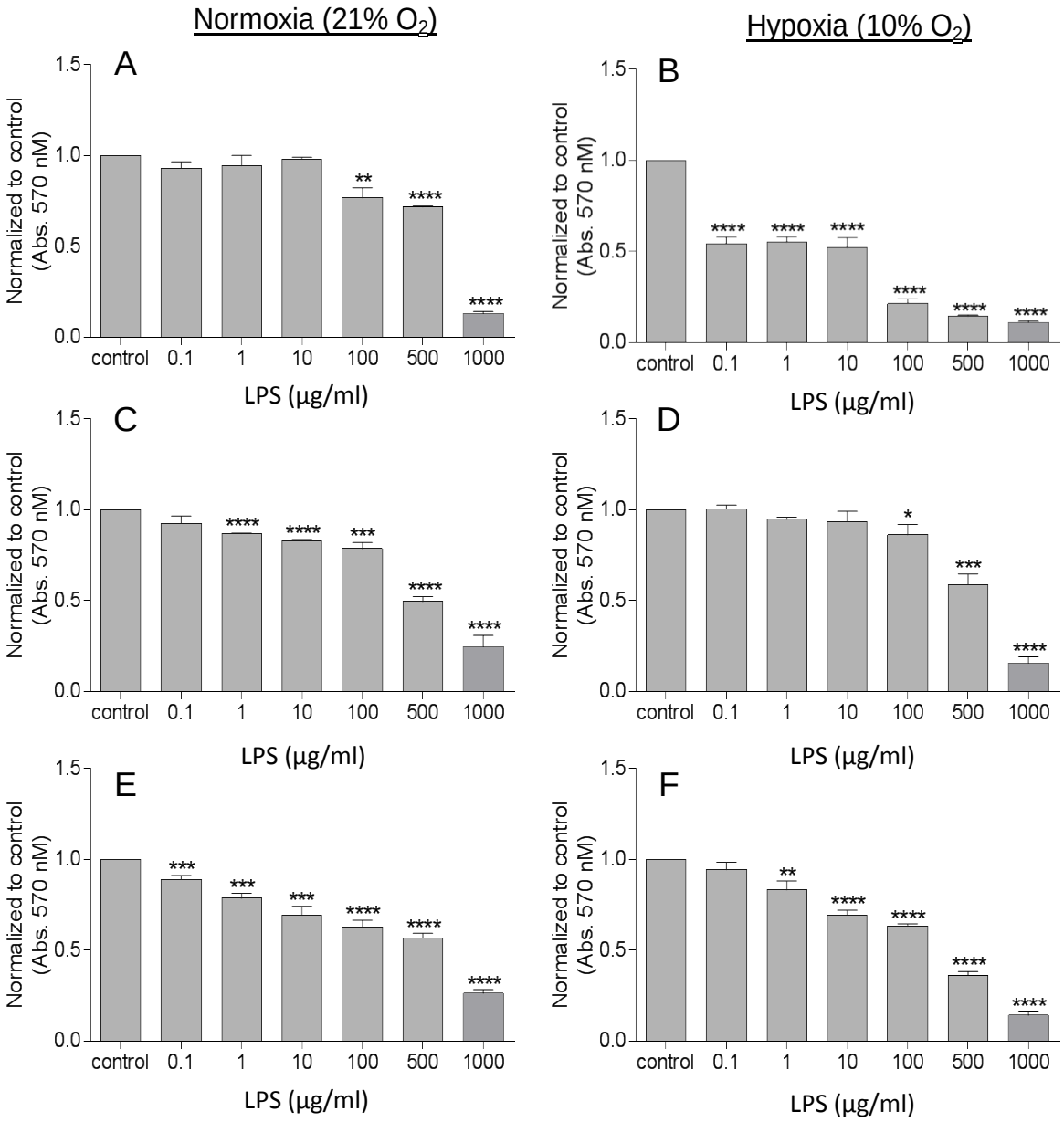

Figure 3.10. Effect of LPS on the viability of pancreatic cells cultured in the absence of serum. Following exposure to rising concentration of LPS for 24 h, the cellular viability of BRIN-BD11 cells (A, B), βTC1.6 cells (C, D) and primary islets (E, F) grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. LPS lipopolysaccharide.

3.3.3 Cytokine-induced apoptosis

To establish if the reduction in cellular viability was due to programmed cell death (apoptosis), the TUNEL assay was performed as outlined in Chapter 2, Section 2.2.5. Significant apoptosis was detected in BRIN-BD11 cells by IFN-γ (1 µg/ml in media with and without serum) grown under normoxia and hypoxia conditions, TNF-α (100 ng/ml for media with serum grown under normoxic and hypoxic conditions, 1 µg/ml for media without serum under normoxic conditions, and 100 ng/ml for cells grown under hypoxic condition), IL-1β (100 ng/ml for media with and without serum grown under normoxic and hypoxic conditions), and LPS (500 µg/ml for media with and without serum grown under normoxic and hypoxic conditions). Apoptosis was induced in βTC1.6 cells in response to IFN-γ (1 µg/ml), TNF-α (1 µg/ml), IL-1β (100 ng/ml) and LPS (500 µg/ml) grown in media with and without serum under normoxic and hypoxic conditions. H_2O_2 was used as a positive control in all instances (Figures 3.11-3.18).

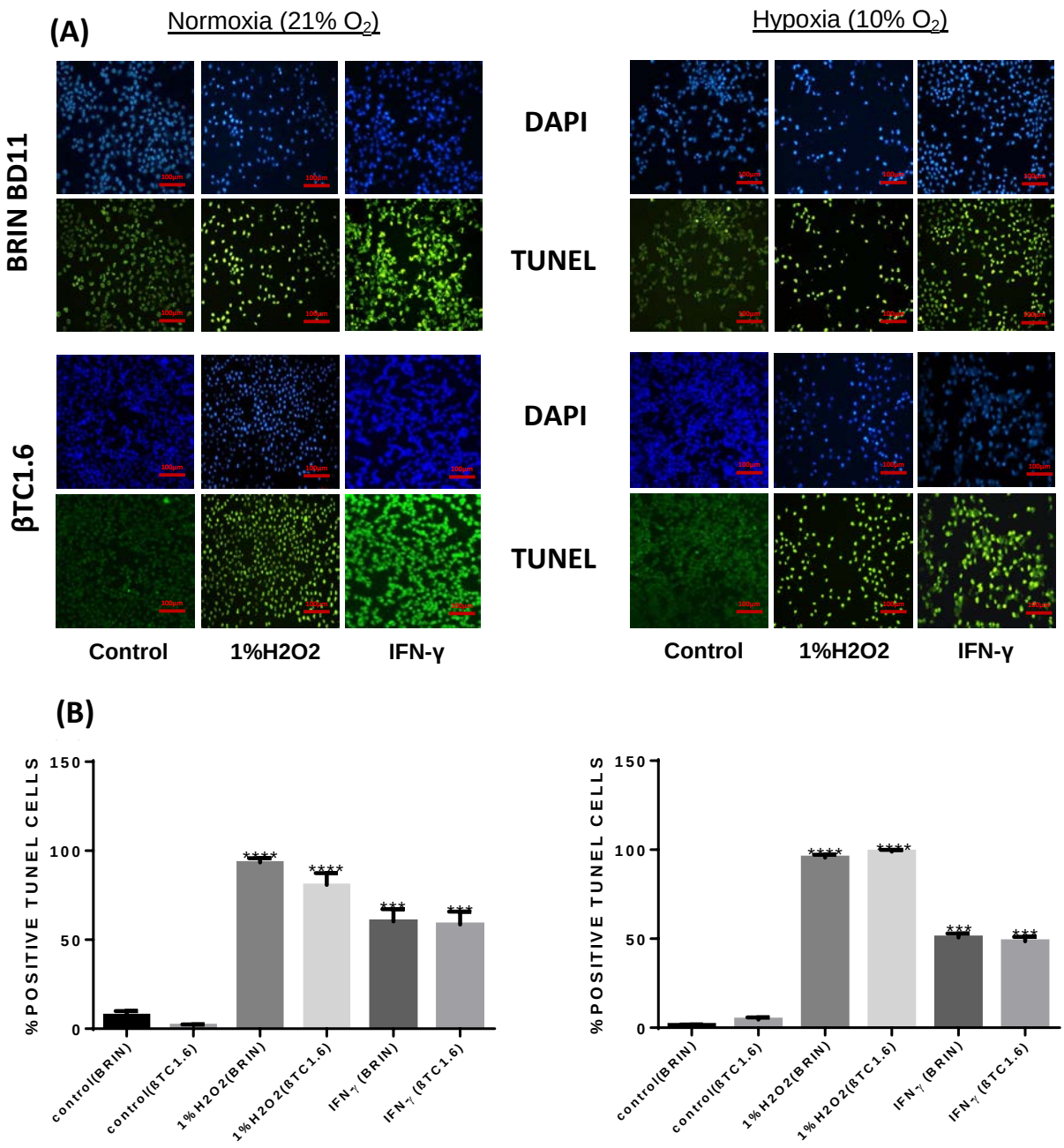

***Figure 3.11. Assessment of IFN-γ-induced apoptosis in pancreatic cell lines grown under normoxic and hypoxic conditions in the presence of serum*. (A)** Fluorescent images of IFN-γ-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines grown under normoxia and hypoxia after 24 hours of exposure to 1 µg/ml of IFN-γ. Blue staining represents DAPI staining of the nuclei, while green staining indicates TUNEL positive cells. **(B)** The % positive TUNEL cells was measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****P*<0.001 and *****P*<0.0001 compared with untreated controls.

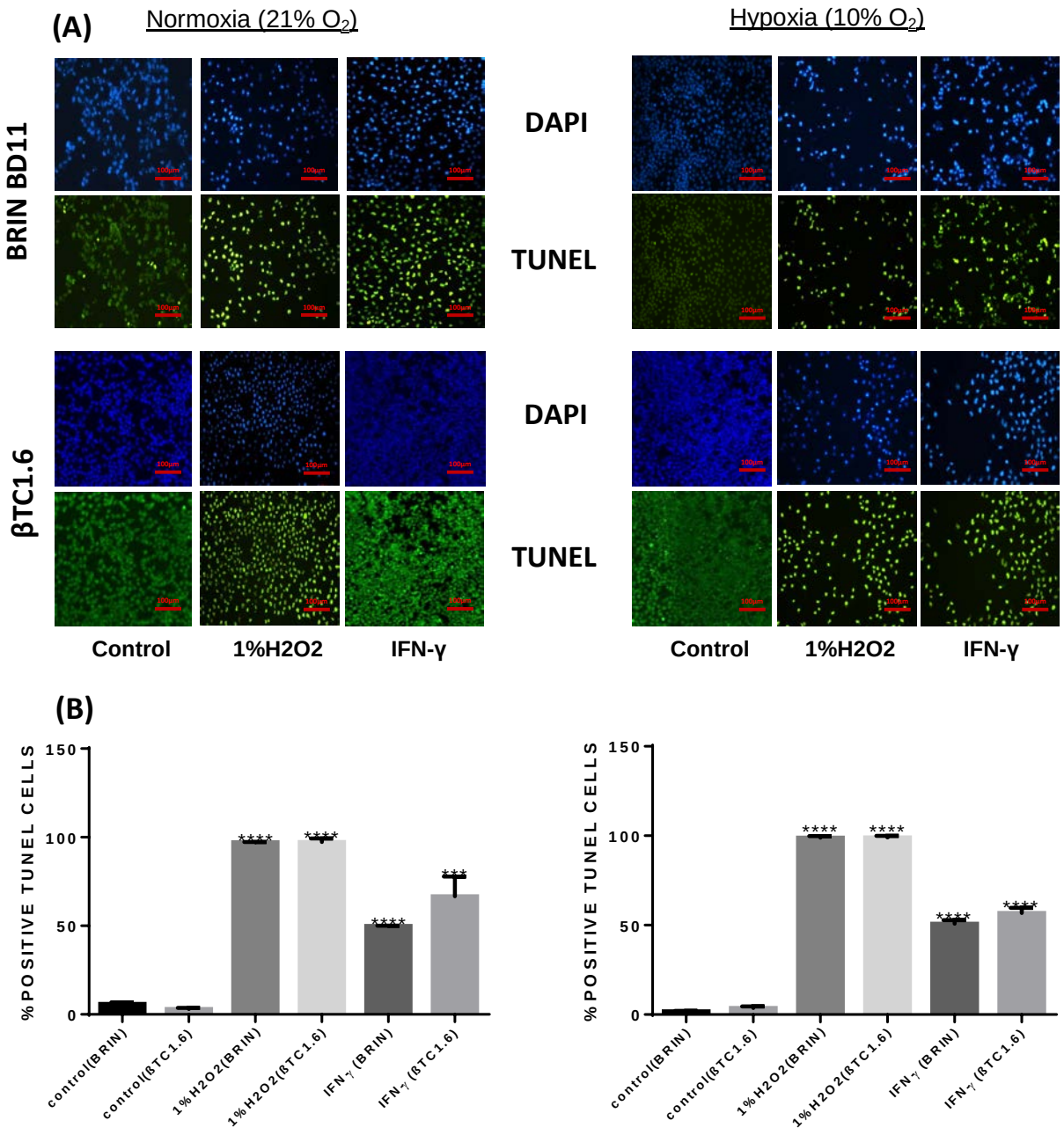

Figure 3.12. Assessment of IFN-γ-induced apoptosis in pancreatic cell lines grown under normoxic and hypoxic conditions in the absence of serum. **(A)** Fluorescent images of IFN-γ-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines grown under normoxic and hypoxic conditions after 24 hours of exposure to 1 µg/ml of IFN-γ. Blue staining represents DAPI staining of the nuclei while green staining indicates TUNEL positive cells. **(B)** The %positive TUNEL cells was measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ***P<0.001 and ****P<0.0001 compared with untreated controls.

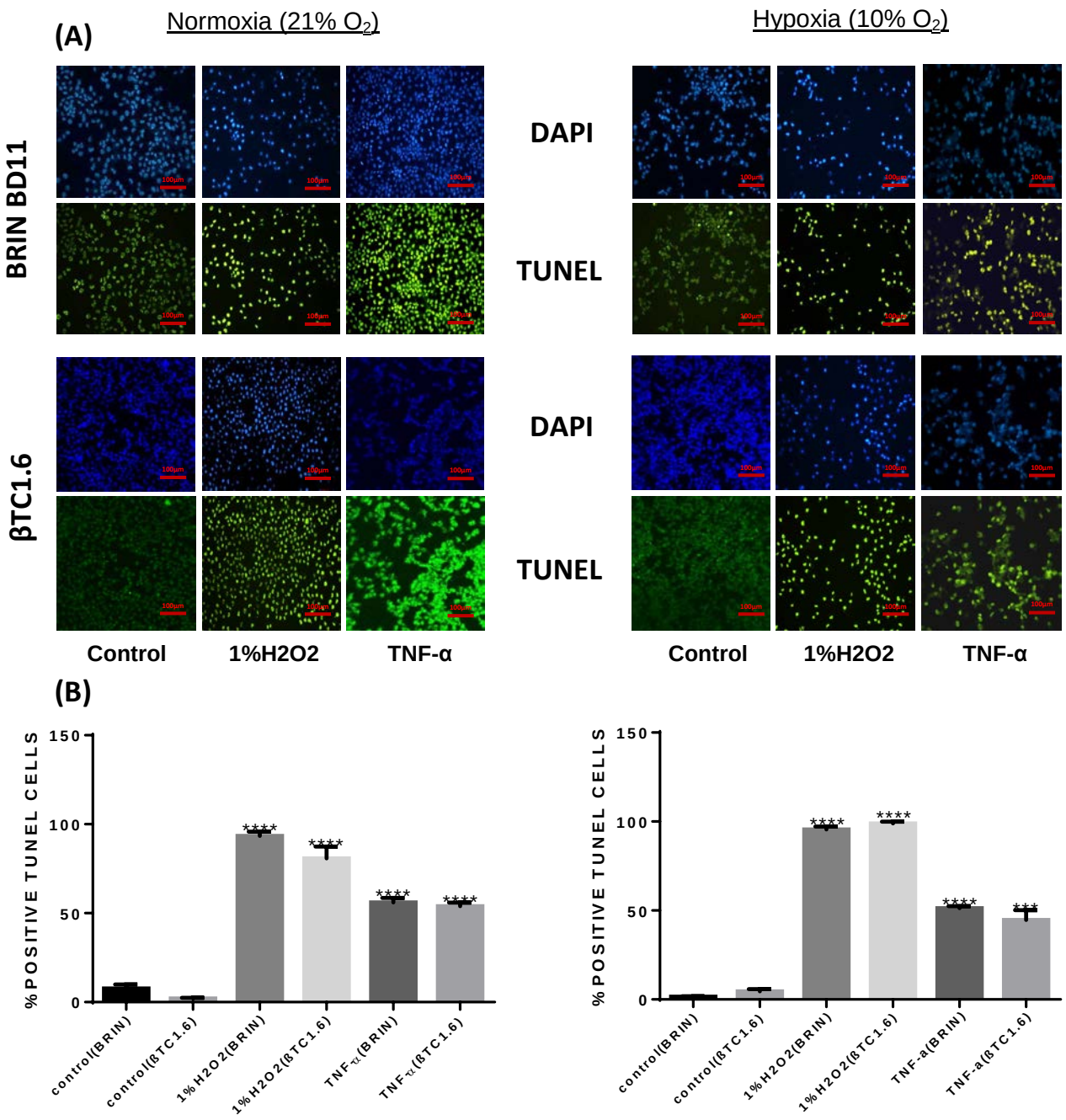

Figure 3.13. Assessment TNF-α-induced apoptosis in pancreatic cell lines grown under normoxic and hypoxic conditions in the presence of serum. **(A)** Fluorescent images of TNF-α-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines grown under normoxia and hypoxia after 24 hours of exposure to 100 ng/ml and 1 µg/ml of TNF-α respectively. Blue nuclei are DAPI staining and the green nuclei are TUNEL positive nuclei. **(B)** The %positive TUNEL cells was measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ***P<0.001 and ****P<0.0001 compared with untreated controls.

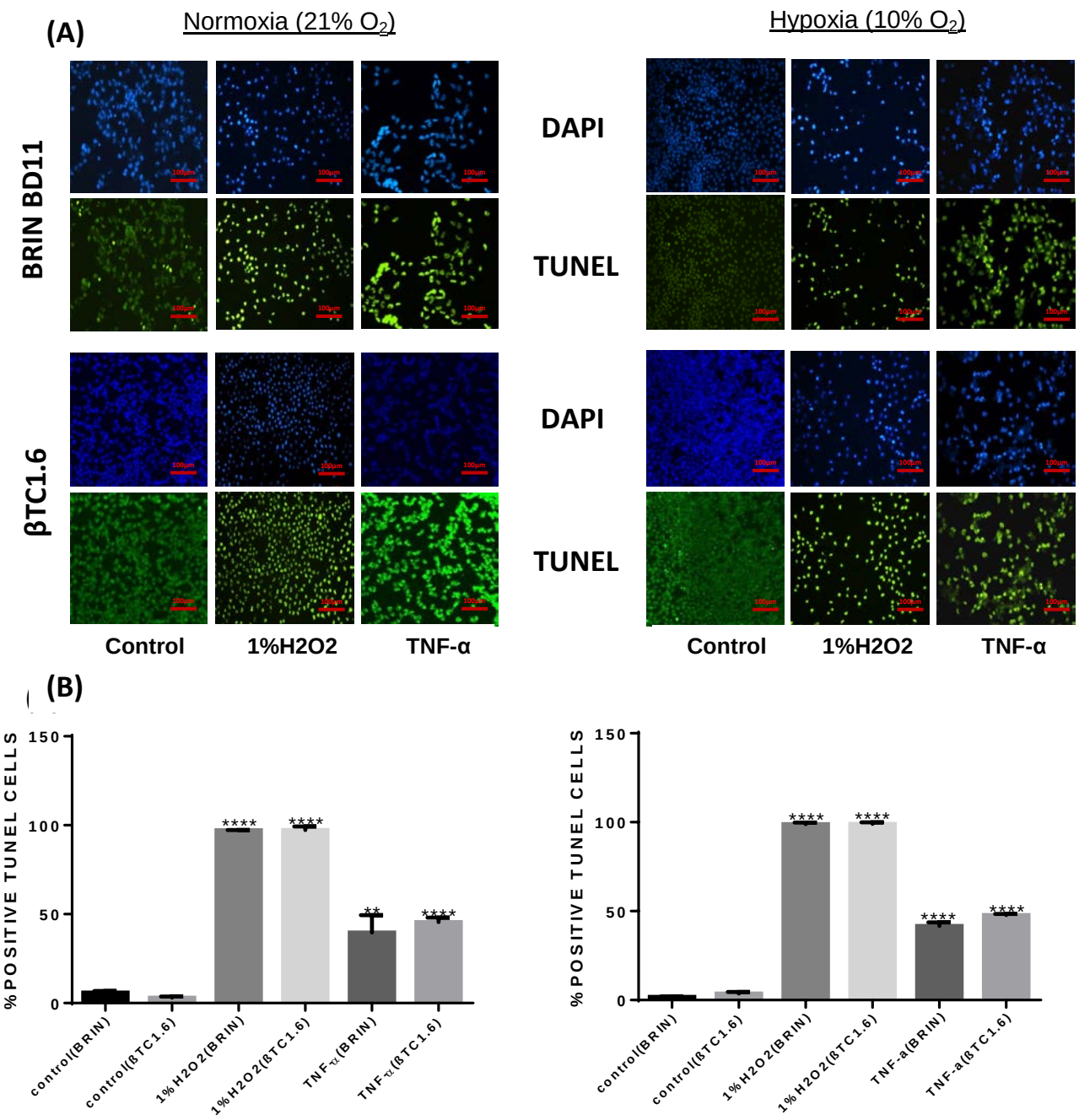

Figure 3.14. Assessment of TNF-α-induced apoptosis in pancreatic cell lines grown under normoxic and hypoxic conditions in the absence of serum. **(A)** Fluorescent images of TNF-α-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines grown under normoxic conditions after 24 hours of exposure to 1 µg/ml of TNF-α. Cells grown under hypoxic conditions were exposed to 100 ng/ml (BRIN-BD11 cells) or 1 µg/ml (βTC1.6) of TNF-α. Blue nuclei are DAPI staining and the green nuclei are TUNEL positive nuclei. **(B)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls.

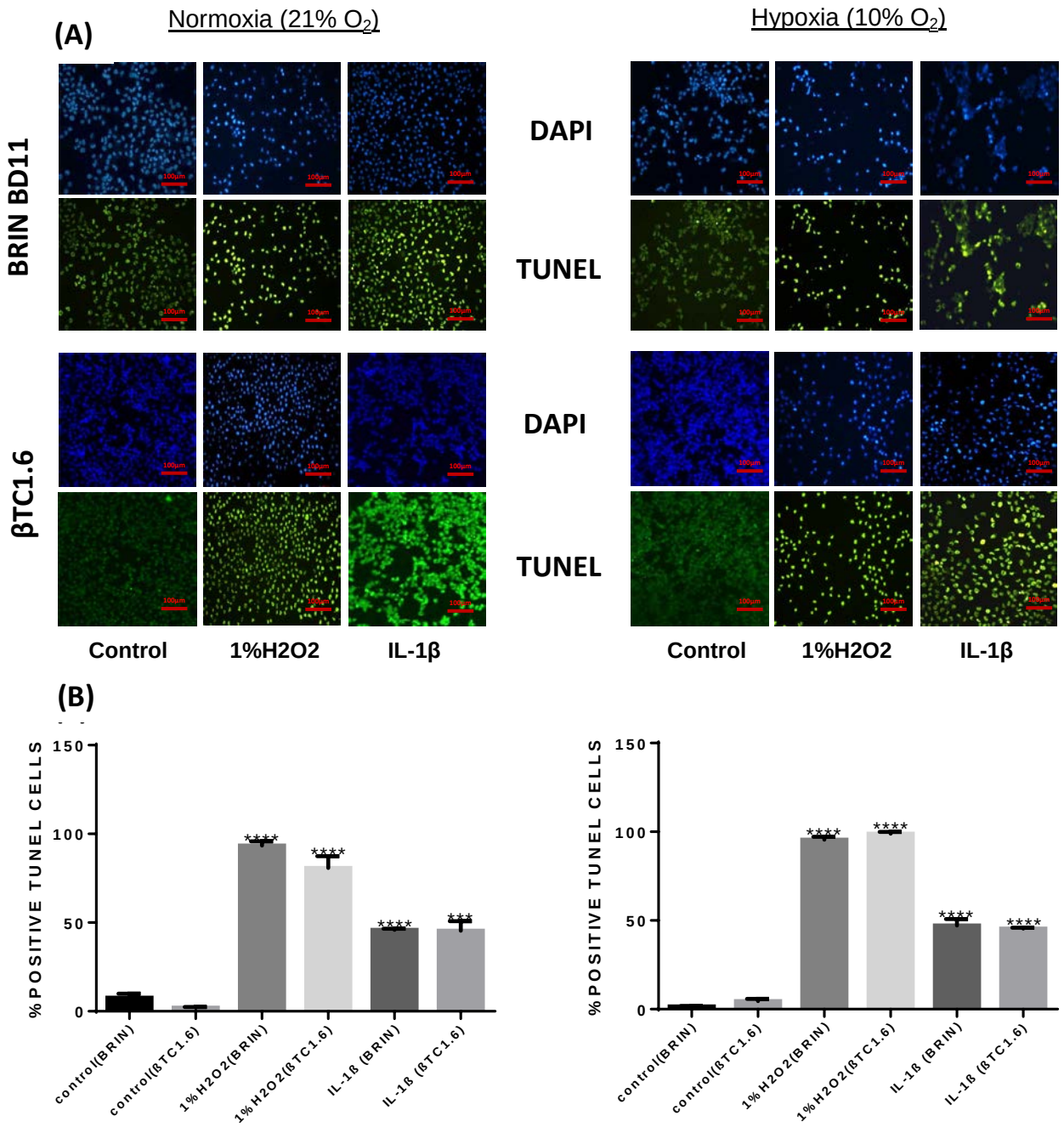

Figure 3.15. Assessment of IL-1β-induced apoptosis in pancreatic cell lines grown under normoxic and hypoxic conditions in the presence of serum. **(A)** Fluorescent images of IL-1β-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines grown under normoxia and hypoxia after 24 hours of exposure to 100 ng/ml of IL-1β. Blue nuclei are DAPI staining and the green nuclei are TUNEL positive nuclei. **(B)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****P*<0.001 and *****P*<0.0001 compared with untreated controls.

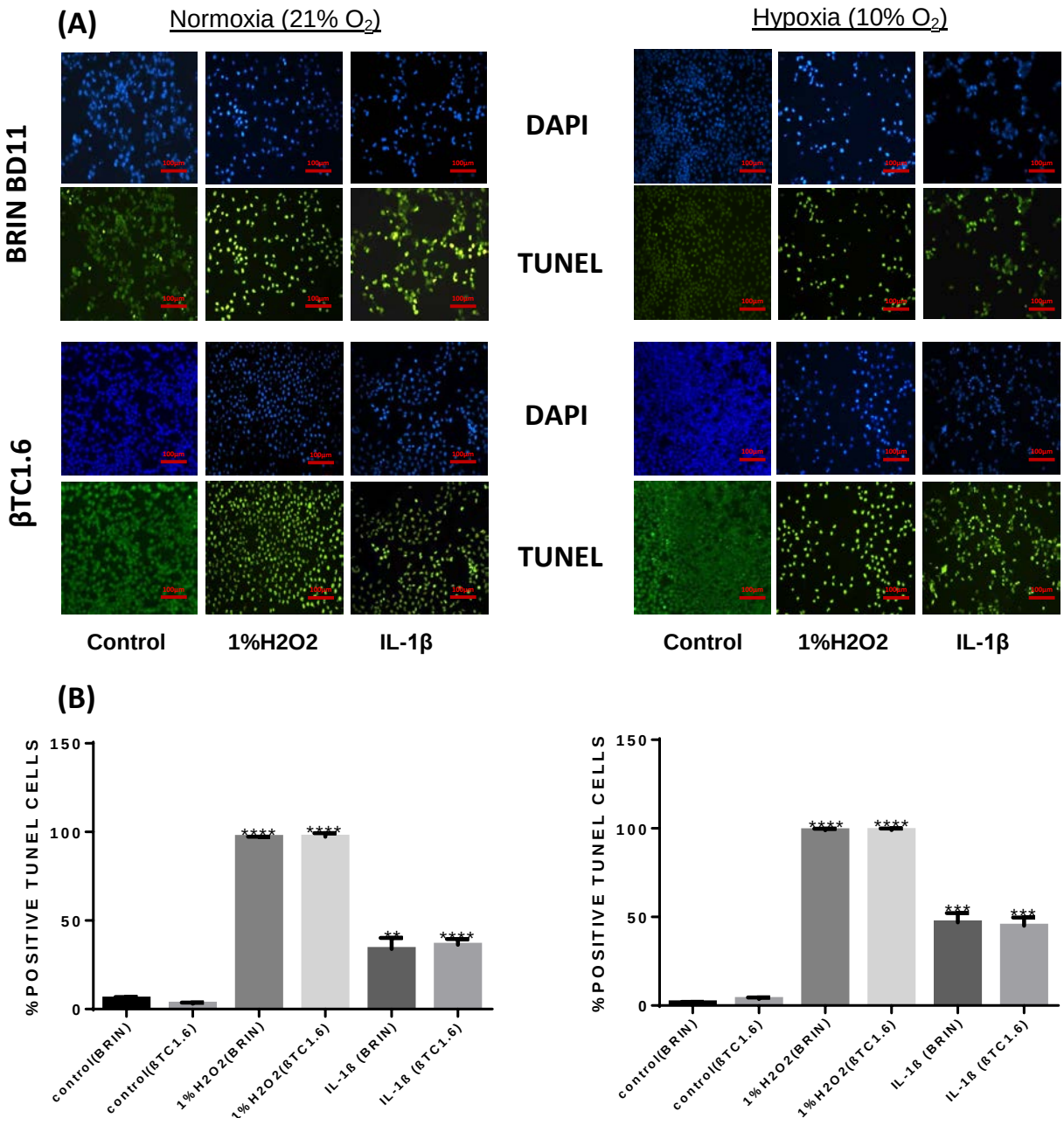

Figure 3.16. Assessment of IL-1β-induced apoptosis in pancreatic cell lines grown under normoxic and hypoxic conditions in the absence of serum. **(A)** Fluorescent images of IL-1β-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines grown under normoxic and hypoxic conditions after 24 hours of exposure to 100 ng/ml of IL-1β. Blue nuclei are DAPI staining and the green nuclei are TUNEL positive nuclei. **(B)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls.

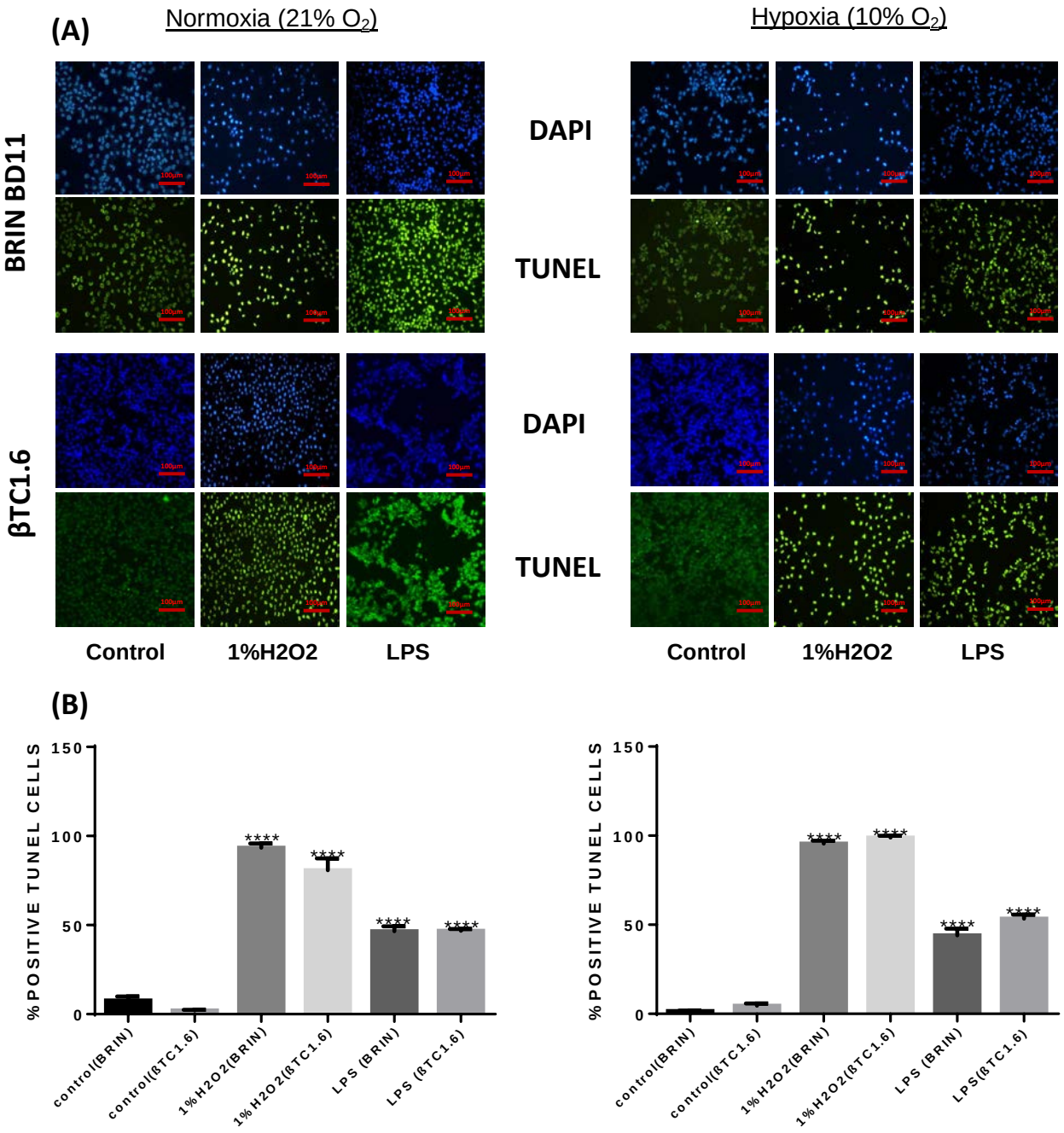

Figure 3.17. Assessment of LPS-induced apoptosis in pancreatic cell lines grown under normoxic and hypoxic conditions in the presence of serum. **(A)** Fluorescent images of LPS-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines grown under normoxic and hypoxic conditions after 24 hours of exposure to 500 µg/ml of LPS. Blue nuclei are DAPI staining and the green nuclei are TUNEL positive nuclei. **(B)** The %positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****P<0.0001 compared with untreated controls.

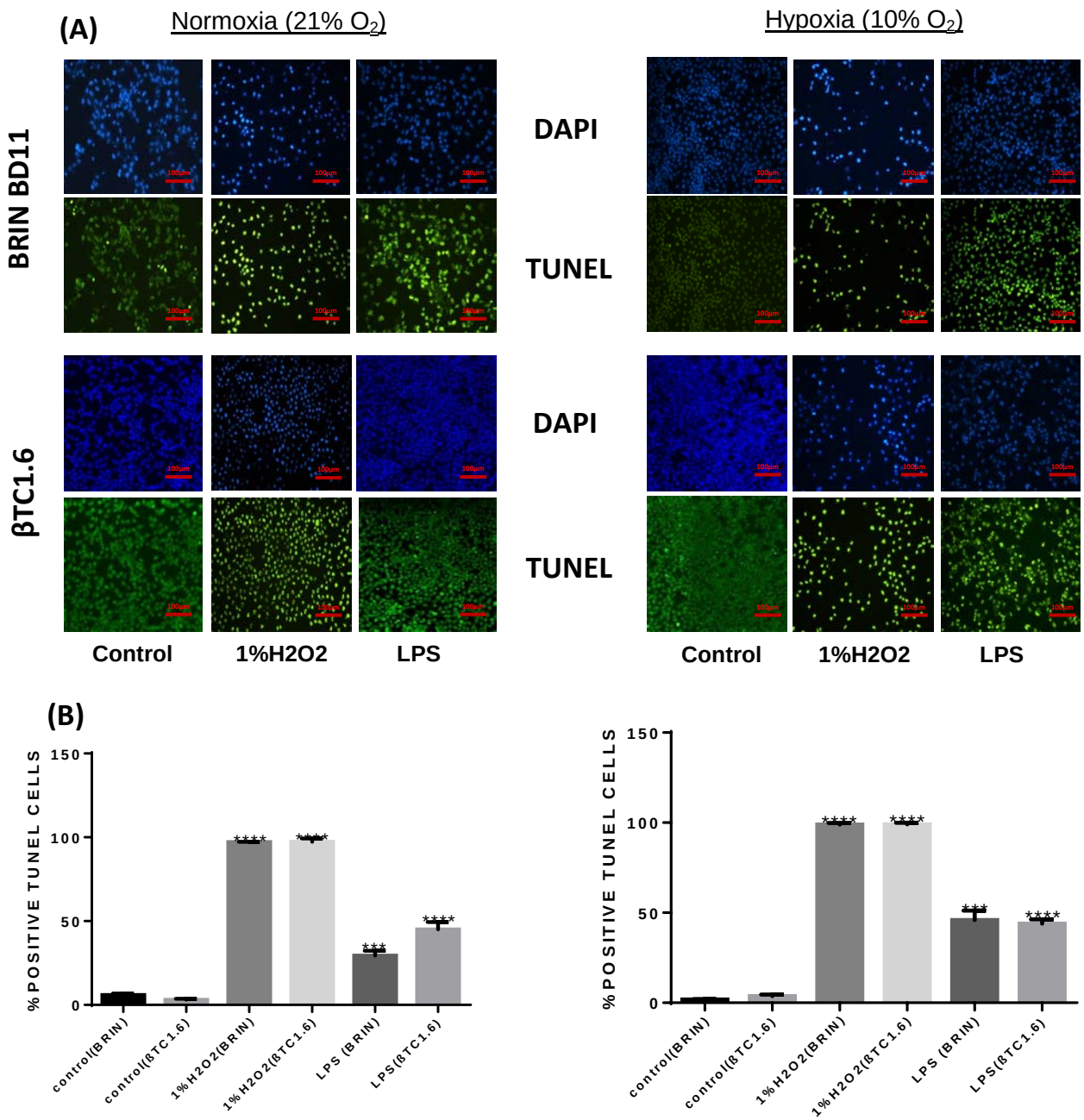

***Figure 3.18. Assessment of LPS-induced apoptosis in pancreatic cell lines grown under normoxia and hypoxia condition in the absence of serum*. (A)** Fluorescent images of LPS-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines grown under normoxic conditions after 24 hours of exposure to 500 µg/ml LPS. Cells grown under hypoxic conditions were exposed to 10 µg/ml (BRIN-BD11) or 500 µg/ml (βTC1.6) LPS. Blue nuclei are DAPI staining and the green nuclei are TUNEL positive nuclei. **(B)** The %positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****P*<0.001 and *****P*<0.0001 compared with untreated controls.

Apoptosis was also assessed in pancreatic islets isolated from CD-1 mice following the protocol outlined in Chapter 2 Section 2.2.5. Significant increases in apoptosis were noted in response to the cytokines tested in both normoxia and hypoxia and in the presence/ absence of serum (Figure 3.19, 3.20).

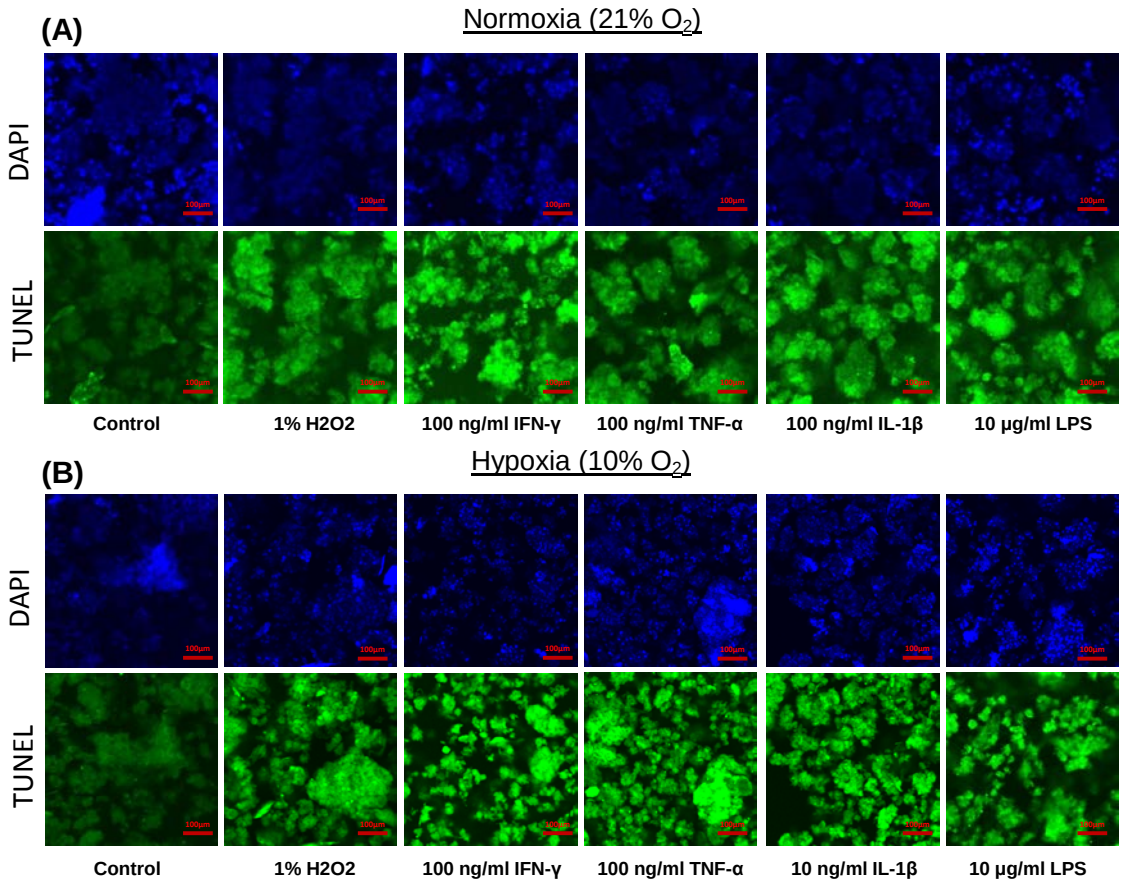

Figure 3.19. Assessment of apoptosis in pancreatic islets grown under normoxic and hypoxic conditions in the presence of serum. Fluorescent images of cytokine-induced apoptosis in mice islets after 24 hours of exposure to different cytokines in (A) normoxic and (B) hypoxic conditions. Blue nuclei are DAPI staining and the green nuclei are TUNEL positive nuclei. Microscope magnification used is 20X and the scale bar in all images is 100 µm.

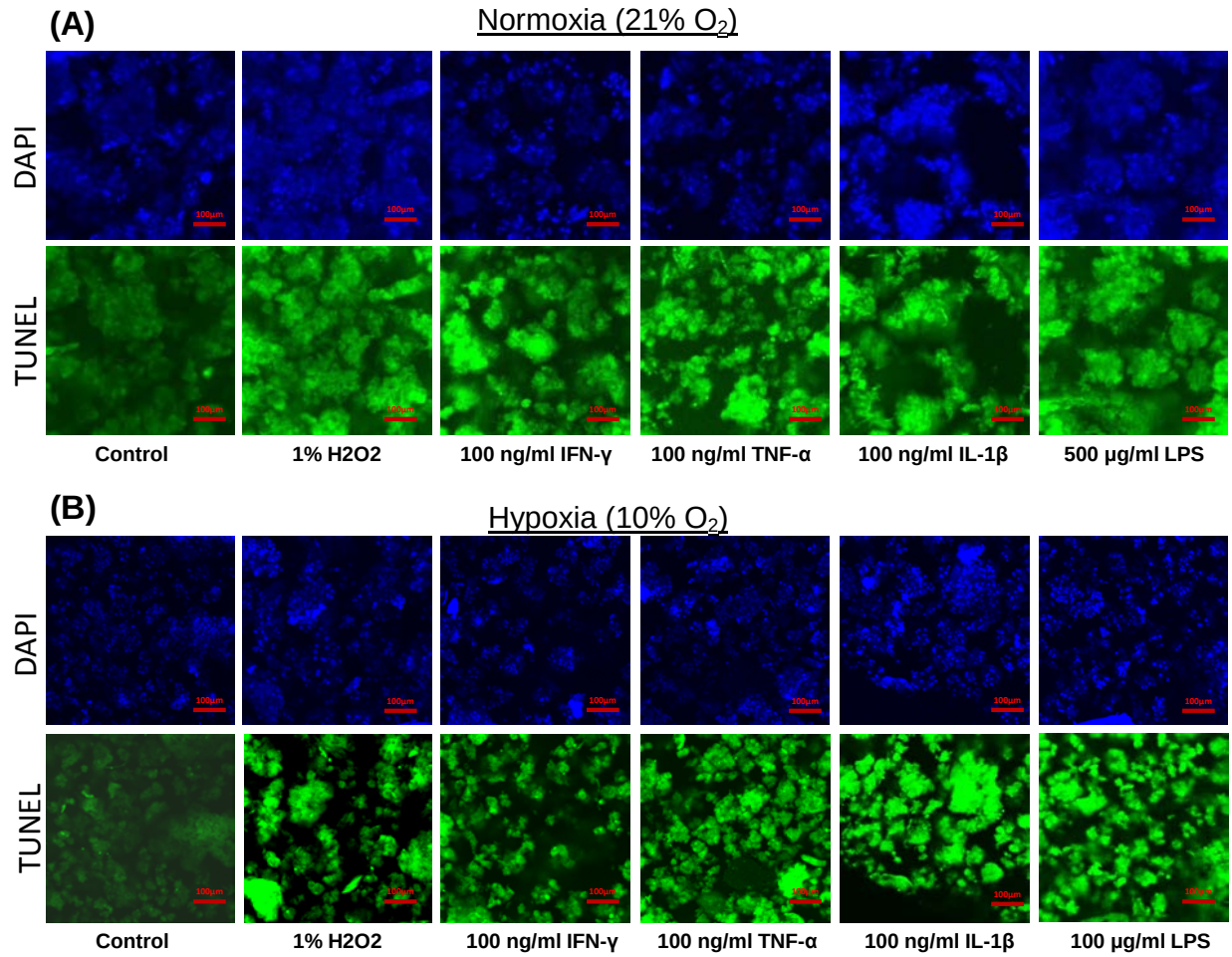

Figure 3.20. Assessment of apoptosis in pancreatic islets grown under normoxic and hypoxic conditions in the absence of serum. Fluorescent images of cytokine-induced apoptosis in mice islets after 24 hours of exposure to different types of cytokines in (A) normoxic and (B) hypoxic conditions. Blue nuclei are DAPI staining and the green nuclei are TUNEL positive nuclei. Microscope magnification used is 20X and the scale bar in all images is 100 µm.

3.3.4 Assessment of gene expression by qRT-PCR

3.3.4.1 Induction of early response genes in β-cell apoptosis

Relative gene expression was determined by qRT-PCR for the anti-apoptotic gene tumor necrosis factor alpha-inducible protein 3 (TNFAIP3), also known as A20, and pro-apoptotic TNF-apoptosis inducing ligand (TRAIL). Both genes are recognized as being important early response genes in the apoptotic pathways in the pancreatic β-cell.[189, 190] Expression was determined relative to the housekeeping gene β-actin with the untreated controls standardized to 1. The induction of both genes following cytokine or LPS treatment is shown alongside time dependent reductions in β-cell viability as measured by MTT assay (Figures 3.21 – 3.36).

3.3.4.1.1 Time-dependent induction of A20 and TRAIL following exposure to IFN-γ

BRIN-BD11 cell exposure to 1 µg/ml IFN-γ, in the presence of serum, resulted in a time-dependent reduction in cell viability, which achieved significance after 15 min ($P<0.01$) in normoxia and after 4 h ($P<0.01$) in hypoxia (Figure 3.21A, B). This was accompanied by a time-dependent increase in the expression of TRAIL, which was most pronounced under hypoxic conditions (Figure 3.21C, D). Maximal A20 induction was observed in BRIN-BD11 cells after 2 h ($P<0.0001$) exposure to IFN-γ under normoxic conditions (Figure 3.21E). However, culture of BRIN-BD11 cells under hypoxic conditions resulted in a time-dependent increase in A20 expression following IFN-γ exposure (Figure 3.21F). Similar results were observed in BRIN-BD11 cells grown in the absence of serum (Figure 3.22). However, in this instance, peak induction of A20 was observed after 15 mins ($P<0.001$) of IFN-γ treatment (Figure 3.22E). All other findings followed a similar trend to that described for BRIN-BD11 cells grown in the presence of serum.

Exposure of βTC1.6 cells cultured in the presence of serum to 1 µg/ml IFN-γ resulted in a time-dependent reduction in cell viability, which achieved significance after 4 h ($P<0.05$) in normoxia and after 2 h ($P<0.001$) in hypoxia (Figure 3.23A, B). This was associated with a significant ($P<0.05$-$P<0.0001$) time-dependent upregulation in TRAIL (Figure 3.23C, D) and A20 (Figure 3.23E, F). Consistent trends were observed in all instances in βTC1.6 cells grown in the absence of serum and exposed to 1 µg/ml IFN-γ (Figure 3.24).

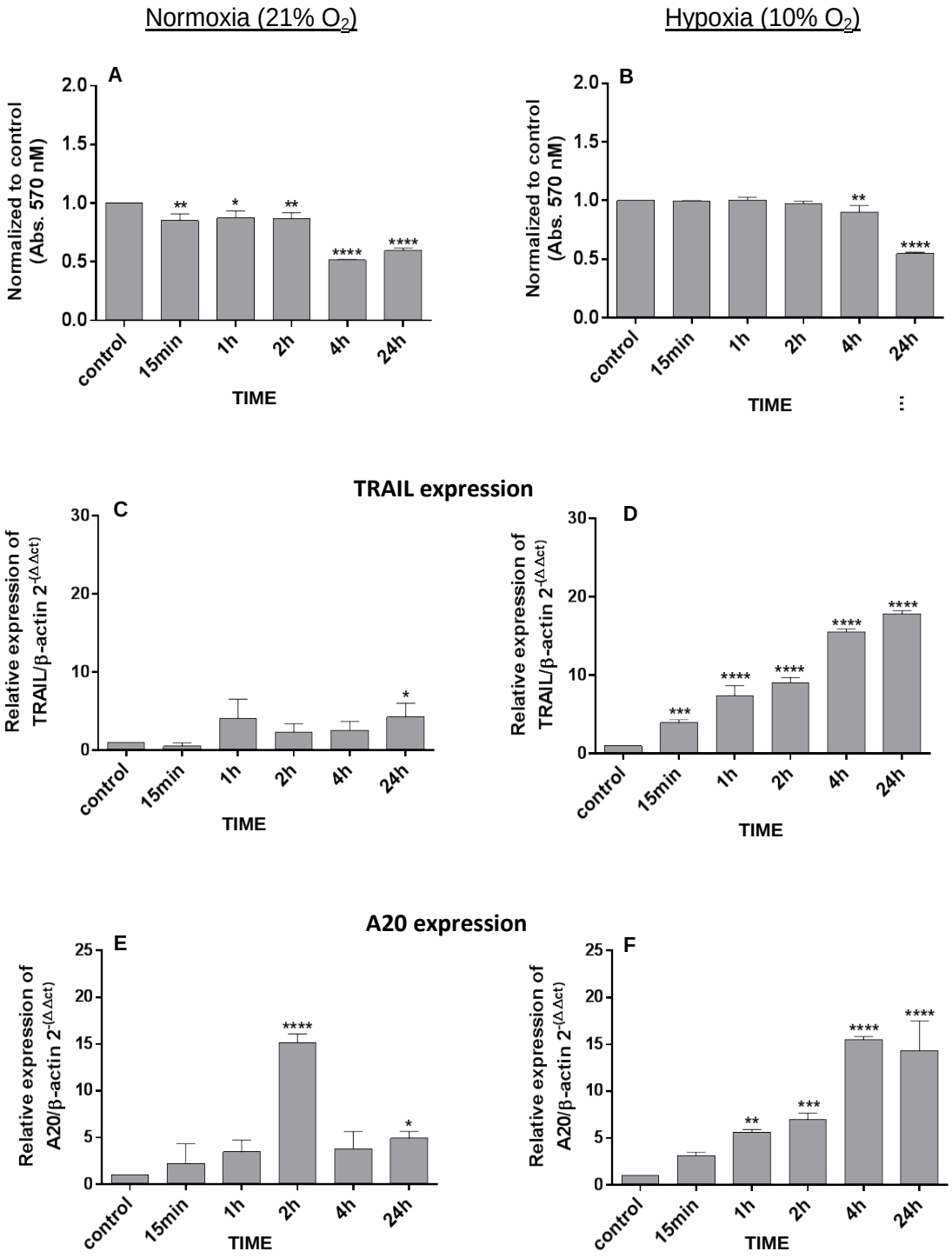

Figure 3.21 Effect of IFN-γ on the viability and relative gene expression over time in BRIN-BD11 cells cultured in the presence of serum. Following exposure to 1 µg/ml of IFN-γ for 0-24 h, the cellular viability of BRIN-BD11 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. IFN-γ, interferon gamma.

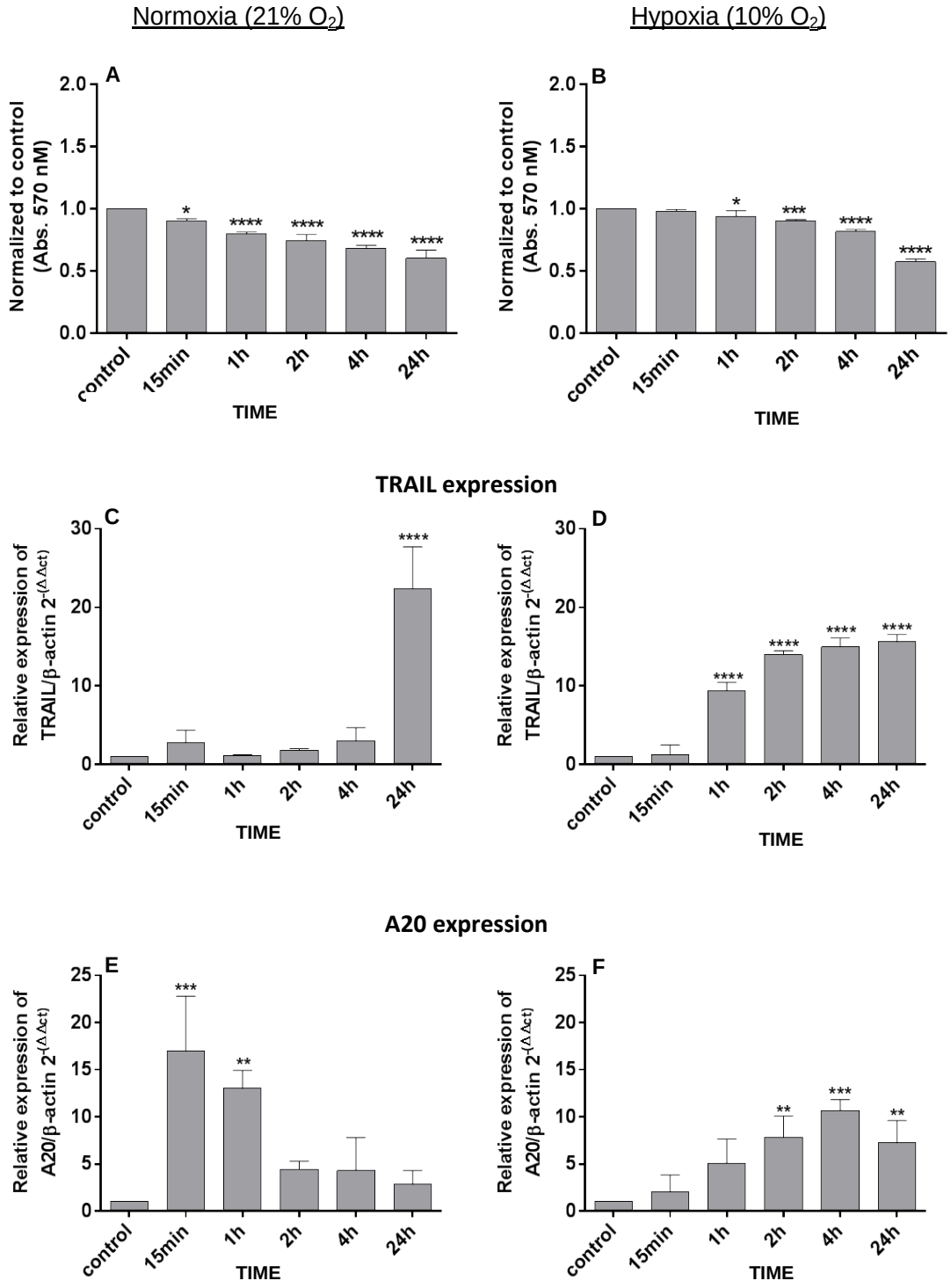

Figure 3.22 Effect of IFN-γ on the viability and relative gene expression over time in BRIN-BD11 cells cultured in the absence of serum. Following exposure to 1 µg/ml of IFN-γ for 0-24 h, the cellular viability of BRIN-BD11 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. IFN-γ, interferon gamma.

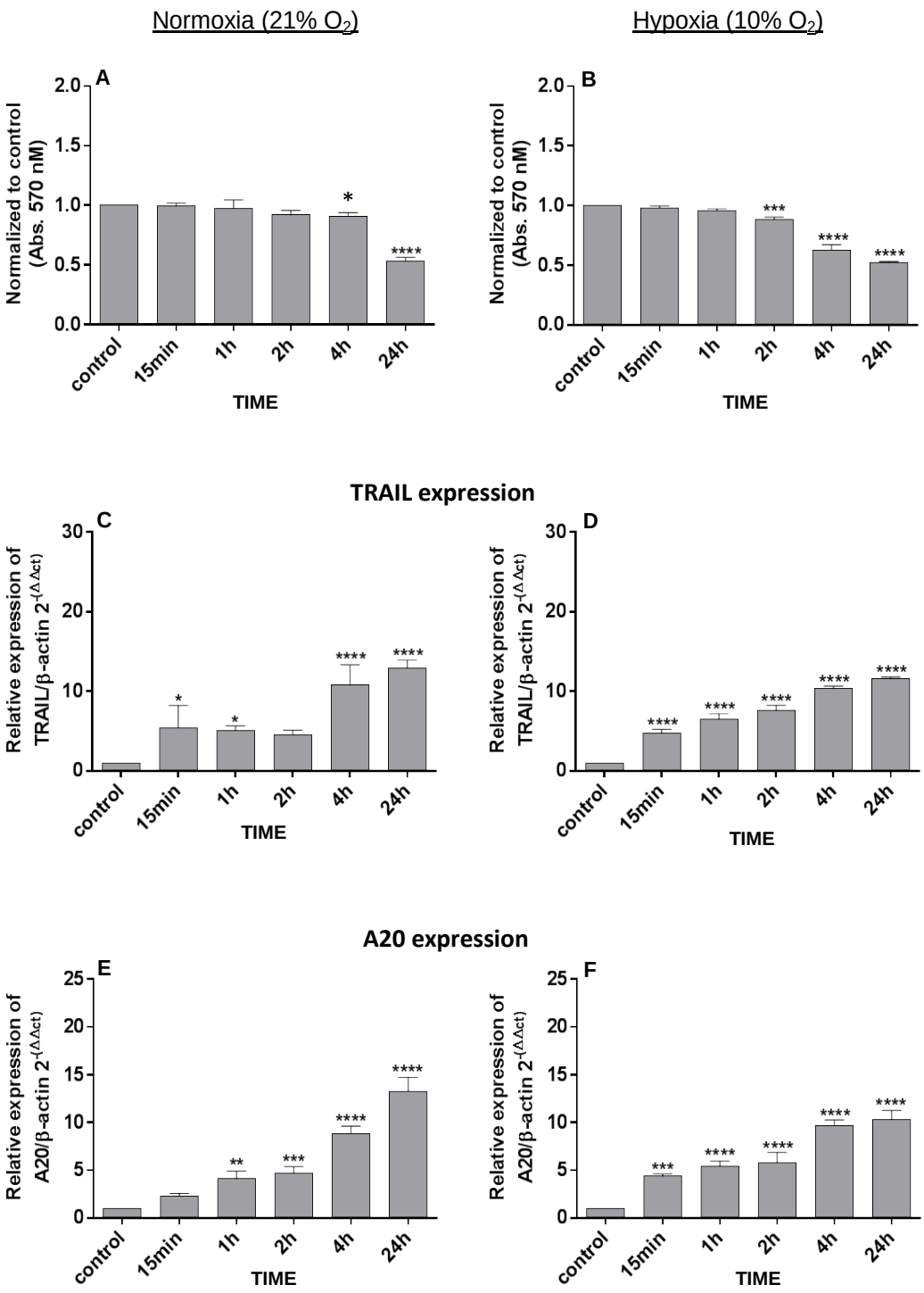

Figure 3.23. Effect of IFN-γ on the viability and relative gene expression over time in βTC1.6 cells cultured in the presence of serum Following exposure to 1 µg/ml of IFN-γ for 0-24 h, the cellular viability of βTC1.6 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. IFN-γ, interferon gamma.

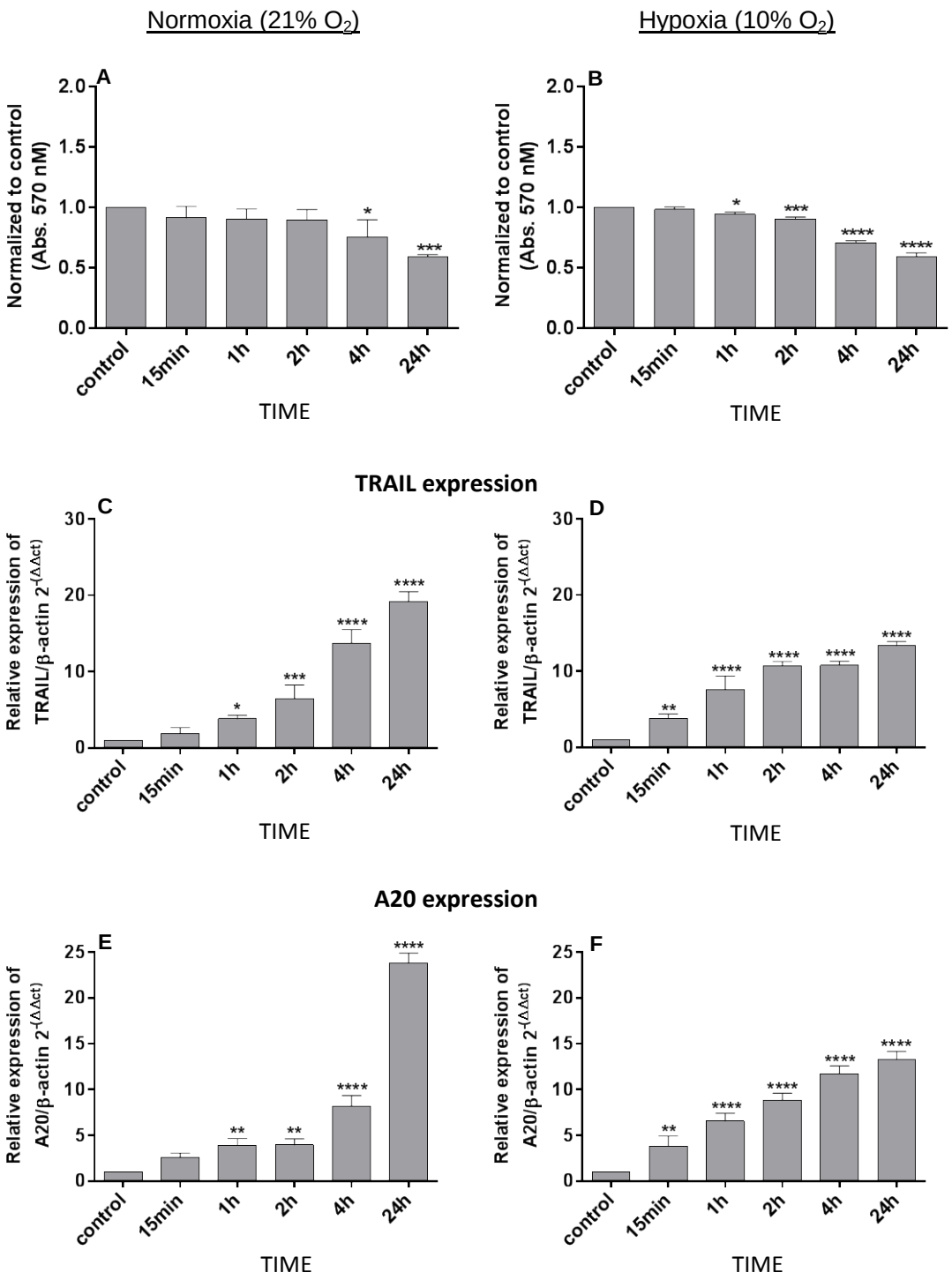

Figure 3.24. Effect of IFN-γ on the viability and relative gene over time in βTC1.6 cells cultured in the absence of serum Following exposure to 1 µg/ml of IFN-γ for 0-24 h, the cellular viability of βTC1.6 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. IFN-γ, interferon gamma.

3.3.4.1.2 Time-dependent induction of A20 and TRAIL following exposure to TNF-α

Exposure of BRIN-BD11 cells to 100 ng/ml of TNF-α in the presence of serum stimulated a time-dependent reduction in cellular viability, which achieved significance after 1 h of exposure under the normoxic conditions ($P<0.01$) and after 4 h in hypoxia ($P<0.0001$) (Figure 3.25A, B). The expression of TRAIL increased in a time dependent manner ($P<0.01$-$P<0.0001$) following TNF-α exposure regardless of whether cells were grown in normoxia or hypoxia (Figure 3.25C, D). Maximal induction of A20 was observed after 1 h exposure ($P<0.0001$) to TNF-α in BRIN-BD11 cells grown under normoxic or hypoxic conditions (Figure 3.25 E, F). Consistent trends were observed for cellular viability (Figure 3.26A, B) and TRAIL induction (Figure 3.26C, D) in BRIN-BD11 cells exposed to TNF-α in the absence of serum. However, in this instance, maximal induction of A20 was delayed until 4 h ($P<0.0001$) post TNF-α treatment in cells grown in both normoxic and hypoxic conditions (Figure 3.26E, F).

Exposure of βTC1.6 cells to 1 μg/ml of TNF-α in the presence of serum stimulated a time-dependent reduction in cellular viability, which achieved significance after 4 h of exposure under normoxic conditions ($P<0.01$) and after 1 h in hypoxia ($P<0.05$) (Figure 3.27A, B). TRAIL (Figure 3.27C, D) and A20 (Figure 3.27E, F) expression increased in a time-dependent manner ($P<0.05$-$P<0.0001$) regardless of whether cells were grown in normoxia or hypoxia. Consistent trends were observed for βTC1.6 cells grown in the absence of serum (Figure 3.28).

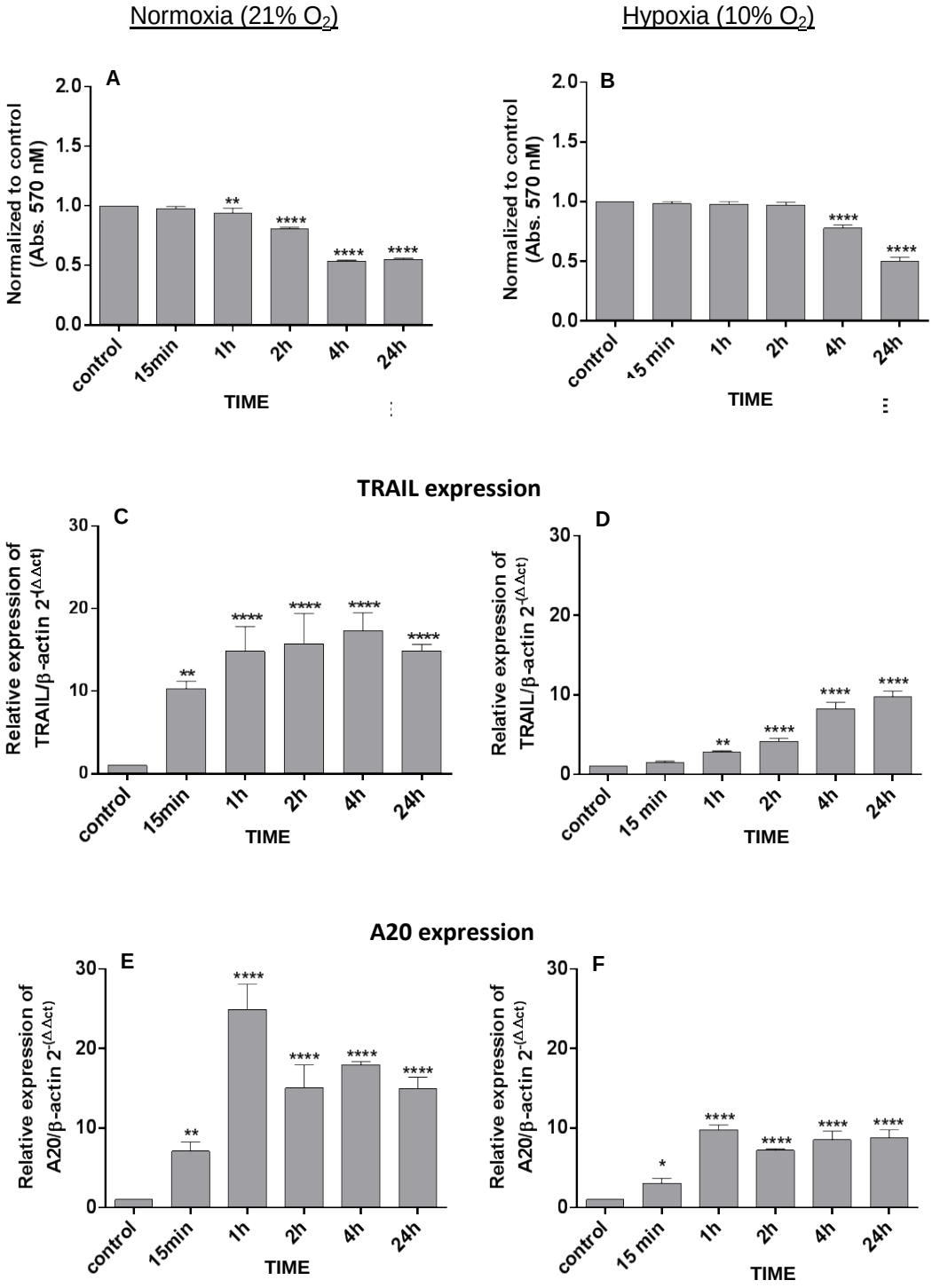

Figure 3.25. Effect of TNF-α on the viability and relative gene expression over time in BRIN-BD11 cells cultured in the presence of serum Following exposure to 100 ng/ml of TNF-α for 0-24 h, the cellular viability of BRIN-BD11 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. TNF-α, tumour necrosis factor-alpha.

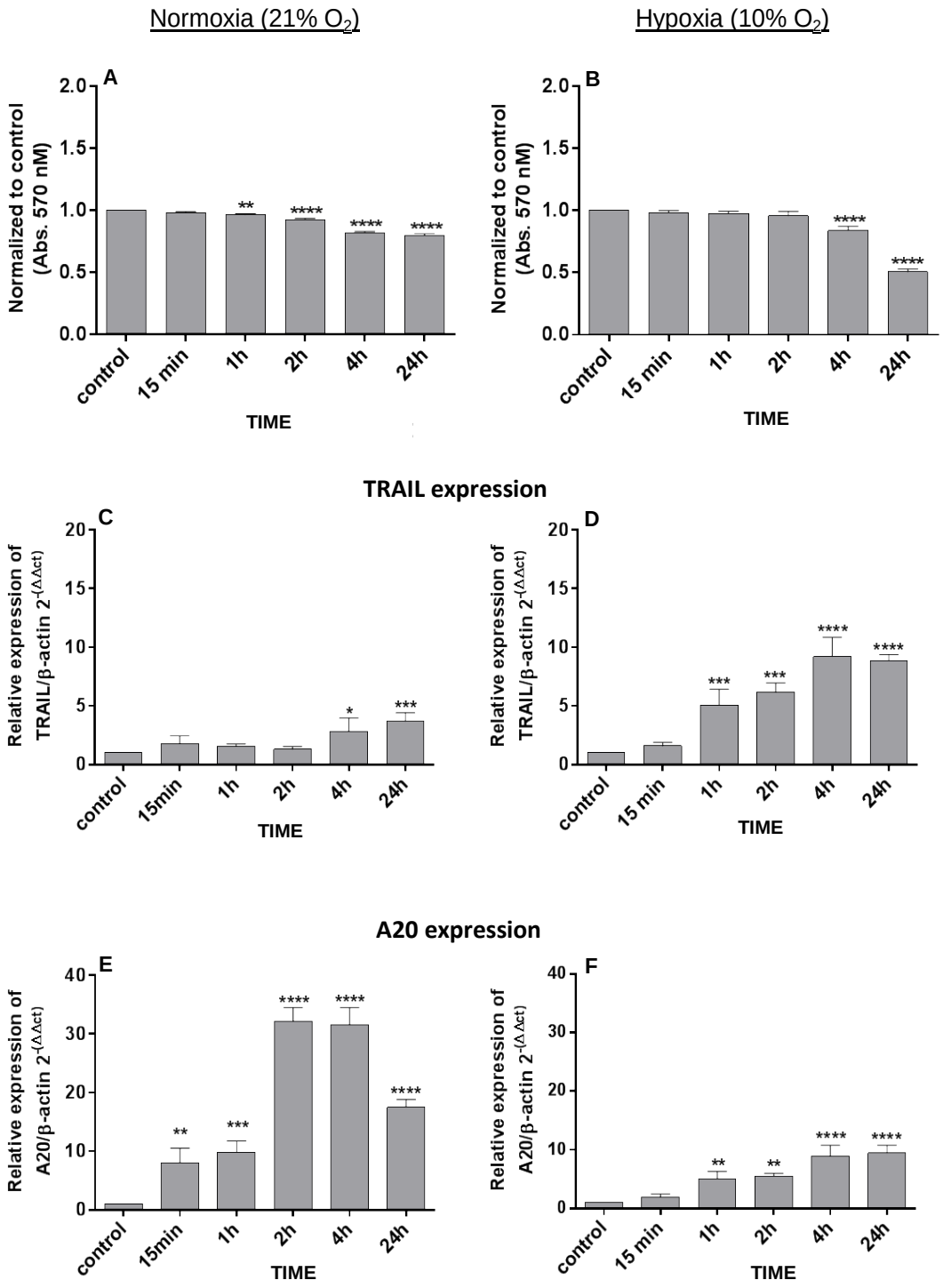

***Figure 3.26. Effect of TNF-α on the viability and relative gene expression over time in BRIN-BD11 cells cultured in the absence of* serum** Following exposure to 1 µg/ml TNF-α for 0-24 h, the cellular viability of BRIN-BD11 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. TNF-α, tumour necrosis factor-alpha.

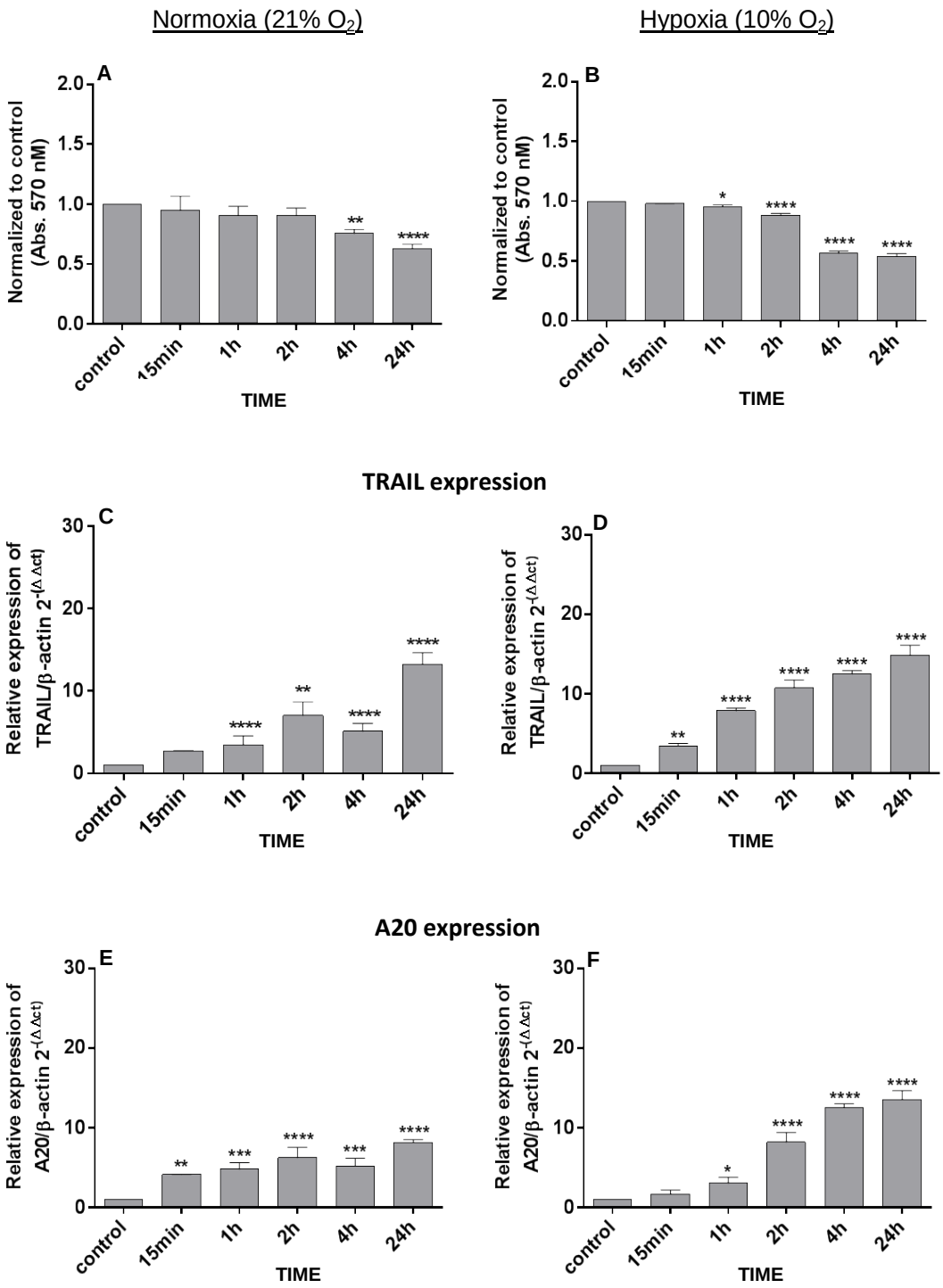

Figure 3.27. Effect of TNF-α on the viability and relative gene expression over time in βTC1.6 cells cultured in presence of serum Following exposure to 1 µg/ml of TNF-α for 0-24 h, the cellular viability of βTC1.6 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. TNF-α, tumour necrosis factor-alpha.

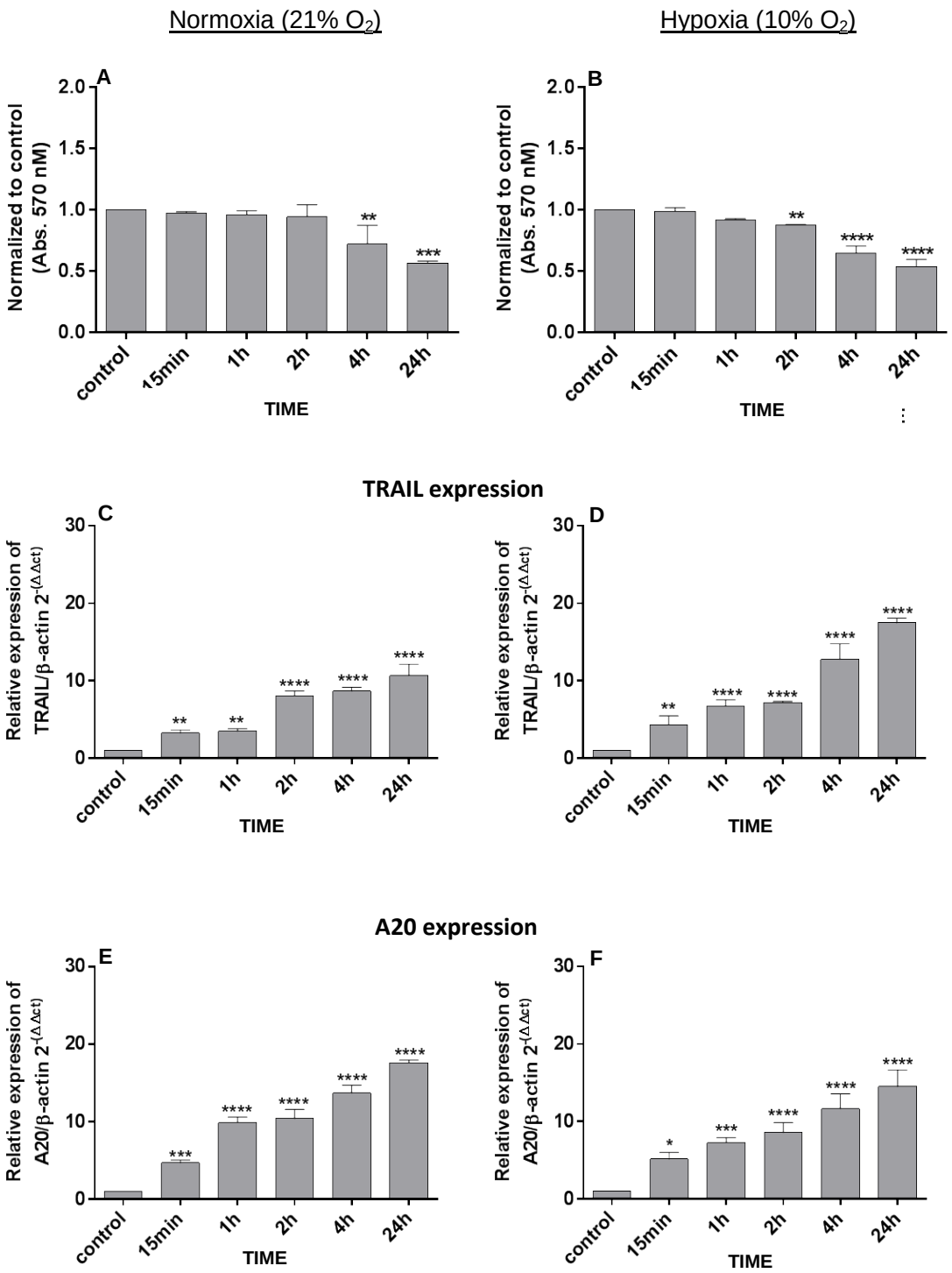

Figure 3.28. Effect of TNF-α on the viability and relative gene expression over time in βTC1.6 cells cultured in the absence of serum Following exposure to 1 µg/ml of TNF-α for 0-24 h, the cellular viability of βTC1.6 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. TNF-α, tumour necrosis factor-alpha.

3.3.4.1.3 Time-dependent induction of A20 and TRAIL following exposure to IL-1β

Exposure of BRIN-BD11 cells to 100 ng/ml of IL-1β in the presence of serum stimulated a time-dependent reduction in cellular viability, which achieved significance after 2 h of exposure under the normoxic conditions ($P<0.0001$) and after 1 h in hypoxia ($P<0.001$) (Figure 3.29A, B). The expression of TRAIL increased in a time dependent manner ($P<0.0001$) following IL-1β exposure regardless of whether cells were grown in normoxia or hypoxia (Figure 3.29C, D). Maximal induction of A20 was observed after 2 h exposure ($P<0.0001$) to IL-1β in BRIN-BD11 cells grown under normoxic conditions ($P<0.0001$) and after 4 h in hypoxic conditions ($P<0.0001$) (Figure 3.29E, F). Consistent trends were observed for cellular viability (Figure 3.30A, B) and TRAIL induction (Figure 3.30C, D) in BRIN-BD11 cells exposed to IL-1β in the absence of serum. However, in this instance, maximal induction of A20 was observed earlier (after 1h; $P<0.05$-$P<0.0001$) in cells grown in both normoxic and hypoxic conditions (Figure 3.30E, F).

Exposure of βTC1.6 cells to 100 ng/ml IL-1β in the presence of serum stimulated a time-dependent reduction in cellular viability, which achieved significance after 2 h of exposure under normoxic conditions ($P<0.01$) and after 1 h in hypoxia ($P<0.001$) (Figure 3.31A, B). TRAIL (Figure 3.31C, D) and A20 (Figure 3.31E, F) expression increased in a time-dependent manner ($P<0.05$-$P<0.0001$) regardless of whether cells were grown in normoxia or hypoxia. Consistent trends were observed for βTC1.6 cells grown in the absence of serum (Figure 3.32).

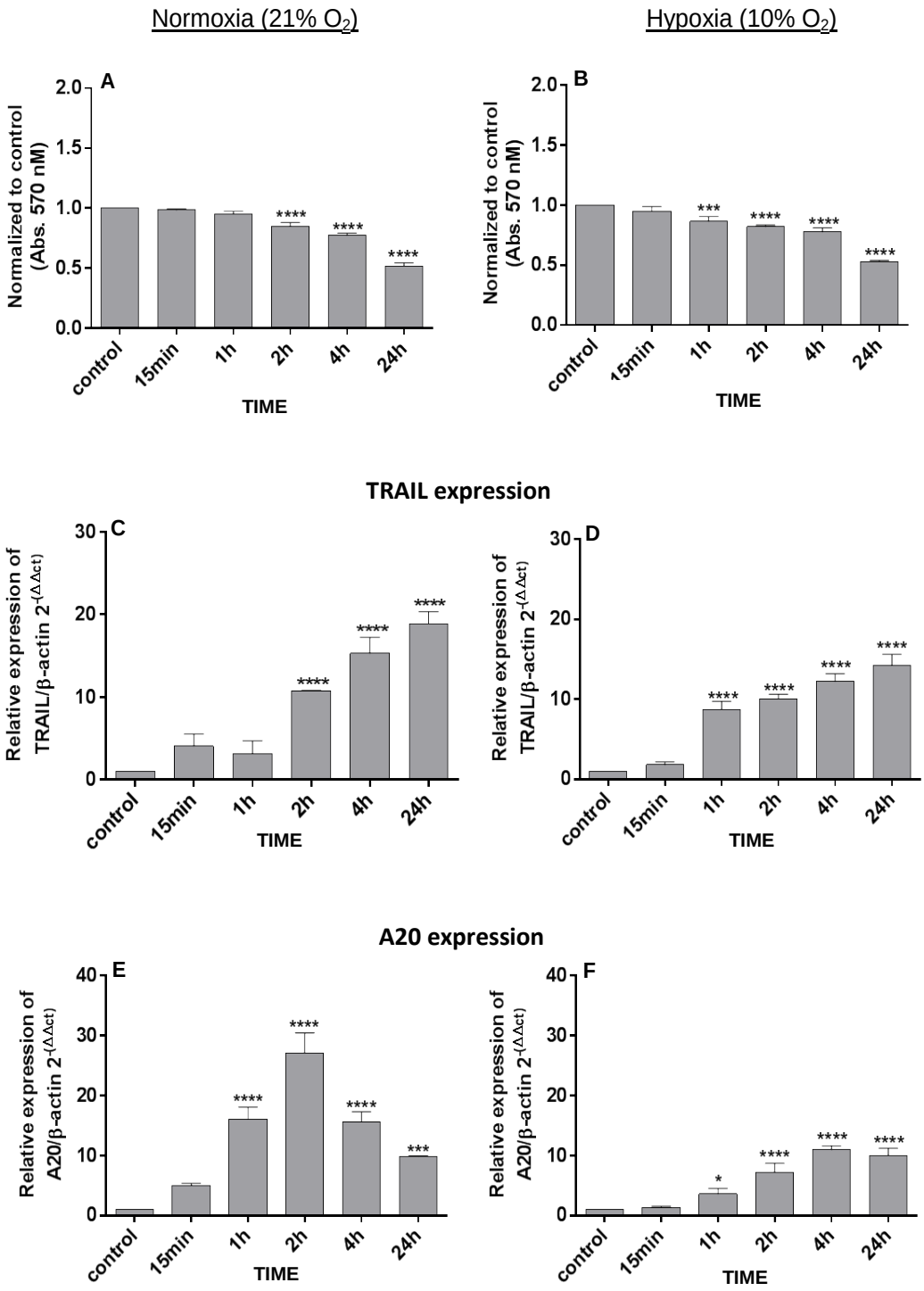

Figure 3.29. Effect of IL-1β on the viability and relative gene expression over time in BRIN-BD11 cells cultured in the presence of serum Following exposure to 100 ng/ml of IL-1β for 0-24 h, the cellular viability of BRIN-BD11 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. IL-1β, interleukin-1beta.

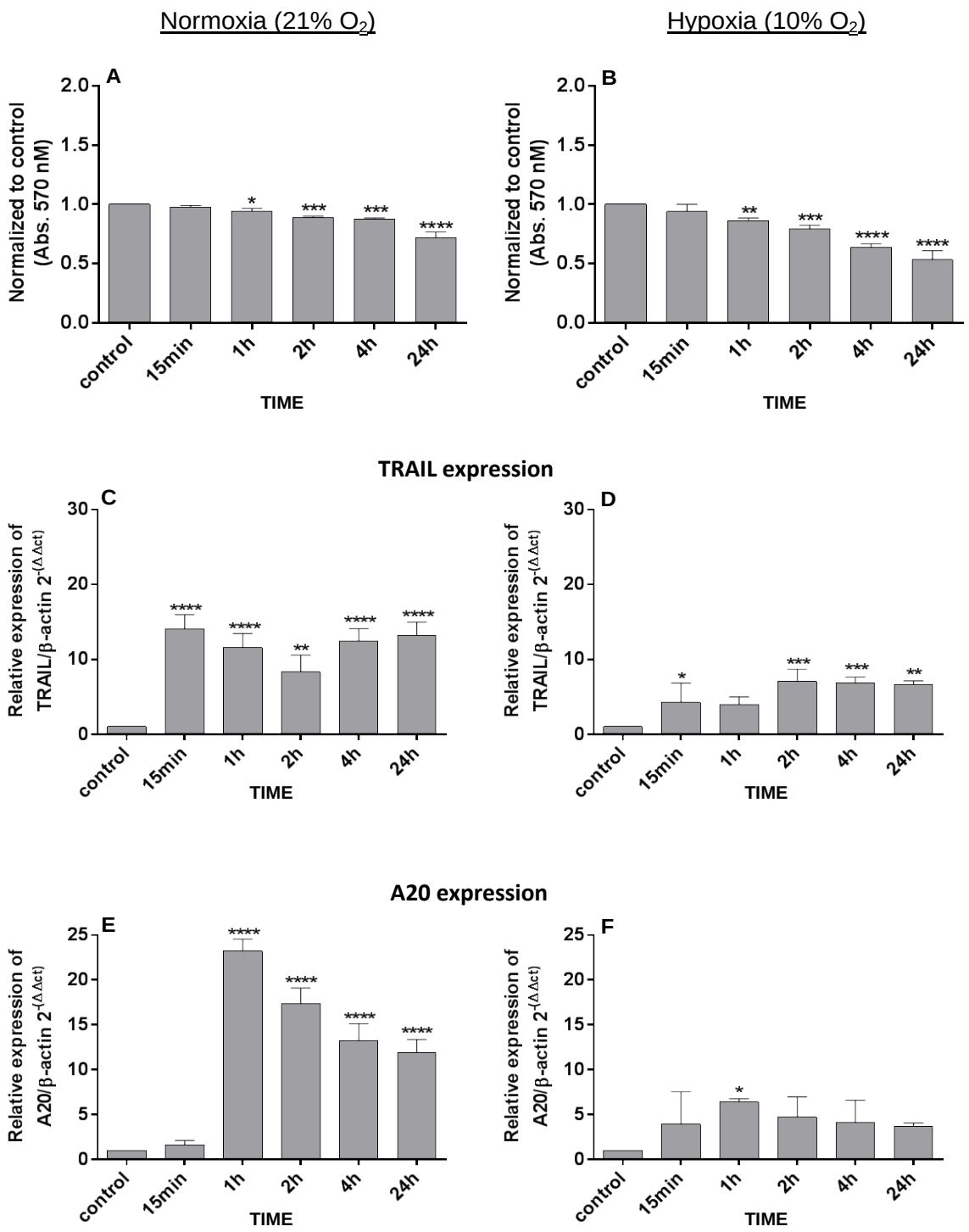

***Figure 3.30. Effect of IL-1β on the viability and relative gene expression over time in BRIN-BD11 cells cultured in the absence of* serum** Following exposure to 100 ng/ml of IL-1β for 0-24 h, the cellular viability of BRIN-BD11 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. IL-1β, Interleukin-1beta.

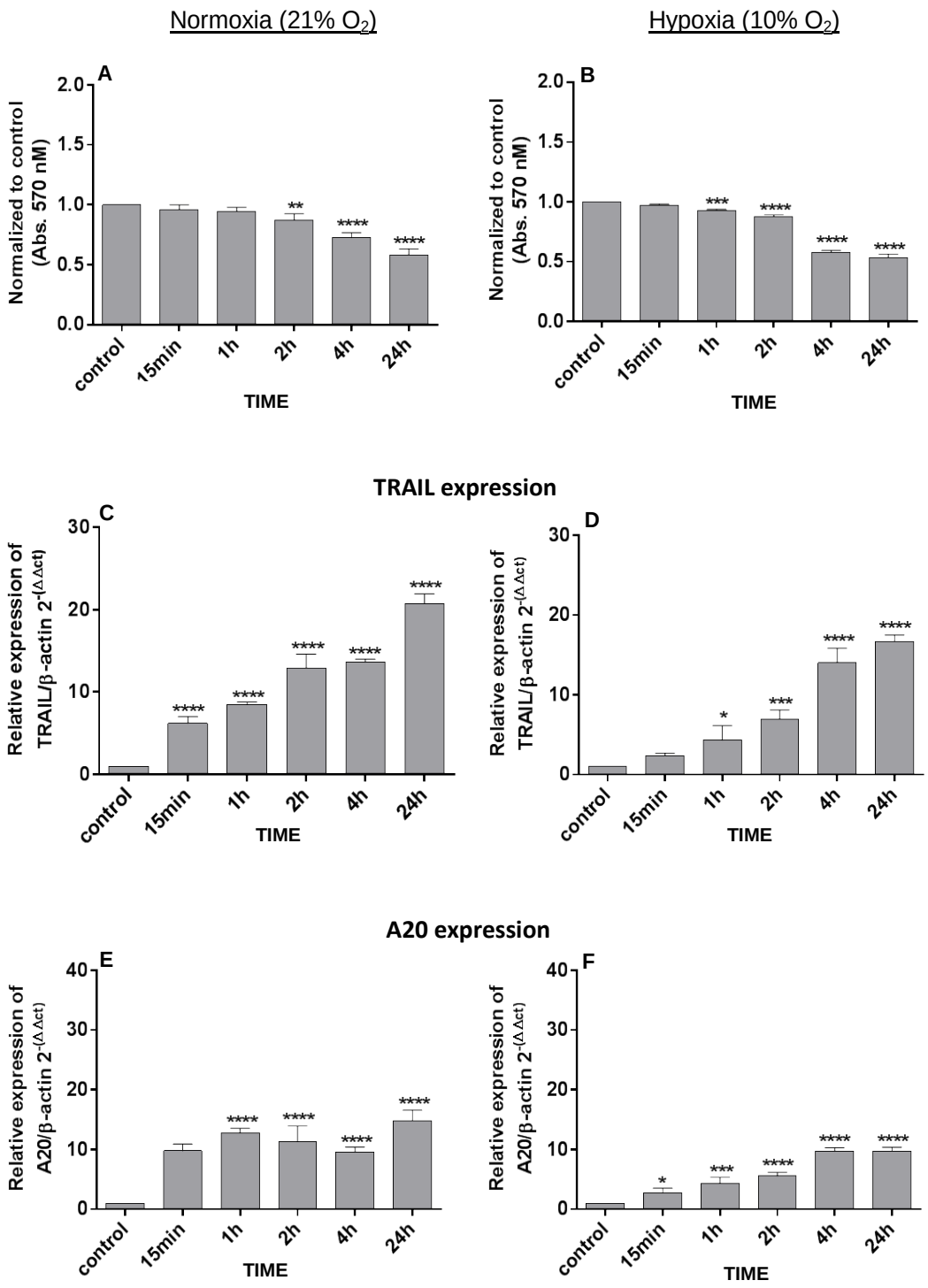

Figure 3.31. Effect of IL-1β on the viability and relative gene expression over time in βTC1.6 cells cultured in the presence of serum Following exposure to 100 ng/ml of IL-1β for 0-24 h, the cellular viability of βTC1.6 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. IL-1β, interleukin-1beta.

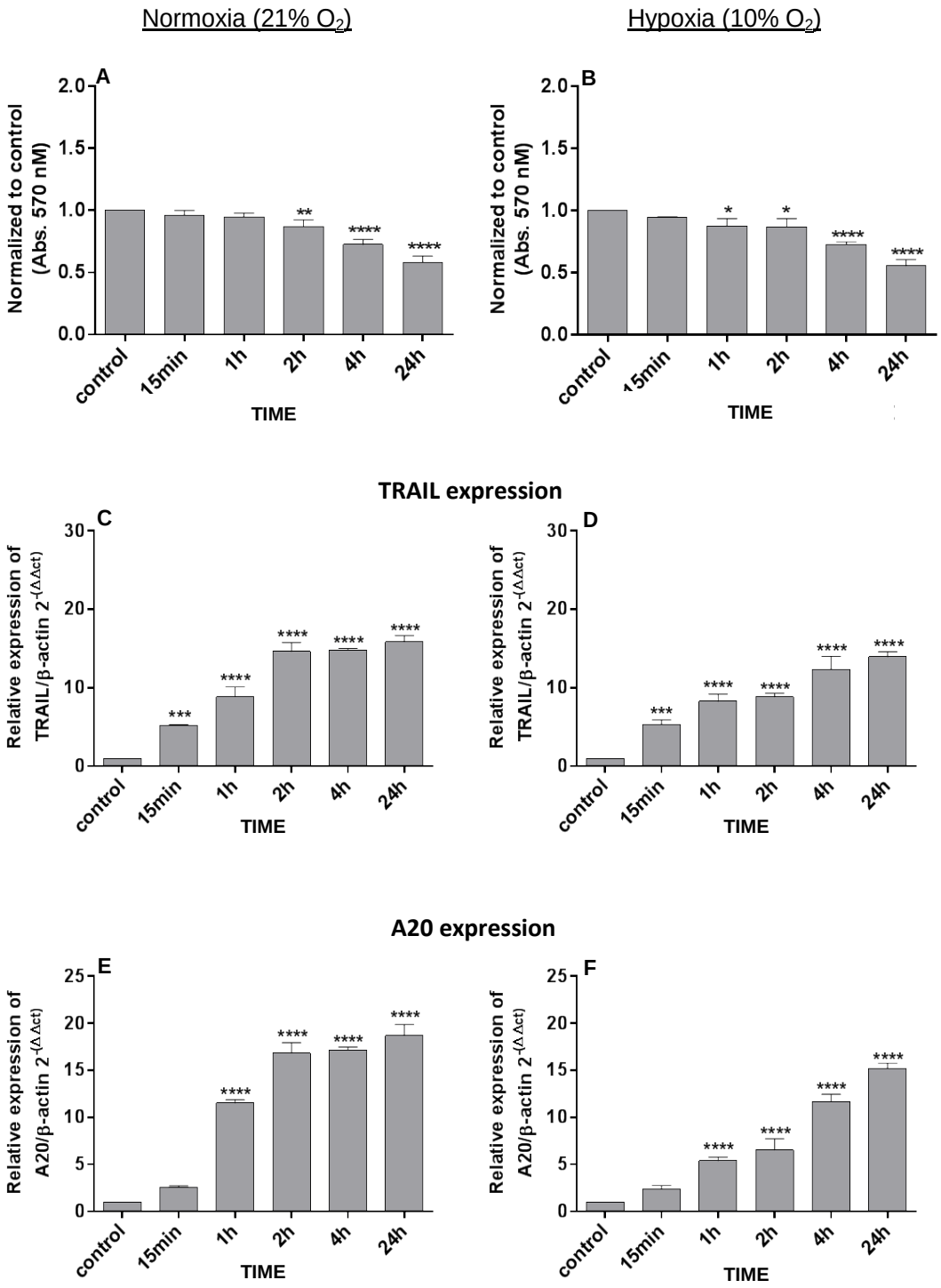

Figure 3.32. Effect of IL-1β on the viability and relative gene expression over time in βTC1.6 cells cultured in the absence of serum. Following exposure to 100 ng/ml of IL-1β for 0-24 h, the cellular viability of βTC1.6 cells grown under normoxic or hypoxia conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. IL-1β, Interleukin-1beta.

3.3.4.1.4 Time-dependent induction of A20 and TRAIL following exposure to LPS

BRIN-BD11 cells grown in the presence of serum were stimulated with 500 µg/ml of LPS for 24 h, the normalized MTT assay data showed a significant (P<0.001-P<0.0001) reduction in cells viability after 15 min of exposure in normoxia versus 1 h in hypoxia, (Figure 3.33A, B). TRAIL was significantly (*P*<0.0001) upregulated after 2 h of exposure in normoxia versus 15 min in hypoxia (Figure 3.33C, D). A20 was highly expressed (P<0.05-P<0.001) after 4 h of exposure, however, the lower level was detected after 24 h in normoxia (Figure 3.33E). In hypoxia there was significant (P<0.001-P<0.0001) upregulation of A20 expression during the different exposure time (Figure 3.33F). In the absence of serum the reduction in cellular viability was significant (P<0.05-P<0.0001) after 1 h of exposure to 500 µg/ml of LPS in normoxia and after 15 min of exposure to 10 µg/ml in hypoxia (Figure 3.34A, B). The TRAIL was significantly (P<0.01-P<0.0001) upregulated after 15 min of exposure in both normoxia and hypoxia (Figure 3.34C, D). There was significant (P<0.05-P<0.0001) upregulation for A20 after 1 h of exposure in normoxia versus 15 min in hypoxia (Figure 3.34E, F).

The exposure of βTC1.6 cells to 500 µg/ml of LPS significantly (P<0.01-P<0.0001) reduced the cellular viability after 2 h of exposure in normoxia versus 4 h in hypoxia (Figure 3.35A, B). TRAIL (Figure 3.35C, D). and A20 (Figure 3.35E, F). were significantly (P<0.05-P<0.0001) upregulated in both normoxia and hypoxia in the presence of serum. In the absence of serum a significant (P<0.05-P<0.0001) reduction in cellular viability was noted after 1 h of exposure to 500 µg/ml for normoxia versus 15 min in hypoxia (Figure 3.35A, B). A significant (P<0.05-P<0.0001) upregulation was noted for TRAIL and A20 in both normoxia and hypoxia (Figure 3.36).

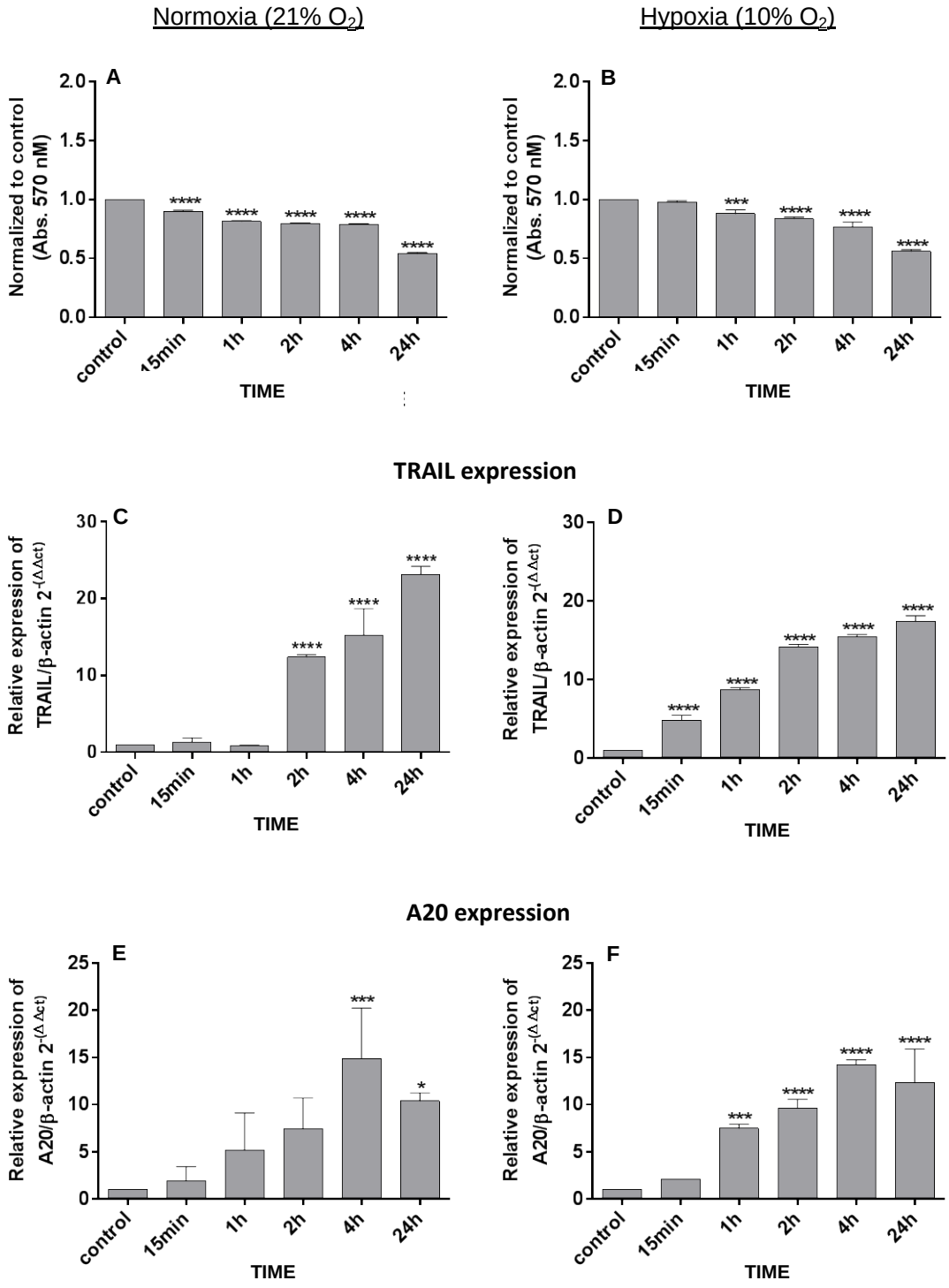

Figure 3.33. Effect of LPS on the viability and relative gene expression over time in BRIN-BD11 cells cultured in the presence of serum. Following exposure to 500 µg/ml of LPS for 0-24 h, the cellular viability of BRIN-BD11 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. LPS, lipopolysaccharide.

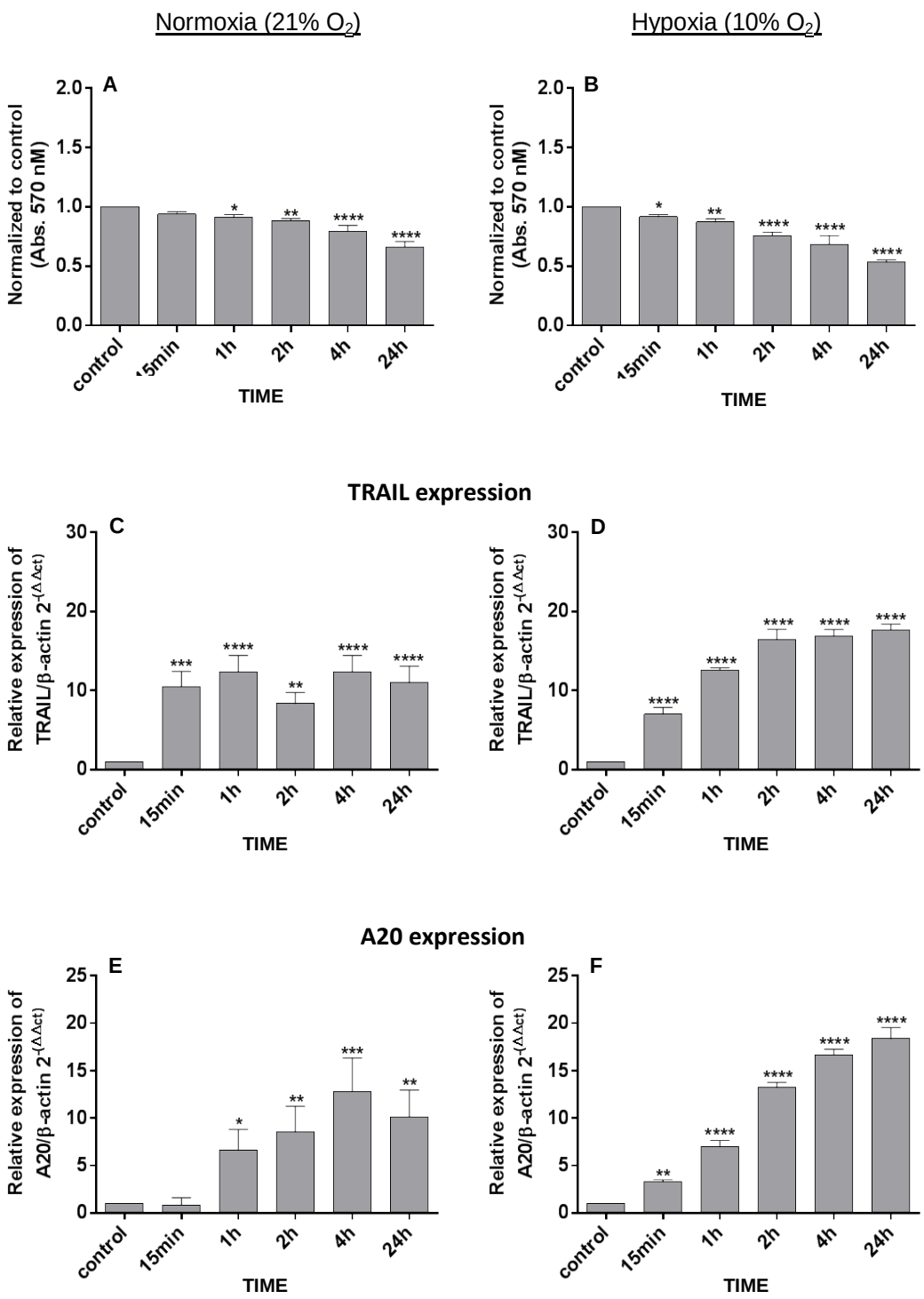

Figure 3.34. Effect of LPS on the viability and relative gene expression over time in BRIN-BD11 cells cultured in the absence of serum. Following exposure to 500 µg/ml and 10 µg/ml of IL-1β for 0-24 h, the cellular viability of BRIN-BD11 cells grown under normoxic or hypoxic conditions respectively was assessed by colorimetric MTT assay (A, B). This was associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. LPS, lipopolysaccharide.

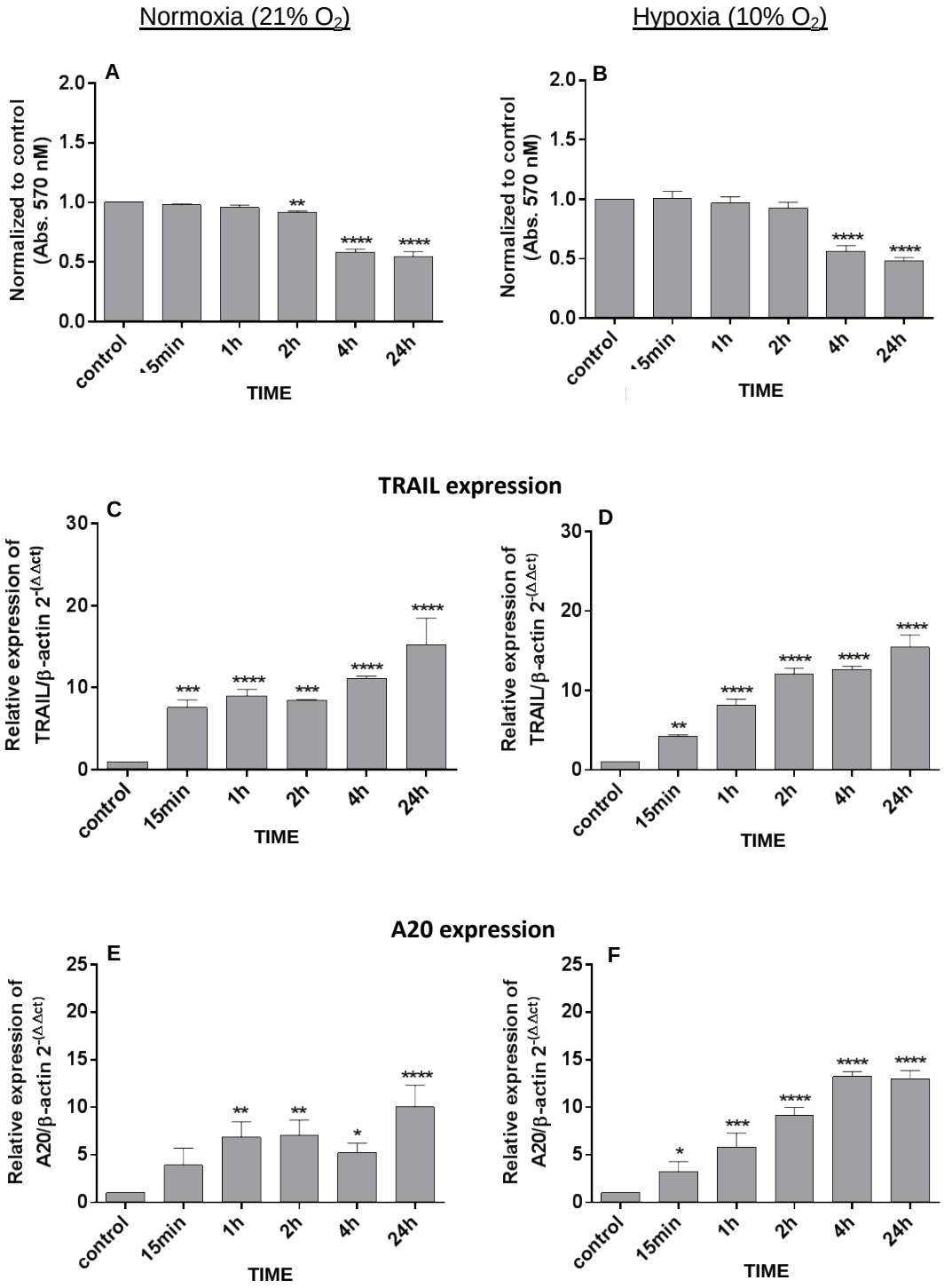

Figure 3.35. Effect of LPS on the viability and relative gene expression over time in βTC1.6 cells cultured in the presence of serum. Following exposure to 500 µg/ml of LPS for 0-24 h, the cellular viability of βTC1.6 cells grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001 compared with untreated controls. LPS, lipopolysaccharide.

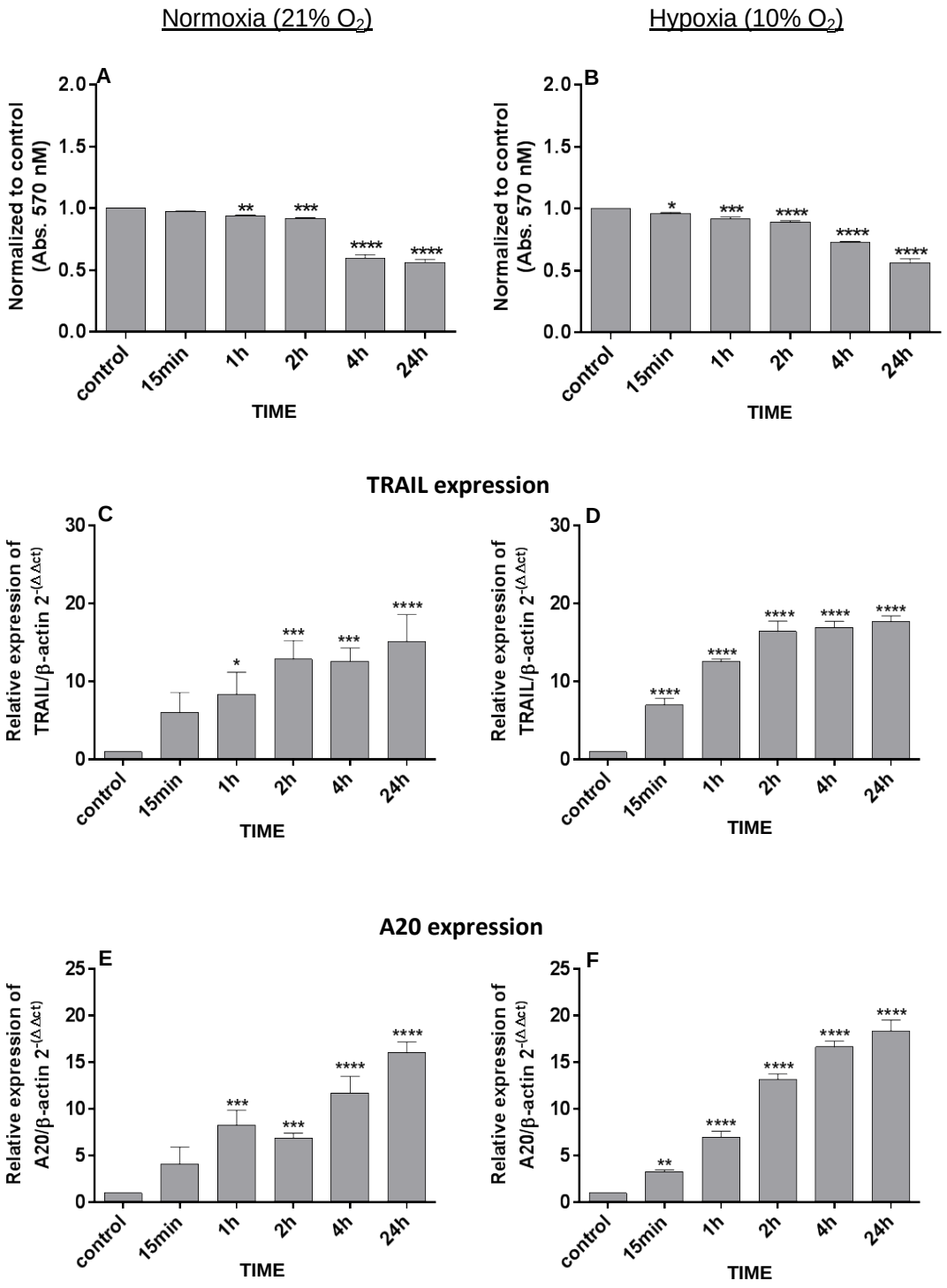

Figure 3.36. Effect of LPS on the viability and relative gene expression over time in βTC1.6 cells cultured in the absence of serum. Following exposure to 500 µg/ml of LPS for 0-24h, the cellular viability of βTC1.6 **cells** grown under normoxic or hypoxic conditions was assessed by colorimetric MTT assay (A, B) and associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this time course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared with untreated controls. LPS, lipopolysaccharide.

3.3.5 Assessment of pancreatic islets viability over time

Islets were exposed to 100 ng/ml of IFN-γ in the presence and absence of serum for 24 h. Normalized MTT data showed a significant (P<0.05-P<0.0001) decrease in cellular viability after 1 h of exposure in normoxia versus 15 min in hypoxia in the presence and absence of serum (Figure 3.37 and 3.38). The pancreatic islets were also exposed to 100 ng/ml of TNF-α the reduction in viability was significant (P<0.05-P<0.0001) after 1h in normoxia versus 15 min in hypoxia in the presence and absence of serum (Figure 3.37 and 3.38). For IL-1β the pancreatic islets were stimulated with 100 ng/ml in normoxia versus 10 ng/ml in hypoxia in the presence of serum and 100 ng/ml in the absence of serum, the reduction in cellular viability was significant (P<0.05-P<0.0001) after 15 min of exposure in normoxia and hypoxia in the presence of serum and also in normoxia in the absence of serum, while in hypoxia in the absence of serum the reduction in cellular viability was significant after 1h. The pancreatic islets cultured in the presence of serum were stimulated with 10 µg/ml of LPS in normoxic and hypoxia condition. The results showed a significant (P<0.01-P<0.0001) reduction in cellular viability after 15 min of exposure in both normoxia and hypoxia (Figure 3.37). In the absence of serum, the pancreatic islets were stimulated with 500 µg/ml in normoxia and 100 µg/ml in hypoxia. The reduction in viability was significant (P<0.01-P<0.0001) after 1 h of exposure in both normoxia and hypoxia (Figure 3.38).

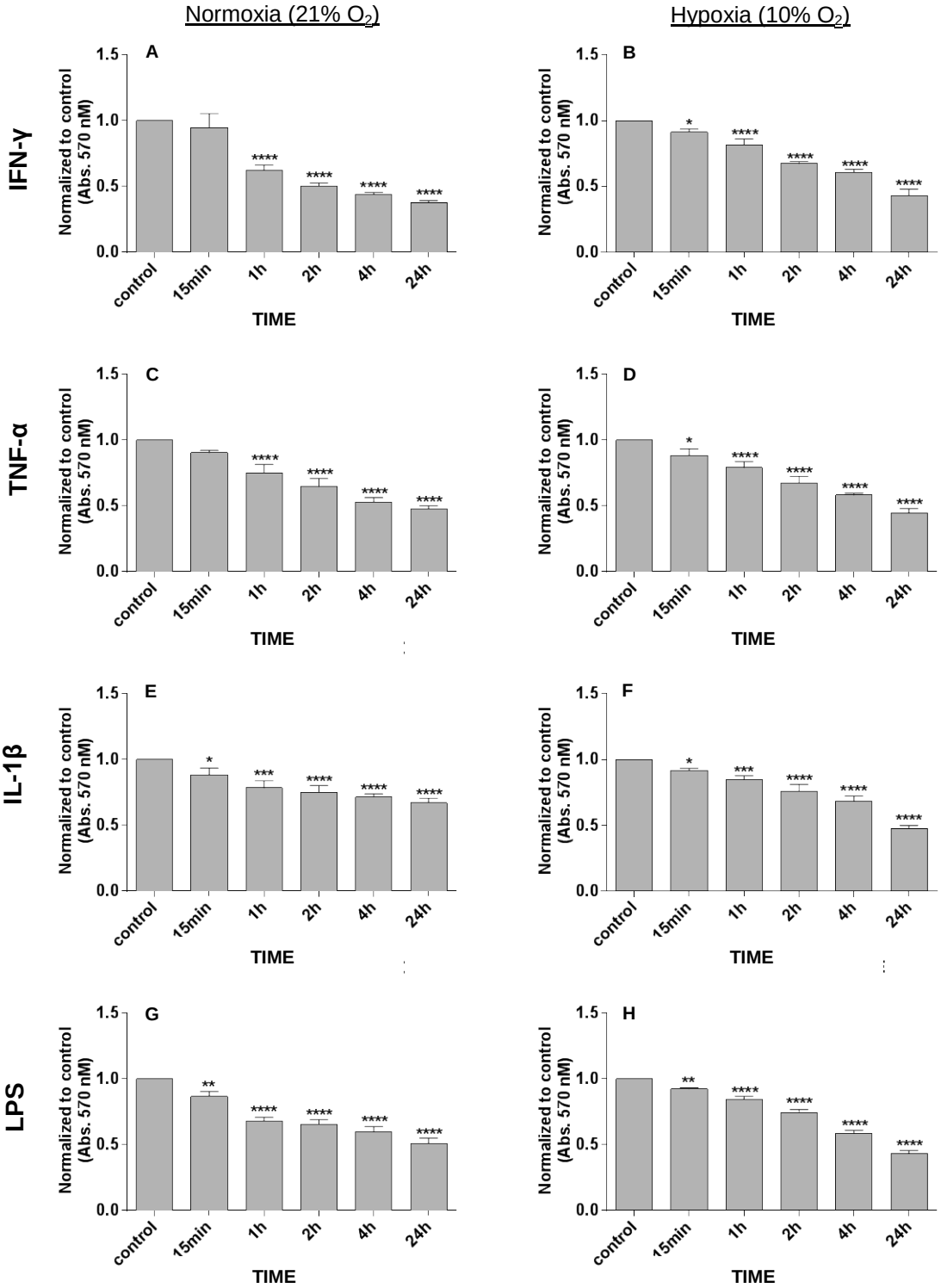

Figure 3.37. Effect of pro-inflammatory cytokines and endotoxin on the viability of pancreatic islets cultured in the presence of serum over time. Pancreatic islets were exposed to 100 ng/ml of IFN-γ (A, B), 100 ng/ml of TNF-α (C, D) and 100 ng/ml of IL-1β (E, F) for 0-24 h in normoxic and hypoxic conditions. The dose of LPS was 10µg/ml in normoxia (G) and 100 µg/ml in hypoxia (H). The viability of the islets was assessed by colorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with untreated controls.

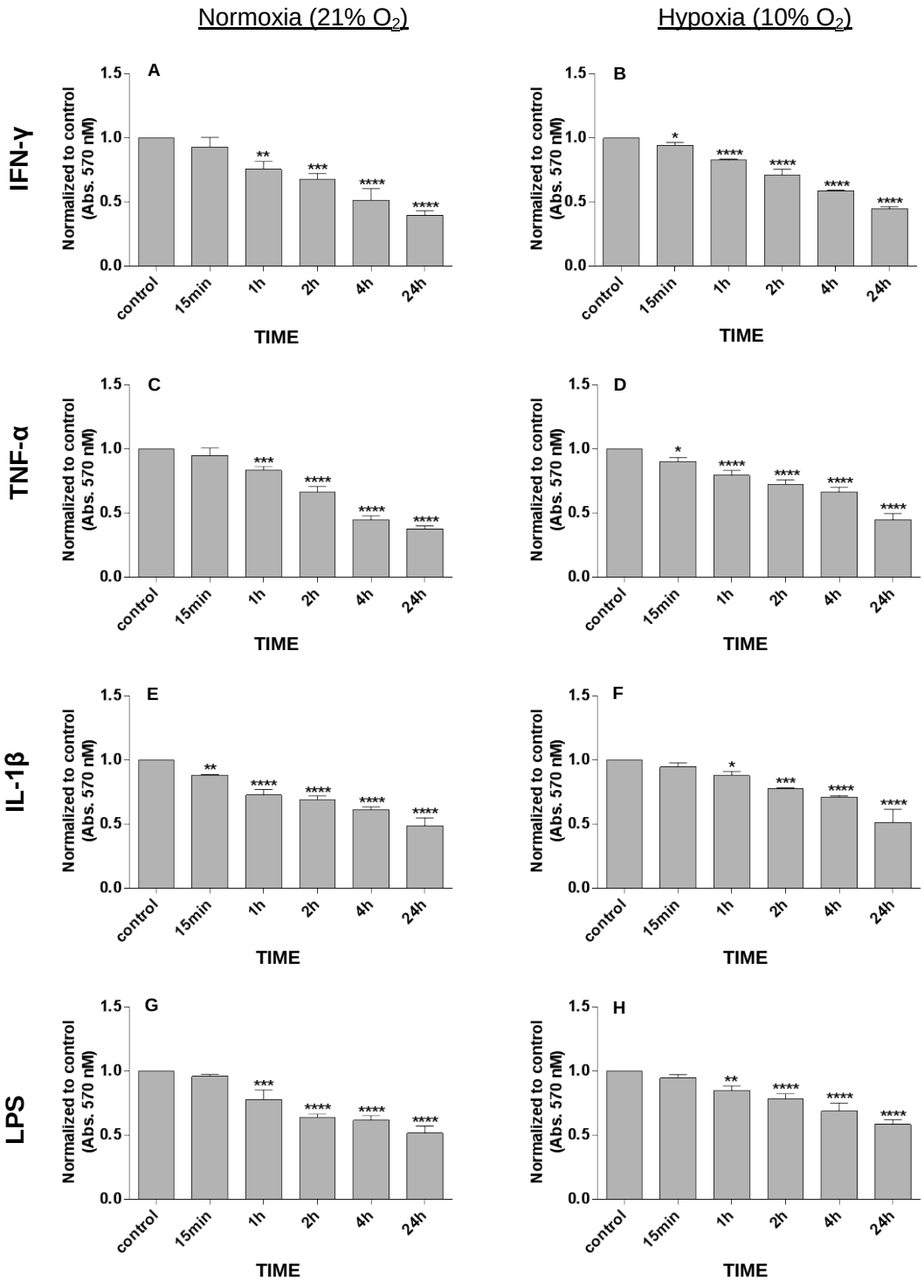

Figure 3.38. Effect of pro-inflammatory cytokines and endotoxin on the viability of pancreatic islets cultured in the the absence of serum over time Pancreatic islets were exposed to 100 ng/ml of IFN-γ (A, B), 100 ng/ml of TNF-α (C, D) and 100 ng/ml of IL-1β (E, F) for 0-24 h in normoxic and hypoxic conditions. The dose of LPS was 10µg/ml in normoxia (G) and 100 µg/ml in hypoxia (H). The viability of the islets was assessed by colorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **P*<0.05, ***P*<0.01, ****P*<0.001 compared with untreated controls

3.4 Discussion

There are many reports that confirm the role of pro-inflammatory cytokines in β-cell dysfunction and development of T1DM.[173] It is also well documented that pro-inflammatory cytokines can be cytotoxic to pancreatic islets *in vitro*. Most of these reports indicated a synergic action of these cytokines that make them lethal.[173],[183] However, the composition of and interaction among pro-inflammatory cytokines are thought to vary during the development of T1DM, which might explain the different level of protection achieved through blocking the action of these cytokines in rat models of autoimmune diabetes.[184] Therefore, understanding the apoptotic effect of each cytokine would be useful in elucidation of the mechanisms of β-cell apoptosis. In this study, we tested the response of pancreatic β-cell lines and pancreatic islets to different individual pro-inflammatory cytokines in rising concentrations under both normoxic and hypoxic conditions and in the presence/absence of serum, to (1) establish cellular models of β-cell apoptosis and (2) determine if oxygen concentration or serum in the culture media sensitized the β-cells to the effects of cytokines.[185]

One of the most important pro-inflammatory cytokines is IL-1β, which is considered a pro-apoptotic cytokine that is known to be responsible for β-cell dysfunction and death.[182] The action of IL-1β as a single agent on β-cells has been mostly studied. Some studies described its action as cytostatic (i.e. the effect will disappear when it is removed) and suggest that it must be used in combination with other cytokines to cause apoptosis in β-cells,[184] this suggests that using IL-1β alone will only prime these cells for apoptosis that would later develop. The finding in this study confirms that using IL-1β alone was enough to produced apoptosis in pancreatic β-cell lines and primary islets. This result agrees with several other studies that demonstrate the ability of IL-1β to induce β-cells apoptosis.[187],[185].

Other studies combined IFN-γ and TNF-α to induced apoptosis in human islets and rat primary β-cells.[184] The mechanism of β-cell damage by these cytokines is realized by a synergic action of both cytokines through activation of calcium channels that results in mitochondrial dysfunction and activation of several caspases.[188] However, the pathway for each cytokine is not clear. The individual use of each cytokine in this study leads to β-cell apoptosis at different concentrations. Moreover, we tried to explore the impact of pro-inflammatory cytokines on β-cells viability under moderate hypoxia. To date, we have had little direct knowledge regarding the impact of hypoxia on β-cells function and insulin secretion.[189]

Pancreatic β-cells consume a large amount of oxygen in order to perform their action this fact renders β-cells very sensitive to hypoxia.[179] Some studies suggest that exposing β-cells to 1% O_2 for 24 h will induce apoptosis.[179]. Other stated a detrimental effect of hypoxia after 18 h of exposure,[188] even the moderate hypoxia (5% or 7%) induced apoptosis in MIN6 cells.[174] In this study, we aimed to expose the pancreatic cell lines (BRIN-BD11, βTC1.6) and pancreatic islets to moderate hypoxia to demonstrate the response of these cells to pro-inflammatory cytokines and compare it with normoxic conditions. At first, we tested the survival of our β-cells in 2% and 5% O_2. However, after 24 h, all cells had died. Therefore, we increased the O_2 level to 10%. The BRIN-BD11 and βTC1.6 cell lines were able to survive and proliferate under this level of moderate hypoxia and after 3 days we were able to perform further experiments.

The assessment of cell viability after exposure to rising concentrations of pro-inflammatory cytokines and endotoxin demonstrate no difference in cell response, IFN-γ, TNF-α and IL-1β along with the endotoxin LPS reduced the viability of cells, in both normoxia and hypoxia condition in the presence and absence of serum. Furthermore, we measured the fold change

in gene expression for the pro-apoptotic and anti-apoptotic genes in both normoxia and hypoxia.

A20 is believed to be a critical negative regulator of the NF-kB signalling pathway that response to different stimuli, including pro-inflammatory cytokines.[180] Moreover, A20 has been found to be highly upregulated anti-apoptotic protein in cytokine-stimulated primary islets and insulinoma cell lines. Consistent with this, overexpression of A20 in islets confers resistance to cytokines mediated activation of NF-kB, protecting them from apoptosis in the early period after islets transplantation.[180] Some studies suggest that A20 cytoprotective effect against apoptosis depends on the abrogation of cytokines-induced NO production.[189][180]. The ability of A20 to inhibit NO production is due to transcriptional blockade of iNOS induction. A20 inhibit NF-kB at a level upstream of IKB degradation, which demonstrates an anti-apoptotic and anti-inflammatory function for A20. These data suggest an important role for A20 in β-cell function and T1DM.[189] In this study, we found that A20 was upregulated in both BRIN-BD11 and βTC1.6 cell lines in normoxia and hypoxia after exposing them to IFN-γ, TNF-α, IL-1β and LPS alone with some variation in the time at which maximal induction was observed. For example, A20 was upregulated in BRIN-BD11 cells 2 hours after exposure to IFN-γ in the presence of serum and after 15 min in absence of serum, while in βTC1.6 it was found after 1 h of exposure, This was also true for the rest of the cytokines; in fact a consistent difference was noted between BRIN BD11 and βTC1.6 cell lines regarding the expression of A20 while the βTC1.6 showed a maximum upregulation after 24 hours in all instance, the BRIN BD11 followed a different manner where the maximum A20 expression was found in the early hours after exposure to cytokines, this may be related to the origin of each cell line and the way they response to cytokines (Table 1 and 2 in appendix 2).

On another hand, an upregulation of TRAIL was found in insulin-secreting MIN6 islets upon exposure to TNF-α and IFN-γ *in vitro.*[190] TRAIL was found to be able to induce a strong cytotoxicity and a high rate of apoptosis in a different type of β-cell lines.[191] The protein encoded by this gene is structurally related to the TNF family of proteins including TNF-α and FasL. It acts through binding to type 1 membrane receptor that mediates apoptosis via a cytoplasmic death domain like DR4 and DR5. There is a controversial opinion regarding the ability of TRAIL to induce apoptosis in pancreatic β-cells while some are confirming this ability.[191] other are suggesting that some β-cells like INS-1 are resistant to TRAIL.[192] In our study, we can confirm that BRIN-BD11 and βTC1.6 cells showed TRAIL expression upregulated after exposure to different types of pro-inflammatory cytokines.

There was a variation in response to cytokines between primary islets and cell lines. The islets appear to be more sensitive than the cell lines used in the current study. For example both cell lines showed an approximate 50% reduction in cell viability after exposure to 1000 ng/ml of IFN-γ while in the islets the 100 ng/ml was enough to produce an approximate 50% reduction in cell viability. Consistent results were found for TNF-α, IL-1β and LPS in the presence of serum. A similar trend was also found in the absence of serum indicating an increase in the sensitivity of the primary islets cells. Although more sensitive to the effects of cytokine treatment, results from primary islets followed consistent trends to those observed in cell lines. It may be speculated that the transformation process undertaken to generate immortal and continually replicating cell lines has resulted in enhanced resistance to insult

3.5 Conclusion

This study confirms the ability of individual pro-inflammatory cytokines to induce apoptosis in pancreatic β-cell lines and primary islets *in vitro*, which was associated with an increase in the expression of TRAIL and A20 in the pancreatic β-cell lines. We also found no significant

difference in the response of β-cells to the addition of pro-inflammatory cytokines and LPS under normoxic and hypoxic conditions, but the primary islets cells were more sensitive to cytokines addition in all instance.

Although the study has reached it is aims limitations were identified, for example, not knowing the actual *in vivo* oxygen concentration experienced by β-cells in the pancreas could be considered one of the limitations of this study. In addition, due to the limited time available the effect of the conditioned media under hypoxia condition wasn't investigated, but this is one of our future works as mentioned in Chapter 6.

Chapter 4

Human bone marrow mesenchymal stem cell conditioned media attenuates cytokine-induced apoptosis in islets and pancreatic β-cell lines.

4.1 Introduction

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) propose minimal criteria to define human mesenchymal stem cells (MSCs). These are the ability to adhere to a plastic surface when maintained in standard culture conditions, positive expression of CD73, CD90 and CD105 surface markers, and negative expression of CD45, CD19, CD14, CD45, and HLA-DR, and an ability to differentiate into adipocytes, chondrocytes, and osteocytes *in vitro* after treatment with differentiation-inducing agents.[193] MSCs can be purified from different sources including bone marrow, adipose tissue, and cord blood.[194] *In vivo,* MSCs are believed to act as precursors for stromal cells that make up the hematopoietic stem cell microenvironment.[195]**.** The first clinical use of MSCs was described in 1995 where they served to accelerate hematopoietic recovery after bone marrow ablation in the context of post-chemotherapy, hematopoietic stem cells transplantation into 15 patients suffering from hematological malignancy.[196] This has been followed by many studies with established techniques for *ex-vivo* expansion and administration of MSCs in a series of clinical trials in a wide range of major diseases including stroke,[197] heart failure,[198] chronic obstructive pulmonary disease (COPD),[199] and liver failure.[200]

The ability of MSCs to rapidly expand from a small clinical sample in to a clinically significant cell number, and their safety profile and relative ease of administration, has made MSCs of significant clinical interest.[201] Many clinical trials testing MSCs are currently recorded in the international registry (www.clinicaltrials.gov). In spite of the promising results for many studies, the mechanism of action is still unclear. Some early research suggested the ability of certain MSC types to differentiate into functional tissue has helped in restoring the injured cells and tissue.[202] However, accumulating evidence now suggests that much of the disease modulating activity of these MSCs results from the actions of their secretome.[203]

Soluble factors secreted by MSCs are believed to alter the secretion profile of dendritic cells which leads to increased production of anti-inflammatory cytokines like IL-10 and decreased production of inflammatory cytokines like IFN-γ and TNF-α.[204] MSCs are also capable of blocking T-cell proliferation through the engagement of the inhibitory molecule programmed death 1 (PD-1) to their ligands, thereby secreting soluble factors that inhibit T-cell proliferation like TGF-β or IL-10. MSCs are able to increase the number of T-regulatory cells that inhibit the immune response.[205] Some reports have stated a relationship between susceptibility to diabetes induction and development with T-regulatory cell activity and expansion of Th17 cells.[206] MSC are capable of rendering T-cells anergic by suppressing the differentiation of monocytes to dendritic cells or by blocking dendritic cell maturation.[204] MSC can suppress proliferation and IgG production of B-cells through secretion of soluble factors.[207] Therefore it seems that MSC therapeutic ability could be based primarily on their production of trophic and immunomodulatory factors.

All of the above features make MSCs attractive for treating T1DM. The immunomodulatory ability of MSCs has been explored in the past decade to treat immune diseases. A study on using MSCs as a treatment for graft-versus-host disease revealed a powerful immunomodulatory capacity for the MSCs.[208] Recent clinical trials involving injection of MSCs via liver puncture demonstrated a significant reduction in islet cell antibodies (ICA), glutamic acid decarboxylase (GAD) and insulin antibodies of two patients over a twelve month period indicating a successful immunomodulatory effect on pancreatic β-cells.[209] However, the results must be interpreted with caution because of the limited number of patients involved. Other studies suggest the possibility of differentiating MSCs into insulin producing cells under certain conditions.[210] However, a study done by Melton and colleagues suggested that new β-cells can only be generated from pre-existing β-cells and not from MSCs.[211]. Given the above evidence, we speculated that mesenchymal stem cell conditioned media (MSC-CM) may exert beneficial immunomodulatory effects on

pancreatic β-cell challenged with inflammatory cytokines. Therefore, the aim of this study was to explore the therapeutic effectiveness of MSC-CM in protecting β-cells from pro-inflammatory cytokines.

4.2 Methods

4.2.1 Materials

Reagents of analytical grade and deionized water were sourced from the same supplier (Sigma, UK). All chemicals employed are listed in Chapter 2, Section 2.1.

4.2.2 Cell models

Human Bone Marrow Mononuclear cells were cultured, maintained and passaged as outlined in Chapter 2, Section 2.2.1.3 in addition to the pancreatic β-cell lines described in Chapter 2, Section 2.2.1.1 and Primary murine islets mentioned in Section 2.2.1.2. All cell models were maintained in an incubator at an atmosphere of 37 °C and 5% CO_2 under normoxia (21% O_2).

4.2.3 Preparation of MSC-CM

MSC-CM was prepared as outlined in Chapter 2, Section 2.2.1.3. The media was applied along with cytokines and LPS as described in Chapter 2, Section 2.2.3.

4.2.4 Measurement of cellular viability and apoptosis

Changes in cellular metabolic activity following the addition of MSC-CM along with cytokines and LPS was assessed by the calorimetric MTT assay as a surrogate marker of viability as outlined in Chapter 2, Section 2.2.4, whilst induction of apoptosis was measured by TUNEL assay as described in Section 2.2.5.

4.2.5 Assessment of early response anti-apoptotic gene induction

After treatment of cells with the optimal concentration of cytokines and LPS in the presence of MSC-CM as outlined in Chapter 2, Section 2.2.3, the induction of early response anti-apoptotic genes was assessed by PCR as outlined in Section 2.2.6.

4.2.6 Assessment of insulin secretion and protein concentration

Insulin release and protein concentration for BRIN-BD11 monolayer cells were determined as outlined in Chapter 2, Section 2.2.7.

4.3 Results

4.3.1 Characterization of Bone Marrow-derived Mesenchymal Stem Cells

As mentioned in Section 2.2.1. human Bone Marrow Mesenchymal stem cells (hMSC) are characterized by trilineage differentiation into adipocyte, chondrocyte and osteocyte. As shown in Figure 4.1A, MSCs were positive for alcian blue staining for chondrogensis, alizarin red staining for osteogensis, and oil red O staining for adipogensis. In addition cell immunophenotype was determined using FACS in which the cells were positive for CD90 (94%), CD73 (90%) and CD105 (80%) while lack the expression for CD14 (7%), CD19 (6%), CD45 (6%), CD34 (5%) and HLA-DR (7%) surface markers (Figure 4.1B, C).

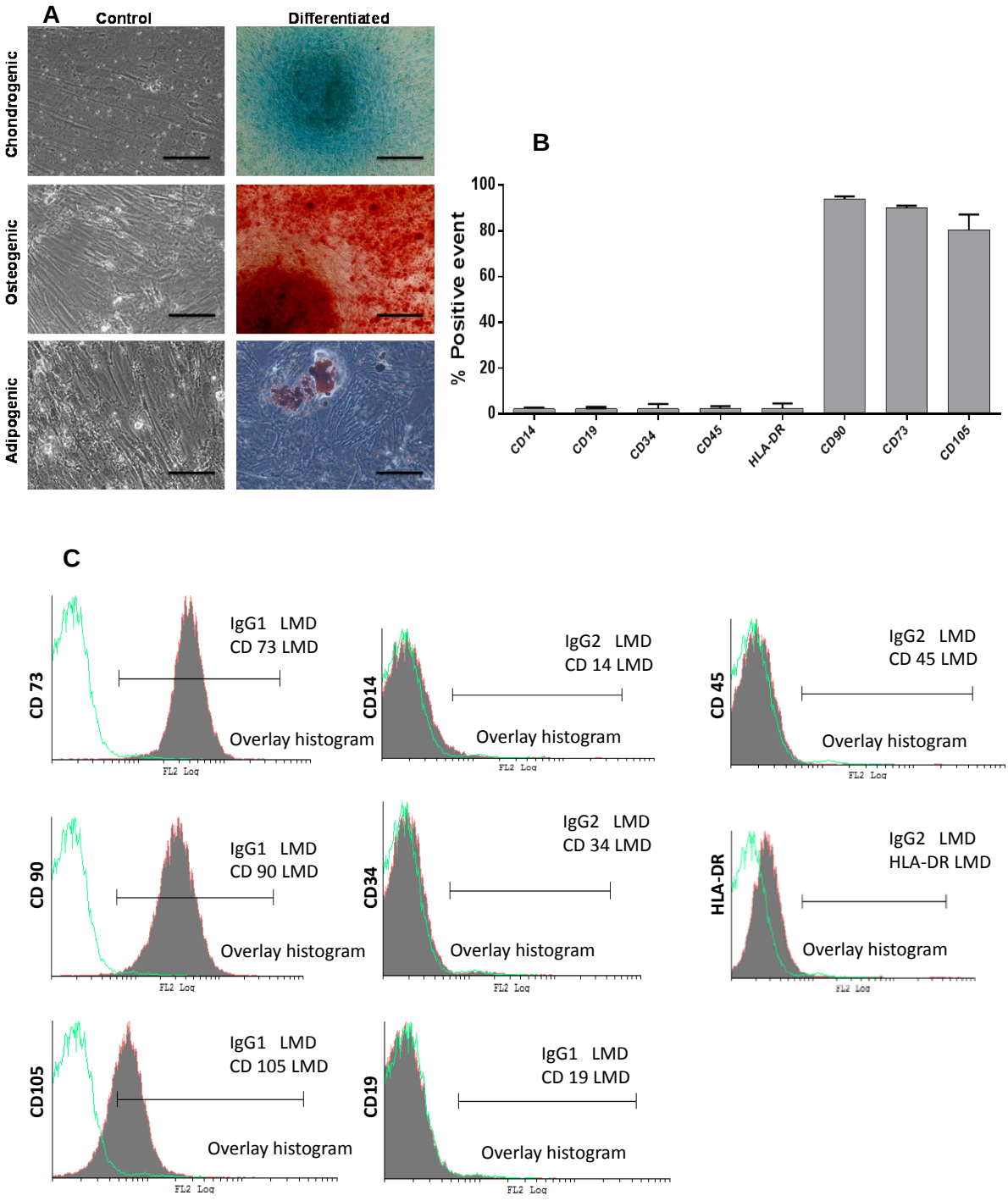

Figure 4.1. Characterization of human Bone Marrow-derived Mesenchymal Stem Cells (hMSCs) (**A**) Classical Tri-lineage differentiation of MSC into adipocyte, osteocyte, and chondrocyte the scale bar equal 100 µm for all images(**B**) The percentage positive event was quantified relative to the relevant isotype control marker; IgG1 (CD73, CD90, CD105, and CD19) or IgG2 (CD14, CD34, CD45, and HLA-DR). **(C)** Expression of typically negative (CD14, CD19, CD37, CD45, HLA-DR) and typically positive (CD90, CD73 and CD105) MSC markers in the human mononuclear mesenchymal stem cells used in this study.

4.3.2 MSC-CM addition increased the resistance of cells towards cytokines and endotoxin

To examine the effect of MSC-CM on cell viability, BRIN-BD11, βTC1.6, and primary islets were treated with cytokines and LPS in the presence and absence of MSC-CM. In all instances, MSC-CM increased the viability of β-cell lines and primary islets. However, the increase in viability of the BRIN-BD11 was greater than that of βTC1.6 and primary islets.

4.3.2.1 Determination of pancreatic cell sensitivity to IFN-γ in the presence of MSC-CM

In all instances the presence of MSC-CM with serum resulted in a significant increase ($P<0.05$-0.001) in viability of pancreatic cell lines following addition of IFN-γ. The highest increase was noted at 1 μg/ml for BRIN-BD11 ($P<0.01$, +62% Figure 4.2A) and βTC1.6 ($P<0.01$, +30% Figure 4.2C). In addition, the presence of MSC-CM significantly increased ($P<0.05$) the resistance of primary islets to the addition of 100 ng/ml of IFN-γ (+26% Figure 4.2E) as compared to non-conditioned media in the presence of serum. Serum free MSC-CM also significantly increase ($P<0.05$-$P<0.01$) the viability for BRIN-BD11 (Figure 4.2B) and βTC1.6 (Figure 4.2D) cell lines. Significant increases in the viability of primary islets were observed in the presence of MSC-CM and 100 ng/ml ($P<0.01$; +31%), or 1 μg/ml ($P<0.05$; +23%) of IFN-γ as compared to non-conditioned media (Figure 4.2F).

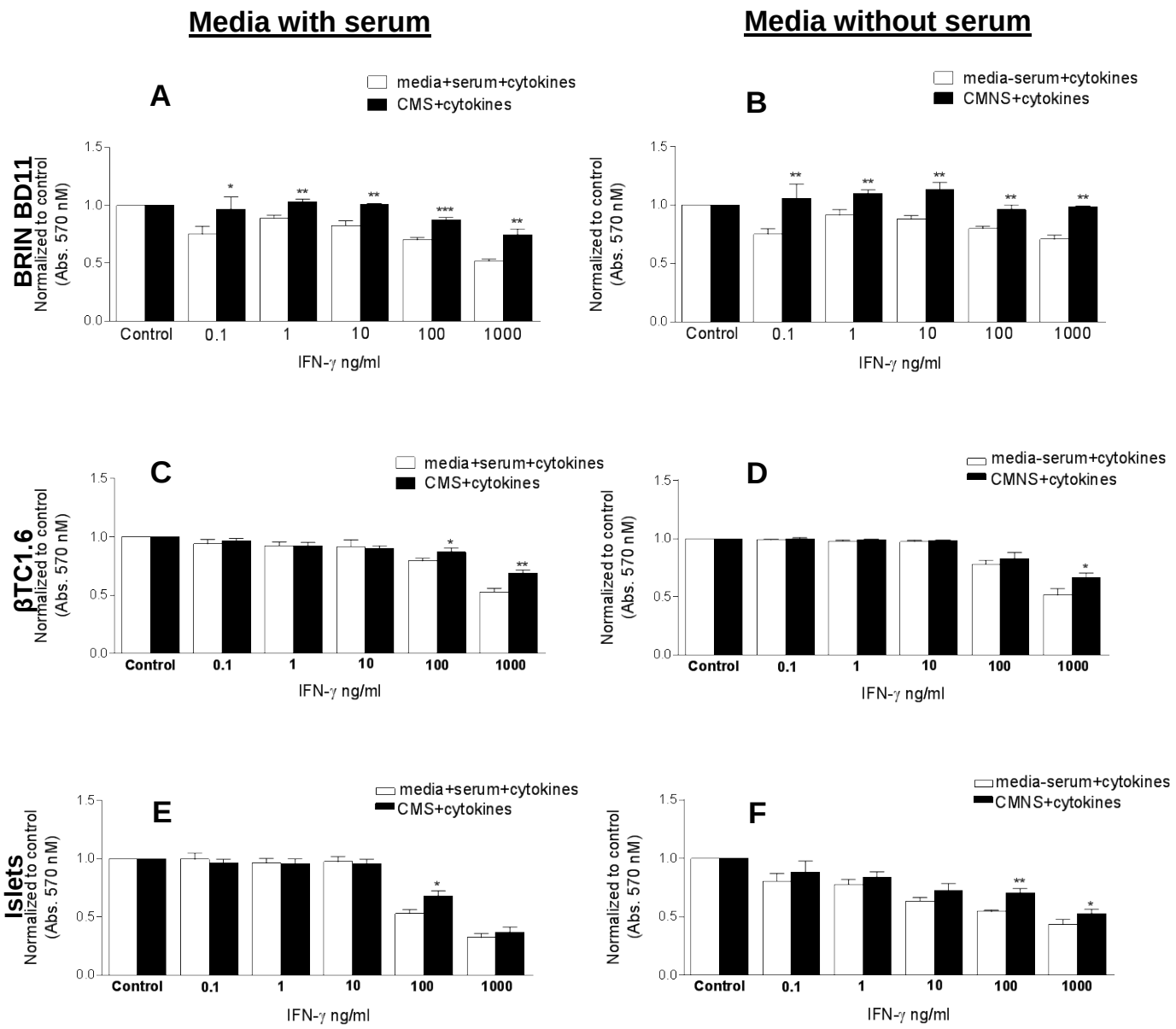

Figure 4.2. Evaluation of IFN-γ effect on cell viability after the addition of MSC-CM with and without serum. Following exposure to rising concentrations of IFN-γ for 24 h, the cellular viability of BRIN-BD11 cells (A, B), βTC1.6 cells (C, D) and primary islets (E, F) grown in MSC-CM with and without serum was assessed by calorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001 compared to non-conditioned media. CMS, MSC-CM with serum; CMNS, MSC-CM no serum; IFN-γ, interferon gamma.

4.3.2.2 Determination of pancreatic cell sensitivity to TNF-α in the presence of MSC-CM

The use of MSC-CM significantly increased (P<0.01-0.001) the viability of BRIN-BD11 treated with TNF-α at a concentrations ≥ 0.1 ng/ml in the presence of serum. However, the highest increase was noted at 100 ng/ml of TNF-α (P<0.001; +67%) as compared to non-conditioned media (Figure 4.3A). In βTC1.6 cells the increase in viability following addition of MSC-CM was significant (P<0.05; +33%) at 1 µg/ml TNF-α in the presence of serum (Figure 4.3C). Under the same conditions, islets showed a significant increase in viability (P<0.01; +14%) at 100 ng/ml TNF-α only as compared to non-conditioned media in presence of serum (Figure 4.3E). The culture of cells in MSC-CM without serum did not significantly affect the results. As such, MSC-CM was still capable of significantly increasing (P<0.05-0.0001) the viability of BRIN-BD11 cells treated with TNF-α, with the highest increase noted at 1 µg/ml TNF-α (P<0.01; +55%; Figure 4.3B). In βTC1.6 the increase in viability was significant (P<0.05; +26%, Figure 4.3D) at 1 µg/ml TNF-α, while in islets, a significant increase in viability (P<0.01; +24%) was noted at 100 ng/ml TNF-α as compared to non-conditioned media in the absence of serum (Figure 4.3F).

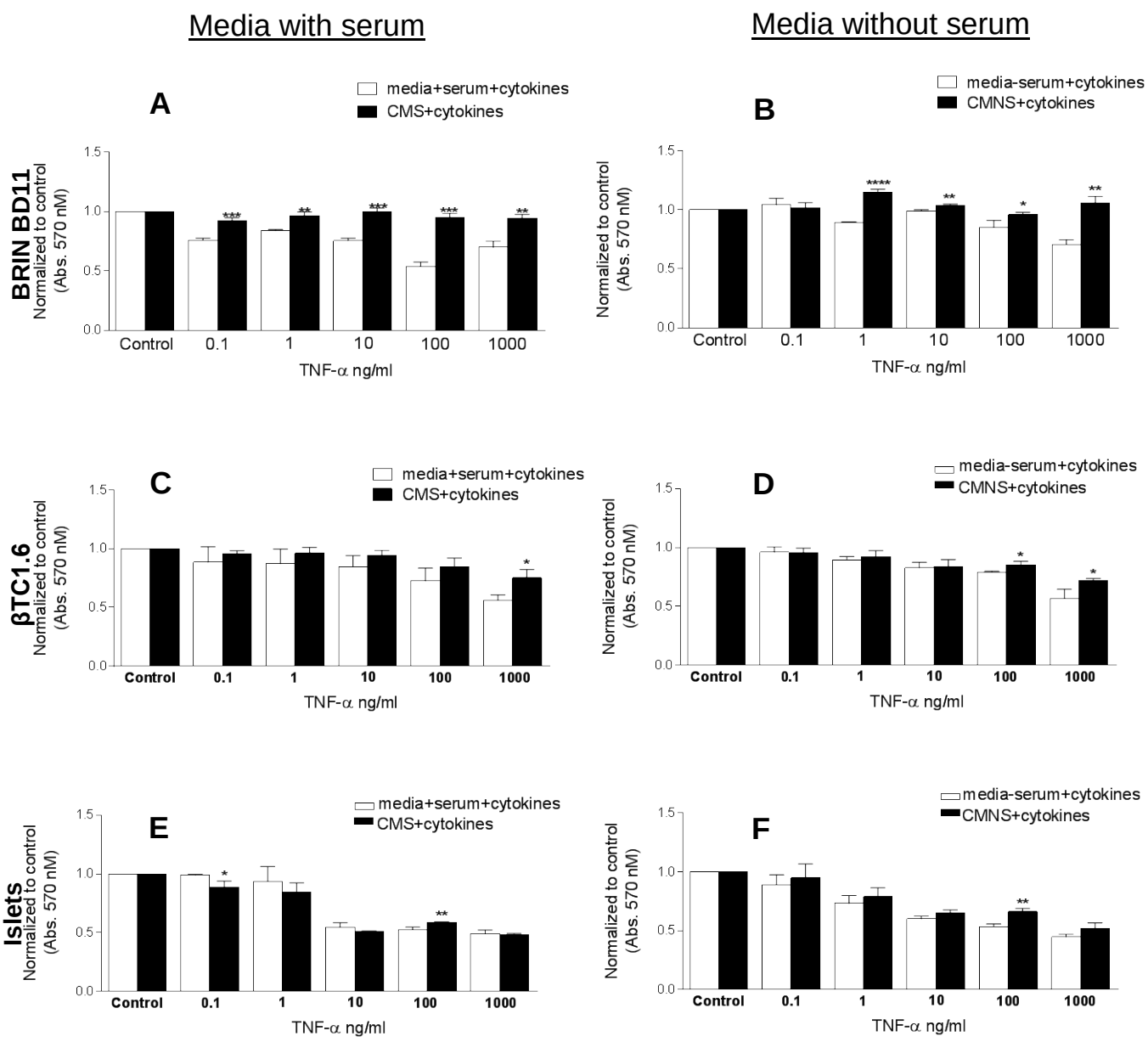

Figure 4.3. Evaluation of TNF-α effect on cell viability after the addition of MSC-CM with and without serum. Following exposure to rising concentrations of TNF-α for 24 h, the cellular viability of BRIN-BD11 cells (A, B), βTC1.6 cells (C, D) and primary islets (E, F) grown in MSC-CM with and without serum was assessed by calorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **p*<0.05, ***p*<0.01,and ****p*<0.001 compared with non-conditioned media. CMS, MSC-CM serum; CMNS, MSC-CM no serum; TNF-α, tumour necrosis factor alpha.

4.3.2.3 Determination of pancreatic cell sensitivity to IL-1β in the presence of MSC-CM

Consistent with observations for IFN-γ and TNF-α, MSC-CM also significantly increased the viability of BRIN-BD11 ($P<0.001$; +76%; Figure 4.4A), βTC1.6 ($P<0.05$, +33%; Figure 4.4C) and primary islets ($P<0.05$; +21%; Figure 4.4E) treated with 100 ng/ml of IL-1β as compared to non-conditioned media in the presence of serum. In the absence of serum significant increases in viability were also noted at 100 ng/ml in BRIN-BD11 cells ($P<0.05$; +32%; Figure 4.4B), βTC1.6 cells ($P<0.05$; +33%; Figure 4.4D), and primary islets ($P<0.05$; +16%; Figure 4.4F).

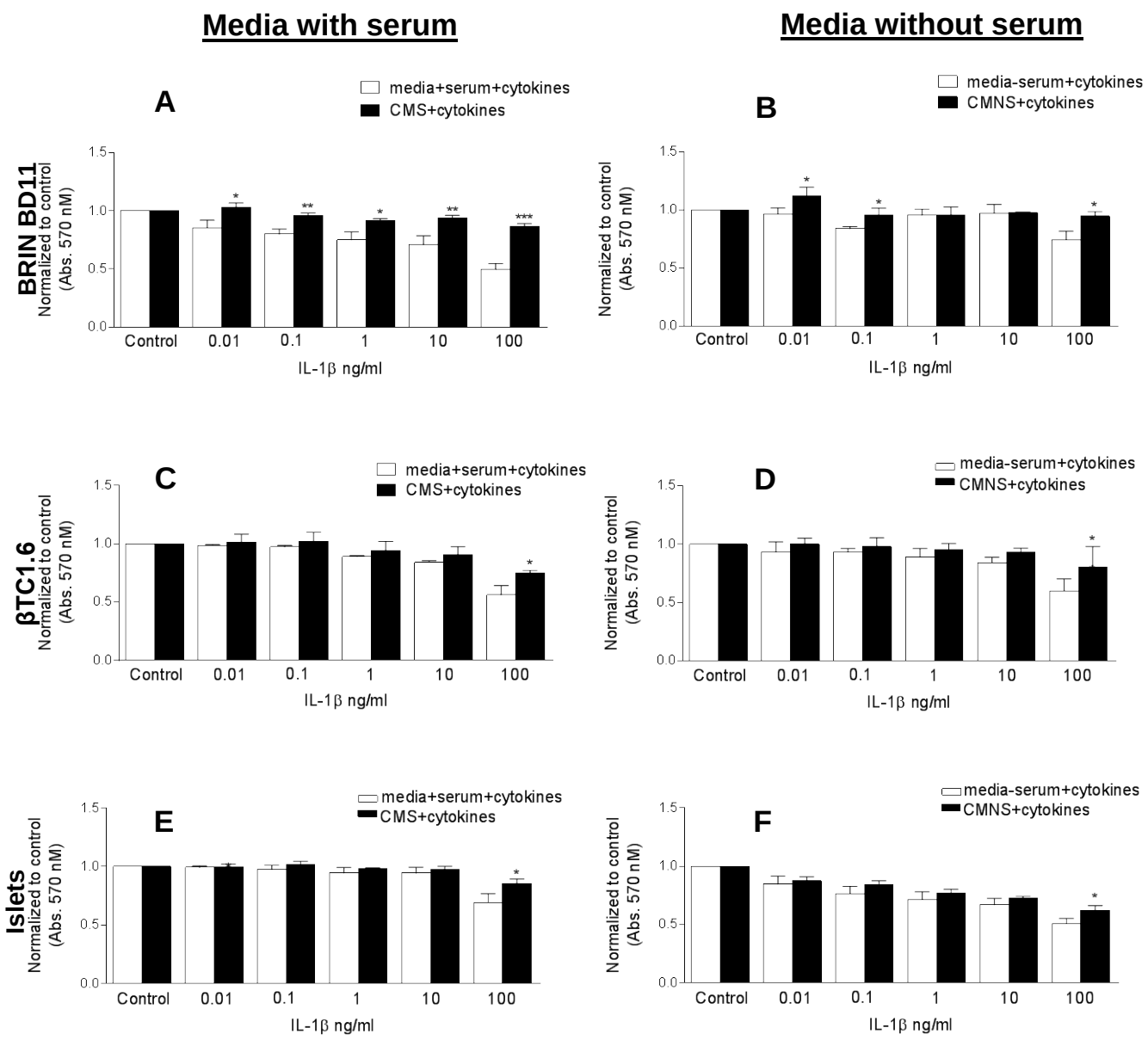

Figure 4.4. Evaluation of IL-1β effect on cell viability after the addition of MSC-CM with and without serum. Following exposure to rising concentrations of IL-1β for 24 h, the cellular viability of BRIN-BD11 cells (A, B), βTC1.6 cells (C, D) and primary islets (E, F) grown in MSC-CM with and without serum was assessed by calorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **p*<0.05, ***p*<0.01, and ****p*<0.001 compared with non-conditioned media. CMS, MSC-CM serum; CMNS, MSC-CM no serum; IL-1β, Interleukin-1β

4.3.2.4 Determination of pancreatic cell sensitivity to LPS in the presence of MSC-CM

The use of MSC-CM significantly increased ($P<0.05$-0.0001) the viability of BRIN-BD11 cells treated with LPS at concentrations ≥ 0.1 µg/ml in the presence of serum (Figure 4.5A). However, the highest increase was noted at 500 µg/ml LPS ($P<0.0001$; +67%; Figure 4.5A) as compared to non-conditioned media. Under the same conditions βTC1.6 cells showed significant enhancements in viability ($P<0.05$-0.01) at doses of 0.1-500 µg/ml LPS with the highest increase noted at 500 µg/ml (+17%; Figure 4.5C). In islets there was a significant increase in viability ($P<0.05$-0.01) at concentrations ≥ 1 µg/ml with the highest increase was noted at 10 µg/ml (+12%; Figure 4.5E) as compared to non-conditioned media in presence of serum. The culture of cells in MSC-CM without serum did not significantly affect the results. As such, MSC-CM was still capable of significantly increasing ($P<0.05$-$P<0.0001$) the viability of BRIN-BD11 cells following the addition of LPS at concentrations ≥100 µg/ml (Figure 4.5B). However in βTC1.6 and primary islets the increase in viability was significant ($P<0.05$) only at 500 µg/ml LPS, resulting in respective (+19% Figure 4.5D), (+21% Figure 4.5F) increases as compared to non-conditioned media.

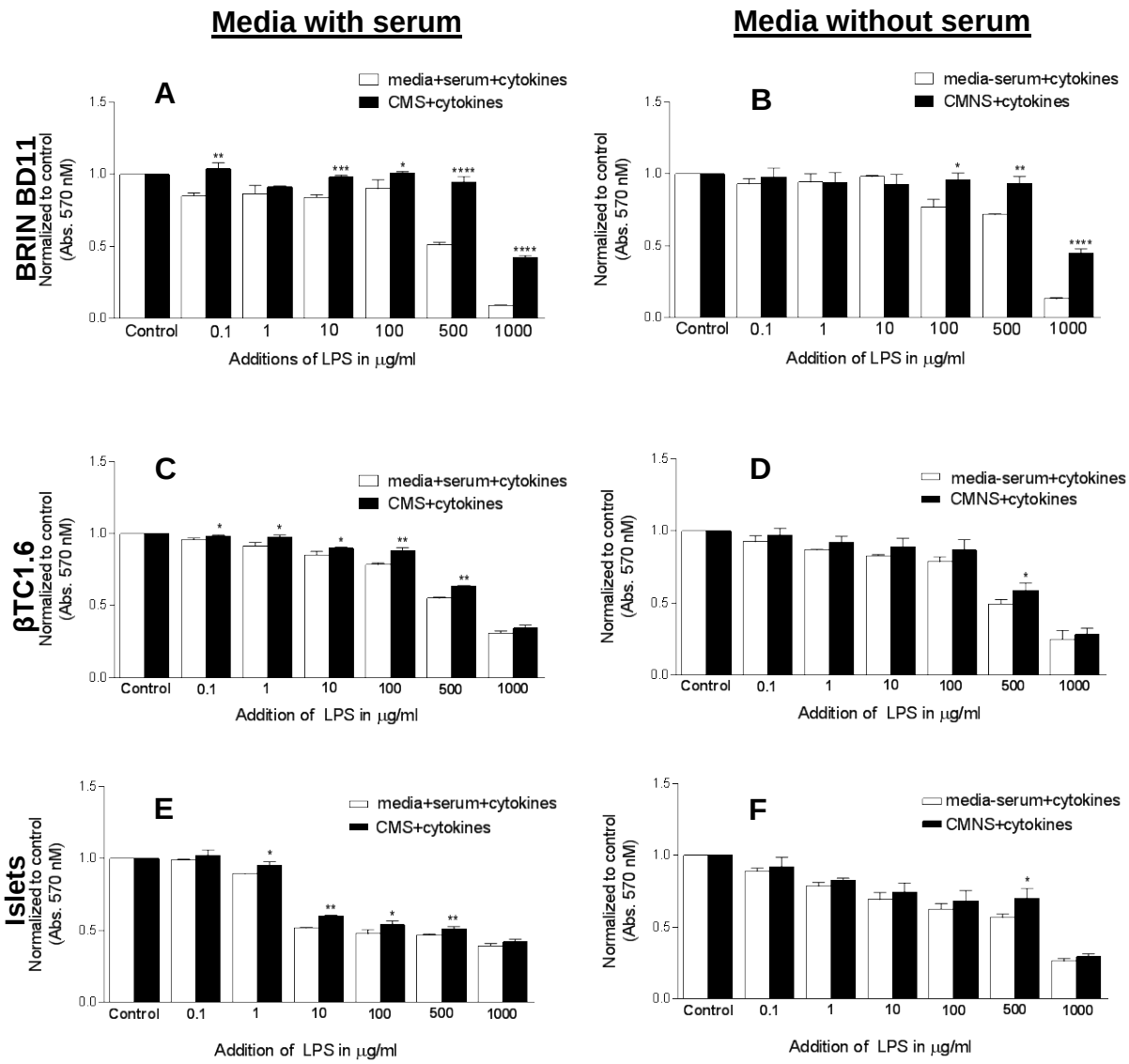

Figure 4.5. Evaluation of LPS effect on cell viability after the addition of MSC-CM with and without serum. Following exposure to rising concentrations of LPS for 24 h, the cellular viability of BRIN-BD11 cells (A, B), βTC1.6 cells (C, D) and primary islets (E, F) grown in MSC-CM with and without serum was assessed by calorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3. **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001 compared with non-conditioned media. CMS, MSC-CM serum; CMNS, MSC-CM no serum; LPS, Lipopolysachharide.

4.3.3 Bone marrow MSC-CM blocks cytokine-induced apoptosis

The above data showed that the addition of MSC-CM enhanced cell viability following the addition of cytokines. In the following experiments, we wished to confirm that the enhancement in cell viability resulted from an inhibition of apoptosis. To evaluate the effectiveness of MSC-CM in this regard the TUNEL assay was performed. MSC-CM blocked cytokine-induced apoptosis in the BRIN-BD11and βTC1.6 cell lines for IFN-γ (Figure 4.6, 4.7), TNF-α (Figure 4.8, 4.9), IL-1β (Figure 4.10, 4.11), and LPS (Figure 4.12, 4.13). H_2O_2 was used as a positive control in all instances.

Please note that in Figures 4.6-4.15, TUNEL images from Chapter 3 have been replicated for comparative purposes. This is not intended as a duplication of data, but rather as reference images to aid the reader in drawing comparisons on the anti-apoptotic effect of MSC-CM treatment, shown in the adjacent panels.

As shown in Figure 4.6, the addition of 1 μg/ml of IFN-γ resulted in the increase in the %positive TUNEL cells (60%) for BRIN BD11 and (56%) for βTC1.6. However, the presence of MSC-CM reduced the %positive TUNEL cells for both cell lines (7%) for BRIN BD11 and (6%) for βTC1.6 indicating the ability of MSC-CM to block IFN-γ induced apoptosis in the presence of serum. A similar trend was found in the absence of serum (Figure 4.7).

Consistent results were observed for cells treated with TNF-α. Figure 4.8 demonstrate the ability of TNF-α to induce apoptosis in BRIN BD11 (56%) and βTC1.6 (53%) cell lines. However, the addition of MSC-CM reduce the % positive TUNEL cells for both cell lines (3%) (6%) respectively in the presence of serum. A similar trend was found in absence of serum (Figure 4.9).

Figure 4.10 demonstrates the ability of 100 ng/ml of IL-1β to induce apoptosis. The %positive TUNEL cells were (46%) for BRIN BD11 and (45%) for βTC1.6. However, the addition of MSC-CM reduce the %positive TUNEL cells to (3%) and (2%) for both cell lines respectively indicating once again the ability of MSC-CM to block apoptosis in the presence of serum. A similar trend was found in absence of serum (Figure 4.11).

Consistent results were found for cells treated with 500 µg/ml of LPS (Figure 4.12). The %positive TUNEL cell was (47%) for both BRIN BD11 and βTC1.6 cell lines. However, the addition of MSC-CM reduces the %positive TUNEL cells for both BRIN BD11 and βTC1.6 (9%) and (6%) respectively in the presence of serum. A similar trend was found in the absence of serum (Figure 4.13)

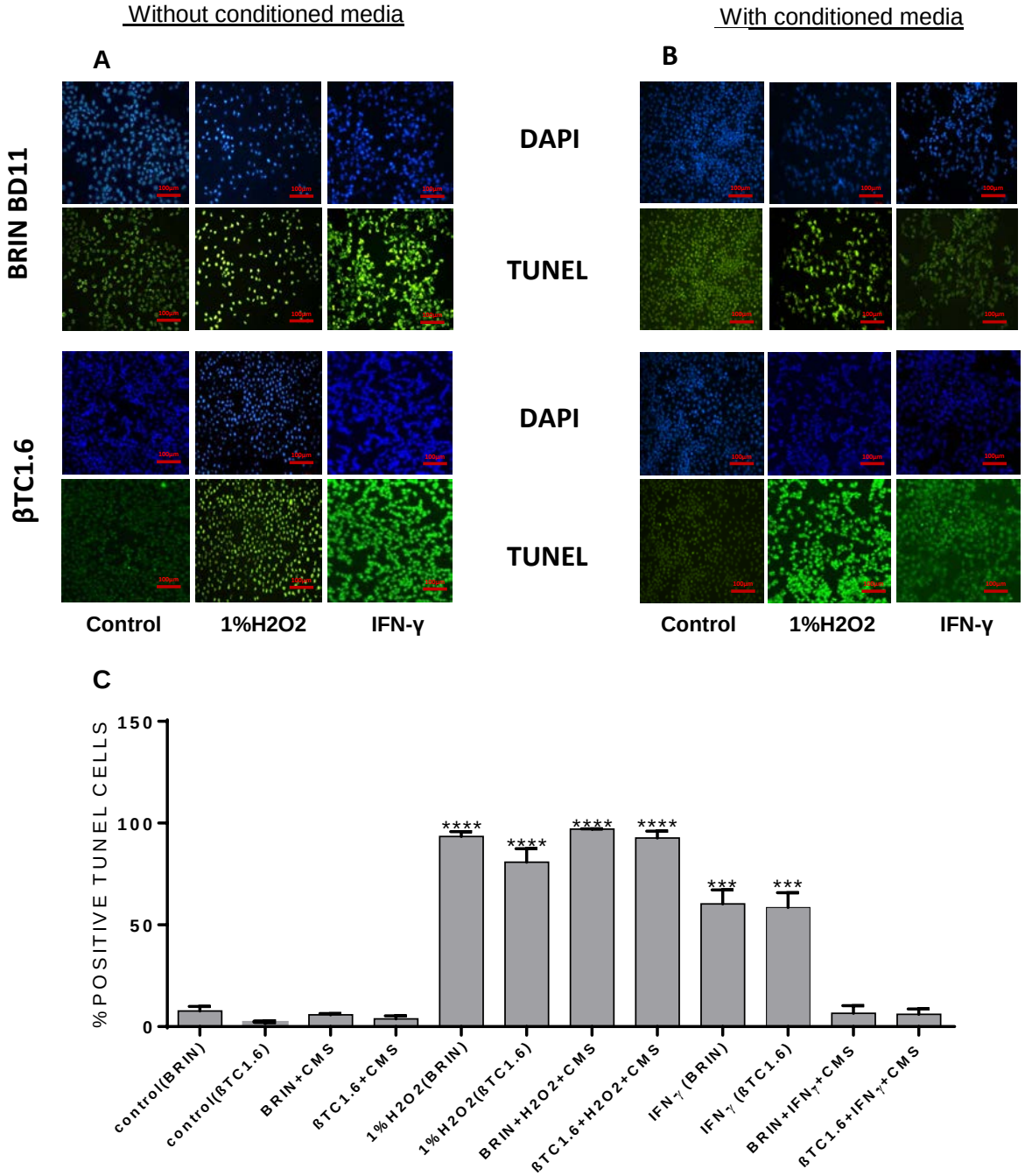

Figure 4.6. Assessment of IFN-γ-induced apoptosis in pancreatic cell lines with and without MSC-CM in the presence of serum. **A.** For comparative purposes, images showing IFN-γ–induced apoptosis in the absence of MSC-CM are repeated here (see Chapter 3). **B.** Fluorescent images showing MSC-CM blockage of IFN-γ-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines after 24 hours of exposure to 1 µg/ml of IFN-γ. **C.** The %positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells (n=3) and presented as mean ± standard deviation (SD). ***p<0.001 and ****p<0.0001 compared with untreated controls. CMS, MSC-CM with serum; H_2O_2, hydrogen peroxide; IFN-γ, interferon gamma; MSC-CM, mesenchymal stem cell conditioned media.

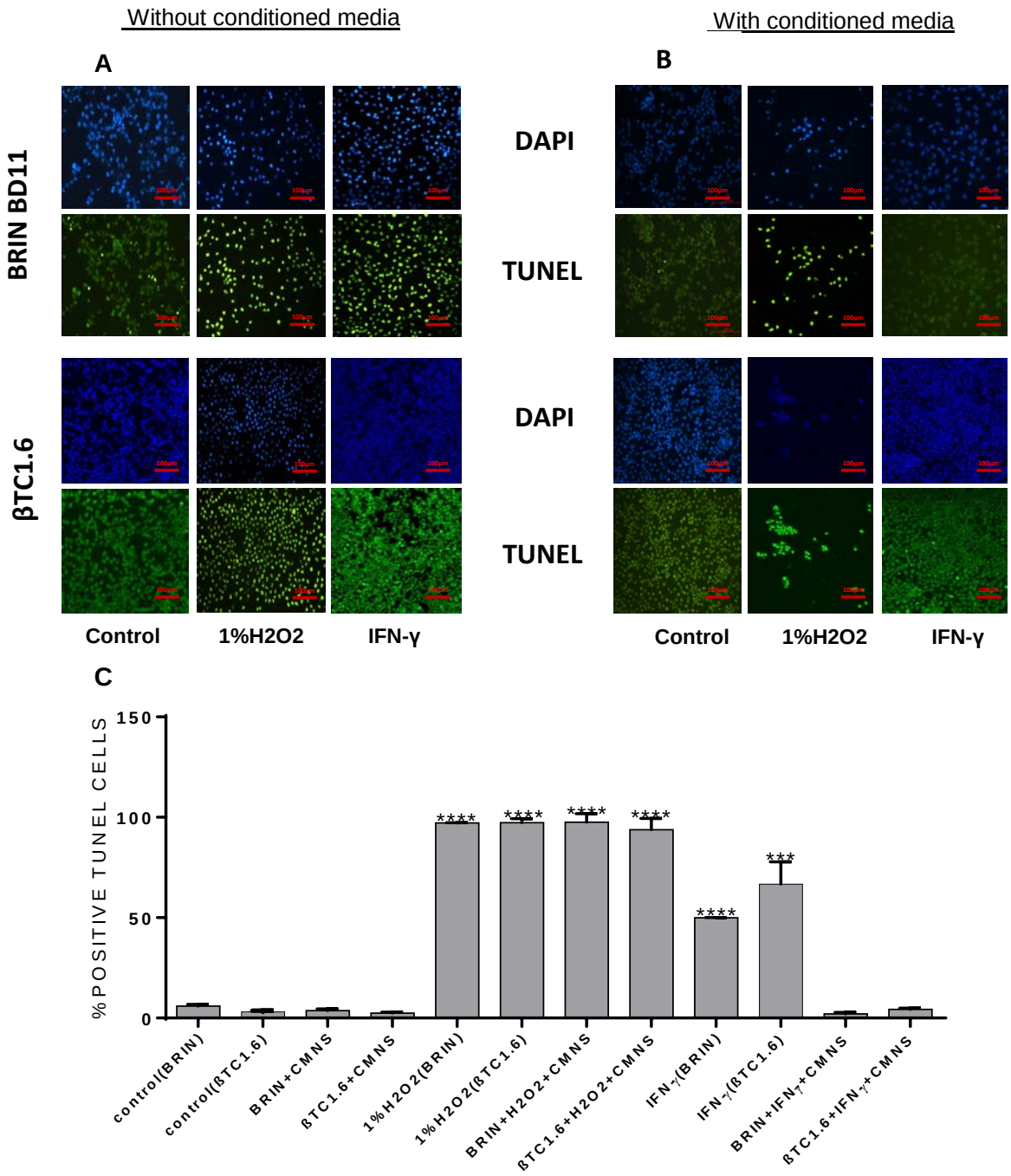

Figure 4.7. Assessment of IFN-γ-induced apoptosis in pancreatic cell lines with and without MSC-CM in the absence of serum. **A.** For comparative purposes, images showing IFN-γ–induced apoptosis in the absence of MSC-CM are repeated here (see Chapter 3). **B.** Fluorescent images showing MSC-CM blockage of IFN-γ-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines after 24 hours of exposure to 1 µg/ml of IFN-γ. **C.** The %positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells (n=3) and presented as mean ± standard deviation (SD). ***p<0.001 and ****p<0.0001 compared with untreated controls. CMNS, MSC-CM without serum; H_2O_2, hydrogen peroxide; IFN-γ, interferon gamma; MSC-CM, mesenchymal stem cell conditioned media.

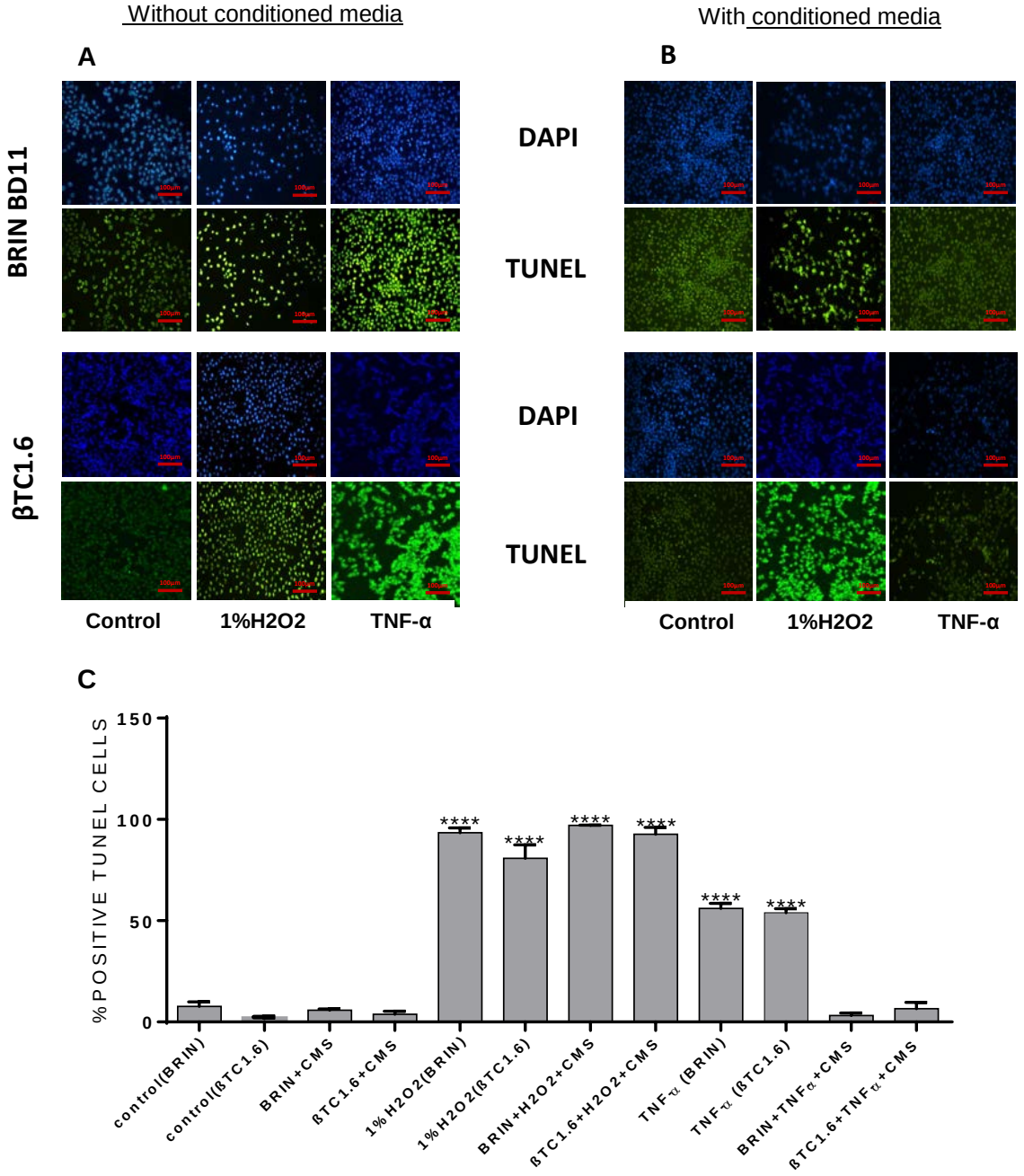

Figure 4.8. Assessment of TNF-α-induced apoptosis in pancreatic cell lines with and without MSC-CM in the presence of serum. **A.** For comparative purposes, images showing TNF-α–induced apoptosis in the absence of MSC-CM are repeated here (see Chapter 3). **B.** Fluorescent images showing MSC-CM blockage of TNF-α-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines after 24 hours of exposure to 100 ng/ml and 1 µg/ml respectively of TNF-α. **C.** The %positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells (n=3) and presented as mean ± standard deviation (SD). ****p<0.0001 compared with untreated controls. CMS, MSC-CM with serum; H2O2, hydrogen peroxide; MSC-CM, mesenchymal stem cell conditioned media; TNF-α, Tumour necrosis factor alpha.

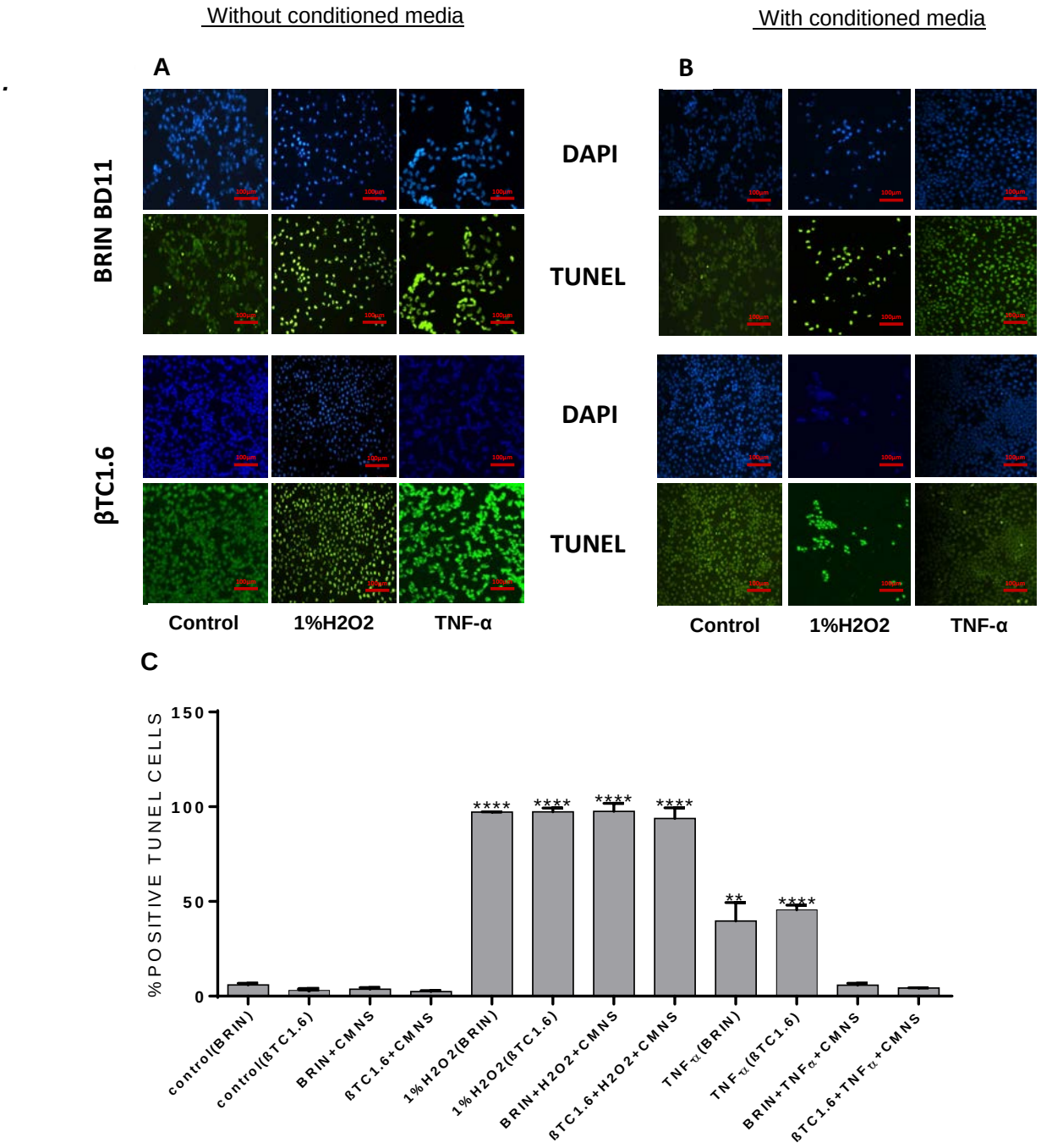

Figure 4.9. Assessment of TNF-α-induced apoptosis in pancreatic cell lines with and without MSC-CM in the absence of serum. **A.** For comparative purposes, images showing TNF-α–induced apoptosis in the absence of MSC-CM are repeated here (see Chapter 3). **B.** Fluorescent images showing MSC-CM blockage of TNF-α-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines after 24 hours of exposure to 1 µg/ml respectively of TNF-α. **C.** The %positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells (n=3) and presented as mean ± standard deviation (SD). ***p*<0.01 and *****p*<0.0001 compared with untreated controls. CMNS, MSC-CM without serum; H2O2, hydrogen peroxide; MSC-CM, mesenchymal stem cell conditioned media; TNF-α, Tumour necrosis factor alpha.

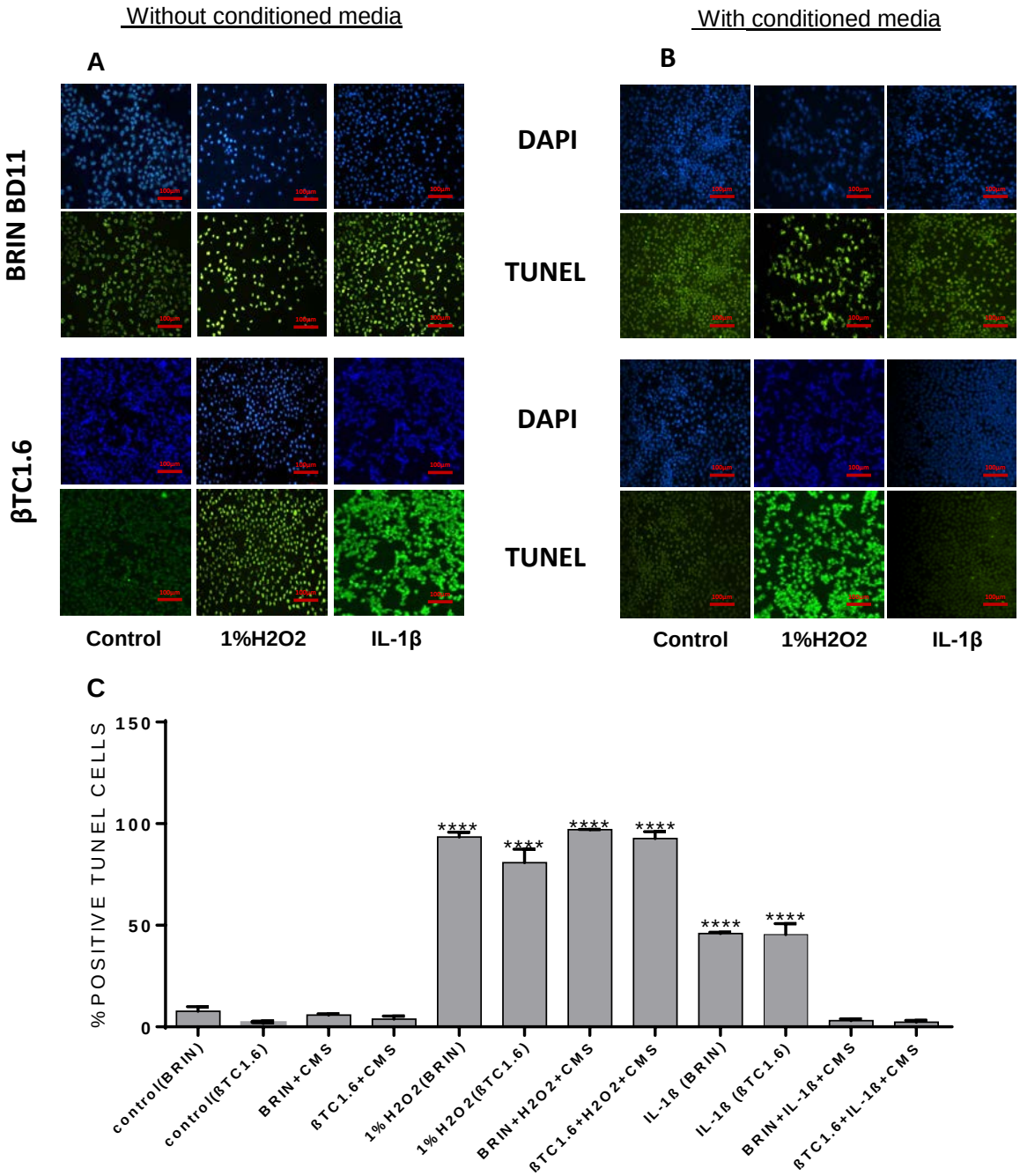

Figure 4.10. Assessment of IL-1β-induced apoptosis in pancreatic cell lines with and without MSC-CM in the presence of serum. **A.** For comparative purposes, images showing IL-1β–induced apoptosis in the absence of MSC-CM are repeated here (see Chapter 3). **B.** Fluorescent images showing MSC-CM blockage of IL-1β–induced apoptosis in BRIN-BD11 and βTC1.6 cell lines after 24 hours of exposure to 100 ng/ml of IL-1β. **C.** The %positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells (n=3) and presented as mean ± standard deviation (SD). ****p<0.0001 compared with untreated controls. CMS, MSC-CM with serum; H2O2, hydrogen peroxide; IL-1β, interleukin-1 beta; MSC-CM, mesenchymal stem cell conditioned media.

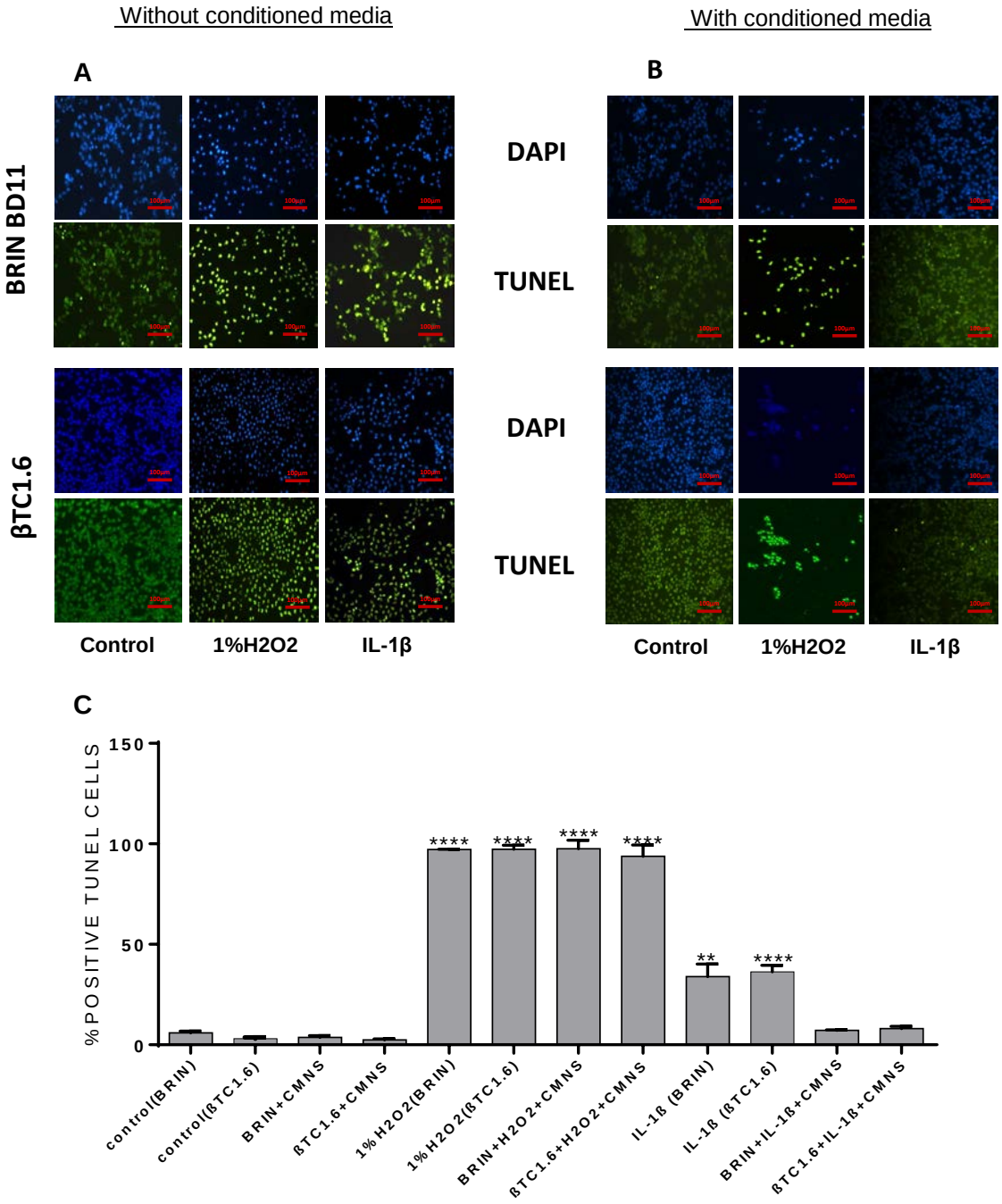

Figure 4.11. Assessment of IL-1β-induced apoptosis in pancreatic cell lines with and without MSC-CM in the absence of serum. **A.** For comparative purposes, images showing IL-1β–induced apoptosis in the absence of MSC-CM are repeated here (see Chapter 3). **B.** Fluorescent images showing MSC-CM blockage of IL-1β–induced apoptosis in BRIN-BD11 and βTC1.6 cell lines after 24 hours of exposure to 100 ng/ml of IL-1β. **C.** The %positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells (n=3) and presented as mean ± standard deviation (SD). ***p*<0.01, ****p*<0.001 and *****p*<0.0001 compared with untreated controls. CMNS, MSC-CM without serum; H2O2, hydrogen peroxide; IL-1β, interleukin-1 beta; MSC-CM, mesenchymal stem cell conditioned media.

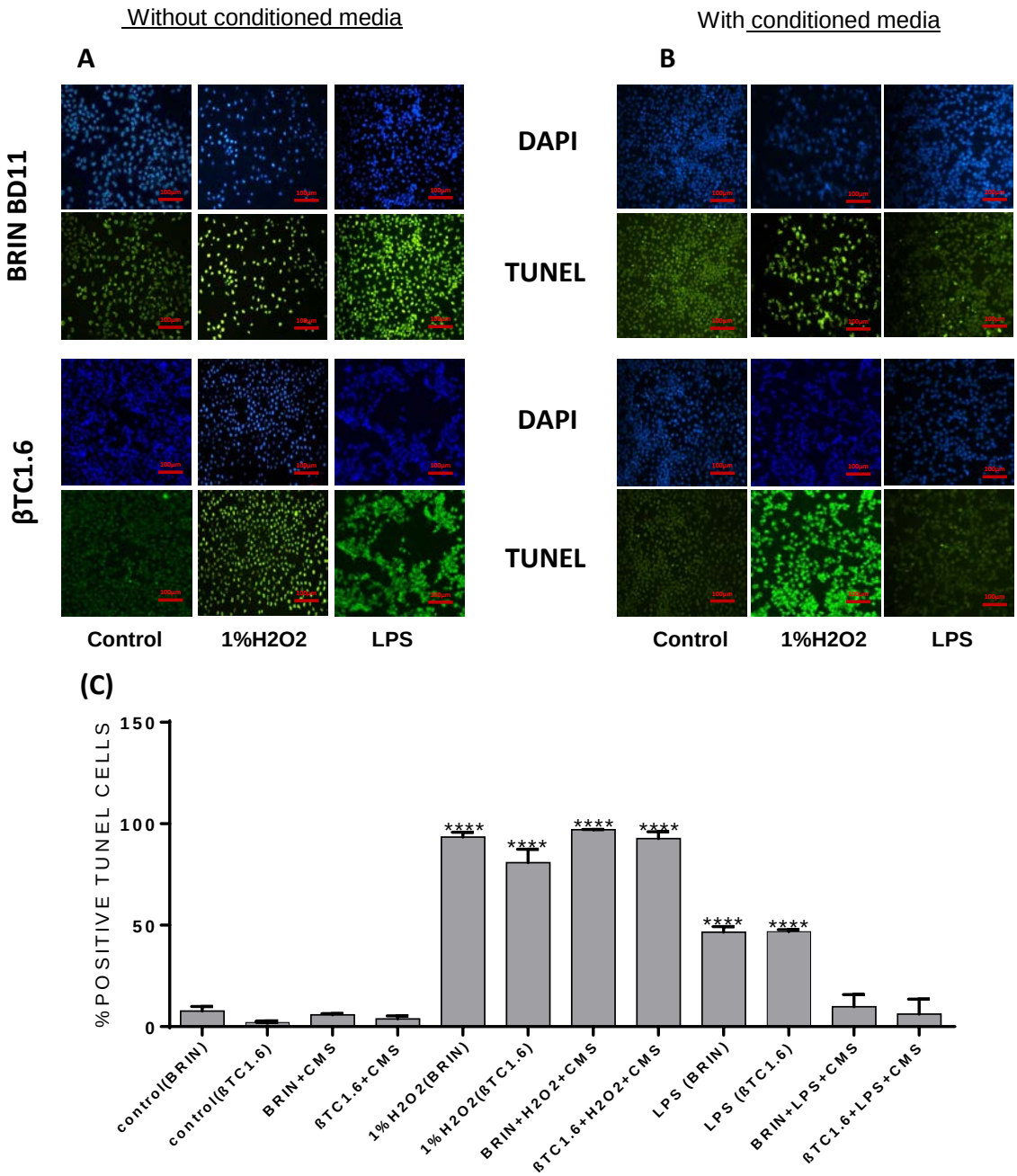

Figure 4.12. Assessment of LPS-induced apoptosis in pancreatic cell lines with and without MSC-CM in the presence of serum. **A.** For comparative purposes, images showing LPS–induced apoptosis in the absence of MSC-CM are repeated here (see Chapter 3). **B.** Fluorescent images showing MSC-CM blockage of LPS–induced apoptosis in BRIN-BD11 and βTC1.6 cell lines after 24 hours of exposure to 500 µg/ml of IL-1β. **C.** The %positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells (n=3) and presented as mean ± standard deviation (SD). ****p<0.0001 compared with untreated controls. CMS, MSC-CM with serum; H2O2, hydrogen peroxide; LPS, lipopolysaccharide; MSC-CM, mesenchymal stem cell conditioned media.

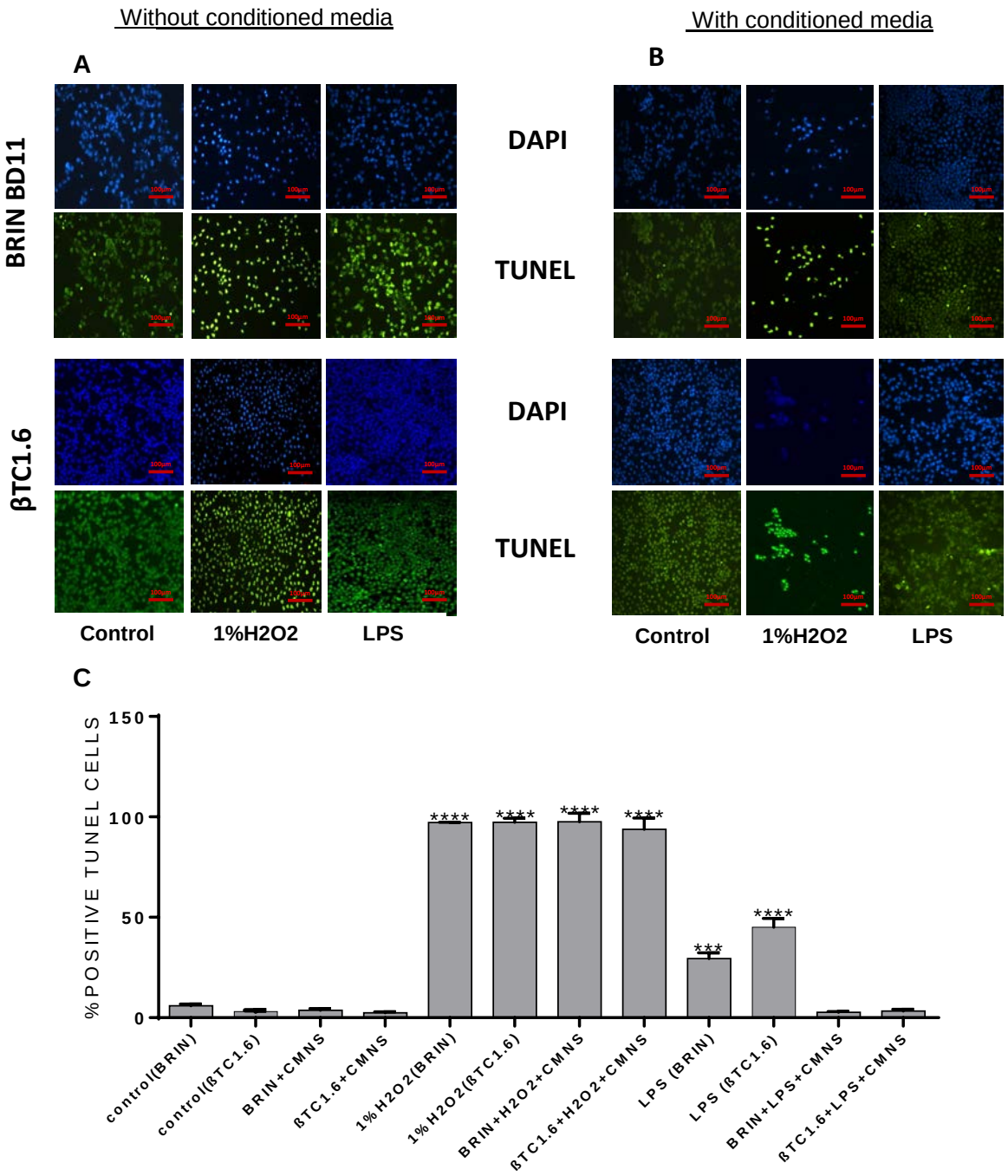

Figure 4.13. Assessment of LPS-induced apoptosis in pancreatic cell lines with and without MSC-CM in the absence of serum. **A.** For comparative purposes, images showing LPS–induced apoptosis in the absence of MSC-CM are repeated here (see Chapter 3). **B.** Fluorescent images showing MSC-CM blockage of LPS–induced apoptosis in BRIN-BD11 and βTC1.6 cell lines after 24 hours of exposure to 500 µg/ml of IL-1β. **C.** The %positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells (n=3) and presented as mean ± standard deviation (SD). *** p<0.001 and **** p<0.0001 compared with untreated controls. CMNS, MSC-CM without serum; H2O2, hydrogen peroxide; LPS, lipopolysaccharide; MSC-CM, mesenchymal stem cell conditioned media.

The MSC-CM was also able to block cytokine-induced apoptosis in primary islets as shown in Figures 4.14 and 4.15. It was difficult to count the actual number of positive TUNEL cells due to the clustering of islets, but the fluorescent images show a clear apoptosis in the islets which was reduced after the addition of MSC-CM.

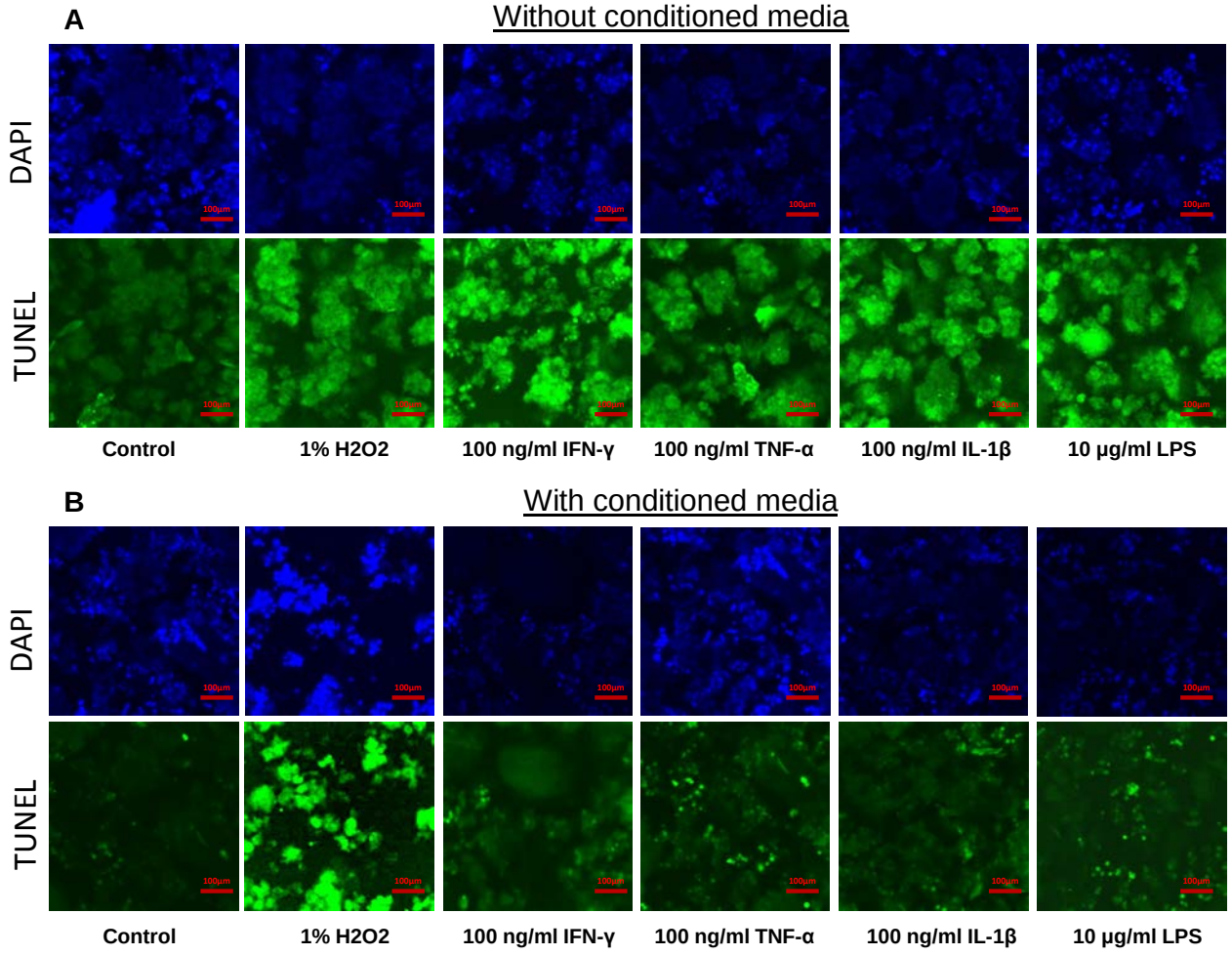

Figure 4.14. Assessment of cytokine-induced apoptosis in pancreatic islets before and after the addition of MSC-CM in the presence of serum. **(A)** Fluorescent images of cytokine–induced apoptosis in CD-1 mice islets after 24 hours of exposure to different cytokines are shown here again for comparison purpose only. **(B)** Images of islets after the addition of MSC-CM. The MSC-CM was able to block cytokine-inducedapoptosis, the blue nuclei are DAPI staining and the green nuclei are TUNEL positive nuclei. IFN-γ (Interferon-gamma), TNF-α (Tumour necrosis factor-alpha), IL-1β (interleukin-1 beta), and LPS (lipopolysaccharide).

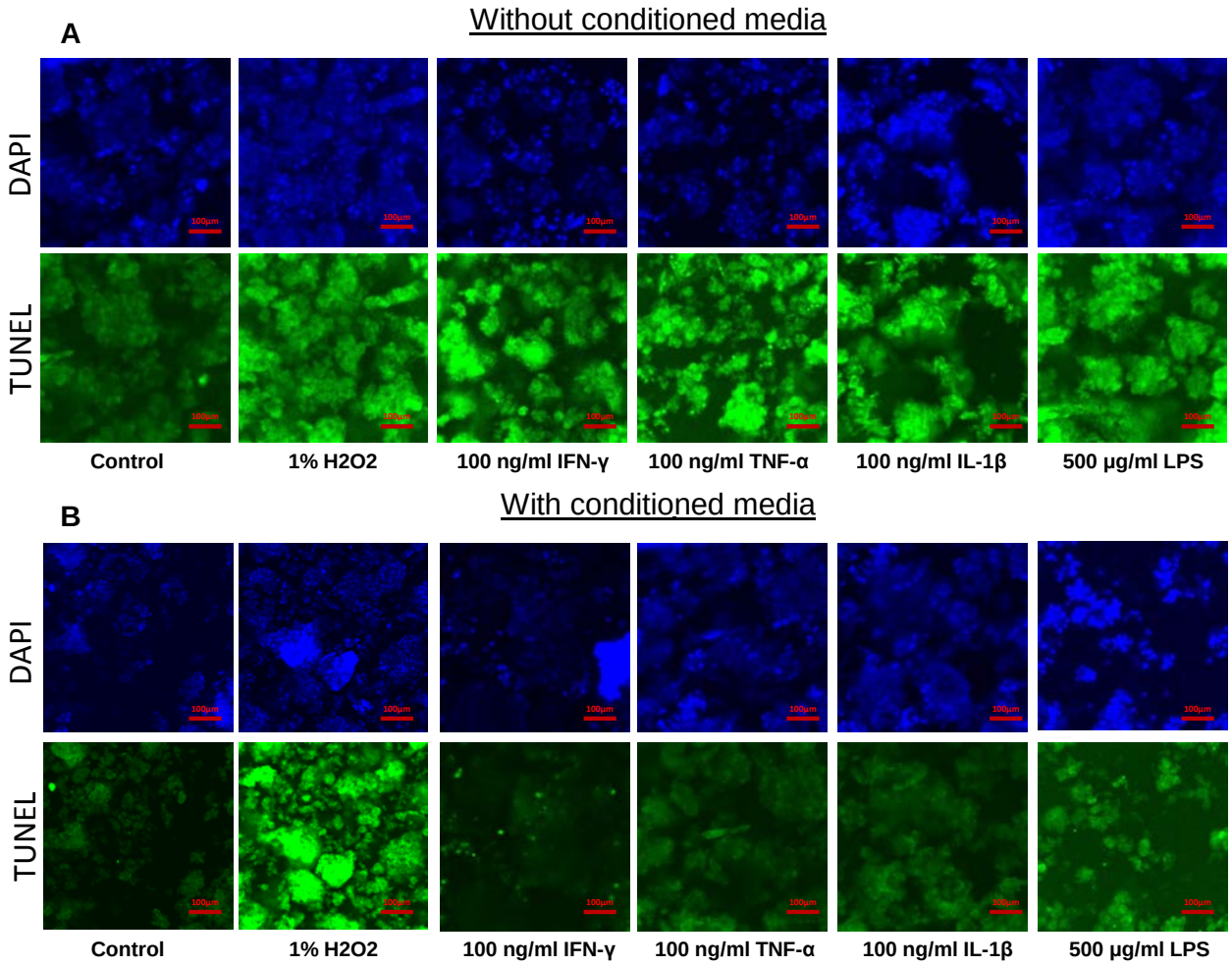

Figure 4.15. Assessment of cytokine-induced apoptosis in pancreatic islets before and after the addition of MSC-CM in the absence of serum. **(A)** Fluorescent images of cytokine-inducedapoptosis in CD-1 mice islets after 24 hours of exposure to different types of cytokines are shown here again for comparative purposes only. **(B)** Images of islets after the addition of MSC-CM. The MSC-CM was able to block cytokine-inducedapoptosis, the blue nuclei are DAPI staining and the green nuclei are TUNEL positive nuclei. IFN-γ (Interferon-gamma), TNF-α (Tumour necrosis factor-alpha), IL-1β (interleukin-1 beta), and LPS (lipopolysaccharide).

4.3.4 Induction of early response genes important in mediating β-cell apoptosis

To assess the induction of early response genes important in the mediation of β-cell apoptosis, qPCR was performed to assess the expression of TRAIL and A20 mRNA before and after the addition of MSC-CM and following exposure of the BRIN-BD11 and βTC1.6 cell lines to cytokines or LPS (Figures 4.16-4.23). This was accompanied by assessments of cellular viability over times, following exposure to cytokines or LPS in the presence or absence of MSC-CM.

4.3.4.1 Times-dependent induction of A20 and TRAIL following exposure to IFN-γ in the presence of MSC-CM

Following exposure to 1 µg/ml IFN-γ, maximal expression of TRAIL was observed after 24 h, whilst maximal expression of A20 was observed after 2 h in BRIN-BD11 cells cultured in the presence of serum, but absence of MSC-CM. Under serum-free conditions, maximal expression of TRAIL and A20 was observed after 24 h and 15 min respectively (Figure 4.16). However, the addition of MSC-CM significantly ($P<0.05$-$P<0.001$) reduced the induction of both genes in all instances regardless of whether cells were grown in the presence or absence of serum (Figure 4.16). This was accompanied by an IFN-γ-mediated times-dependent reduction in cell viability, which was reversed through the addition of MSC-CM.

The exposure of βTC1.6 to 1 µg/ml of IFN-γ resulted in an increase in the expression of TRAIL and A20, the maximal expression was noted after 24 h of exposure for both genes and in both media with and without serum (Figure 4.17). However, the addition of MSC-CM significantly ($P<0.05$-$P<0.0001$) reduced the induction of both genes in all instances regardless of whether cells were grown in the presence or absence of serum (Figure 4.17). This again was associated with IFN-γ-mediated times-dependent reduction in cell viability, which was reversed through the addition of MSC-CM

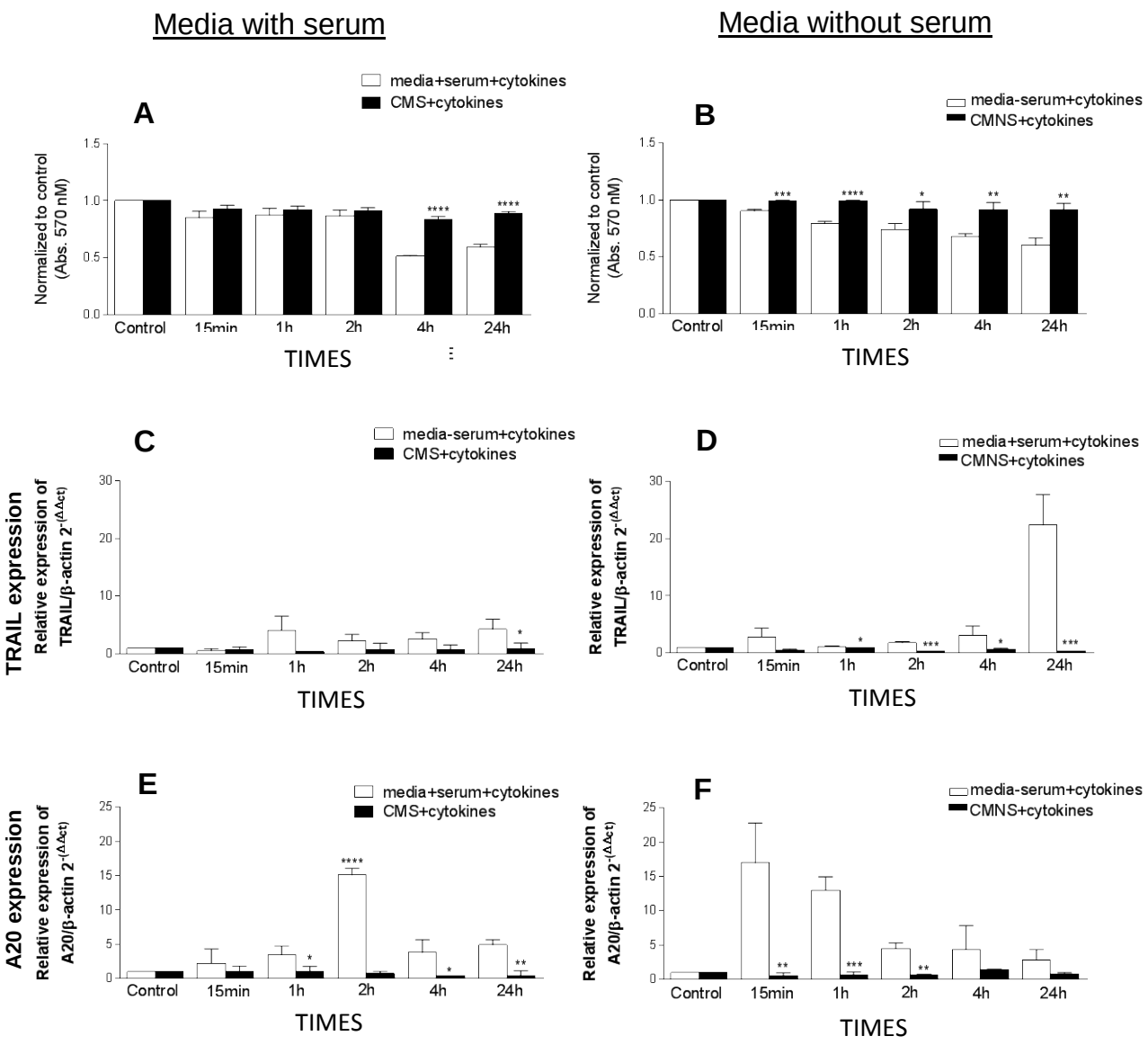

Figure 4.16. Assessment of IFN-γ effect on the viability and relative gene expression at different exposure times in BRIN-BD11 cultured in MSC-CM with and without serum. Following exposure to IFN-γ (1 µg/ml) at different times, the cellular viability of BRIN-BD11 cultured in MSC-CM was assessed by calorimetric MTT assay (A, B) associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this times course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3. **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001 compared with non- conditioned media. IFN-γ, interferon gamma.

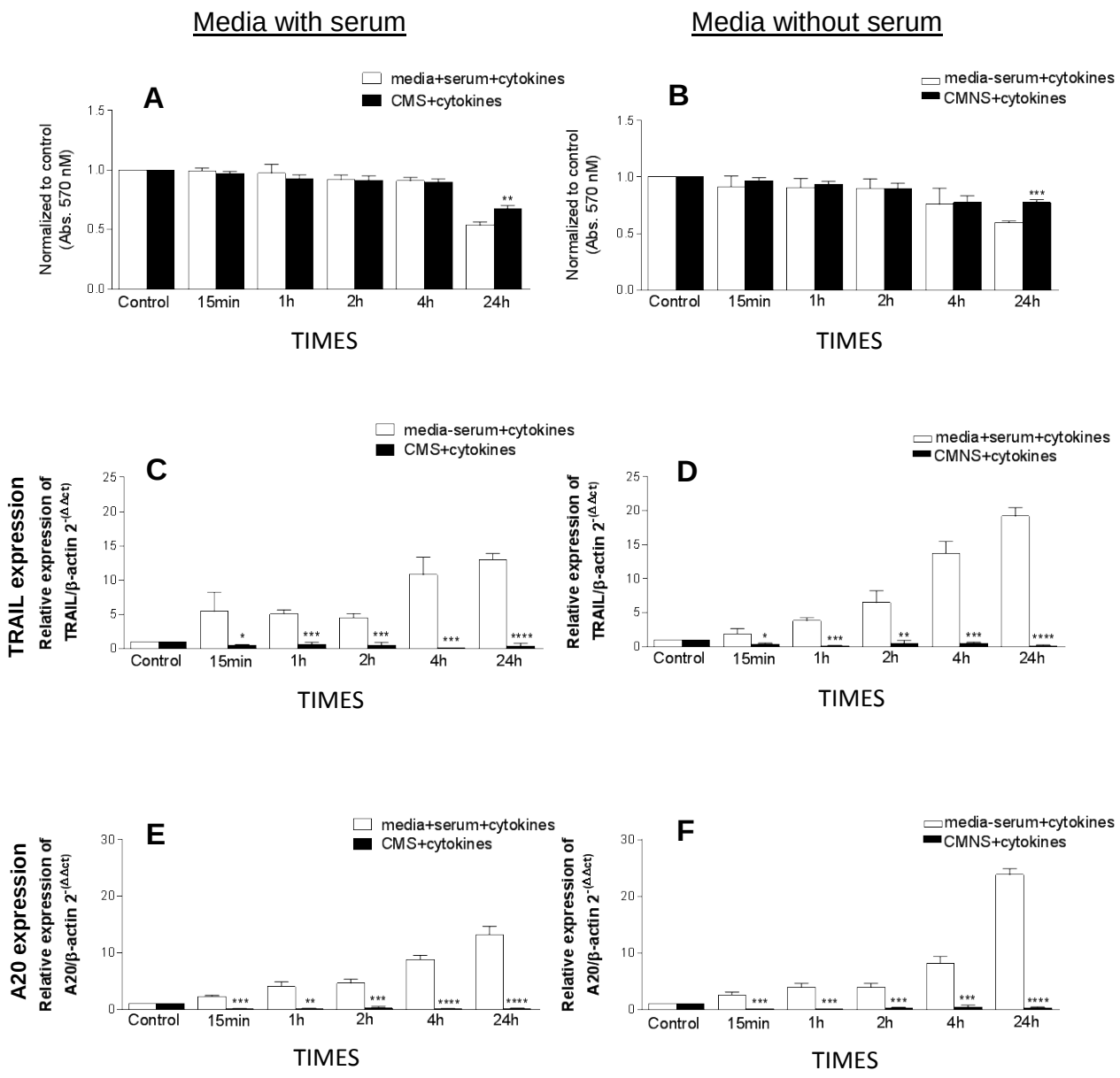

Figure 4.17. Assessment of IFN-γ effect on the viability and relative gene expression at different exposure times in βTC1.6 cultured in MSC-CM with and without serum. Following exposure to IFN-γ (1 µg/ml) at different times, the cellular viability of βTC1.6 cultured in MSC-CM was assessed by calorimetric MTT assay (A, B) associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this times course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared with non-conditioned media. IFN-γ, interferon gamma.

4.3.4.2 Times-dependent induction of A20 and TRAIL following exposure to TNF-α in the presence of MSC-CM

Following exposure of BRIN BD11 to 100 ng/ml of TNF-α maximal expression for TRAIL was noted after 4 h of exposure whilst the maximal expression for A20 was seen after 1 h of exposure in the presence of serum, but absence of MSC-CM. In the absence of serum the maximal expression for TRAIL and A20 was noted after 24 h and 2 h respectively (Figure 4.18). However, the presence of MSC-CM significantly ($P<0.05$-$P<0.0001$) reduced the induction of both gene regardless of whether the cells were grown in media with or without serum as shown in (Figure 4.18). This was accompanied by a TNF-α-mediated times-dependent reduction in cell viability, which was reversed through the addition of MSC-CM.

In the βTC1.6 cell line, exposure to 1 μg/ml of TNF-α induced a maximal expression for TRAIL and A20 after 24 h of exposure in media with and without serum, but absence of MSC-CM. However, the addition of MSC-CM significantly ($P<0.01$-$P<0.0001$) reduced the induction of both as shown (Figure 4.19).

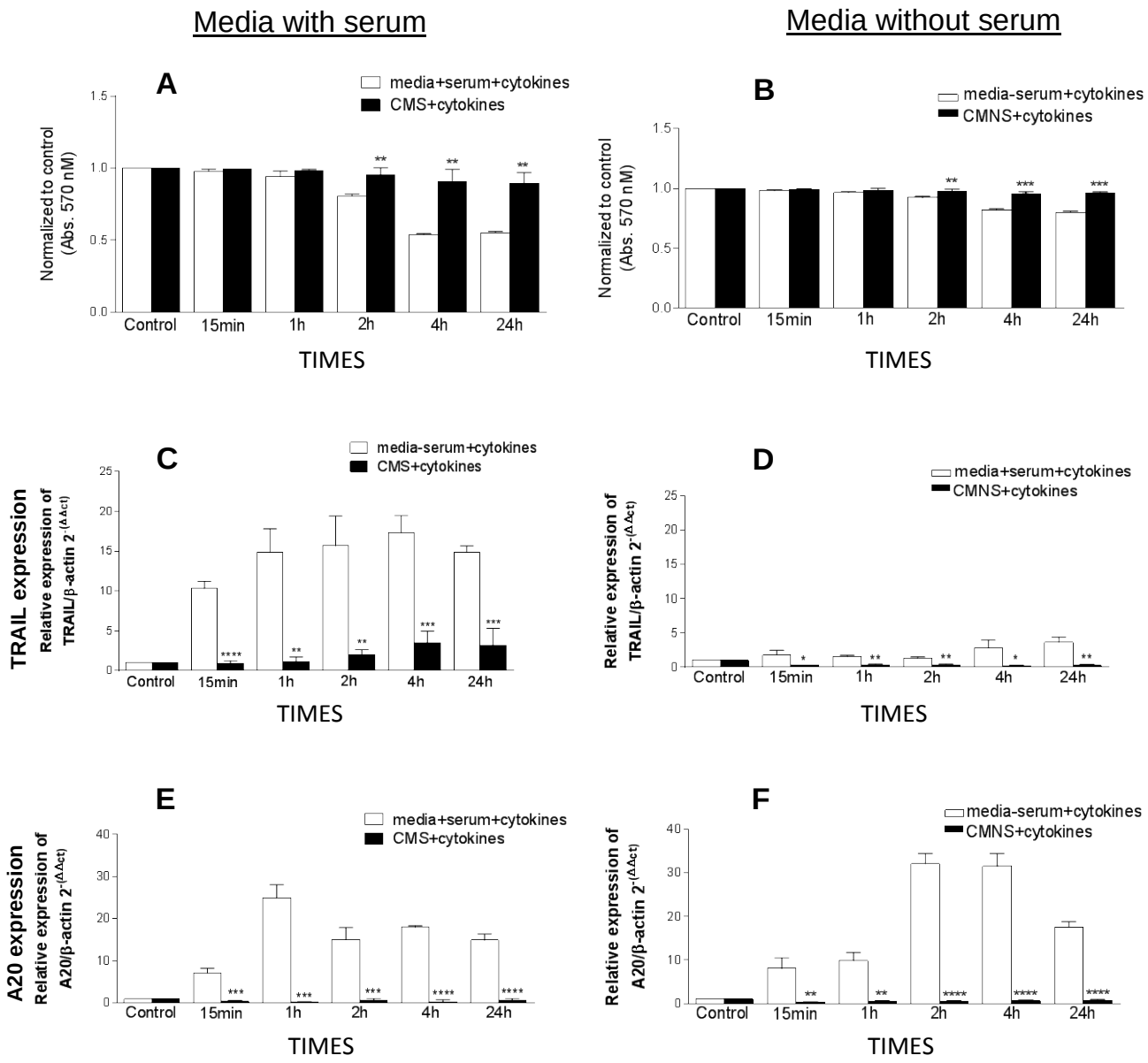

Figure 4.18. Assessment of TNF-α effect on the viability and relative gene expression at different exposure times in BRIN-BD11 cultured in MSC-CM with and without serum. Following exposure to TNF-α (100 ng/ml for media with serum and 1 µg/ml for media without serum) at different times, the cellular viability of BRIN-BD11 cultured under MSC-CM was assessed by calorimetric MTT assay (A, B) associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this times course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared with non-MSC-CM. TNF-α; Tumour necrosis factor alpha.

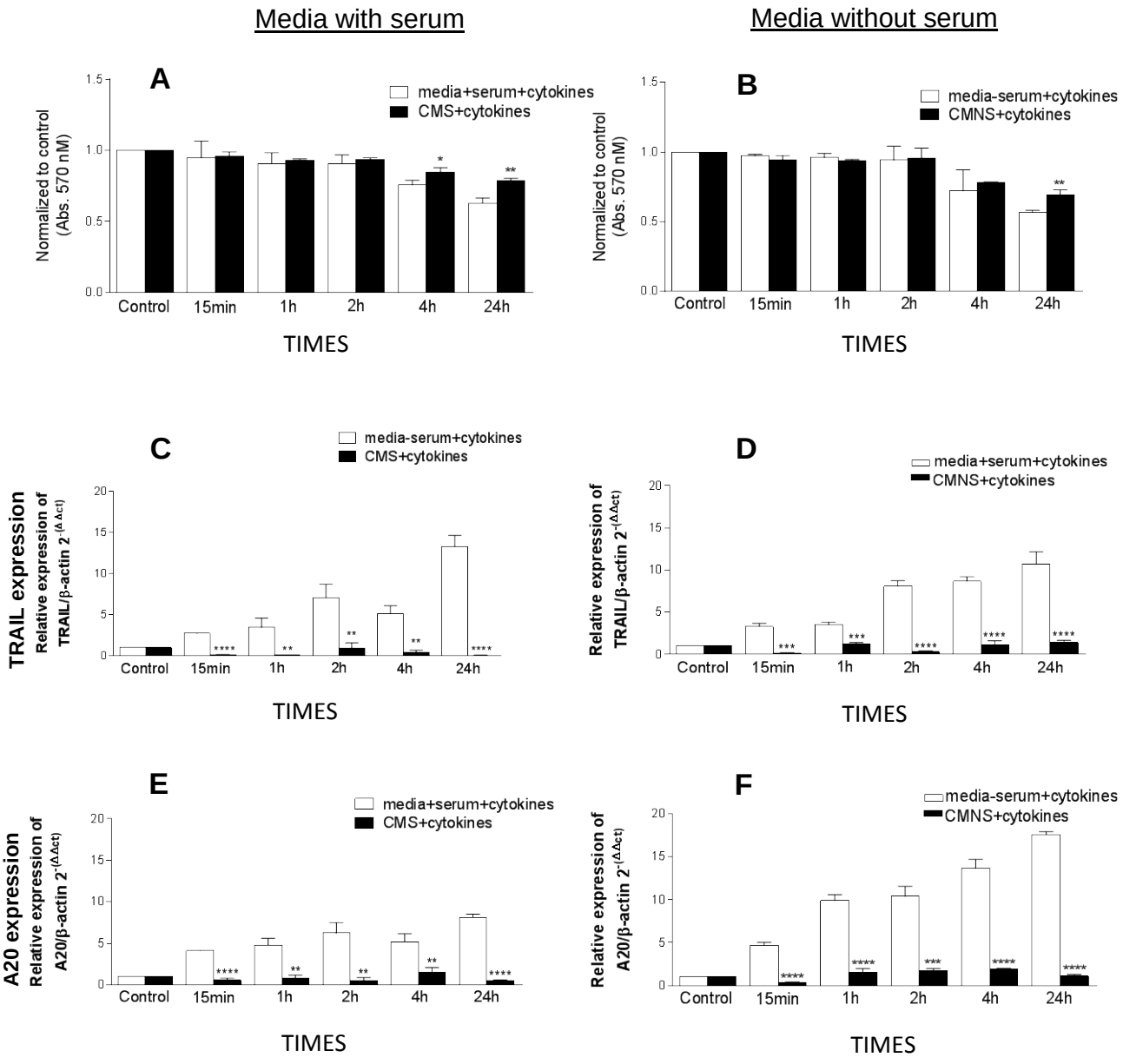

Figure 4.19. Assessment of TNF-α effect on the viability and relative gene expression at different exposure times in βTC1.6 cultured in MSC-CM with and without serum. Following exposure to TNF-α (1 µg/ml) at different times, the cellular viability of βTC1.6 was assessed by calorimetric MTT assay (A, B) associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this times course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001 compared with non-conditioned media. TNF-α, Tumor necrosis factor alpha.

4.3.4.3 Times-dependent induction of A20 and TRAIL following exposure to IL-1β in the presence of MSC-CM

Following exposure to 100 ng/ml of IL-1β, maximal expression of TRAIL was observed after 24 h, whilst maximal expression of A20 was observed after 2 h in BRIN-BD11 cells cultured in the presence of serum, but absence of MSC-CM. Under serum-free conditions, maximal expression of TRAIL and A20 was observed after 15 min and 1 h respectively (Figure 4.20). However, the addition of MSC-CM significantly ($P<0.05$-$P<0.0001$) reduced the induction of both genes in all instances regardless of whether cells were grown in the presence or absence of serum (Figure 4.20). This was accompanied by an IL-1β-mediated times-dependent reduction in cell viability, which was reversed through the addition of MSC-CM.

The exposure of βTC1.6 to 100 ng/ml of IL-1β resulted in increase in the expression of TRAIL and A20, the maximal expression was noted after 24 h of exposure for both genes and in both media with and without serum (Figure 4.21). However, the addition of MSC-CM significantly ($P<0.01$-$P<0.0001$) reduced the induction of both genes in all instances regardless of whether cells were grown in the presence or absence of serum (Figure 4.21). This is again was associated with IL-1β -mediated times-dependent reduction in cell viability, which was reversed through the addition of MSC-CM.

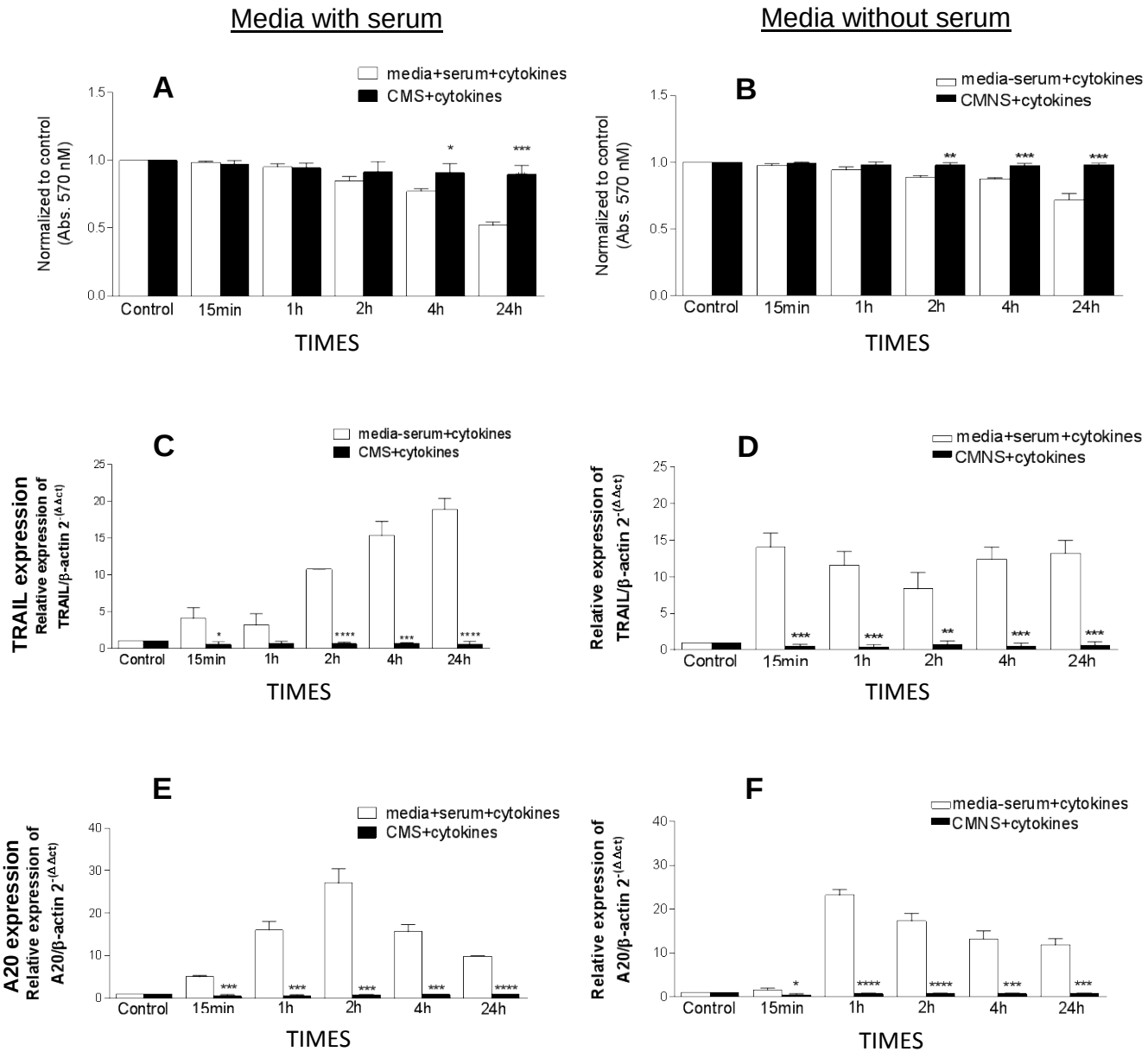

Figure 4.20. Assessment of IL-1β effect on the viability and relative gene expression at different exposure times in BRIN-BD11 cultured in MSC-CM with and without serum. Following exposure to IL-1β (100 ng/ml) at different times, the cellular viability of BRIN-BD11 cultured in MSC-CM was assessed by calorimetric MTT assay (A, B) associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this times course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared with non- conditioned media. IL-1β, interleukin-1beta.

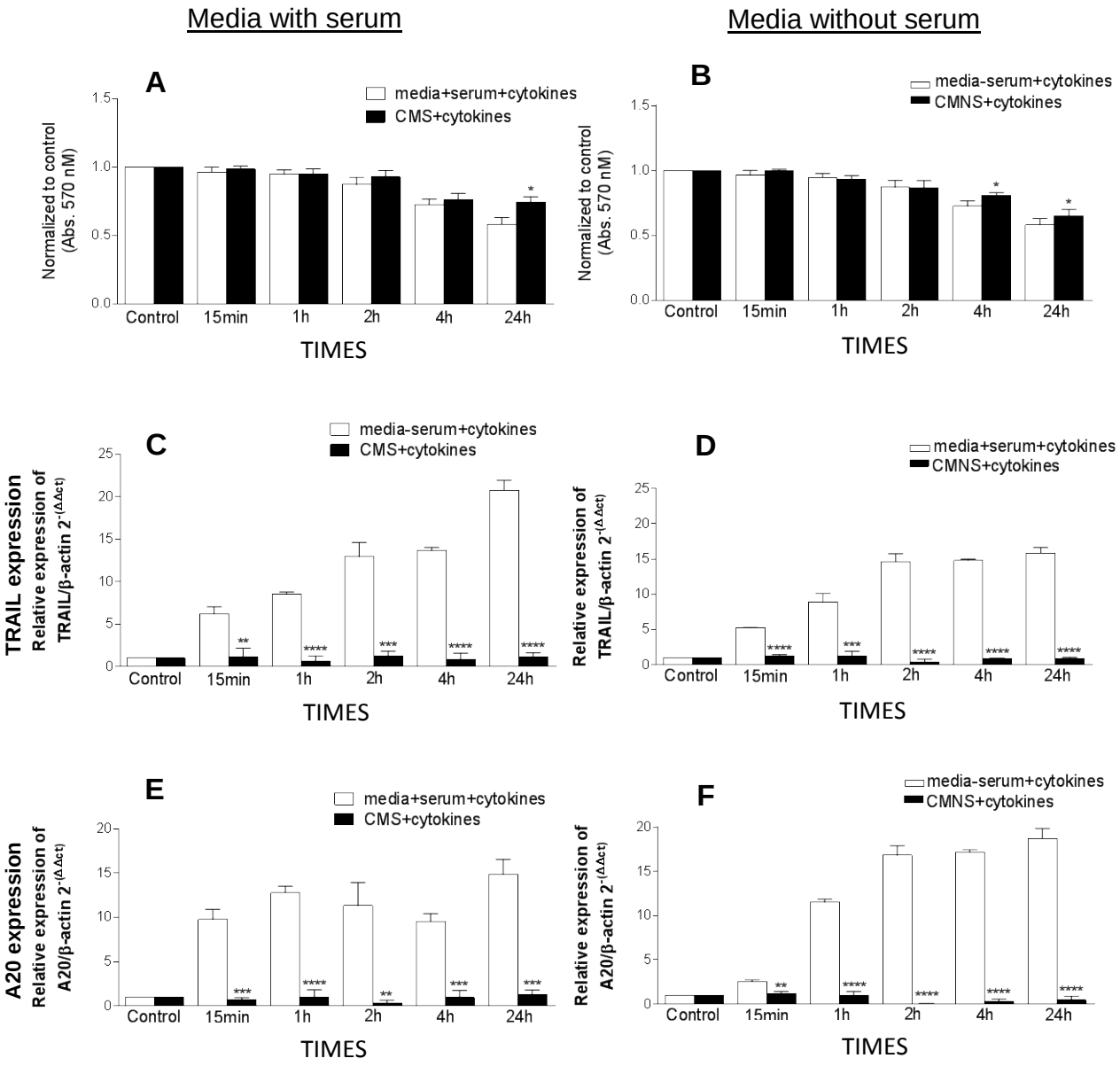

Figure 4.21. Assessment of IL-1β effect on the viability and relative gene expression at different exposure times in βTC1.6 cultured in MSC-CM with and without serum. Following exposure to IL-1β (100 ng/ml) at different times, the cellular viability of βTC1.6 cultured in MSC-CM was assessed by calorimetric MTT assay (A, B) associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this times course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared with non- conditioned media. IL-1β, interleukin-1beta.

4.3.4.4 Times-dependent induction of A20 and TRAIL following exposure to LPS in the presence of MSC-CM

Following exposure to 500 µg/ml of LPS, maximal expression of TRAIL was observed after 24 h, whilst maximal expression of A20 was observed after 4 h in BRIN-BD11 cells cultured in the presence of serum, but absence of MSC-CM. Under serum-free conditions, maximal expression of TRAIL and A20 was observed after 4 h for both genes (Figure 4.22). However, the addition of MSC-CM significantly ($P<0.05$-$P<0.0001$) reduced the induction of both genes in all instances regardless of whether cells were grown in the presence or absence of serum (Figure 4.22). This was accompanied by an IFN-γ-mediated times-dependent reduction in cell viability, which was reversed through the addition of MSC-CM.

The exposure of βTC1.6 to 500 µg/ml of LPS resulted in increase in the expression of TRAIL and A20, the maximal expression was noted after 24 h of exposure for both genes and in both media with and without serum (Figure 4.23). However, the addition of MSC-CM significantly ($P<0.05$-$P<0.0001$) reduced the induction of both genes in all instances regardless of whether cells were grown in the presence or absence of serum (Figure 4.23). This is again was associated with LPS-mediated times-dependent reduction in cell viability, which was reversed through the addition of MSC-CM.

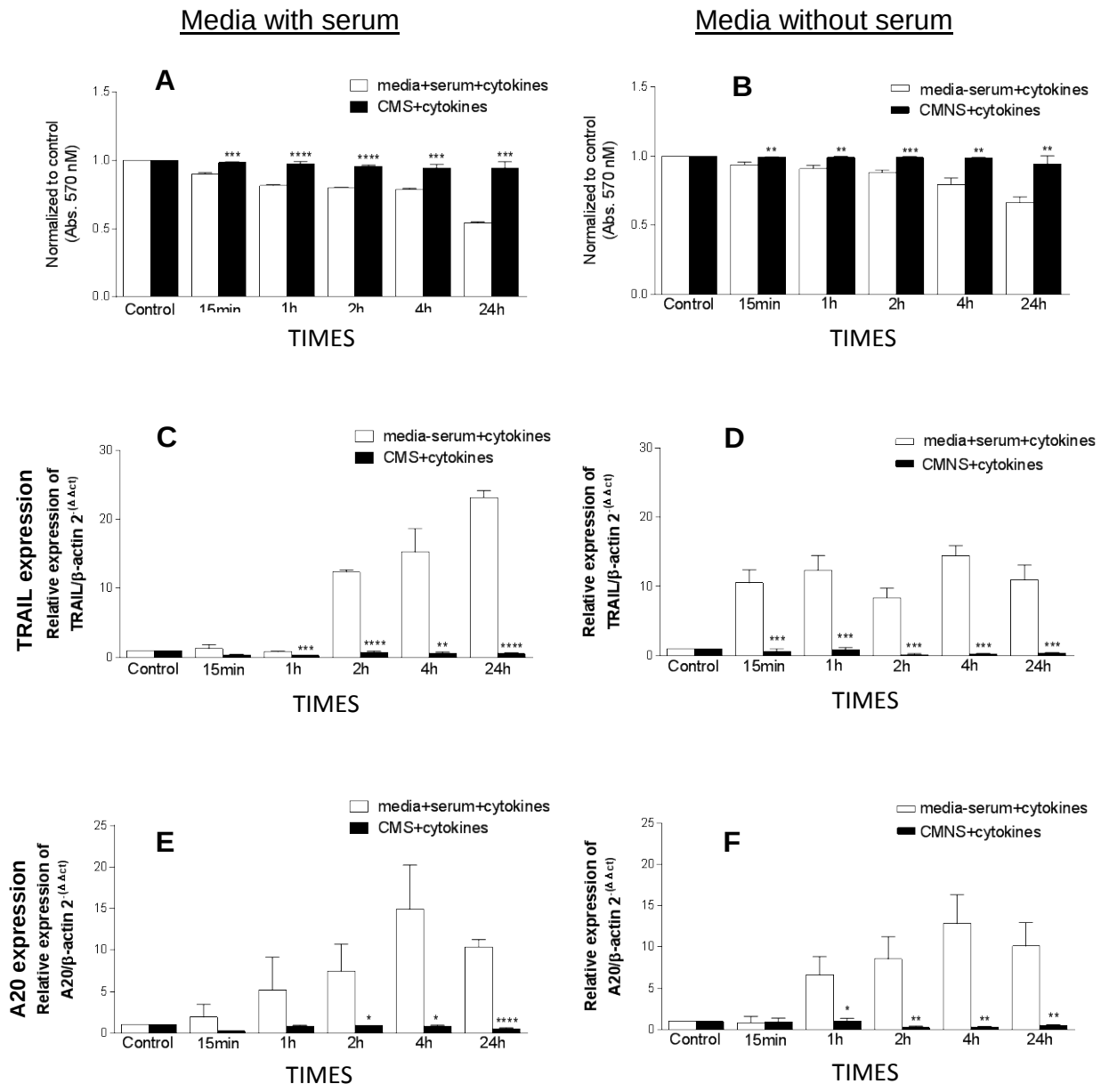

Figure 4.22. Assessment of LPS effect on the viability and relative gene expression at different exposure times in BRIN-BD11 cultured in MSC-CM with and without serum. Following exposure to LPS (500 µg/ml) at different times, the cellular viability of BRIN-BD11 cultured in MSC-CM was assessed by calorimetric MTT assay (A, B) associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this times course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared with non-MSC-CM. LPS, lipopolysaccharides

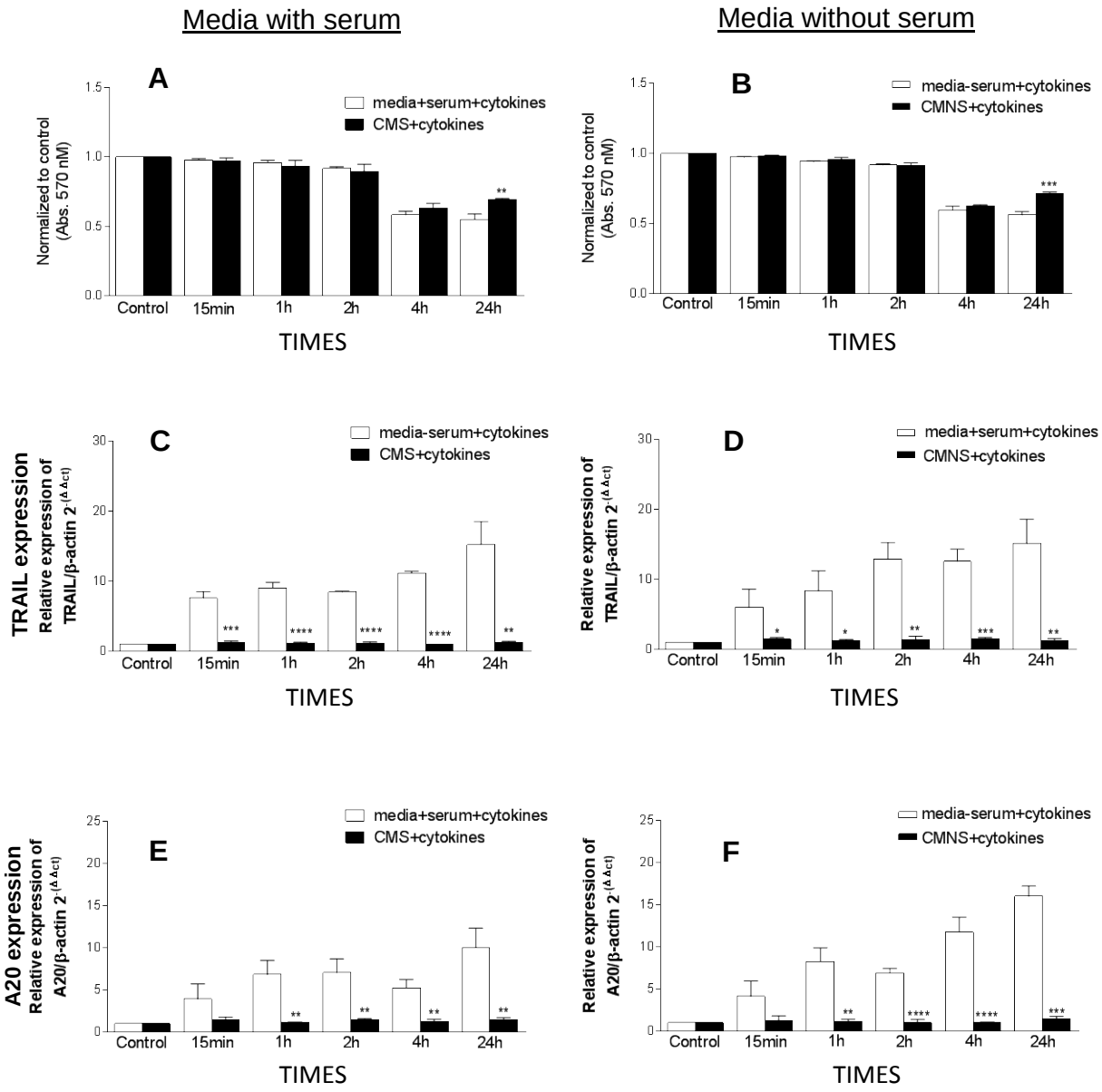

Figure 4.23. Assessment of LPS effect on the viability and relative gene expression at different exposure times in βTC1.6 cultured in MSC-CM with and without serum. Following exposure to LPS (500 µg/ml) at different times, the cellular viability of βTC1.6 cultured in MSC-CM was assessed by calorimetric MTT assay (A, B) associated with evaluation of changes in TRAIL (C, D) and A20 (E, F) gene expression during this times course. Data from MTT are normalized to untreated controls and all the data are presented as mean ± standard deviation (SD). n=3 **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001 compared with non-MSC-CM. LPS, lipopolysaccharides.

4.3.5 Assessment of pancreatic islets viability over times

The islets were treated with 100 ng/ml of IFN-γ in the presence of MSC-CM with and without serum in a times course manner started from 15 min to 24 h. The normalized data from MTT assay showed a significant increase ($P<0.05$-<0.01) in cellular viability after 4 h of exposure in the presence of serum versus 24 h ($P<0.05$). In the absence of serum as compared to non-conditioned media (Figure 4.24A, B). The pancreatic islets were also exposed to 100 ng/ml of TNF-α a significant increase ($P<0.05$-<0.01) in viability was noted after 4 h in presence of serum versus 24 h ($P<0.05$) in absence of serum (Figure 4.24C, D). The viability of the cells significantly increased ($P<0.05$) after 2 h of exposure to 100 ng/ml of IL-1β in presence of MSC-CM with serum versus 1 h ($P<0.05$) of exposure in absence of serum (Figure 4.24E, F). Exposing the islets to 10 µg/ml of LPS in the presence of MSC-CM with serum resulted in the increment of islets viability ($P<0.05$) after 1 h of exposure while in absence of serum the viability increases after 2 h ($P<0.05$-<0.01) of exposure to 500 µg/ml of LPS (Figure 4.24G, H).

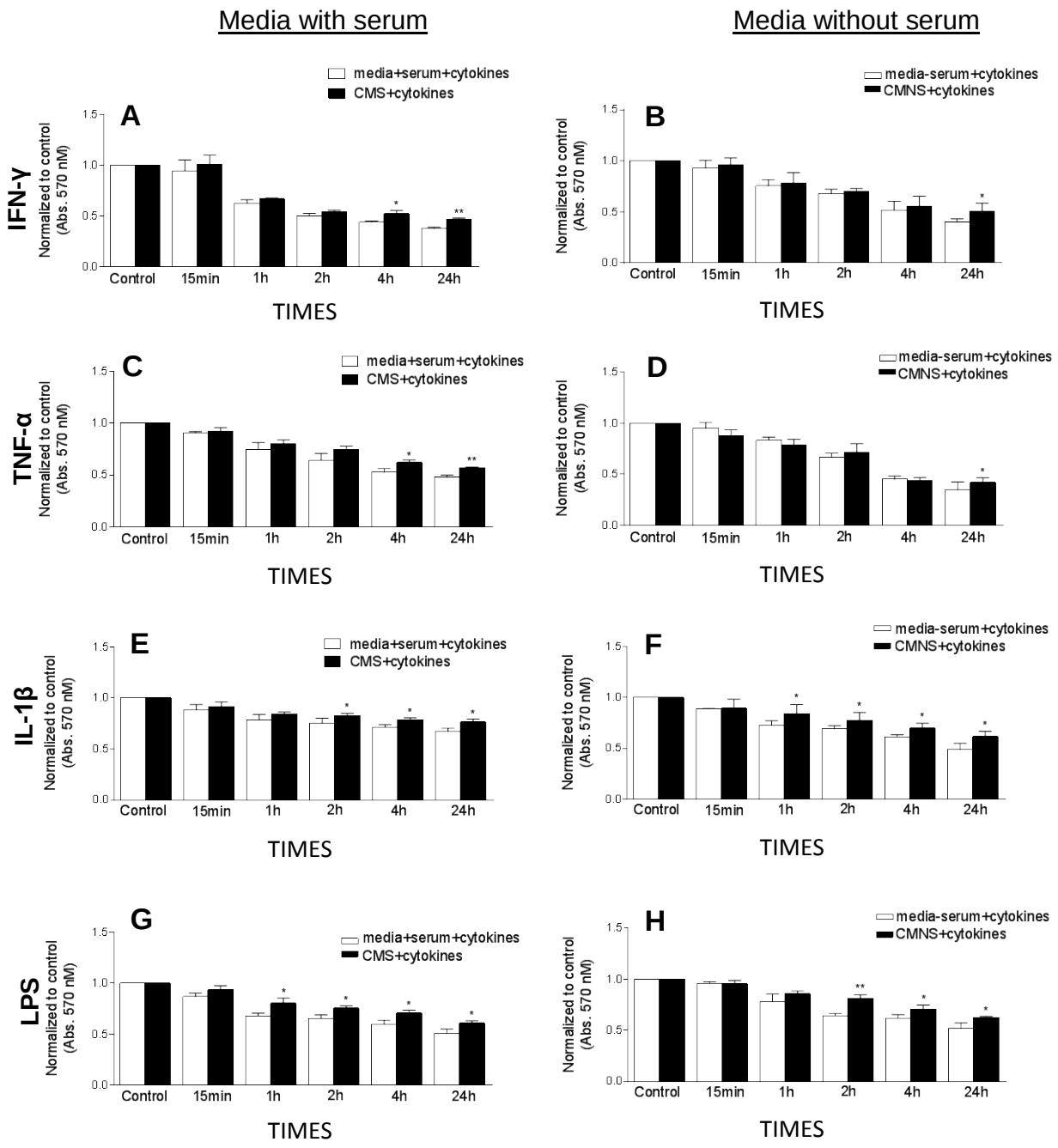

Figure 4.24. Effect of pro-inflammatory cytokines and endotoxin at different times course on the viability of pancreatic islets cultured in MSC-CM in presence and absence of serum. Pancreatic islets were exposed to 100 ng/ml of IFN-γ (A, B), 100 ng/ml of TNF-α (C, D), 100 ng/ml of IL-1β (E, F) and LPS in dose of 10 µg/ml in the presence of serum and 500 µg/ml in absence of serum (G, H). The viability of the islets was assessed by calorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **p*<0.05, ***p*<0.01, ****p*<0.001 as compared to non-conditioned media. IFN-γ, Interferon gamma; TNFα, Tumour Necrosis Factor alpha; IL-1β, Interleukin-1 beta; LPS, Lipopolysaccharide.

4.3.6 Assessment of insulin secretion

Examination of insulin secretion in response to 1.1, 5.6 and 16.7 mM D-glucose before and after the addition of MCS-CM revealed that in all instances BRIN-BD11 monolayer cells treated with cytokines or LPS in the presence of MSC-CM showed a significant ($P<0.01$-$P<0.001$) higher levels of insulin secretion than those treated with non-MSC-CM (Figure 4.25). Since we have shown above that MSC-CM protects against β-cell apoptosis in the BRIN-BD11 cell line, we wished to determine if the observed increase in insulin secretion was a direct consequence of enhanced β-cell survival. Therefore, data was standardized according to protein concentration, which acted as a surrogate for cell number in this instance.

Following standardization of the data, many of the apparent increases in insulin secretion were abolished. However, following stimulation of LPS-treated cells with 1.1 mM D-glucose a modest, but significant ($P<0.001$) increase in insulin secretion was achieved through MSC-CM (with serum) treatment (Figure4.25). Similar trends were observed in cells challenged with IFN-γ ($P<0.01$), TNF-α ($P<0.01$) and LPS ($P<0.05$) prior to exposure to 5.6 mM D-glucose, and for cells challenged with TNF-α ($P<0.05$) and IL-1β ($P<0.01$) prior to exposure to 16.7 mM D-glucose (Figure 4.25). MSC-CM treatment (without serum) resulted in similar increases in insulin secretion prior to standardization for protein concentration. However, after standardization, no significant differences in insulin release were observed (Figure 4.26).

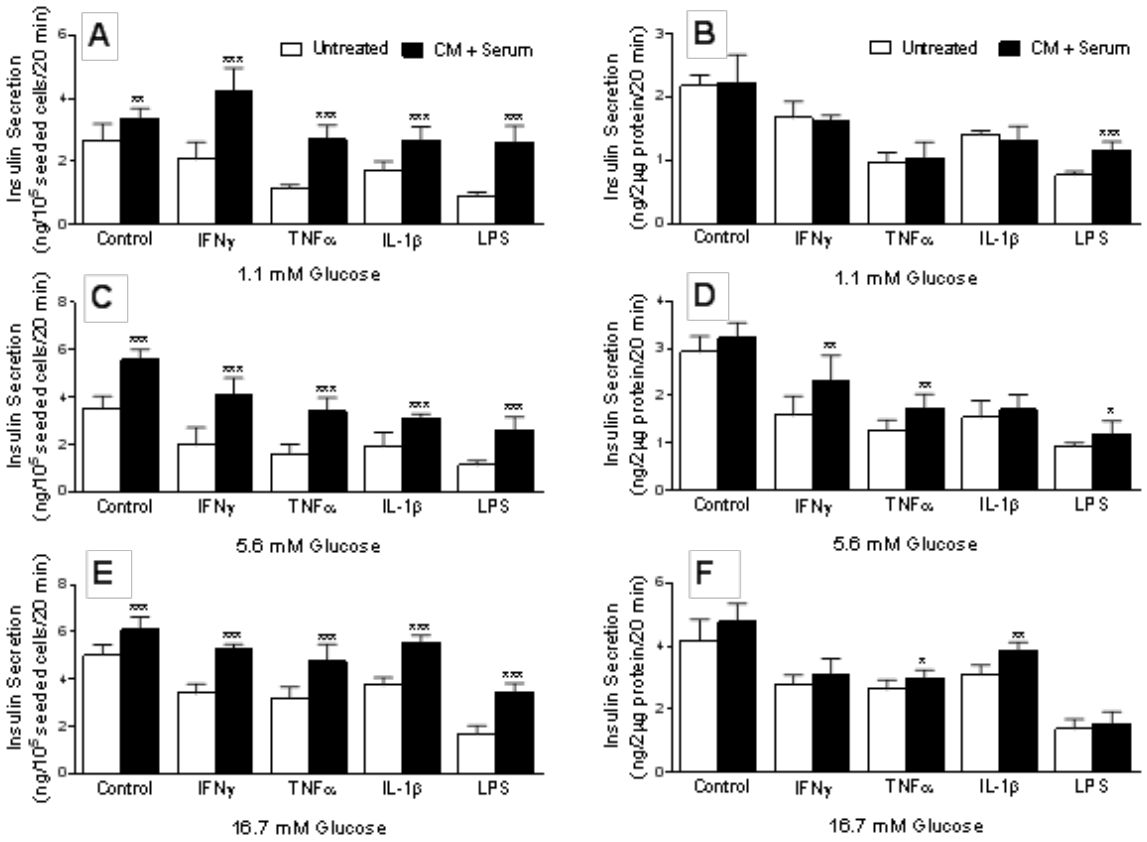

Figure 4.25. Reversal of cytokine-driven reductions in insulin secretion from pancreatic β-cells through addition of MSC-CM (with serum). BRIN-BD11 cells were exposed to 1 µg/ml IFN-γ, 100 ng/ml TNFα, 100 ng/ml IL-1β and 500 µg/ml LPS for 24h prior to exposure to rising concentrations of D-Glucose 1.1 (A, B), 5.6 (C, D), 16.7 (E, F) mM in the presence or absence of MSC-CM with serum. Insulin secretion was measured by ELISA and data presented according to insulin concentration (A, C, E) or insulin concentration as a function of protein concentration (B, D, F). Data are presented as mean ± standard deviation (SD). n=4 *p<0.05, **p<0.01, and ***p<0.001 compared with corresponding treatments in the absence of MSC-CM. IFN-γ, Interferon gamma; TNFα, Tumour Necrosis Factor alpha; IL-1β, Interleukin-1 beta; LPS, Lipopolysaccharide.

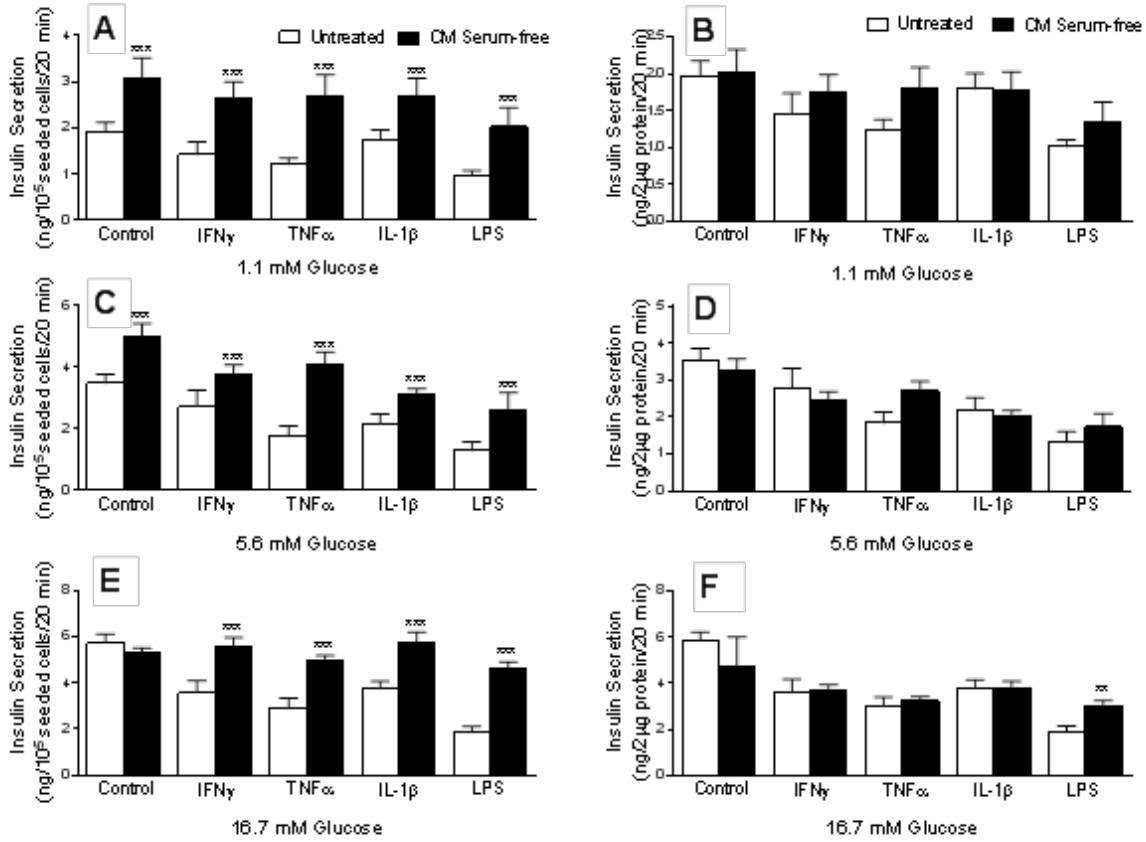

Figure 4.26. Reversal of cytokine-driven reductions in insulin secretion from pancreatic β-cells through addition of MSC-CM (without serum). BRIN-BD11 cells were exposed to 1 µg/ml IFN-γ, 100 ng/ml TNFα, 100 ng/ml IL-1β and 500 µg/ml LPS for 24h prior to exposure to rising concentrations of D-Glucose 1.1 (A, B), 5.6 (C, D), 16.7 (E, F) mM in the presence or absence of MSC-CM without serum. Insulin secretion was measured by ELISA and data presented according to insulin concentration (A, C, E) or insulin concentration as a function of protein concentration (B, D, F). Data are presented as mean ± standard deviation (SD). n=4 ** $p<0.01$, and *** $p<0.001$ compared with corresponding treatments in the absence of MSC-CM. IFN-γ, Interferon gamma; TNFα, Tumour Necrosis Factor alpha; IL-1β, Interleukin-1 beta; LPS, Lipopolysaccharide.

4.4 Discussion

Despite major advances in our understanding of diabetes and ways to treat the disease, it still represents a significant cause of mortality and morbidity worldwide.[212] Indeed, one of the main complications of diabetes is cardiovascular disease, which is also considered as a major cause of death worldwide.[212] Even with the best monitoring for diabetes, people with diabetes will eventually develop different types of complications. Treatment with insulin rarely prevents the development of diabetic complications and in some cases; the injection itself may lead to serious complications if not monitored properly. Therefore, looking for alternative ways to treat or even cure diabetes has been a challenge to all researchers. Transplantation of pancreatic islets has been used to treat diabetes. However, it's wide spread use has been hampered by immune rejection and an insufficient supply of islets.[41]

Mesenchymal stem cells (MSCs) have emerged as a new therapeutic tool in regenerative medicine. The ability of MSCs to differentiate into different cell types from all three germ layer in addition to their high *ex vivo* expansion potency made them an attractive therapeutic tool.[193] Several studies have showed that MSC transplantation improved the metabolic profiles in some diabetic animal models.[210] However, the mechanisms underlying their therapeutic effects are still unclear. Several theories arise to explain the therapeutic ability of MSCs in diabetes. One of them demonstrates the potential trans-differentiation of MSCs into insulin producing cells by using a specific culture medium supported by insulin promoting factors like glucose, this was followed by identification of insulin producing cells based on their ability to express genes related to pancreatic functions and development like GLUT2 and insulin I and II.[213],[214] However, other studies estimated that there is no significant *in vivo* differentiation of MSCs into pancreatic β-cells in adult mice, neither under steady-state conditions, nor after administration of STZ that results in tissue injury.[215] Many studies suggested that MSCs exert paracrine mechanisms in which MSC-secreted soluble factors are capable of promoting the survival of surrounding cells.[216]

The first observations of MSC-mediated paracrine effects was in murine models of heart disease in which the administration of MSC derived from bone marrow prevented cellular differentiation into cardiomyocytes. However, the cardiac function was re-established after 72 h post injection giving rise to the possibility of paracrine effects.[216] Later Gnecchi and colleagues showed that MSC-CM alone enhanced the recovery of ischemic cardiomyocyte *in vitro*.[217]

Other studies suggest that the MSC effect is not only due to secretion of regenerative factors, but also due to their ability to produce some factors in response to stimuli. For example, MSCs were found to produce immunomodulatory and regenerative factors in response to inflammatory stimuli like IFN-γ,[218] a pro-inflammatory cytokine released during the innate immune response and associated with autoimmune diseases like T1DM.[219] The exposure of MSCs to IFN-γ induced the secretion of anti-inflammatory factors like TGF-β and HGF.[148]

Another inflammatory mediator known to induce regenerative activity is TNF-α. Some studies suggested that MSC pre-treatment with TNF-α increased proliferation, mobilization and osteogenic differentiation of MSCs.[220] Recently some studies showed that activators of innate immunity like LPS and toll like receptor agonists also stimulated the production of paracrine factors such as VEGF.[221] Also recently it has been established that pre-conditioning of MSCs with IFN-γ and TNF-α *in vitro* prior to cell transplantation may provide a potential strategy for activating and increasing MSC immunosuppressive transplantation.[220]

It is well documented that pro-inflammatory cytokines like IFN-γ, TNF-α and IL-1β play a role in the development of T1DM.[180] Many studies confirm the capability of pro-inflammatory cytokines to induce apoptosis to pancreatic β-cell lines and primary islets *in vitro*. An increase in the expression of pro-apoptotic gene TRAIL was found in MIN6 β-cell line after exposure to TNF-α and IFN-γ *in vitro*.[181],[180] In addition to that a study on mice models showed an over expression of anti-apoptotic gene A20 in β-cell of pancreatic islets that exhibited resistant to IFN-γ, TNF-α, and IL-1β which was associated with decreased NF-$_k$B induced nitric oxide (NO) production.[189]

Given this evidence, we hypothesized that MSC-CM have the ability to protect pancreatic β-cell lines from apoptosis and restore their normal function. To prove our hypothesis we prepared MSC-CM and applied it to BRIN-BD11, βTC1.6 pancreatic β-cell lines and isolated primary islets treated with IFN-γ, TNF-α, IL-1β and LPS *in vitro*. Our findings confirm the following: MSC-CM had protective effects on pancreatic β-cell challenged with cytokines or LPS. This increase in cellular viability was associated with an inhibition of cytokine/LPS-driven apoptosis in all instances and a reduction in the expression of early response genes important in β-cell apoptosis. Furthermore, the inhibition of cellular apoptosis also appeared to result in enhancements in insulin secretion in BRIN-BD11 cells.

Research into the potential effect of MSC-CM has focused on understanding the mechanism behind the paracrine actions of the MSC-CM. Recent reports on methods to isolate and concentrate MSC-CM demonstrate experimental protocols for using the trophic factors produced by these cells.[222] However, there are still limitations to the therapeutic use of the MSC-CM that include possible contamination from animal products, the half-lives of molecules secreted by MSCs and the effective dosage needed to produce functional response *in vivo.*[222]

Many studies reported the use of MSC-CM in the treatment of different types of diseases however, the culture and collection methods used to prepare the MSC-CM varies,[223],[224] for example some MSC-CM was prepared using fetal bovine serum or adding other supplement containing complete media.[225] others were prepared as serum free media.[226] The basal media and types of cells used also vary.[226] Preparation of MSC-CM also varies in culture duration from several hours up to five days under different culture condition some used normoxia O2 level 20- 21% or under hypoxia condition ranging from 0.5-2%.[227] Nearly all of these studies showed successful and promising results however, standardized methods for various MSC-CM formation are still undefined.

In our study we used two types of basal media RPMI1640 and DMEM media applied it on the bone marrow mesenchymal stem cells monolayer for 24 h in the presence and absence of serum. The use of MSC-CM has several advantage over the use of MSC infusion, as MSC-CM can be formed, packaged, stored and transported more easily than cells. Moreover, there is no need to match the donor and the recipient to avoid rejection problems.

4.5 Conclusion

Based on clinical trials, MSC-CM are relatively safer than intravenous administration of cells that could cause complication like vascular occlusion or long term maldifferentiation of injected MSCs. The understanding of the paracrine action of stem cell and learning to modulate and use them properly provide researchers with a wide range of treatment options for different types of disease and the forthcoming advance in defining and applying paracrine actions will significantly benefit the regenerative medicine applications in the coming years.

All the techniques used to culture MSCs and prepare the conditioned media to date are far from being standardized. We aim to be consistent in culturing the MSC, preparing and

collecting the conditioned media. However, testing different culture methods and various protocols for conditioned media preparation will be our aim in our future works.

Chapter 5

Conditioned media from mesenchymal stem cells protect β-cells from TRAIL-induced apoptosis

5.1 Introduction

It is believed that β-cell mass has been reduced by 70-80% at the time of diagnosis of T1DM. It has also been suggested that β-cell loss occurs slowly over many years due to the absence of detectable β-cell necrosis and variable degrees of insulitis.[228] This is supported by the detection of insulin antibodies years before the appearance of clinical symptoms in susceptible individuals.[229]

β-cell apoptosis was found to be the primary reason behind β-cell loss in rodent models of T1DM.[230]Apoptosis is a highly complex sophisticated process that is activated by a various types of signals, which include extracellular signals, phosphorylation cascades, intracellular ATP levels, and expression of pro and anti-apoptotic genes.[231] Although the mechanism is still not completely clarified, there are several proposed mechanisms that include: expression of Apo-1 and Apo-2L receptors, release of perforin and granzyme by activated $CD8_+$T-lymphocytes, secretion of cytokines including IL-1β, IFN-γ, TNF-α by the immune cells that attack the islets, and production of nitric oxide by dendritic cells, macrophages, and the β-cell themselves, which secrete chemokine following viral infection or exposure to cytokines.[24]

Several studies have revealed the ability of cytokines to induce stress response genes that are either protective or deleterious for β-cell survival like a nitric oxide-dependent gene that induces nitric oxide synthesis, which interferes with electron transfer and inhibits ATP synthesis in mitochondria affecting insulin secretion and resulting in β-cell dysfunction.[232],[84] It is thought that after receptor binding by IL-1β, TNF-α, and IFN-γ, signalling cascades are activated leading to the activation of different transcription factors including NF-κB. In addition, IFN-γ signalling involves the activation of Janus kinases (JAK_s) leading to the activation of signal transducer and activator of transcription (STAT-1). These transcription

factors will alter gene expression resulting in deleterious effects on β-cells, culminating in apoptosis and death.[233] Another pathway of apoptosis involves the TNF-related apoptosis-inducing ligand (TRAIL), which is a pro-apoptotic ligand of the TNF family that has the ability to initiate apoptosis through the trimerization of its transmembrane receptor and the formation of the death-inducing signalling complex (DISC). DISC activates the Fas-associated death domain (FADD) that results in subsequent activation of several caspases specially caspases 3 and 8 resulting in apoptosis and cell death.[234]

Reports describing a balance between pro- and anti-inflammatory cytokines are becoming more commonplace.[180] It is agreed that this relation is a very complicated where most anti-inflammatory cytokines have at least some pro-inflammatory properties.[180), (235] Therefore, physiological functions could be determined by several factors including the timing of cytokine release and the presence of competing or synergistic factors.[235]

One of the most important anti-inflammatory cytokines is IL-10, which is also known as human cytokines synthesis inhibitory factor (CSIF). It is a homodimer protein found within the human immune response. CD4+Th2, monocytes, and B-cells are described as the primary source of IL-10, which acts as a potent deactivator of macrophage and monocyte pro-inflammatory cytokine synthesis.[236] IL-10 downregulates the expression of major histocompatibility complex class II, Th1 cytokines, and co-stimulatory molecules on macrophages.[236] IL-10 also enhances B-cell survival, proliferation and antibody formation. In addition, it blocks the NF-κB pathway and regulates the JAK and STAT signalling pathways.[237] Furthermore, it is believed that IL-10 modulates surface expression of the TNF receptor family, which could interfere with the biological activity of TNF-α and result in a reduction of the pro-inflammatory potential of TNF-α.[238]

IL-10 has two receptors; IL-10R1 (also known as IL-10RA) and IL-10R2 (IL-10RB), both of which consist of two subunits that are members of interferon receptor family (IFNR).[239] The mRNA for IL-10R1 is found in all IL-10 responsive cells, and the neutralization of this receptor by anti-IL-10R1 antibody inhibits all IL-10 known activities indicating an important role for this receptor in mediating the action of IL-10.[240] IL-10R2 is also found in most tissues and cells tested, but its role in IL-10 signalling is not known, some researchers have suggested that the principle function of IL-10R2 is to recruit the JAK kinase (TYK2) into the signalling complex.[241] The binding of IL-10 to its receptor forms a functional tetramer complex consisting of two IL-10R1 subunits and two IL-10R2 subunits (Figure 5.1). This will in turn activate phosphorylation of JAK1 and TYK2, which are associated with IL-10R1 and IL-10R2, respectively. This, in turn, will stimulate the latent transcription factors STAT3, STAT1, and STAT5.[242] Several studies demonstrate a role for STAT3 in mediating IL-10 expression.[243], [244] The activation and translocation of STAT3 into the nucleus leads to the binding of STAT3 to STAT binding elements (SBE), which induce responses including induction of suppressor of cytokines signals gene-3(SOCS-3), which will inhibit JAK/STAT-dependent signal.[243] The role of STAT1 and STAT5 are not completely understood.[244]

Previous work performed within our group characterized the secretome of MSCs (see appendix 1). In this Chapter we sought to determine the pathway by which cytokines induce apoptosis in β-cells and to identify individual candidate molecules from the MSC secretome with a role in overcoming the effects of pro-inflammatory cytokines.

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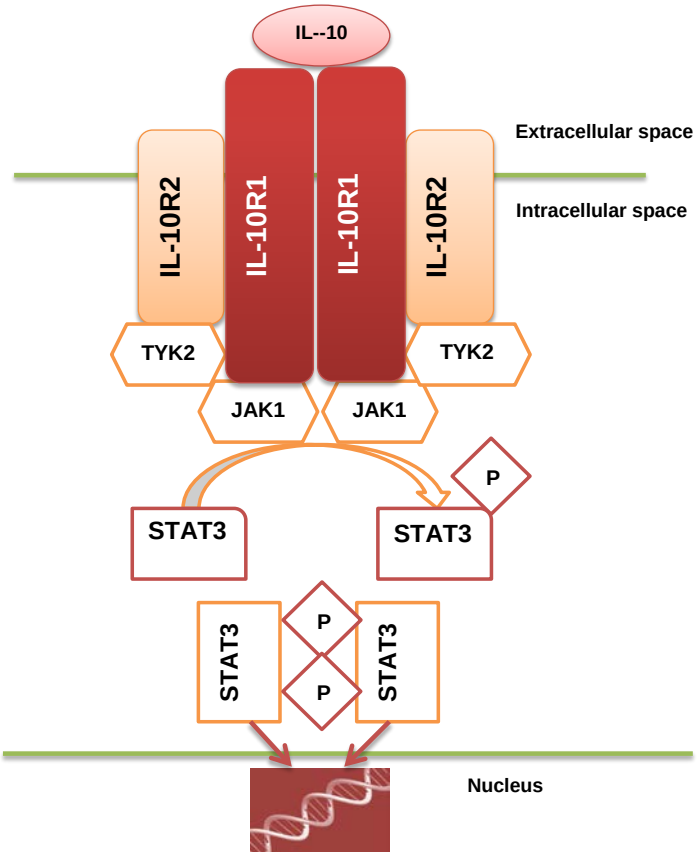

Figure 5.1 IL-10 binding to it is ligand initiating a signal transduction resulted in activation and translocation of STAT 3. This figure demonstrates the signal transduction pathway activated by IL-10 after receptor binding when it will form a homodimeric complex that activates TYK2 and JAK1, which in turn stimulates the translocation of STAT3, which promotes transcription of a wide range of IL-10 responsive genes like SOCS-3

5.2 Methods

5.2.1 Materials

All chemicals and reagent employed are listed in Chapter 2, Section 2.1.

5.2.2 Cell models

Pancreatic β-cell lines and Human Bone Marrow Mononuclear cells were cultured, maintained and passaged as outlined in Chapter 2, Section 2.2.1.1 and 2.2.1.3 respectively. All cell models were maintained in an incubator at an atmosphere of 37 °C and 5% CO_2 under normoxia (21% O_2).

5.2.3 Preparation of MSC-CM

MSC-CM was prepared as outlined in Chapter 2, Section 2.2.1.3.

5.2.4 Measurement of cellular viability and apoptosis

Changes in cellular metabolic activity were assessed by colorimetric MTT assay as a surrogate of viability as outlined in Chapter 2, Section 2.2.4, whilst induction of apoptosis was measured by TUNEL assay as described in Section 2.2.5.

5.2.5 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to quantify specific candidates from a secretome screen of MSC-CM as described in Chapter 2 Section 2.2.8.

5.2.6 Assessment of TRAIL and Anti-TRAIL effect on pancreatic β-cell lines

To determine the role of TRAIL in cytokine-induced apoptosis, the pancreatic β-cell lines BRIN-BD11 and βTC1.6 were seeded as described in Chapter 2 Section 2.2.3. Following

that, cell lines were treated with recombinant murine TRAIL for 24 h after which, cellular viability was determined using colorimetric MTT assay and in order to ensure that any reduction in viability is due to apoptosis TUNEL assay was performed as outlined in Chapter 2, Section 2.2.5. The specificity of TRAIL-induced apoptosis was established by inhibition of apoptotic response using anti-TRAIL antibody.

5.3 Results

5.3.1 Recombinant TRAIL reduces the viability of pancreatic β-cell lines.

After treating BRIN-BD11 and βTC1.6 cells with rising concentrations of recombinant murine TRAIL (200, 400, and 800 ng/ml) for 24 h, the cellular viability was assessed using a colorimetric MTT assay. The viability of BRIN-BD11 and βTC1.6 cells was significantly reduced (P<0.001) in all instances (53%-69% reduction in BRIN-BD11) and (54%-70% reduction in βTC1.6) in the presence of serum. The absence of serum did not significantly alter the pattern of the results (Figure 5.2). As such, 200 ng/ml of recombinant murine TRAIL was chosen for subsequent experiments.

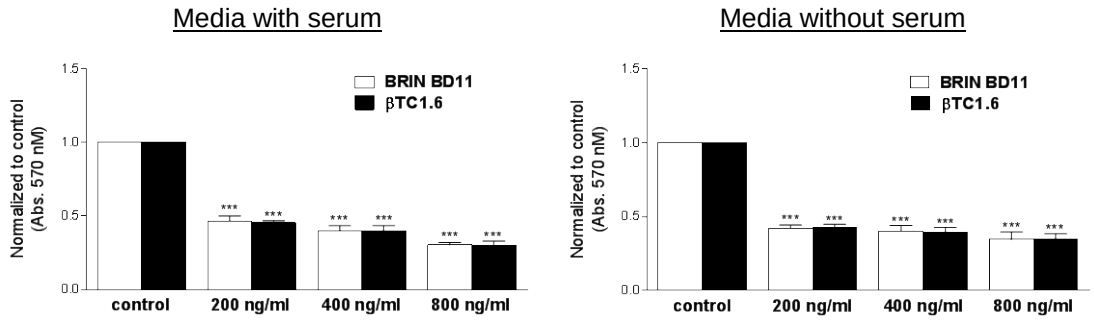

Figure 5.2. Effect of TRAIL on β-cells viability. Normalized data from MTT assay showing the effect of 200, 400, and 800 ng/ml of recombinant murine TRAIL on the viability of BRIN-BD11 and βTC1.6 cells. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 ****p*<0.001 compared with untreated controls.

We next aimed to neutralize TRAIL activity via an anti-TRAIL antibody at different concentrations (1, 5, and 10.µg/ml). The results showed that 5 and 10 µg/ml of murine anti-TRAIL antibody was able to reverse the reduction in the viability of the cells after treating them with 200 ng/ml of recombinant murine TRAIL (Figure 5.3), 5 µg/ml was chosen for subsequent experiments.

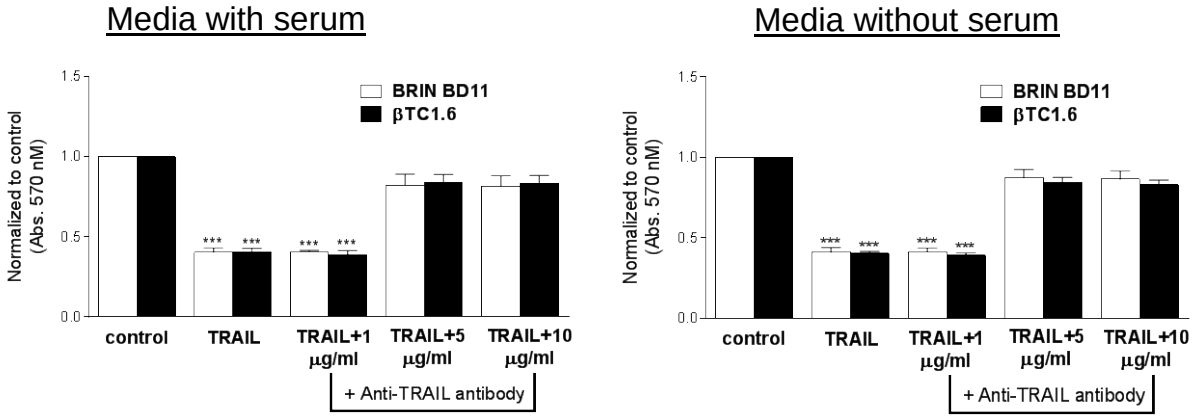

Figure 5.3 Evaluation of anti-TRAIL antibody effect on viability of cells. Following the addition of rising concentrations of murine anti-TRAIL antibody (1, 5, 10 µg/ml) along with 200 ng/ml of recombinant murine TRAIL, the viability of BRIN-BD11 and βTC1.6 cells was assessed by colorimetric MTT assay. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 ****p*<0.001 compared with untreated controls.

5.3.2 Anti-TRAIL antibody increases the viability of β-cell lines treated with cytokines

To evaluate the effect of anti-TRAIL antibody on viability of BRIN-BD11 and βTC1.6 cell lines treated with cytokines, 5 µg/ml of murine anti-TRAIL antibody was added along with 1 µg/ml of IFN-γ, 100 ng/ml of IL-1β, 500 µg/ml of LPS for both cell lines in media with and without serum. TNF-α was added to BRIN-BD11 cells at concentrations of 100 ng/ml in the presence of serum and 1 µg/ml in media without serum. TNF-α addition to βTC1.6 cells was at concentrations of 1 µg/ml in media with and without serum. Following this, cellular viability was assessed by colorimetric MTT assay. The results showed that in all instances, blocking TRAIL resulted in a significant reversal of cytokine-driven reductions in cell viability, for BRIN BD11(IFN-γ +61%, P<0.001), (TNF-α +50%, P<0.001), (IL-1β +50%, P<0.001), and (LPS +59, P<0.0001) (Figure 5.4A) and βTC1.6 (IFN-γ +64%, P<0.0001), (TNF-α +53%, P<0.001), (IL-1β +57%, P<0.0001), and (LPS +60%, P<0.01) (Figure 5.4C) in the presence of serum. A similar trend was found in the absence of serum (Figure 5.4B, D).

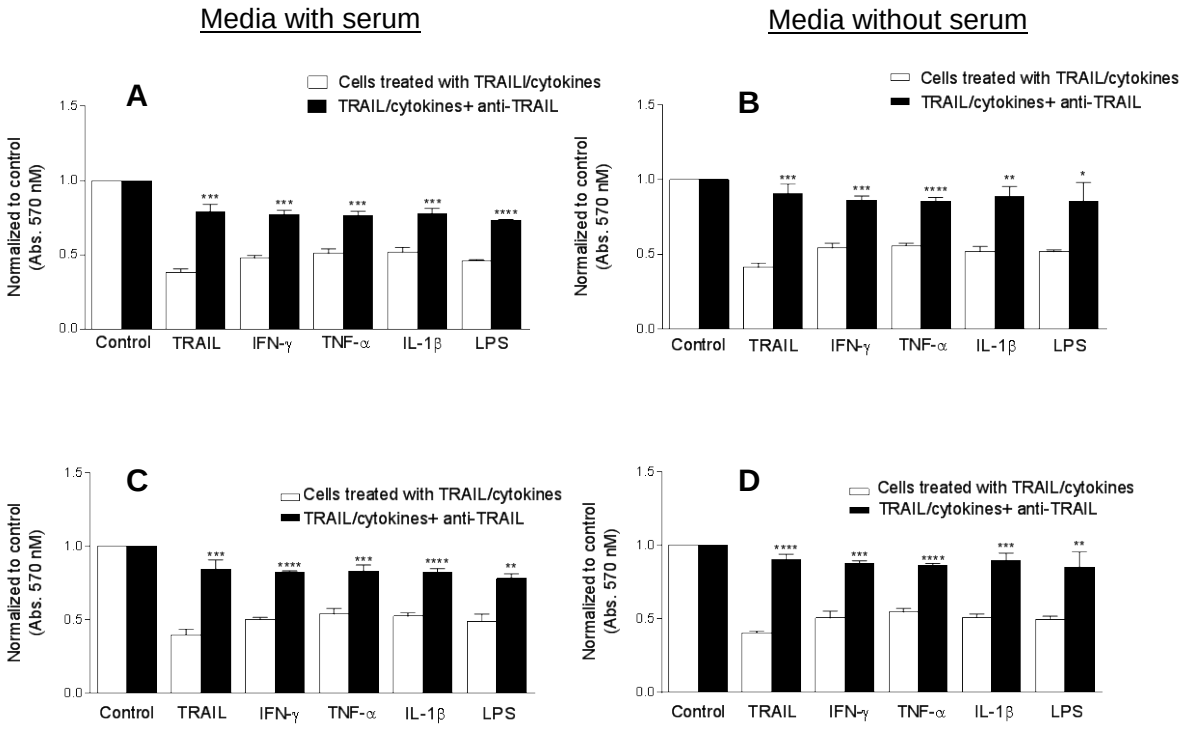

Figure 5.4. Effect of anti-TRAIL on cytokine-driven reductions in β-cell viability. Normalized data from MTT assay showing the effect of 200 ng/ml of recombinant murine TRAIL, 1 µg/ml of IFN-γ, 100 ng/ml of IL-1β, 500 µg/ml of LPS and TNF-α (100 ng/ml in media with serum vs 1 µg/ml in media without serum for BRIN-BD11 and 1 µg/ml for βTC1.6 in media with and without serum) on the viability of BRIN-BD11 cells (A, B) and βTC1.6 cells (C, D) before and after the addition of 5 µg/ml of murine anti-TRAIL antibody. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **p*<0.05, ***p*<0.01 ****p*<0.001and *****p*<0.0001 comparison between before and after anti-TRAIL antibody addition. IFN-γ interferon gamma, TNF-α tumour necrosis factor alpha, IL-1β interleukin-1beta, and LPS lipopolysaccharide.

5.3.3 TRAIL-induced apoptosis in pancreatic β-cell lines

In order to determine if the reduction in cellular viability was due to programmed cell death (apoptosis), the TUNEL assay was performed. Significant apoptosis was noted in BRIN-BD11 and βTC1.6 cells after treatment with 200 ng/ml of recombinant murine TRAIL. However, the addition of 5 µg/ml of murine anti-TRAIL antibody significantly reduced ($P<0.001$-$P<0.0001$) the % positive TUNEL cells (Figure 5.5).

The addition of 5 µg/ml of anti-TRAIL antibody along with 1 µg/ml of IFN-γ (Figure 5.6), 100 ng/ml of IL-1β (Figure 5.8), 500 µg/ml of LPS (Figure 5.9) and for TNF-α the BRIN BD11 was treated with 100 ng/ml in media with serum vs 1 µg/ml in media without serum, while 1 µg/ml was used for βTC1.6 in media with and without serum (Figure 5.7), resulted in significant reduction ($P<0.001$-$P<0.0001$) of % positive TUNEL cells. There was some variation in the %positive TUNEL cells among cytokines and endotoxin for both BRIN BD11 (IFN-γ 66%, TNF-α 65%, IL-1β 48%, and LPS 52%) and βTC1.6 (IFN-γ 58%, TNF-α 61%, IL-1β 50%, and LPS 52%). However, the addition of anti-TRAIL antibody blocked all cytokine and endotoxin-induced apoptosis in serum free media suggesting that cytokines may utilize the TRAIL ligand in induction of apoptosis. Similar trends were noted in the absence of serum indicating that the presence of serum did not sensitized cells to the effects of cytokines (Figures 5.6-5.9).

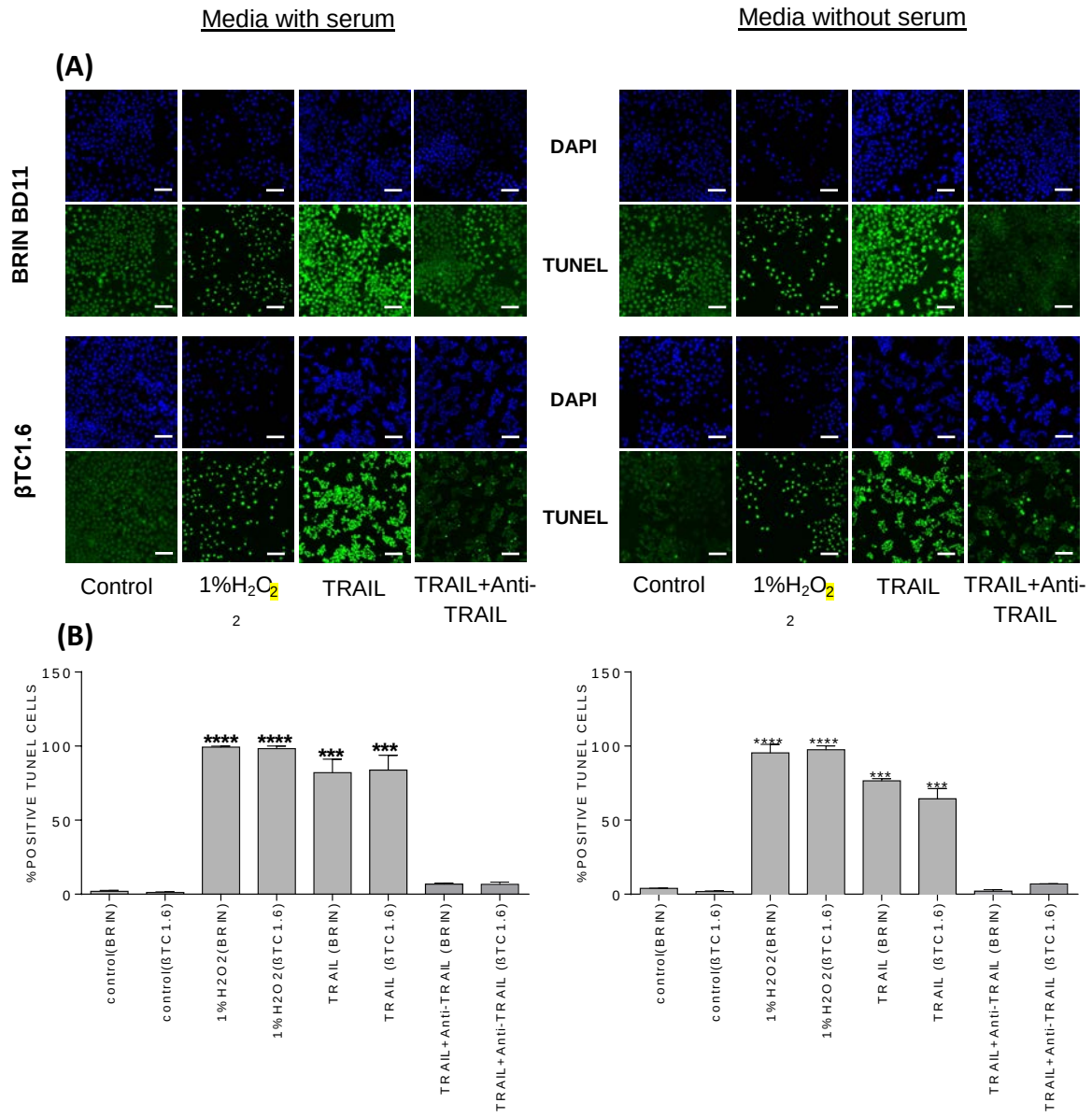

Figure 5.5. Assessment of TRAIL-induced apoptosis in pancreatic cell lines. **(A)** Fluorescent images showing TRAIL-induced apoptosis in BRIN-BD11 and βTC1.6 cells after 24 h of exposure to 200 ng/ml of recombinant murine TRAIL. Blue staining represents DAPI staining of the nuclei while green staining indicates TUNEL positive cells. **(B)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****p*<0.001 and *****p*<0.0001 compared with untreated controls. The scale bar in all images equals 100μm

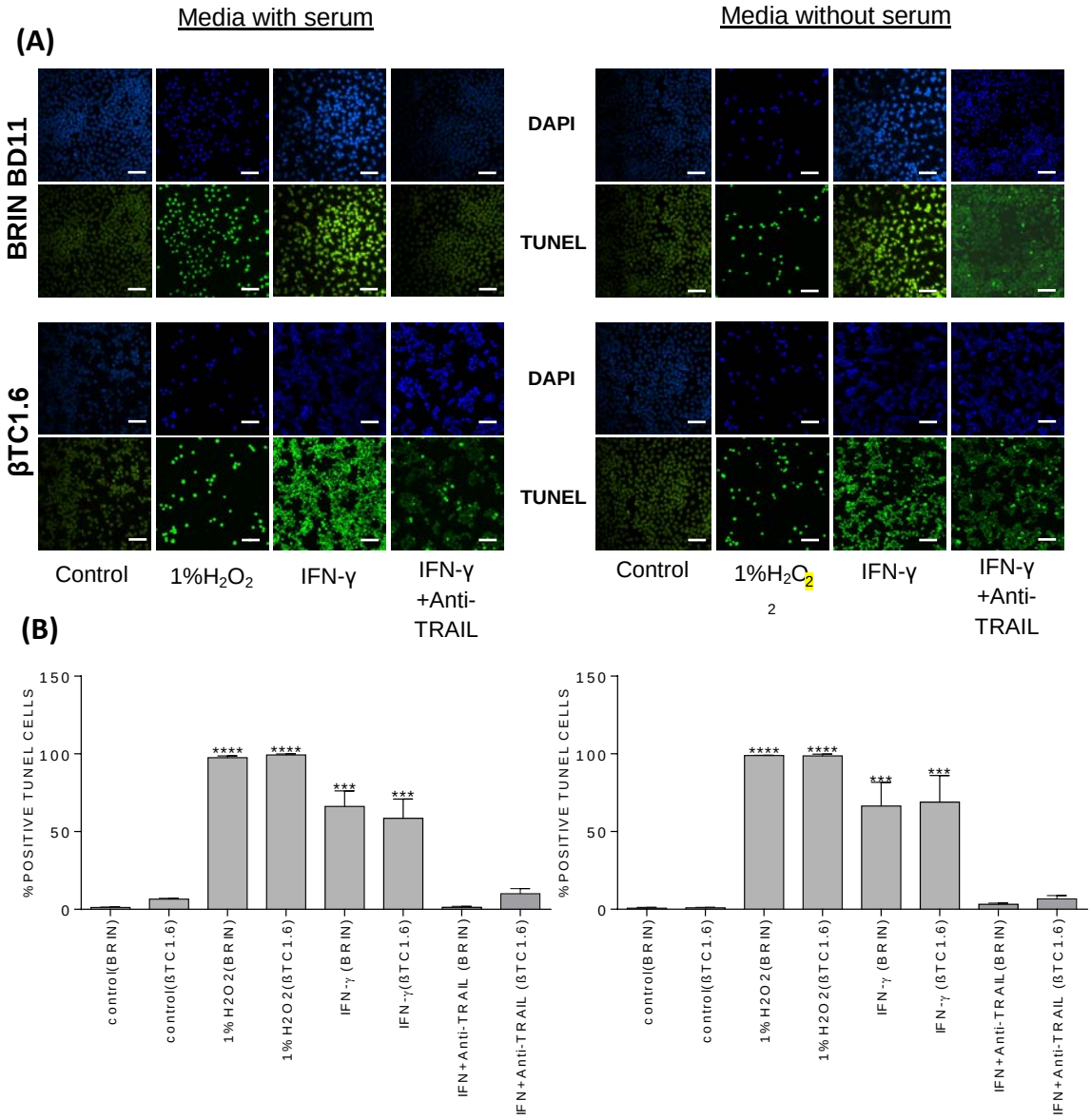

Figure 5.6. Assessment of IFN-γ-induced apoptosis in pancreatic cell lines. **(A)** Fluorescent images showing IFN-γ-induced apoptosis in BRIN-BD11 and βTC1.6 cells after 24 h of exposure to 1 µg/ml of IFN-γ. Blue staining represents DAPI staining of the nuclei, while green indicates TUNEL positive cells. **(B)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ***p<0.001 and ****p<0.0001 compared with untreated controls. The scale bar in all images equals 100 µm.

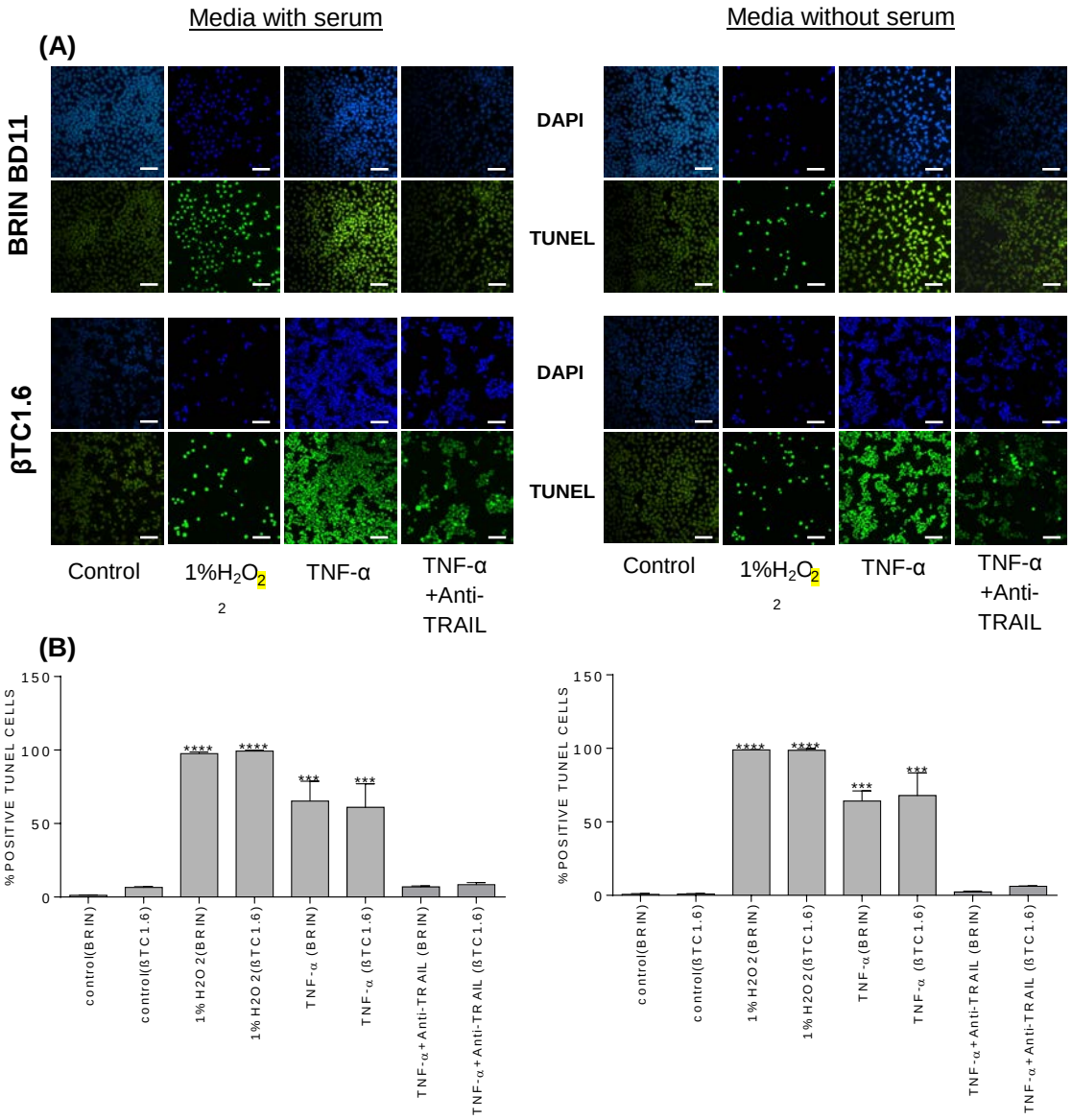

Figure 5.7.Assessment of TNF-α-induced apoptosis in pancreatic cell lines. **(A)** Fluorescent images showing the TNF-α-induced apoptosis in BRIN-BD11 and βTC1.6 cells after 24 h of exposure to 100 ng/ml in media with serum vs 1 µg/ml in media without serum for BRIN-BD11 and 1 µg/ml for βTC1.6 in media with and without serum. Blue staining represents DAPI staining of the nuclei while green staining indicates TUNEL positive cells. **(B)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****p*<0.001 and *****p*<0.0001 compared with untreated controls. The scale bar in all images equals 100 µm

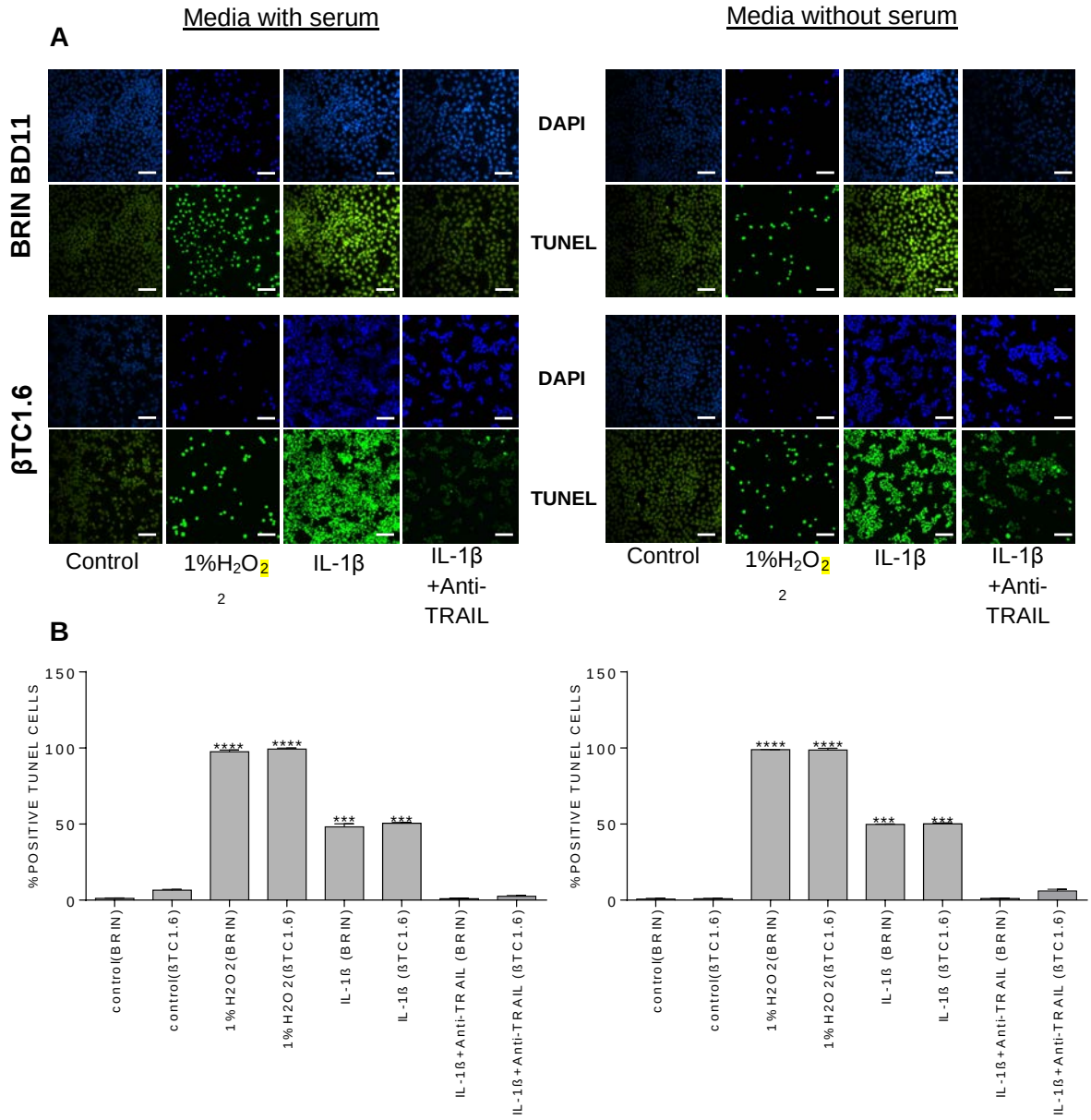

***Figure 5.8.Assessment of IL-1β-induced apoptosis in pancreatic cell lines cultured in media with and without serum*. (A)** Fluorescent images showing IL-1β-induced apoptosis in BRIN-BD11 and βTC1.6 cells after 24 h of exposure to100 ng/ml of IL-1β. Blue staining represents DAPI staining of the nuclei while green staining indicates TUNEL positive cells. **(B)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****p*<0.001 and *****p*<0.0001 compared with untreated controls. The scale bar in all images equals 100 µm.

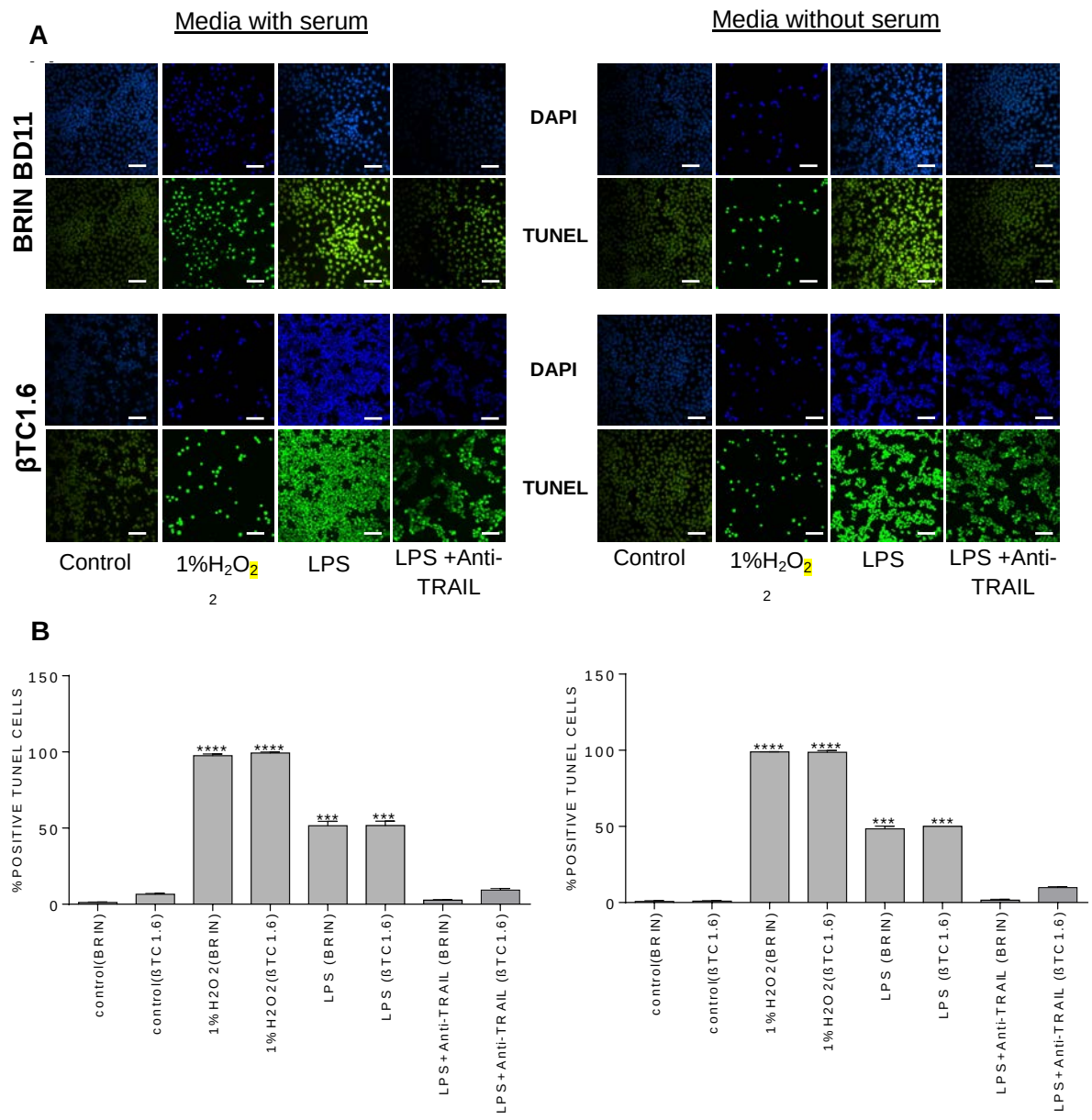

Figure 5.9.Assessment of LPS-induced apoptosis in pancreatic cell lines. **(A)** Fluorescent images showing LPS-induced apoptosis in BRIN-BD11 and βTC1.6 cells after 24 h of exposure to 500 µg/ml of LPS. Blue staining represents DAPI staining of the nuclei while green staining indicates TUNEL positive cells. **(B)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****p*<0.001 and *****p*<0.0001 compared with untreated controls. The scale bar in all images equals 100 µm.

5.3.4 MSC-CM increases the viability of β-cell lines treated with TRAIL

In order to assess the ability of MSC-CM to protect pancreatic β-cell lines from TRAIL they were treated with 200 ng/ml of recombinant murine TRAIL, in the absence and presence of MSC-CM, with and without serum. Thereafter, a colorimetric MTT assay was performed. The results showed a significant increase (P<0.01-P<0.0001) in the viability of BRIN-BD11 cultured in MSC-CM with (+80%) and without (+82%) serum when compared with cells cultured without MSC-CM (Figure 5.10). Consistent data was observed in the βTC1.6 cell line (Figure 5.10).

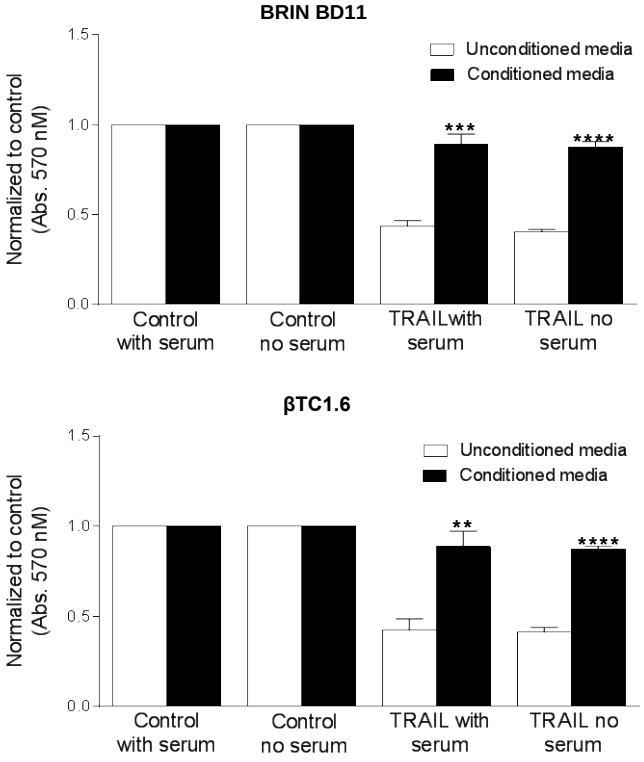

Figure 5.10.Evaluation of TRAIL effects on viability of cells in the presence of MSC-CM
Normalized data from MTT assays showing the effect of 200 ng/ml of recombinant murine TRAIL on the viability of BRIN-BD11 cells and βTC1.6 cells in the absence and presence of MSC-CM with and without serum. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 ***p*<0.01 ****p*<0.001and *****p*<0.0001 compared to non-MSC-CM treated.

5.3.5 MSC-CM blocks TRAIL-induced apoptosis

To determine the potential role of MSC-CM in the amelioration of TRAIL-induced apoptosis cells were exposed to 200 ng/ml of recombinant murine TRAIL in the presence of MSC-CM, and compared with those treated in non-MSC-CM. TUNEL results showed a significant reduction ($P<0.001$-$P<0.0001$) in % positive TUNEL cells in the presence of MSC-CM. Treating the BRIN BD11 with 200 ng/ml of TRAIL in the absence of MSC-CM resulted in 68%positive TUNEL cells and for βTC1.6 75% which was reduced to 4% and 9% respectively after MSC-CM addition in media with serum. A similar trend was found in the absence of serum (Figure 5.11, 5.12).

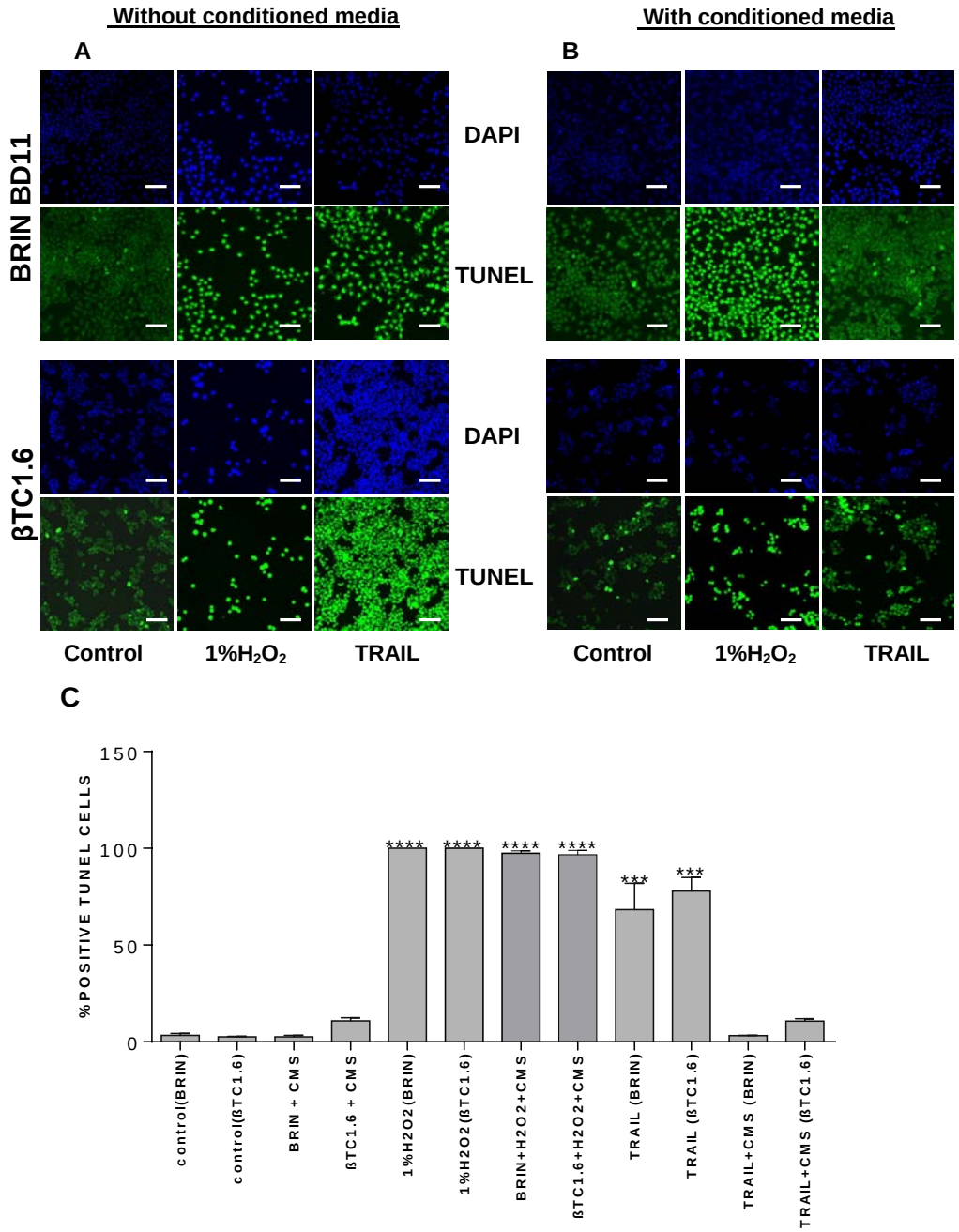

***Figure 5.11.Assessment of TRAIL-induced apoptosis in pancreatic cell lines with and without MSC-CM in the presence of serum*. (A)** Fluorescent images showing TRAIL-induced apoptosis in BRIN-BD11 and βTC1.6 cells after 24 h of exposure to 200 ng/ml of recombinant murine TRAIL. **(B)** Fluorescent images showing the MSC-CM block TRAIL-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines. Blue staining represents DAPI staining of the nuclei while green staining indicates TUNEL positive cells**. (C)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****p*<0.001 and *****p*<0.0001 compared with untreated controls. The scale bar in all images equals 100 µm. CMS stand for MSC-CM with serum.

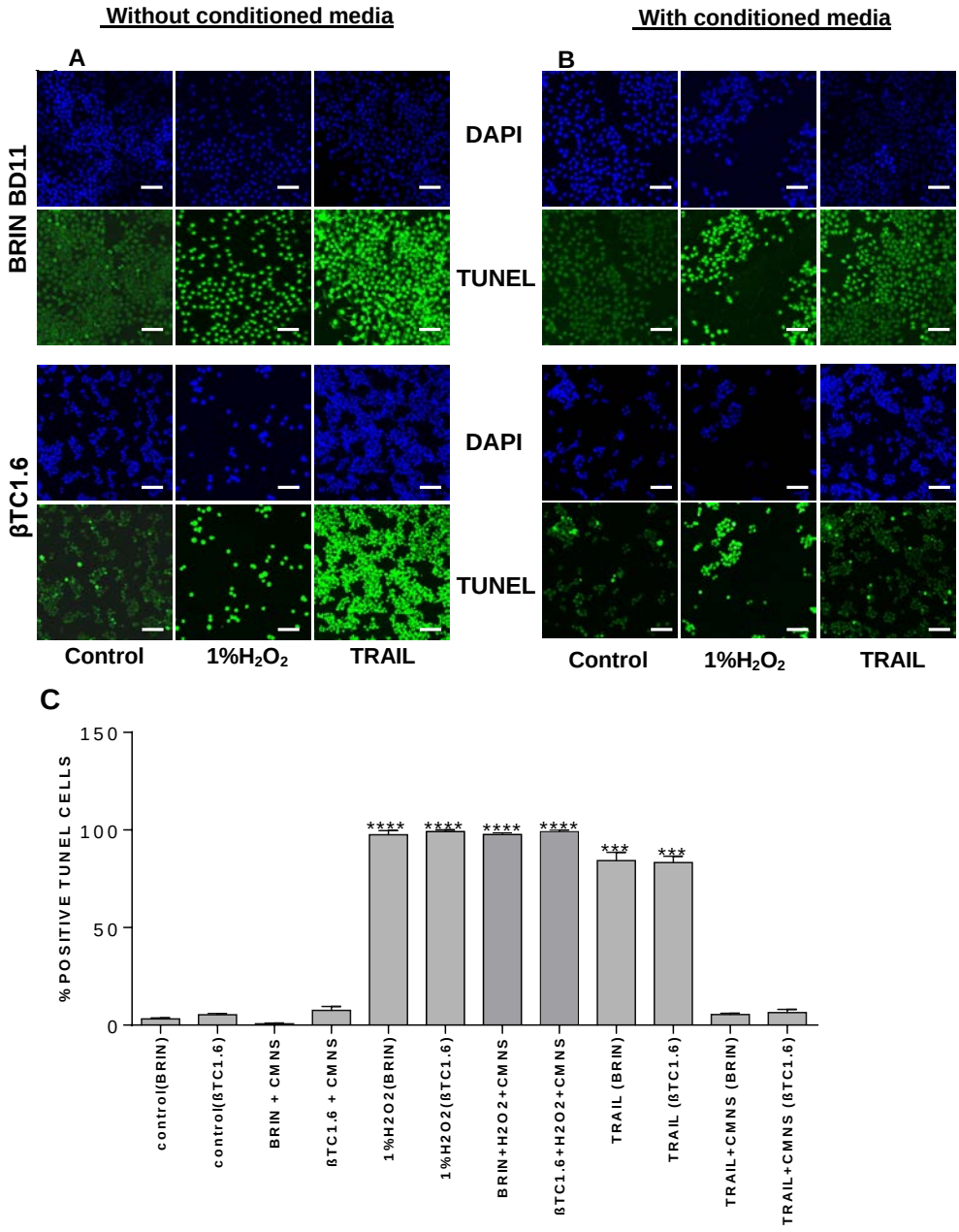

Figure 5.12.Assessment of TRAIL-induced apoptosis in pancreatic cell lines with and without MSC-CM in the absence of serum. **(A)** Fluorescent images showing TRAIL–induced apoptosis in BRIN-BD11 and βTC1.6 cells after 24 h of exposure to 200 ng/ml of recombinant murine TRAIL. **(B)** Fluorescent images showing the MSC-CM blocked TRAIL-induced apoptosis in BRIN-BD11 and βTC1.6 cell lines. Blue staining represents DAPI staining of the nuclei while green staining indicates TUNEL positive cells. **(C)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****p*<0.001 and *****p*<0.0001 compared with untreated controls. The scale bar in all images equals 100 µm. CMNS stands for MSC-CM without serum.

5.3.6 MSC-CM contains high concentrations of anti-inflammatory IL-10

Following on from the preceding results with MSC-CM in abrogation of apoptosis we next asked what are the responsible component(s) of the MSC-CM? To explore the content of our MSC-CM, possible candidates were chosen based on data obtained from a cytokine array conducted by one of our colleagues (Appendix 1).

MSC-CM obtained from three independent donors was analyzed for interleukin-4 (IL-4), interleukin-10 (IL-10), vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) content via quantification utilising commercially available ELISA assays. The results showed high concentrations of IL-10 in RPMI1640 (3269 pg/ml) and DMEM media (3038 pg/ml) in media with serum (Figure 5.13 A, B), and RPMI1640 (2847 pg/ml) DMEM (1912 pg/ml) in serum free media (Figure 5.13 C, D). VEGF concentrations in conditioned media were noted as RPMI1640 with serum (2314 pg/ml) (Figure 5.13 A), and in serum free media (1617 pg/ml) (Figure 5.13 C) while, DMEM with serum was 1422 pg/ml (Figure 5.13 B) and, and 106 pg/ml without serum (Figure 5.13 D). We also tested the presence of IL-4 in RPMI1640 and DMEM conditioned media and the results indicated the presence of 93 pg/ml and 106 pg/ml in media with serum, respectively, (Figure 5.13 A, B), and 70 pg/ml and 69 pg/ml in media without serum, respectively (Figure 5.13 C, D). Finally we explored PIGF levels in conditioned RPMI1640 (153 pg/ml) and DMEM (108 pg/ml) in media with serum (Figure 5.13 A, B) and without, RPMI1640 (112 pg/ml) and DMEM (79 pg/ml) (Figure 5.13 C, D).

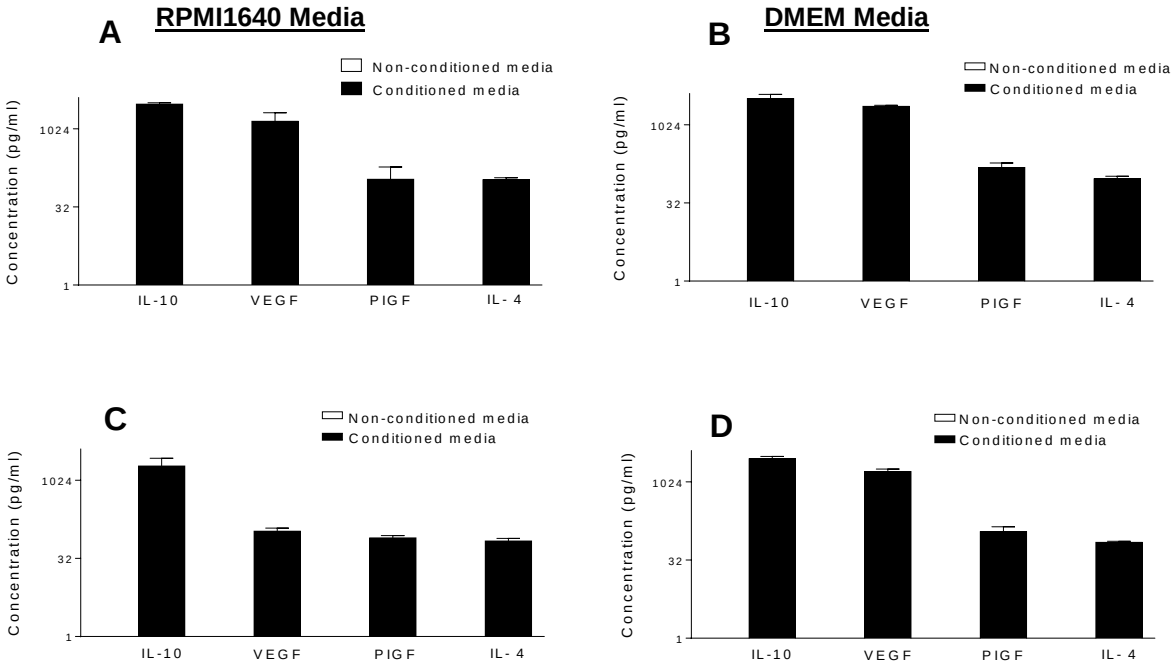

Figure 5.13.Quantifying the content of MSC-CM. The concentration of candidate anti-inflammatory/anti-apoptotic proteins in MSC-CM was quantified by ELISA assays. The results showed a high concentration of IL-10 in MSC-CM while it completely absence from non-conditioned media in the presence of serum (A, B) and absence of serum (C, D). n=3 and presented as mean ± standard deviation (SD).

5.3.7 Testing the effect of candidate anti-inflammatory and anti-apoptotic proteins on β-cell viability

The next step was to evaluate the role of IL-4, PIGF, VIGF, and IL-10 addition along with cytokines and endotoxin on the viability of BRIN-BD11 and βTC1.6 cells by colorimetric MTT assay. These cytokines were randomly chosen from cytokines array done by one of our colleagues to examine the MSC-CM content (Appendix 1).

5.3.7.1 IL-4

The ELISA test confirmed the presence of IL-4 in MSC-CM (0.07-0.1 ng/ml). To test whether IL-4 is behind the protective effect of our MSC-CM, we treated the cells with rising concentrations of IL-4 (0.01-100 ng/ml) mixed with either 1 µg/ml of IFN-γ, 100 ng/ml of IL-1β, 500 µg/ml of LPS and for TNF-α (100 ng/ml in media with serum vs 1 µg/ml in media without serum for BRIN-BD11 cells and 1 µg/ml for βTC1.6 cells in media with and without serum) and measured the viability of the cells by the colorimetric MTT assay. The results showed no significant difference in cell viability between cells treated with cytokines alone and those treated with a mixture of IL-4 and cytokines (Figures 5.14-5.15).

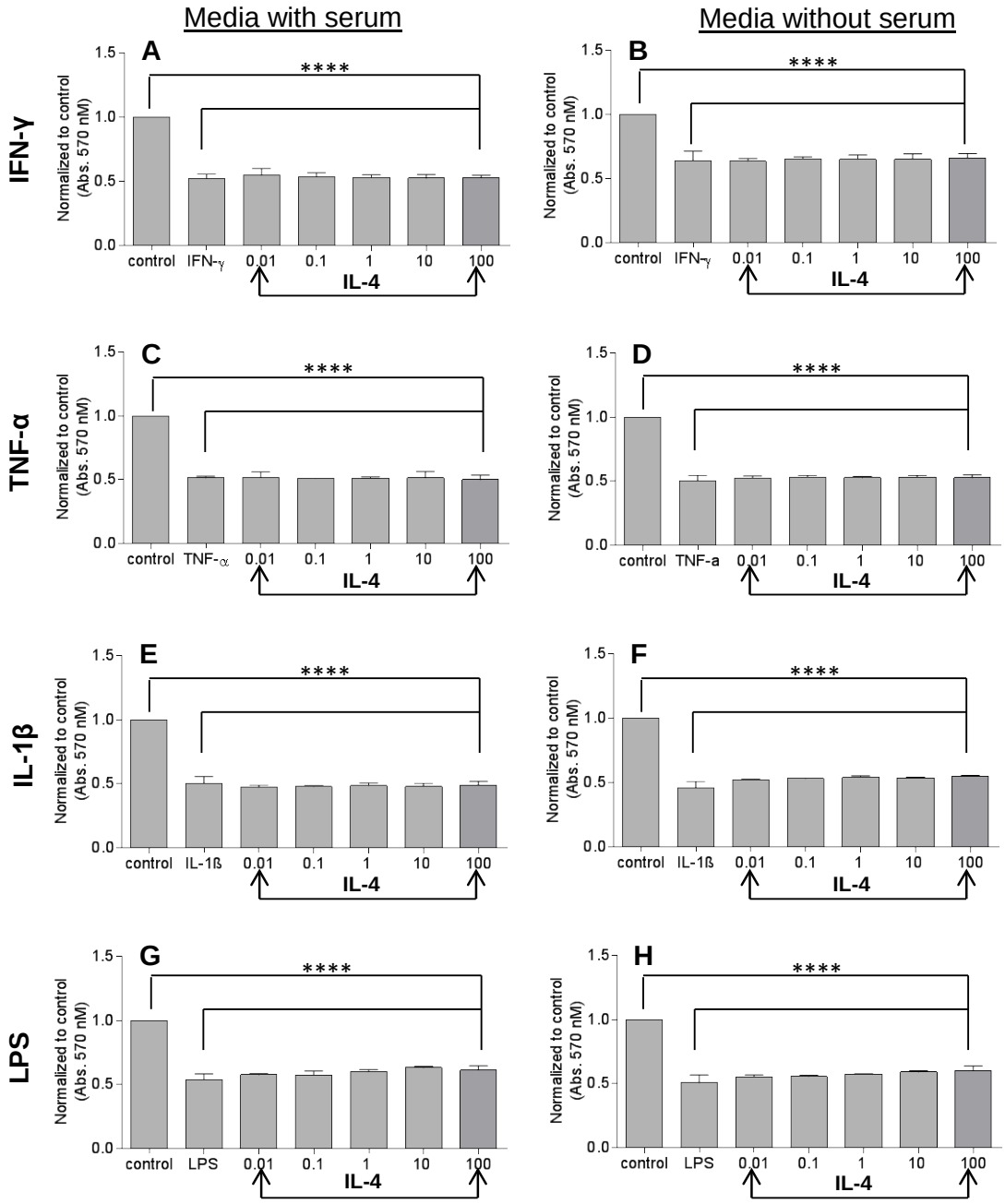

Figure 5.14.Effect of IL-4 on the cellular viability of BRIN-BD11 cells exposed to cytokines and LPS. Evaluation of BRIN-BD11 cell viability after 24 h of exposure to a mixture of IL-4 at different doses in ng/ml with 1 µg/ml of IFN-γ with and without serum (A, B), 100 ng/ml of TNF-α with serum and 1 µg/ml without serum (C, D), 100 ng/ml of IL-1β with and without serum (E, F) and 500 µg/ml of LPS with and without serum (G, H). Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 ****p<0.0001 as compared to control. IFN-γ (Interferon-gamma), TNF-α (Tumour necrosis factor-alpha), IL-1β (Interleukin-1beta), and LPS (Lipopolysaccharide).

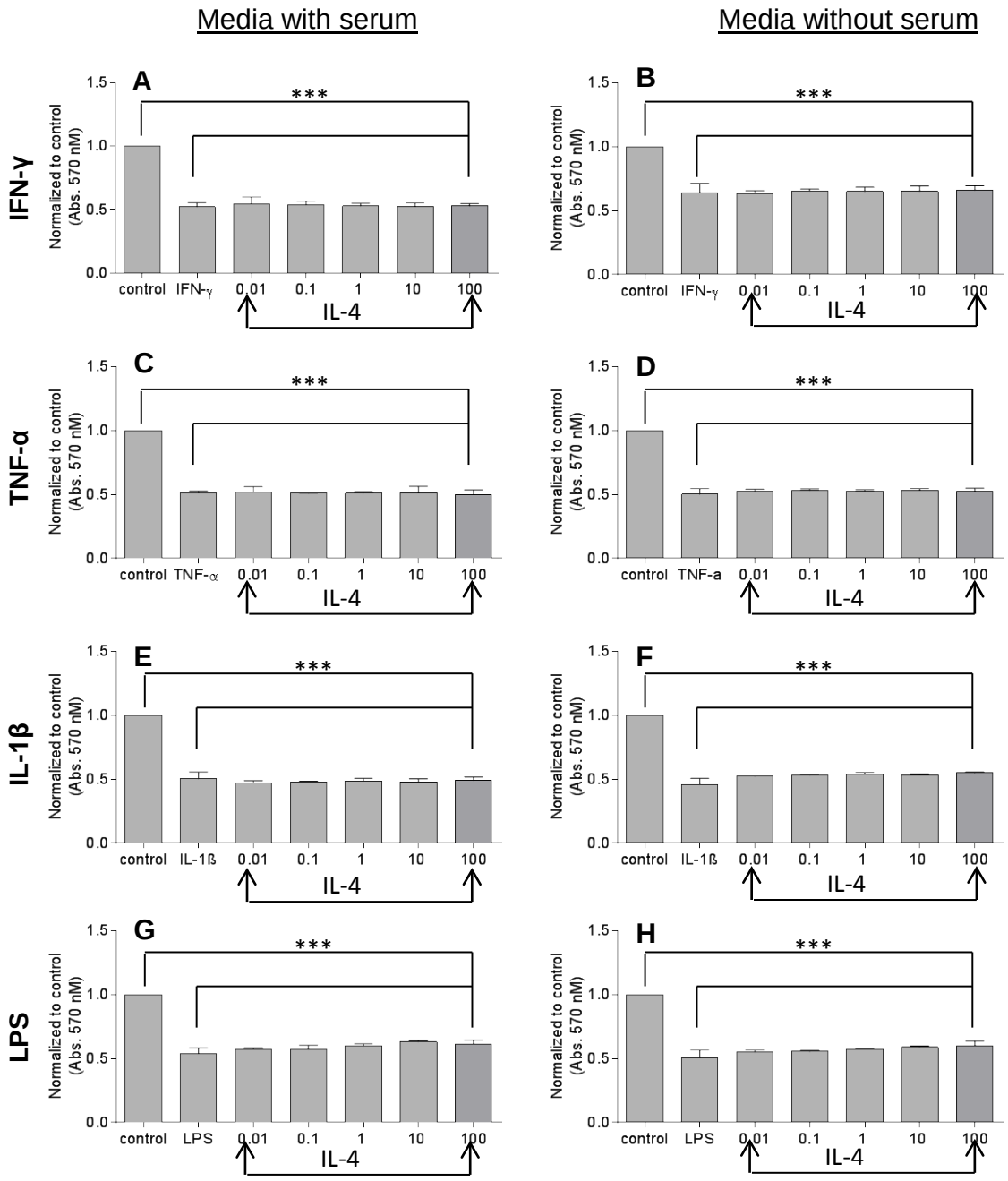

Figure 5.15.Effect of IL-4 on the cellular viability of βTC1.6 cells exposed to cytokines and LPS. Evaluation of βTC1.6 cell viability after 24 h of exposure to a mixture of IL-4 at different doses in ng/ml with 1 µg/ml of IFN-γ with and without serum (A, B), 1 µg/ml of TNF-α with and without serum (C, D), 100 ng/ml of IL-1β with and without serum (E, F) and 500 µg/ml of LPS with and without serum (G, H). Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 $^{***}p<0.001$ as compared to control. IFN-γ (Interferon-gamma), TNF-α (Tumour necrosis factor alpha), IL-1β (Interleukin-1beta), and LPS (Lipopolysaccharide).

5.3.7.2 PIGF

The second candidate tested was placental growth factor (PIGF) the concentration found in the MSC-CM equals (0.07-0.1 ng/ml). The pancreatic β-cell lines were treated with rising concentrations of PIGF (0.01-100 ng/ml) mixed with either 1 µg/ml of IFN-γ, 100 ng/ml of IL-1β, 500 µg/ml of LPS and TNF-α (100 ng/ml in media with serum vs 1 µg/ml in media without serum for BRIN-BD11 cells and 1 µg/ml for βTC1.6 cells in media with and without serum) and then the viability of the cells were measured by the colorimetric MTT assay. The results showed no significant difference in cells viability between cells treated with cytokines alone and those treated with a mixture of PIGF and cytokines (Figures 5.16-5.17).

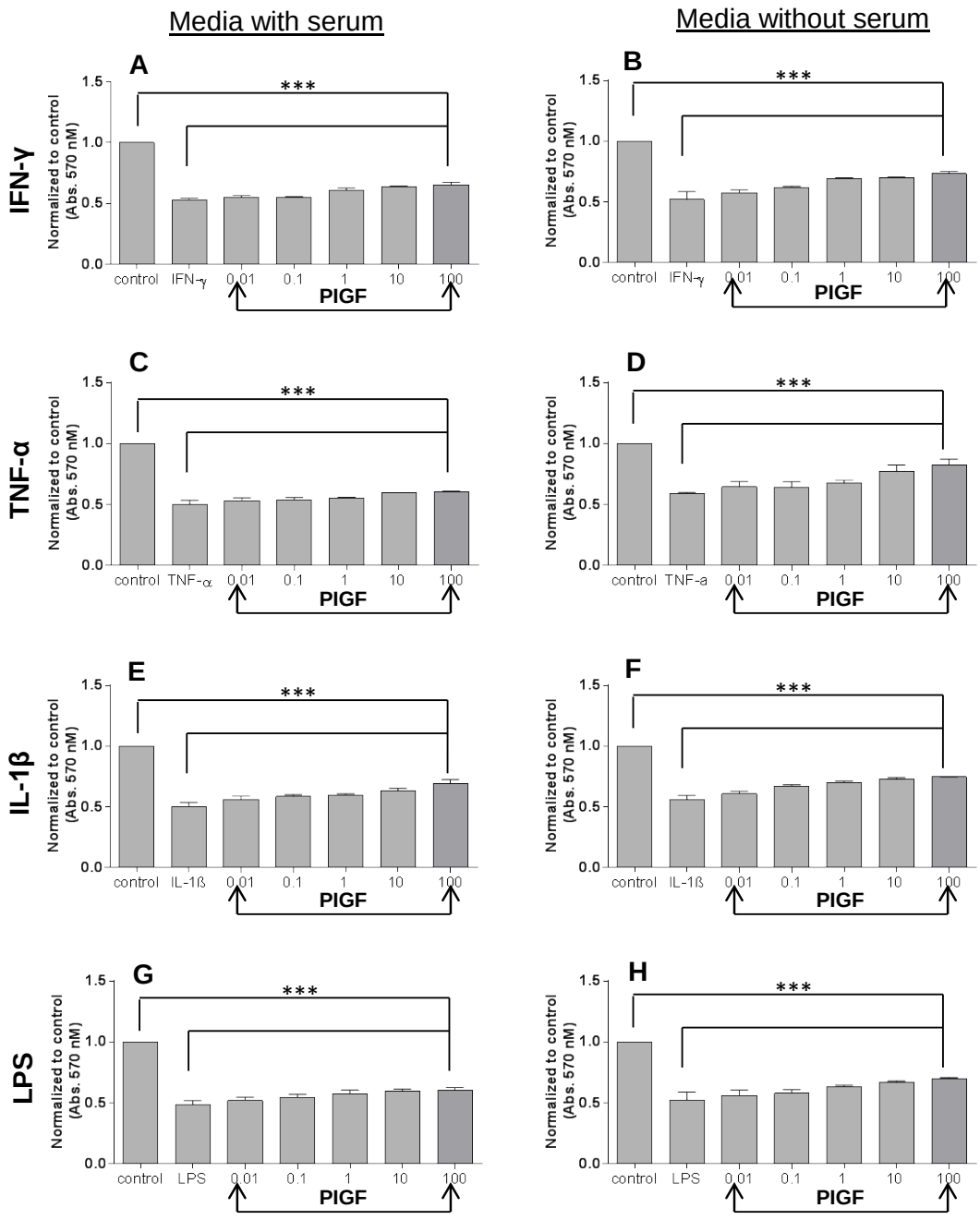

Figure 5.16.Effect of PIGF on the cellular viability of BRIN-BD11 cells exposed to cytokines and LPS. Evaluation of BRIN-BD11 cell viability after 24 h of exposure to a mixture of PIGF at different doses in ng/ml with 1 µg/ml of IFN-γ with and without serum (A, B), 100 ng/ml of TNF-α with serum and 1 µg/ml without serum (C, D), 100 ng/ml of IL-1β with and without serum (E, F) and 500 µg/ml of LPS with and without serum (G, H). Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 *** $p<0.001$ as compared to control. IFN-γ (Interferon-gamma), TNF-α (Tumour necrosis factor alpha), IL-1β (Interleukin-1beta), and LPS (Lipopolysaccharide).

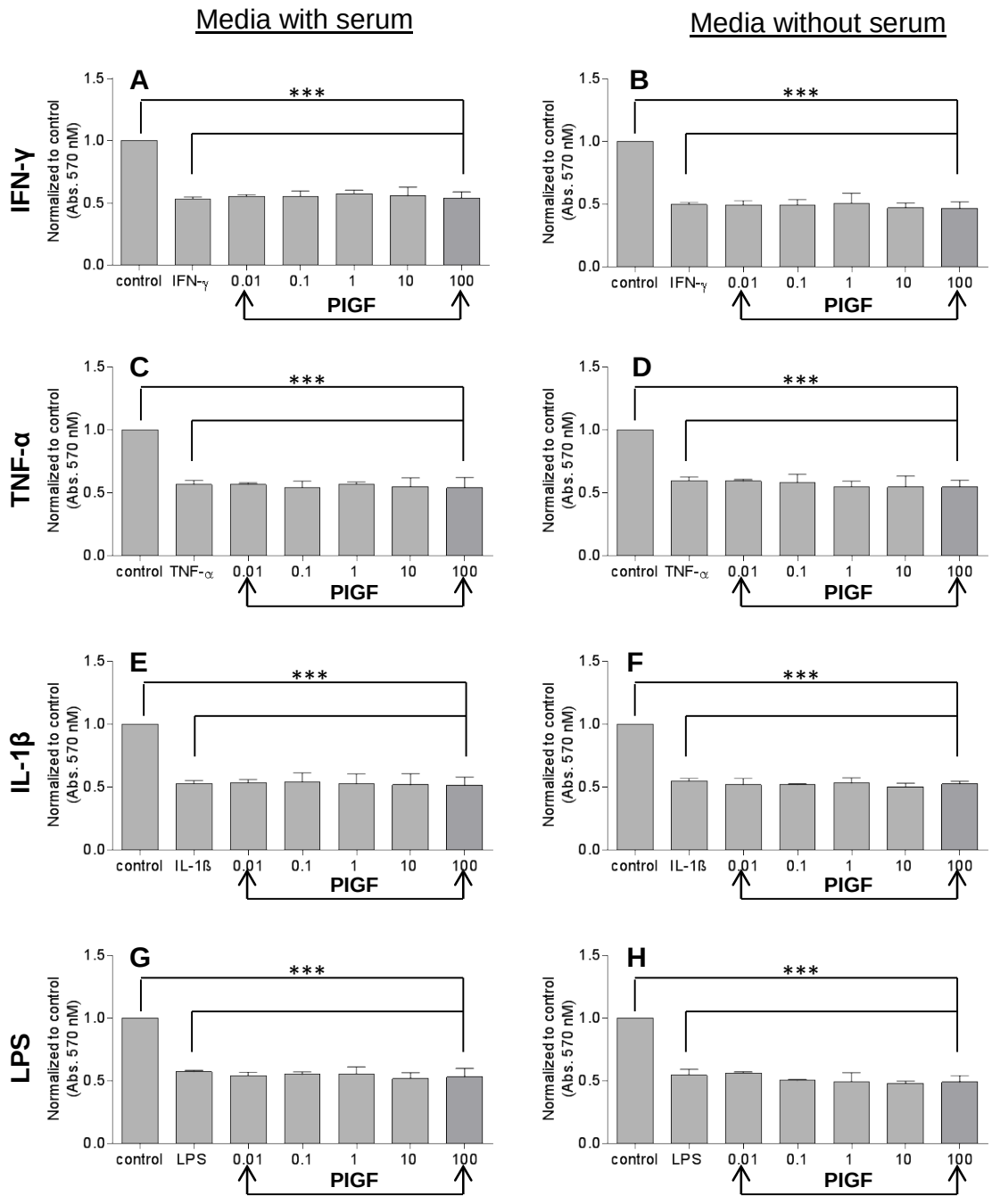

Figure 5.17.Effect of PIGF on the cellular viability of βTC1.6 cells exposed to cytokines and LPS. Evaluation of βTC1.6 cell viability after 24 h of exposure to a mixture of PIGF at different doses in ng/ml with 1 µg/ml of IFN-γ with and without serum (A, B), 1 µg/ml of TNF-α with and without serum (C, D), 100 ng/ml of IL-1β with and without serum (E, F) and 500 µg/ml of LPS with and without serum (G, H). Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 *** $p<0.001$ as compared to control. IFN-γ (Interferon-gamma), TNF-α (Tumour necrosis factor alpha), IL-1β (Interleukin-1beta), and LPS (Lipopolysaccharide).

5.3.7.1 VEGF

The third candidate tested was vascular endothelial growth factor (VEGF) the concentration found was (0.1-2 ng/ml). The pancreatic β-cell lines were treated with rising concentrations of VEGF (0.01-100 ng/ml) mixed with either 1 µg/ml of IFN-γ, 100 ng/ml of IL-1β, 500 µg/ml of LPS and TNF-α (100 ng/ml in media with serum vs 1 µg/ml in media without serum for BRIN-BD11 cells and 1 µg/ml for βTC1.6 cells in media with and without serum) and then the viability of the cells was measured by the colorimetric MTT assay. The results showed no significant difference in cells viability between cells treated with cytokines alone and those treated with a mixture of PIGF and cytokines (Figures 5.18-5.19).

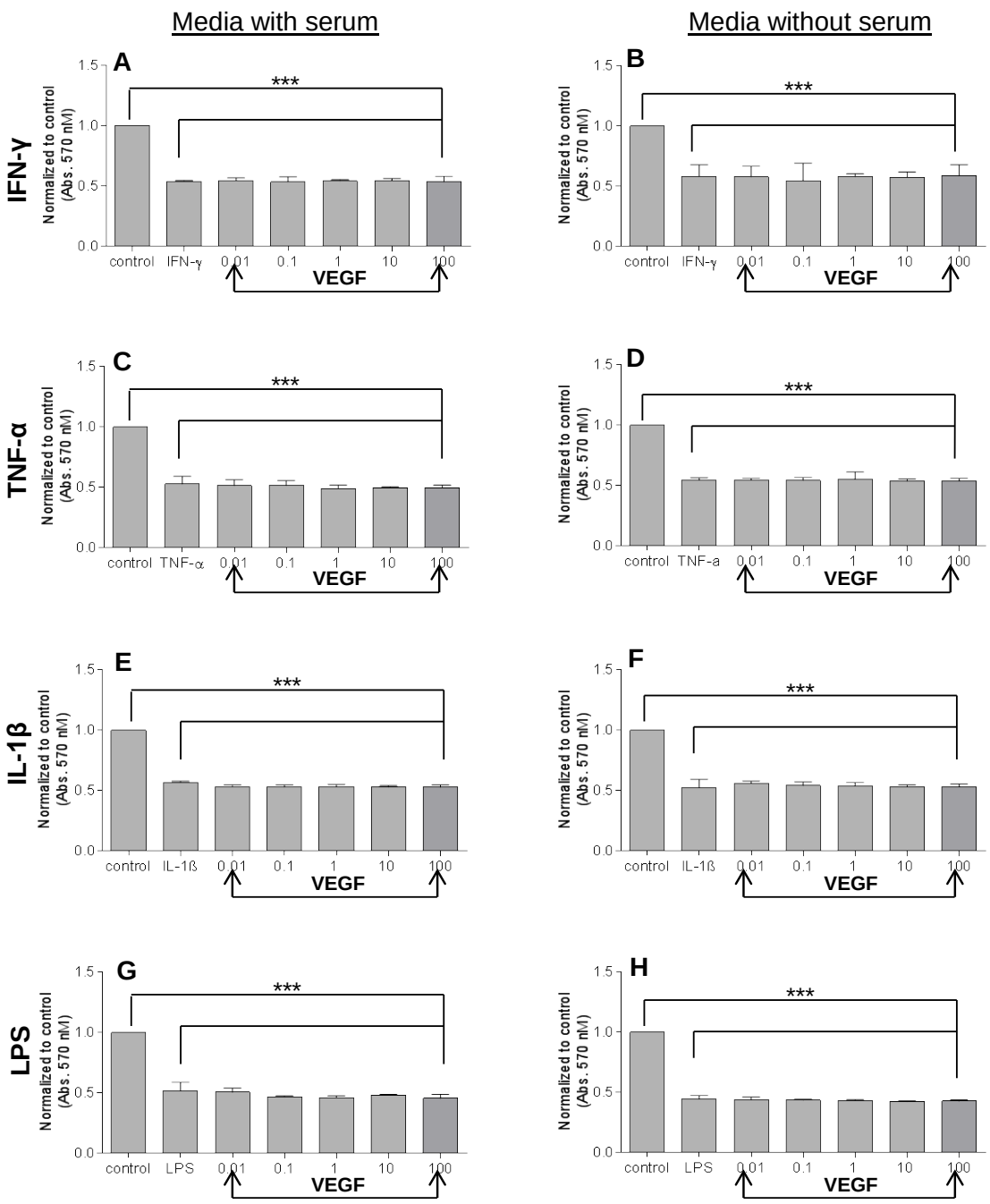

Figure 5.18. Effect of VEGF on the cellular viability of BRIN-BD11 cells exposed to cytokines and LPS. Evaluation of BRIN-BD11 cell viability after 24 h of exposure to a mixture of VEGF at different doses in ng/ml with 1 µg/ml of IFN-γ with and without serum (A, B), 100 ng/ml of TNF-α with serum and 1 µg/ml without serum (C, D), 100 ng/ml of IL-1β with and without serum (E, F) and 500 µg/ml of LPS with and without serum (G, H). Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 $^{***}p<0.001$as compared to control. IFN-γ (Interferon-gamma), TNF-α (Tumour necrosis factor alpha), IL-1β (Interleukin-1 beta), and LPS (Lipopolysaccharide).

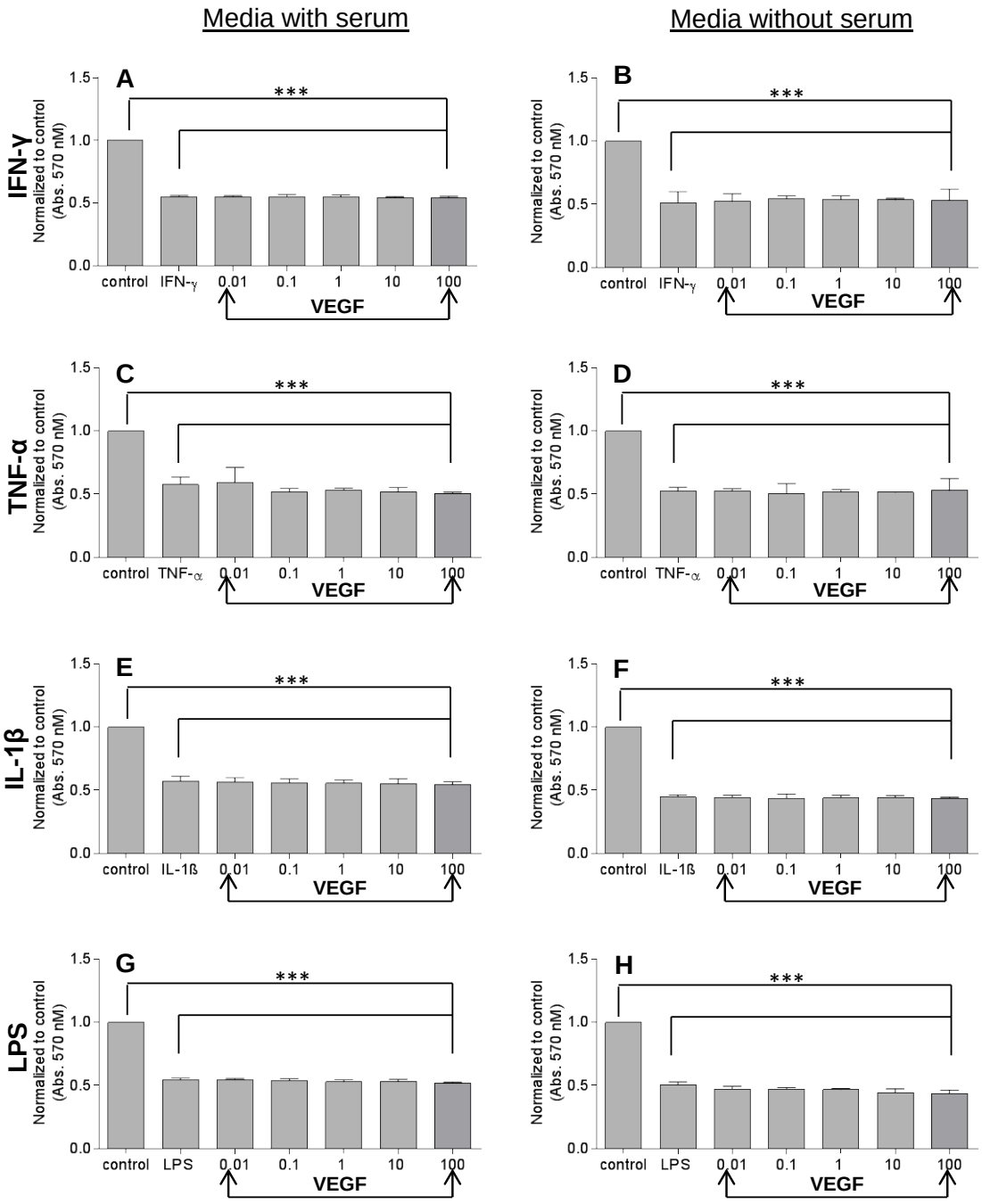

Figure 5.19. Effect of VEGF on the cellular viability of βTC1.6 cells exposed to cytokines and LPS. Evaluation of βTC1.6 cell viability after 24 h of exposure to a mixture of VEGF at different doses in ng/ml with 1 µg/ml of IFN-γ with and without serum (A, B), 1 µg/ml of TNF-α with and without serum (C, D), 100 ng/ml of IL-1β with and without serum (E, F) and 500 µg/ml of LPS with and without serum (G, H). Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 *** p<0.001as compared to control. IFN-γ (Interferon-gamma), TNF-α (Tumour necrosis factor alpha), IL-1β (Interleukin-1 beta),and LPS (Lipopolysaccharide).

5.3.7.4 IL-10

The ELISA test confirmed the presence of IL-10 in MSC-CM (1.9-3 ng/ml). The pancreatic β-cell lines were treated with rising concentrations of IL-10 (0.01-100 ng/ml) mixed with either 1 µg/ml of IFN-γ, 100 ng/ml of IL-1β, 500 µg/ml of LPS and TNF-α (100 ng/ml in media with serum vs 1 µg/ml in media without serum for BRIN-BD11 cells and 1 µg/ml for βTC1.6 cells in media with and without serum). This was followed by measuring the viability of the cells using colorimetric MTT assays. The results showed a significant increase in cell viability in cells treated with IFN-γ or TNF-α mixed with IL-10 than those treated with IFN-γ/TNF-α alone. The largest effect was noted at 1 ng/ml for all cells cultured with and without serum (Figures 5.20-5.21). BRIN-BD11 cells showed a significant increase in viability (+46%; $P<0.001$) with serum and (+64%; $P<0.0001$) without serum for cells treated with IFN-γ and IL-10 compared with IFN-γ alone (Figure 5.20 A, B). Consistently, βTC1.6 cells also showed a significant increase in viability (+47%; $P<0.01$) with serum and (+62%; $P<0.01$) without serum when treated with IFN-γ and IL-10 compared with IFN-γ alone (Figure 5.21 A, B). For cell treated with TNF-α and IL-10, significant increases in cell viability (+25% with serum; $P<0.01$) (+39% without serum; $P<0.01$) were observed in BRIN-BD11 cells (Figure 5.20 C, D) and βTC1.6 cells (+29% with serum; $P<0.01$) (+45% without serum; $P<0.01$) (Figure 5.21 C, D). However, no significant differences were observed in cells treated with IL-1β or LPS for both cell lines (Figures 5.20-5.21).

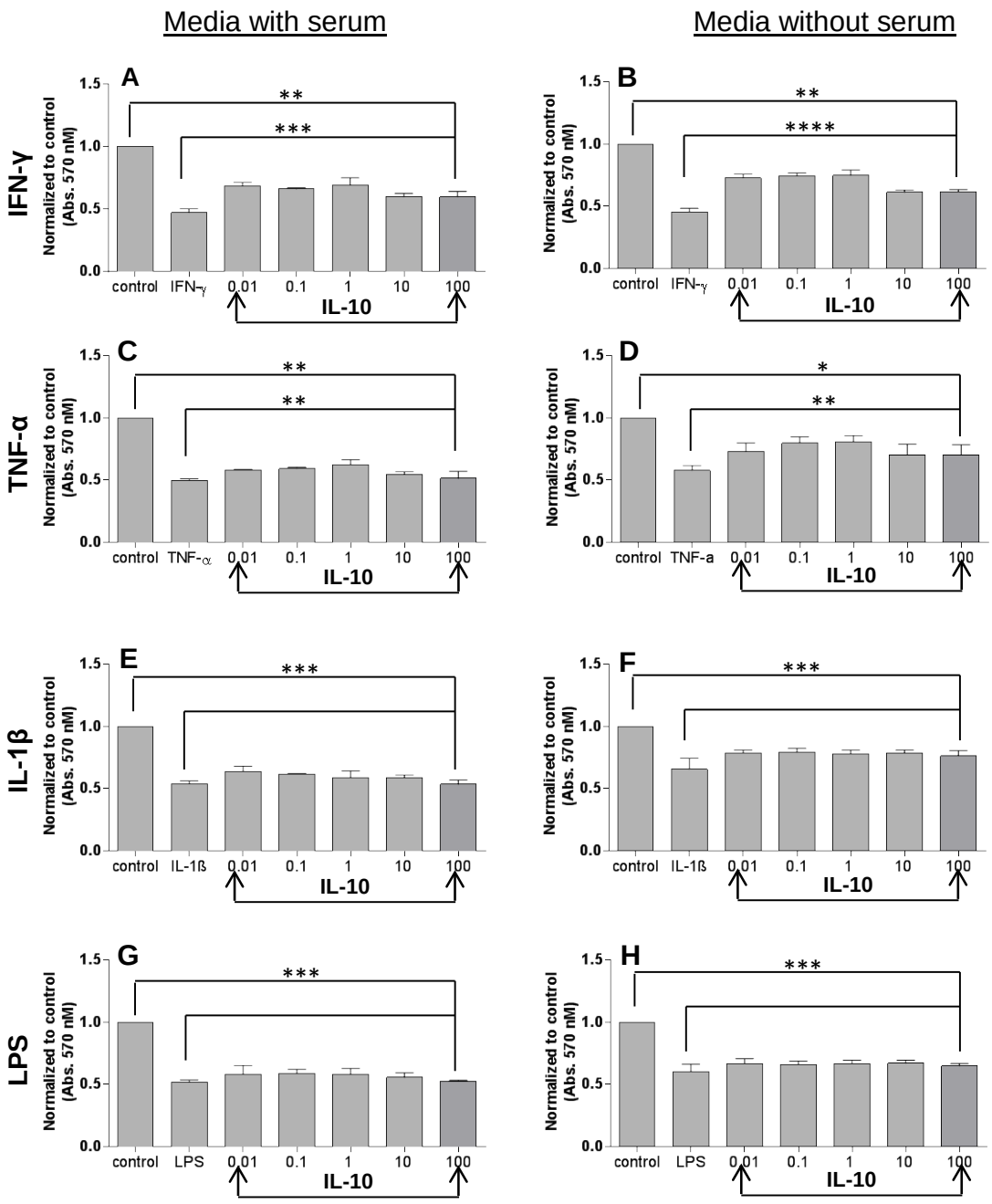

Figure 5.20. Effect of IL-10 on the cellular viability of BRIN-BD11 cells exposed to cytokines and LPS. Evaluation of BRIN-BD11 cell viability after 24 h of exposure to a mixture of IL-10 at different doses in ng/ml with 1 µg/ml of IFN-γ with and without serum (A, B), 100 ng/ml of TNF-α with serum and 1 µg/ml without serum (C, D), 100 ng/ml of IL-1β with and without serum (E, F) and 500 µg/ml of LPS with and without serum (G, H). Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, **** p<0.0001.as compared first to untreated control and second to IFN-γ/TNF-α IFN-γ (Interferon-gamma), TNF-α (Tumour necrosis factor alpha), IL-1β (Interleukin-1beta), and LPS (Lipopolysaccharide).

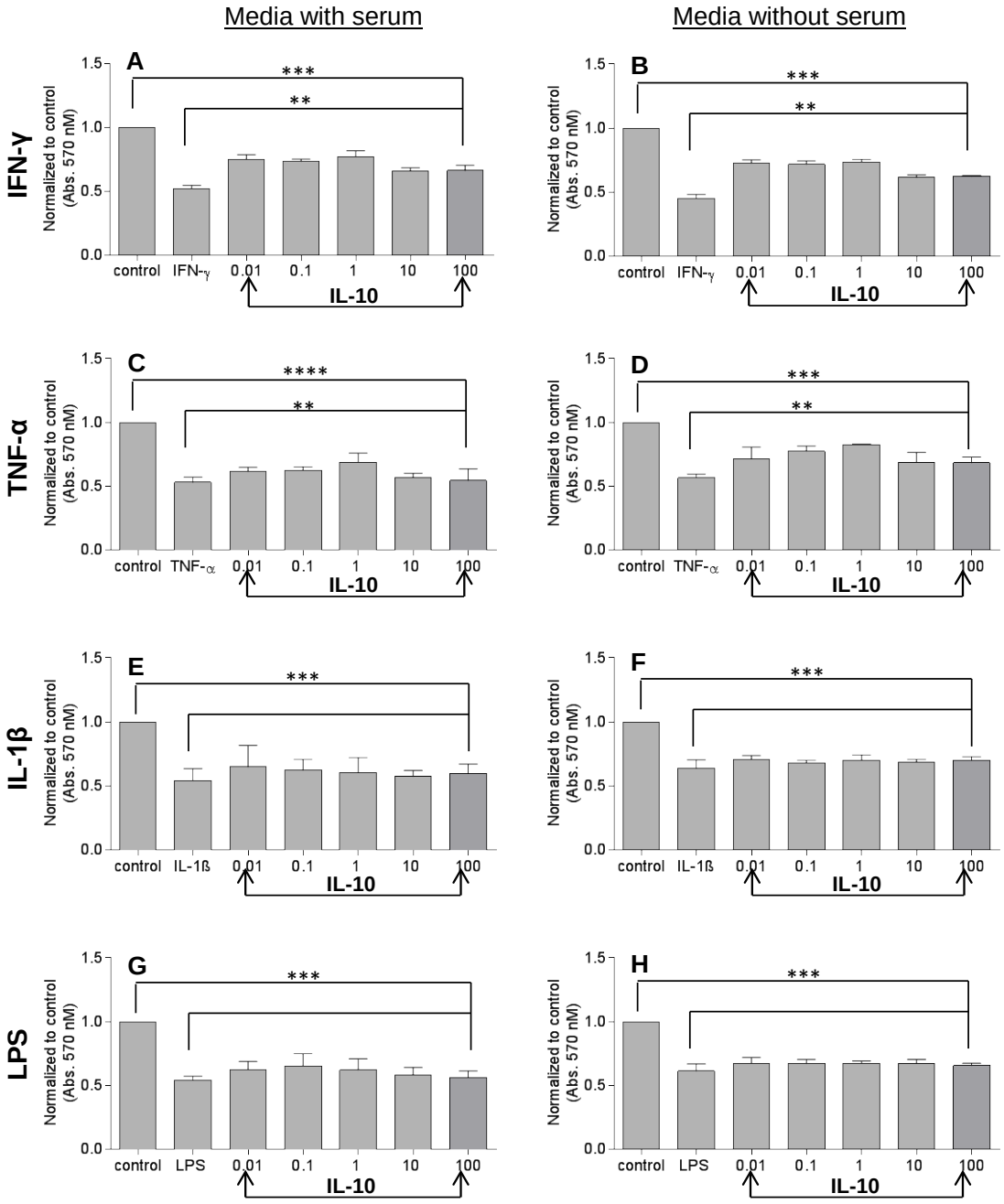

Figure 5.21. Effect of IL-10 on the cellular viability of βTC1.6 cells exposed to cytokines and LPS Evaluation of βTC1.6 cell viability after 24 h of exposure to a mixture of IL-10 at different doses in ng/ml with 1 µg/ml of IFN-γ with and without serum (A, B), 1 µg/ml of TNF-α with and without serum (C, D), 100 ng/ml of IL-1β with and without serum (E, F) and 500 µg/ml of LPS with and without serum (G, H). Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 ** p<0.01, *** p<0.001. as compared first to untreated control and second to IFN-γ/TNF-α. IFN-γ (Interferon-gamma), TNF-α (Tumour necrosis factor alpha), IL-1β(Interleukin-1beta),and LPS (Lipopolysaccharide).

5.3.8 IL-10 blocks IFN-γ and TNF-α-induced apoptosis in pancreatic β-cell lines

IL-10 increases the viability of β-cells treated with IFN-γ or TNF-α. We next sought to determine whether IL-10 conferred this protection via interference with apoptotic processes. Cells were treated with a combination of 1 ng/ml of IL-10 mixed with 1 μg/ml of IFN-γ with and without serum or TNF-α (100 ng/ml in media with serum vs 1 μg/ml in media without serum for BRIN-BD11 and 1 μg/ml for βTC1.6 in media with and without) after which TUNEL assays were performed. In all instances, the addition of IL-10 protected against cytokine-driven β-cell apoptosis and resulted in a significant reduction ($P<0.001$-$P<0.0001$) in the % positive TUNEL cells when compared with cells exposed to cytokines alone (Figures 5.22, 5.23).

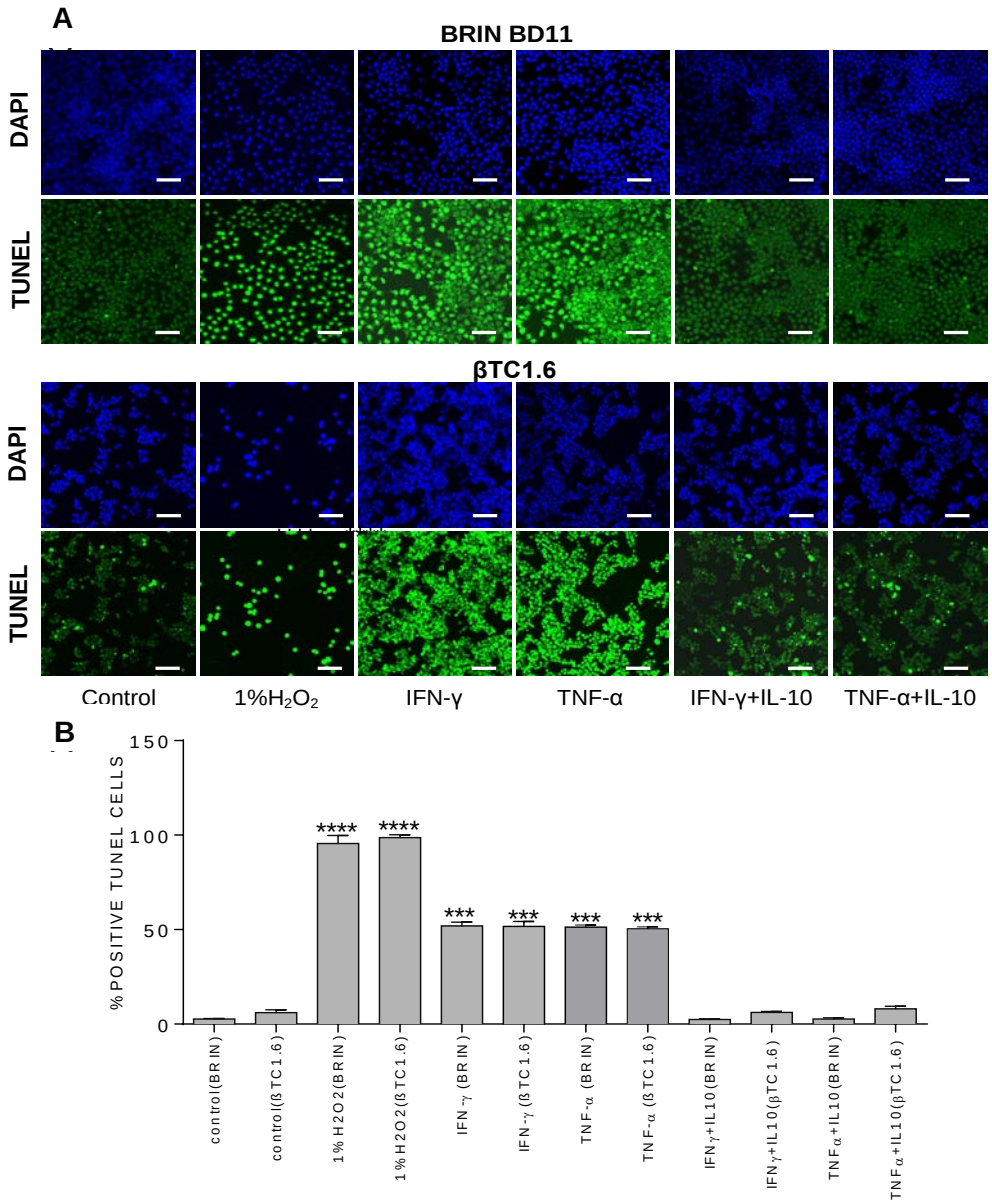

Figure 5.22. Assessment of IFN-γ/TNF-α-induced apoptosis in pancreatic cell lines before and after the addition of IL-10 in the presence of serum. **(A)** Fluorescent images showing the ability of IL-10 (1ng/ml) to reduce the % positive TUNEL cells after treating them with a combination of IL-10 and 1 µg/ml of IFN-γ or 100 ng/ml of TNF-α for BRIN-BD11 and 1 µg/ml of TNF-α for βTC1.6 cells. Blue staining represents DAPI staining of the nuclei while green staining indicates TUNEL positive cells. **(B)**The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****p*<0.001 and *****p*<0.0001 compared with untreated controls. The scale in all images equals 100 µm.

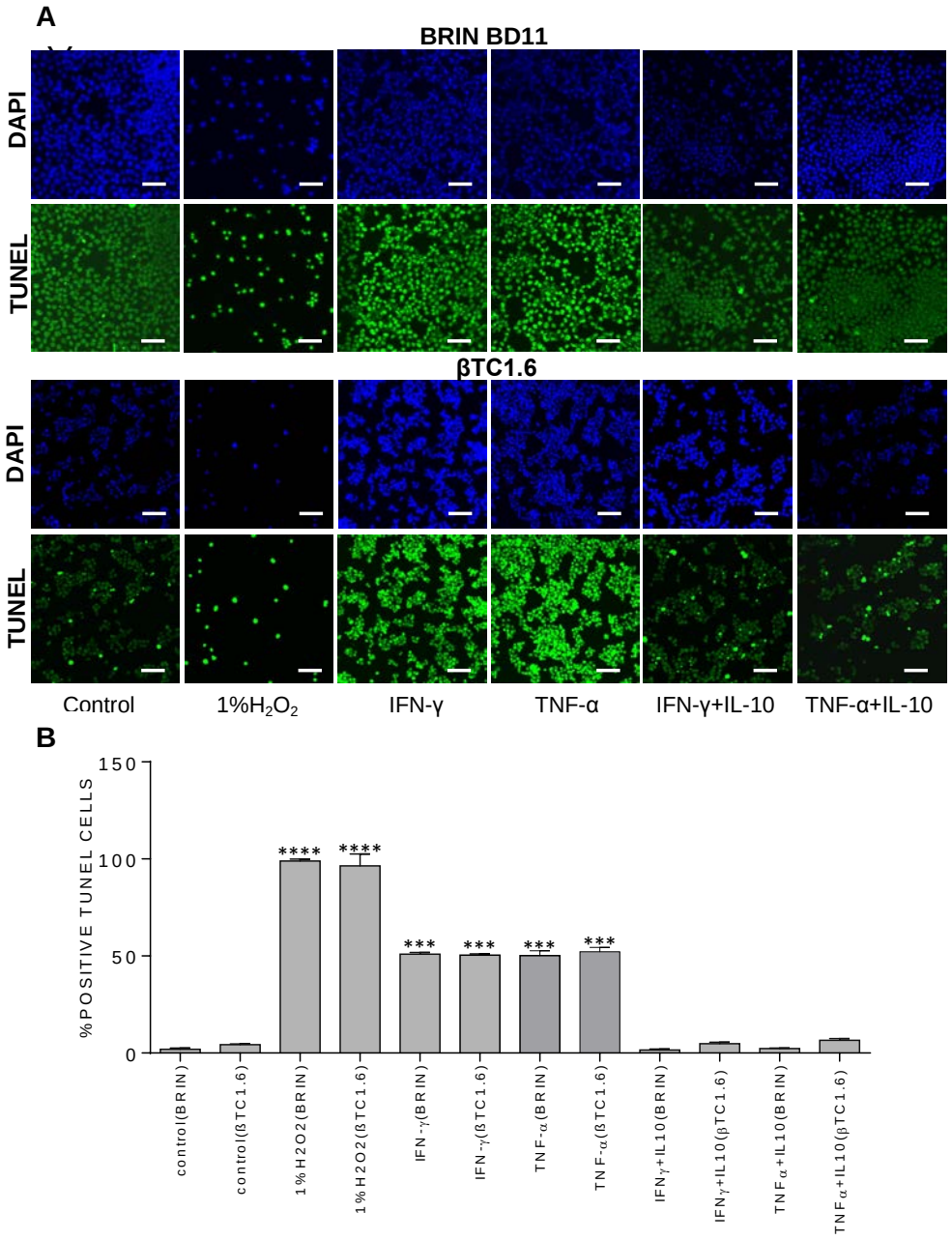

***Figure 5.23. Assessment of IFN-γ/TNF-α-induced apoptosis in pancreatic cell lines before and after the addition of IL-10 in the absence of serum*. (A)** Fluorescent images showing the ability of IL-10 (1ng/ml) to reduce the % positive TUNEL cells after treating them with a combination of IL-10 and 1 µg/ml of IFN-γ or 1 µg/ml of TNF-α. Blue staining represents DAPI staining of the nuclei while green staining indicates TUNEL positive cells **(B)**The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****p*<0.001 and *****p*<0.0001 compared with untreated controls. The scale in all images equals 100 µm.

5.3.9 Blocking IL-10 reduces the viability of cells following the addition of IFN-γ/TNF-α addition

To test whether the high IL-10 concentration in MSC-CM was a major contributor to the protective effect of MSC-CM, we sought to block IL-10 in our MSC-CM by adding 100 ng/ml of anti-IL-10 antibody. Anti-IL-10 treated MSC-CM was applied to the cells along with 1 µg/ml of IFN-γ with and without serum or TNF-α (100 ng/ml in media with serum vs 1 µg/ml in media without serum for BRIN-BD11 cells and 1 µg/ml for βTC1.6 cells in media with and without serum). Results of the MTT assay showed that anti-IL-10 treated MSC-CM was no longer able to protect cells against IFN-γ or TNF-α-induced cell death (Figure 5.24).

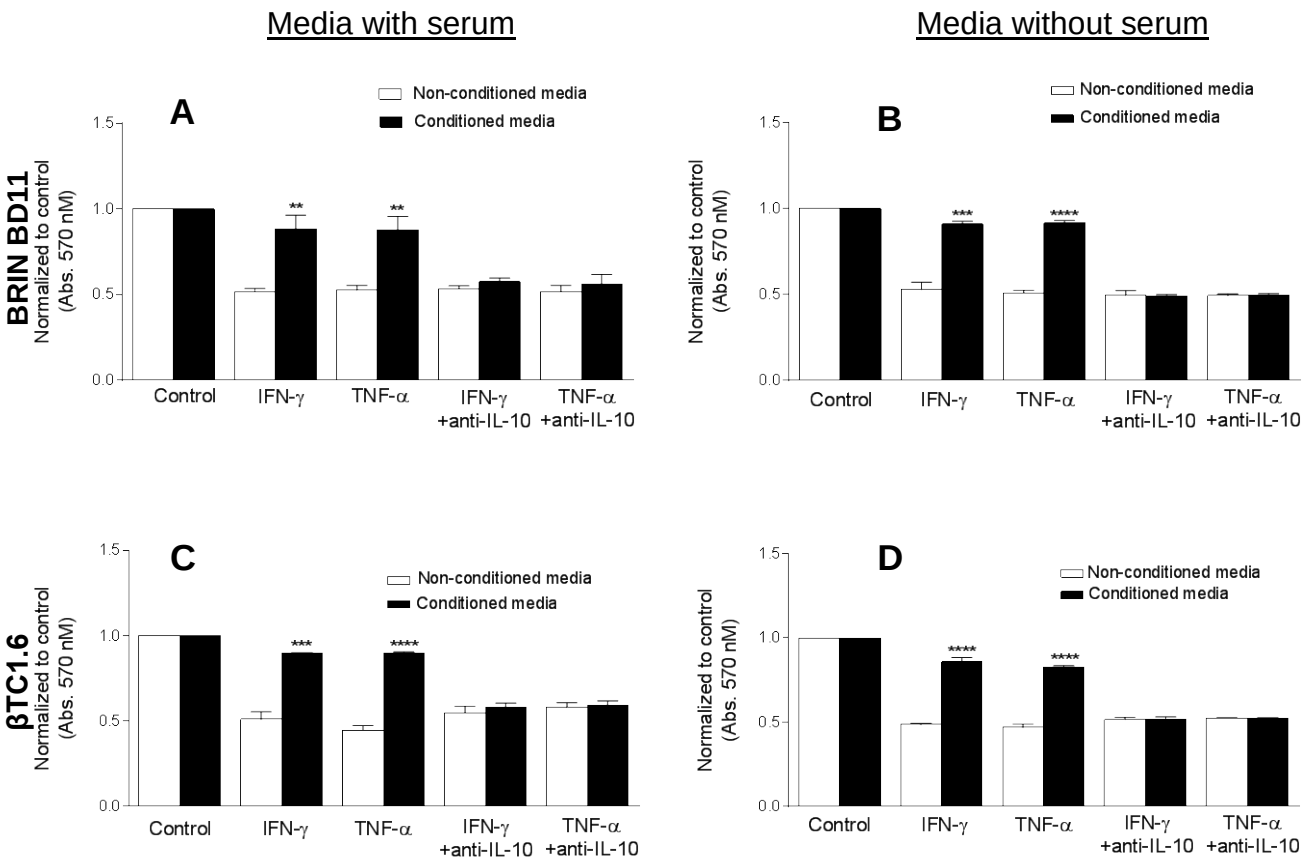

Figure 5.24. Evaluation of cells viability after the addition of anti-IL-10 antibody in the presence of MSC-CM. The viability of BRIN-BD11 (A, B) and βTC1.6 cells (C, D) was assessed by colorimetric MTT assay after the addition of 100 ng/ml of anti-IL-10 along with 1 μg/ml of IFN-γ and TNF-α (100 ng/ml in media with serum vs 1 μg/ml in media without serum for BRIN-BD11 cells and 1 μg/ml for βTC1.6 cells in media with and without serum) in the presence of MSC-CM with and without serum. Data are normalized to untreated controls and presented as mean ± standard deviation (SD). n=3 **p<0.01 ***p<0.001and ****p<0.0001 comparison between conditioned and non-conditioned. IFN-γ interferon gamma, TNF-α tumour necrosis factor alpha.

5.3.10 Blocking IL-10 increases the number of % positive TUNEL cells following the addition of IFN-γ/TNF-α

We next hypothesized that the differences in cell viability in the presence and absence of MSC-CM was due to IL-10 interfering in the IFN-γ/TNF-α-induction of apoptosis and that the addition of an anti-IL-10 antibody would lead to an increased number of % positive TUNEL cells. To test this hypothesis, we treated our cells with 1 µg/ml of IFN-γ or TNF-α (100 ng/ml in media with serum vs 1 µg/ml in media without serum for BRIN-BD11 cells and 1 µg/ml for βTC1.6 cells in media with and without serum) in the presence of MSC-CM with and without an anti-IL-10 antibody and compared it with non-conditioned media. The results showed an increase in % positive TUNEL cells after the addition of 100 ng/ml of anti-IL-10 antibody to the MSC-CM in the presence and absence of serum. Figure 5.25 and 5.26 demonstrate once again the ability of 1 µg/ml of IFN-γ to induce apoptosis in β-cell lines (59%positive TUNEL cell) for BRIN BD11 and (57%positive TUNEL cell) for βTC1.6.However, the addition of MSC-CM reduced the %positive TUNEL cell to (6%) and (7%) respectively in the presence of serum (Figure 25). However, the addition of 100 ng/ml anti-IL-10 antibody increased the %positive TUNEL cells for BRIN BD11 (55.9%) and βTC1.6 (58%) in the presence of MSC-CM with serum. A similar trend was observed in the absence of serum (Figure 26).Cells treated with TNF-α also showed an increase in the %positive TUNEL cells in the presence of MSC-CM after the addition of 100 ng/ml of anti-IL-10 antibody, for BRIN BD11 (56%) in media with serum and (52%) in media without serum and for βTC1.6 (57%) in media with serum and (51%) in media without serum as shown in Figures 5-27 and 5-28.

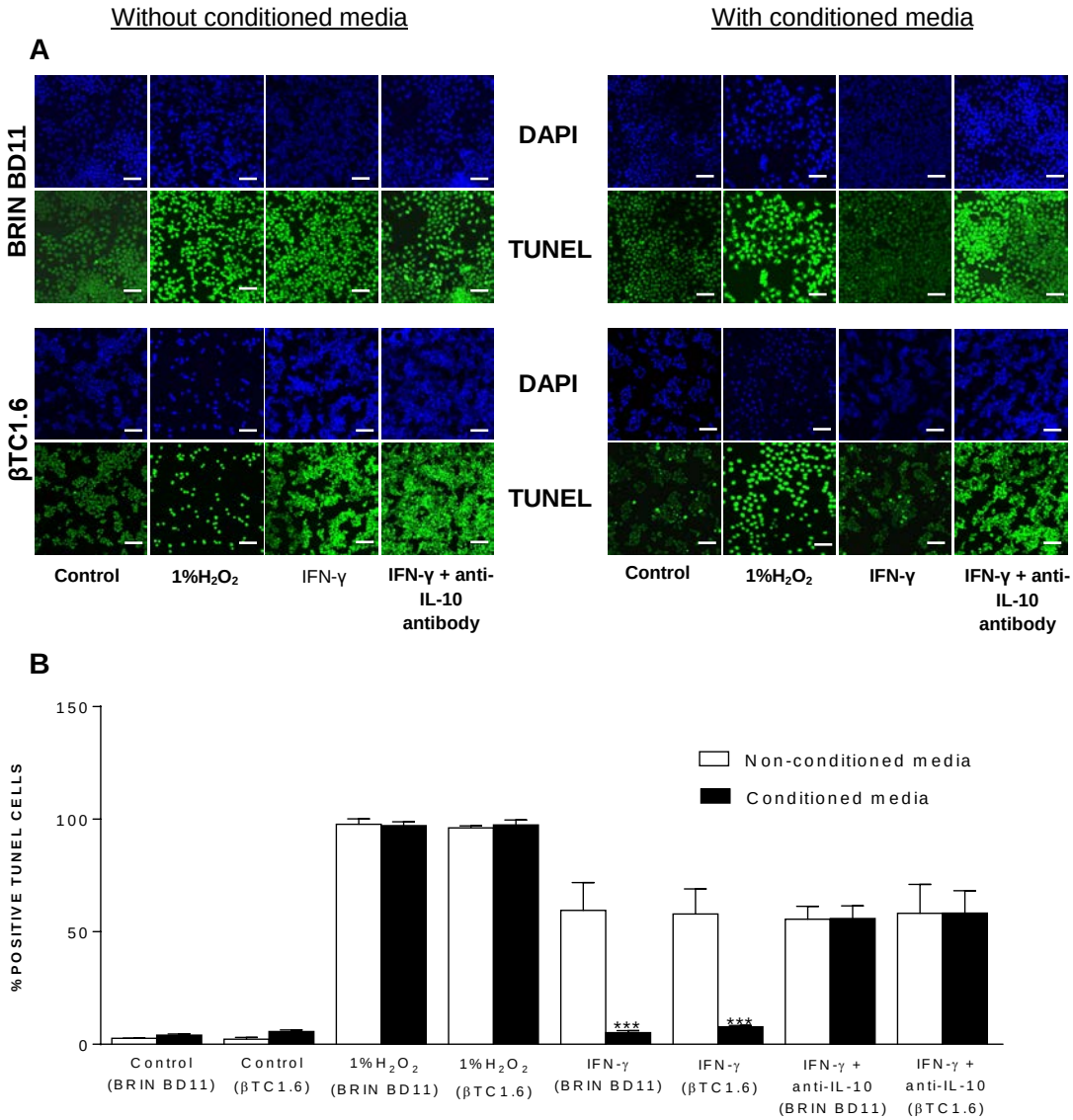

***Figure 5.25. Assessment of IFN-γ-induced apoptosis in pancreatic cell lines before and after the addition of anti-IL-10 antibody in the presence of MSC-CM with serum*.** **(A)** Fluorescent images showing the increase in the number of % positive TUNEL cells treated with 1 µg/ml of IFN-γ in the presence of MSC-CM after the addition of 100 ng/ml of anti-IL-10 to the MSC-CM. Blue staining represents DAPI staining of the nuclei while green staining indicates TUNEL positive cells. **(B)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****p*<0.001 compared with non-conditioned media. The scale bar in all images equals 100 µm. IFN-γ. Interferon gamma

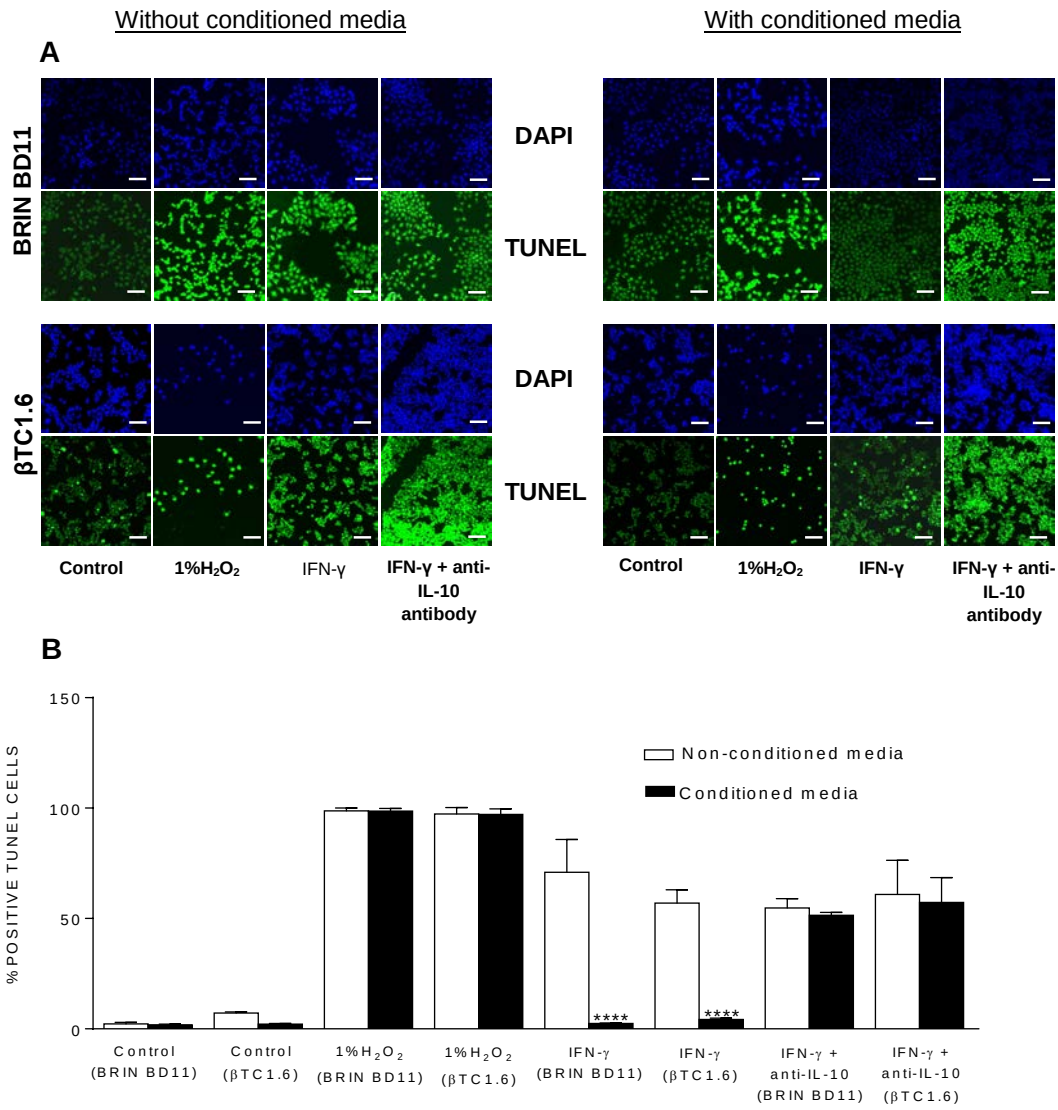

Figure 5.26. Assessment of IFN-γ-induced apoptosis in pancreatic cell lines before and after the addition of anti-IL-10 antibody in the presence of MSC-CM without serum. **(A)** Fluorescent images showing the increase in the number of % positive TUNEL cells treated with 1 µg/ml IFN-γ in the presence of MSC-CM after the addition of 100 ng/ml of anti-IL-10 to the MSC-CM. Blue staining represents DAPI staining of the nuclei while green staining indicates TUNEL positive cells. **(B)**The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****p<0.0001 compared with non-conditioned media. The scale bar in all images equals 100 µm. IFN-γ. Interferon gamma.

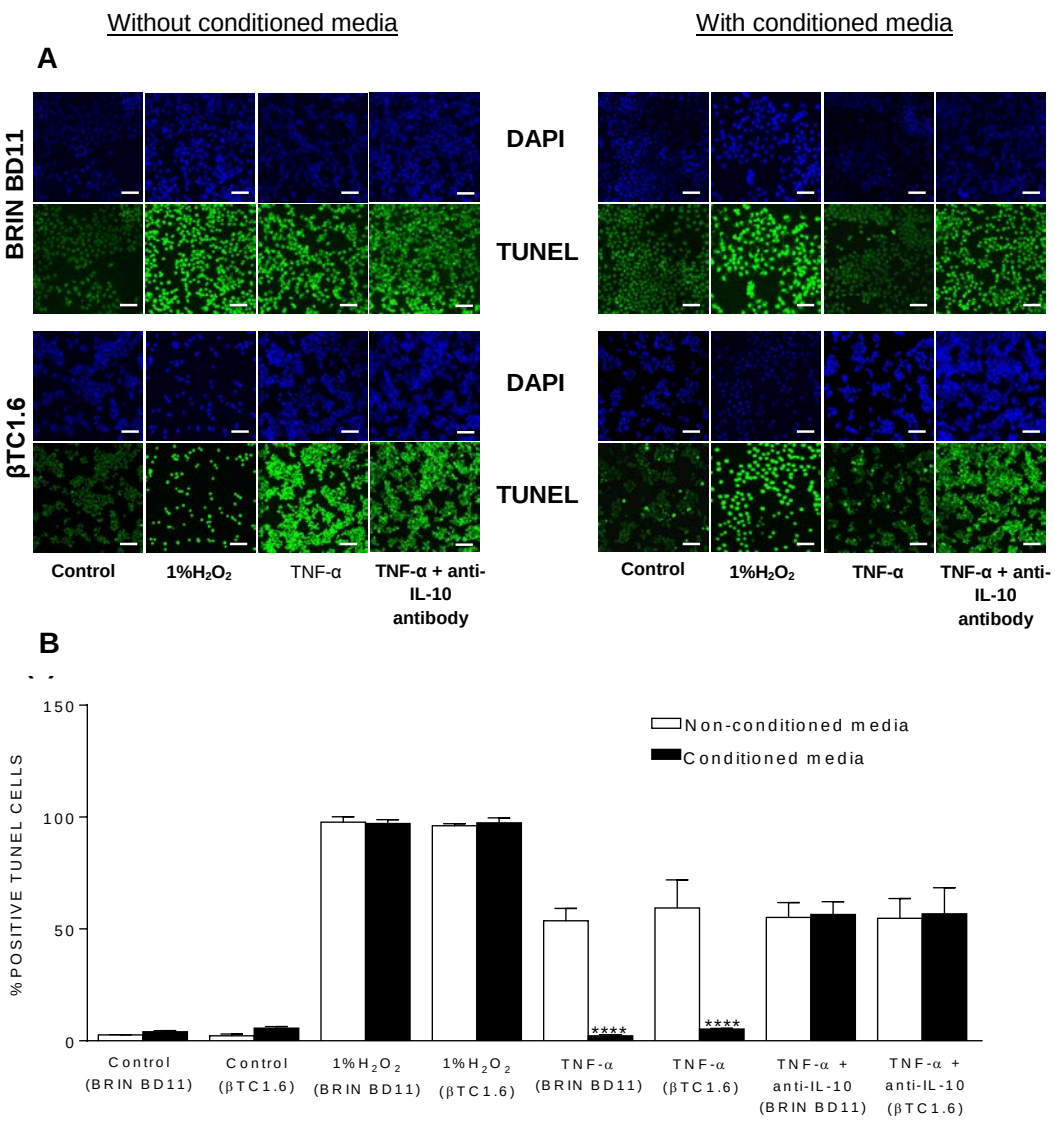

Figure 5.27. Assessment of TNF-α-induced apoptosis in pancreatic cell lines before and after the addition of anti-IL-10 antibody in the presence of MSC-CM with serum. **(A)** Fluorescent images showing the increase in the number of % positive TUNEL cells treated with 100 ng/ml of TNF-α for BRIN-BD11 cells and 1 µg/ml for βTC1.6 cells in the presence of MSC-CM after the addition of 100 ng/ml of anti-IL-10 to the MSC-CM. Blue staining represents DAPI staining of the nuclei while green staining indicates TUNEL positive cells. **(B)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****p<0.0001 compared with non-conditioned media. The scale bar in all images equals 100 µm. TNF-α, tumour necrosis factor alpha.

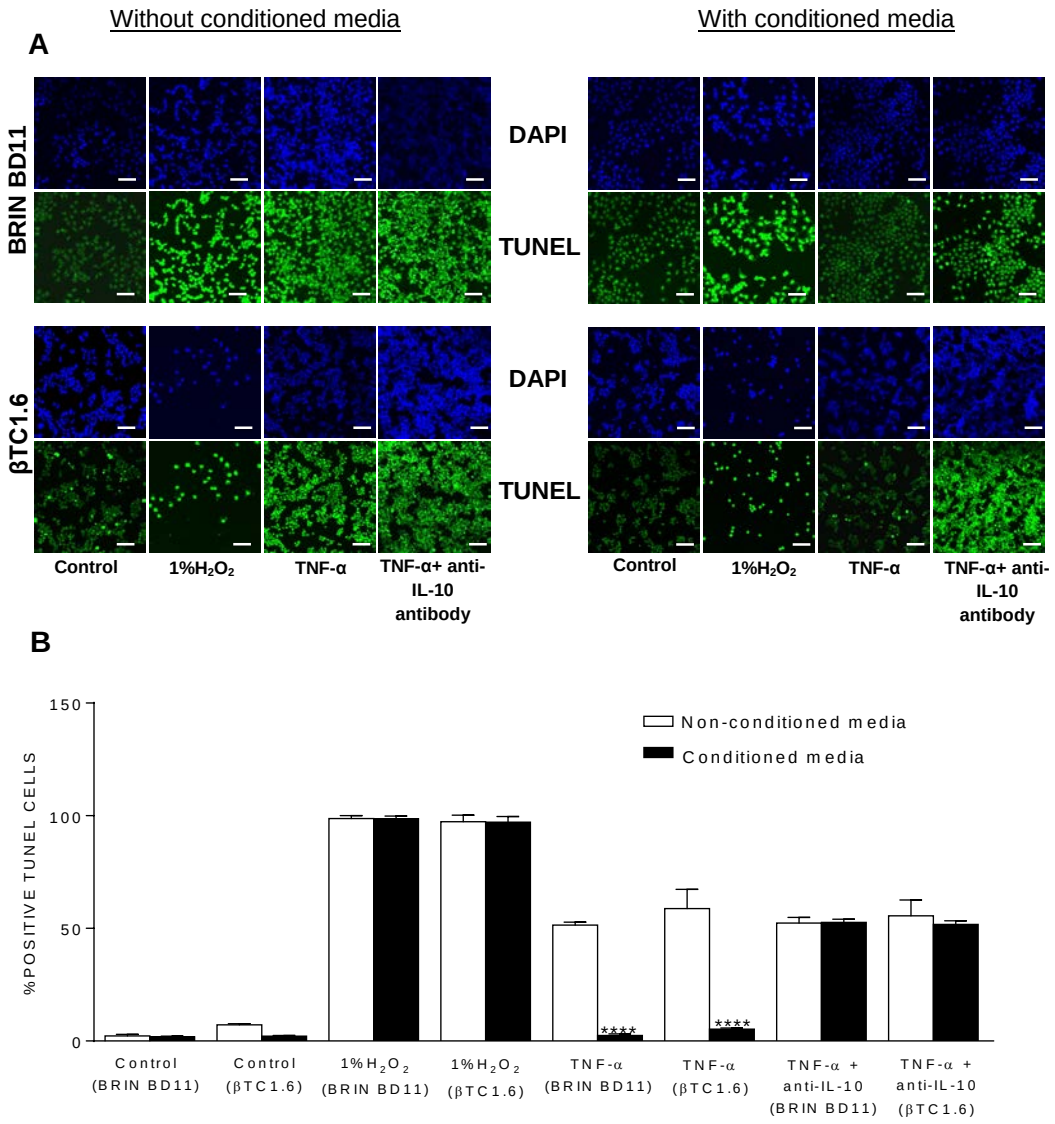

Figure 5.28. Assessment of TNF-α-induced apoptosis in pancreatic cell lines before and after the addition of anti-IL-10 antibody in the presence of MSC-CM without serum. **(A)** Fluorescent images showing the increase in the number of % positive TUNEL cells treated with 1 µg/ml of TNF-α in the presence of MSC-CM after the addition of 100 ng/ml anti-IL-10 to the MSC-CM. Blue staining represents DAPI staining of the nuclei, while green staining indicates TUNEL positive cells **(B)** The % positive TUNEL cells were measured by calculating the number of positive TUNEL cells divided by the total number of cells n=3 and presented as mean ± standard deviation (SD). ****p<0.0001 compared with non-conditioned media. The scale bar in all images equals 100 µm. TNF-α, tumour necrosis factor alpha.

5.3.11 TRAIL and IL-10 receptors expression profile in BRIN-BD11 and βTC1.6 cells

Finally, we confirmed the expression profile of TRAIL receptors (R1, R2) and IL-10 receptors (A, B) in BRIN-BD11 and βTC1.6 cells at the transcriptional level by RT-PCR. The transcriptional analysis confirmed TRAIL-R1, TRAIL-R2, IL-10RA and IL-10RB mRNA expression in both BRIN-BD11 and βTC1.6 cells as shown in (Figure 5.29).

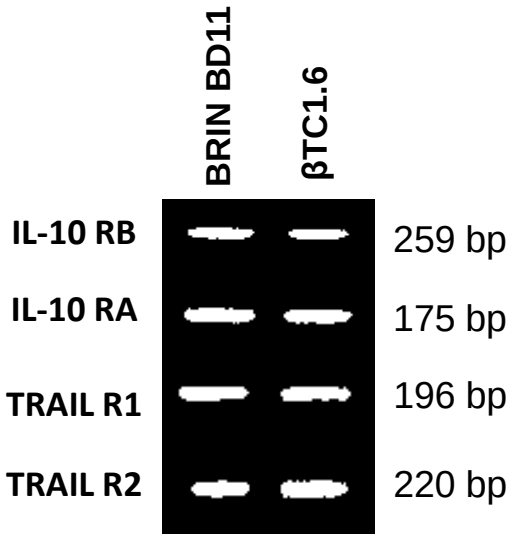

Figure 5.29.TRAIL and IL-10 receptor expression profiles in BRIN-BD11 and βTC1.6 cells. The TRAIL receptors (R1, R2) and IL-10 receptors (A, B) were expressed in BRIN-BD11 and βTC1.6 as confirmed by transcriptional analysis.

5.4 Discussion

Insulin replacement is the current primary therapeutic approach for T1DM, an approach that seeks to treat the symptoms of diabetes rather than curing it. Although the transplantation of islets can now be applied successfully, its widespread use has been hampered by immune rejection and insufficient supply of islets.[245]

The progress in understanding several key features of stems cells made in the last ten years has led to the development of rational cell therapy protocols. Several studies have shown that MSC transplantation can improve the metabolic profile in diabetic animal models.[246] However, the mechanisms underlying their therapeutic ability are not clearly known. Some studies suggested a cardinal role for the secretome and its paracrine signals rather than stem cell differentiation that may mediate many of the regenerative effects observed following therapeutic stem cell administration.[247] It is thought that the secretome consists of a complex set of molecules released by stem cells that are important for many biological functions like replication, cell growth, differentiation, apoptosis and many other functions.[248] These molecules could be proteins, growth factors, cytokines, angiogenic factors, hormones and extracellular matrix proteins.[203] There are some theories that attempt to explain the way by which these soluble factors act. In general, it is thought that they either act directly by mediating intracellular pathways in injured cells, or indirectly by inducing the secretion of functionally active products from adjacent tissues.[249]

In the present study, we sought to evaluate the anti-apoptotic effect of some of the soluble factors secreted by MSCs. Most of the studies within the secretome research community demonstrates an important role for the MSCs in the prevention of cell death not only through their ability to restore local microenvironments, but also by specifically producing proteins that are known for their apoptotic-inhibitory properties like IL-10.[250] Some researchers have

also suggested a role for paracrine effects of MSCs based on the ability of MSCs to reduce apoptosis of alveolar macrophages when co-cultured at appropriate ratios.[251] Tang and colleagues also found that in MSC-treated rats with ischemic hearts the level of pro-apoptotic factors like Bax and cleaved caspase 3 were reduced, while the levels of pro-angiogenic factors like VEGF and fibroblast growth factor (FGF) were increased as compared to medium treated hearts.[252] The findings of Gnecchi and colleagues also confirmed that the ability of MSCs to restore cardiac function is mainly due to secretion of paracrine factors rather than myocardial regeneration.[253][216]

A study that involved the activation of splenocytes in the presence of MSC-CM confirmed the presence of high concentrations of IL-10.[254] In our study, the ELISA assay revealed the presence of a high concentration of IL-10 in both MSC-CM with and without serum. IL-10 is a homodimeric cytokine that modulates the biological activity of immune cells; it binds to a tetrameric transmembrane cytokine receptor composed of IL-10RA and two accessory molecules of IL-10RB.[255] Several studies that used genetically deficient IL-10 mice have illustrated the importance of this cytokine in limiting autoimmune pathology.[256] The molecular mechanism by which IL-10 modulates the secretion of pro-inflammatory cytokines is still incompletely understood. However, there is a general agreement of IL-10-triggered signalling steps in the human and murine system.[257] After binding to its receptor IL-10 starts an intracellular signalling pathway that involves JAK1 and TYK2, which is followed by tyrosine phosphorylation STAT3 and nuclear translocation, which further results in stimulating the expression of the target genes.[161] The activation of STAT3 will activate the suppressor of cytokine signalling 3 (SOCS3), which exerts a negative effect on various cytokines genes. The role of STAT3 and SOCS3 in IL-10 signal transduction has been well established. However, a complete understanding of the molecular mechanisms by which IL-10 inhibits immune responses remains elusive.[258] A study that explored the ability of IL-6R and an engineered erythropoietin receptor (EpoR) to activate STAT3 demonstrated the

ability of EpoR to activate STAT3 and produce an anti-inflammatory effect similar to IL-10 while IL-6R, which also activates STAT3, failed to show anti-inflammatory responses. This finding indicated that STAT3 activation is needed, but not sufficient for the anti-inflammatory ability of IL-10. Other groups suggested that the IL-10 treatment resulted in diminished NF-κB activation in response to many stimuli.[258] This could occur via suppression of NF-κB translocation to the nucleus and DNA binding, which could be specific for the p65 subunit of NF-κB that has a crucial role in inflammatory and immune responses, resulting in induction of p50 monomers and homodimer, which act as a transcriptional repressor.[259] Suppression of NF-κB would also inhibit dendritic cell maturation and diminish antigen-presenting cell function.[260] In the present study treating the β-cells with a mixture of IL-10 and cytokines protected the cells from IFN-γ and TNF-α-induced apoptosis, but not from IL-1β, which may suggest that the apoptotic pathway of each individual cytokines might not be the same.

The pro-inflammatory cytokines; IFN-γ, TNF-α, and IL-1β play a major role in β-cell dysfunction and death. Signal transduction by these cytokines involves binding to a specific receptor that results in activation of kinases and/or phosphatase, which will stimulate and translocate several transcriptional factors like Apo1, STAT, and NF-κB resulting in up-regulation of some genes and downregulation of others that will eventually lead to β-cell apoptosis and death.[261] A detailed understanding of these genes and their signalling pathway and the transcription factor that control them could help us to fully understand the mechanisms of β-cell death and may allow us to stop apoptosis at some point.

In this study, we have clarified that pro-inflammatory cytokines-induced apoptosis occurred through a TRAIL-dependent mechanism. This was supported by the presence of TRAIL-R1 and TRAIL-R2 expression in BRIN-BD11 and βTC1.6 cell lines. TRAIL is a member of TNF superfamily that has the ability to induce apoptosis.[262] In humans, TRAIL interacts with four

transmembrane receptors that belong to the TNF receptor gene family.[263] TRAIL-R1 and TRAIL-R2 possess the ability to produce apoptosis while the other receptors cannot transduce apoptosis signal so they are known as decoy receptors.[264] The binding of TRAIL to TRAIL-R1 and TRAIL-R2 will result in apoptosis via FADD and caspase-dependent pathway.[265]

The expression of TRAIL and its receptors are found in many steady-state human cell types, but, their functions are still unclear.[266]However, TRAIL has been shown to play an important role in intestinal epithelial homeostasis and T-cell-mediated immune modulatory function.[267] Here the observation that anti-TRAIL could block cytokine-induced apoptosis supports the idea of the involvement of TRAIL in mediating β-cell apoptosis. In contrast to our findings, one study demonstrates the ability of TRAIL to downregulate the immune response leading to autoimmune disease.[268] However in our findings, blocking TRAIL through the addition of anti-TRAIL antibody protected our cells from apoptosis. The reasons for this contradictory result could be the poor knowledge of the full physiological function of TRAIL and its receptor.

5.5 Conclusions

The understanding of the process of β-cell apoptosis is still growing. Based on our present findings, cytokine-induced apoptosis is mediated through a TRAIL-dependent pathway. Furthermore, the past ten years have provided new *in vitro* and *in vivo* data on the immunomodulatory abilities of MSCs. Although there is still debate about the ways in which MSCs produce their effect, MSCs are now used to treat many types of diseases including autoimmune diseases, and we therefore speculated that MSC-CM could protect the cells from cytokine-induced apoptosis, which is now confirmed by our results. The analysis of MSC-CM content revealed a high concentration of IL-10. Although treatment of our cells with a mixture of IL-10 and cytokines helped reduce IFN-γ and TNF-α-induced apoptosis, it had little effect on IL-1β or LPS-induced apoptosis. This is despite the fact that our MSC-CM blocked all the cytokines-induced apoptosis, which suggest that additional factors play a key role. Given this, further experiments are still needed for full understanding of MSC-CM capability and content.

Chapter 6

General discussion, conclusions, and future directions

6.1 Discussion

The death and loss of β-cells are the main cause of T1DM. For reasons we do not yet fully understand our immune system starts to target the β-cells towards apoptosis whilst sparing other islet cells. This leads to the death of β-cells, which result in the absence of the insulin hormone that will eventually create hyperglycaemia.

Insulin replacement is the current treatment for T1DM, but this treats only the symptoms of diabetes rather than curing it. Progress in the transplantation of pancreatta and islets as an alternative treatment for T1DM has been made and transplantation can now be applied successfully. However, its widespread use has been hampered by immune rejection and insufficient supply.

MSCs have emerged as a new therapeutic tool in regenerative medicine, largely because of their ability for self-renewal, differentiation into other cell types and immunomodulatory properties. These attributes make them an attractive alternative cell therapy for many disease conditions including T1DM.

The aim of this project was to explore the therapeutic effectiveness of MSC conditioned media (MSC-CM) in protecting and restoring β-cell function. In the first instance, we wanted to establish *in vitro* models of cytokine-induced β-cells apoptosis. To achieve this goal we exposed two β-cell lines and primary islets to different concentrations of pro-inflammatory cytokines. The role of pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-1β in β-cells loss and development of T1DM is well documented.[180), (181), (269)] Many reports suggest that pro-inflammatory cytokines act together in a synergistic way in order to produce apoptosis in β-cells.[(181)] However, the mechanisms underlying pro-inflammatory cytokines' ability to induce β-cell death are not clear.[(270)]. Several signal transduction

pathways have been demonstrated to play a role in pro-inflammatory cytokine-mediated cell death. However, it still unclear how these pathways are linked to each other. Our finding confirms the ability of each individual cytokine to induce apoptosis in pancreatic β-cells and primary islets. This was associated with changes in pro-apoptotic and anti-apoptotic gene expression.

A20 (TNFAIP3) was first known as a TNF-inducible gene.[271], it was cloned from human umbilical vein endothelial cells. It is well known that A20 expression is under the control of NF-κB and for that reason, A20 is up-regulated by NF-κB-inducing factors such as TNF-α, IL-1β, and LPS. When the A20 is up-regulated it acts as a central negative regulator of NF-κB activation, therefore it is believed that A20 act as an anti-inflammatory.[271] The role of A20 as an inhibitor of inflammation is evident in mice.[272] and humans.[273]

The ability of A20 to suppress inflammation, inhibit the NF-κB, and it's reported anti-apoptotic capabilities when overexpressed in β-cells, associated with knowledge that β-cell loss in T1DM is mainly due to apoptosis,[230] lend a strong evidence for A20 modulator ability in T1DM. Some reports demonstrate that A20 mRNA is highly expressed in human and rat islets after cytokine stimulation, which indicates a physiological protective function for A20 in protecting β-cells.[274] Furthermore, some reports demonstrate an up-regulation of TRAIL (Apo2 ligand) which is a transmembrane protein related to TNF superfamily and play a role in apoptosis via activation of caspase cascade and release of cytochrome c from mitochondria.

In this study, we confirmed that A20 and TRAIL are highly expressed in β-cell lines after stimulating them with pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-1β) and endotoxin LPS. Also, culturing the cells under hypoxic conditions did not affect the results as

stimulating the cells with the same levels of cytokines promoted apoptosis, which was accompanied by upregulation of TRAIL and A20 expression.

The use of MSCs as a treatment for T1DM has been the focus of many researchers. Some reports demonstrate the ability of MSCs to differentiate into insulin-producing cells through using a specific culture media provided with elements that promote insulin secretion like glucose, and they confirm that the differentiated islet-like cells express insulin at both the mRNA and protein level. Additionally, they are able to control glucose level in non-obese diabetic mice (NOD)[275] However, other studies contradict this result and stated that there is no significant *in vivo* differentiation of MSCs into insulin-producing cells in adult mice.[215]

Various studies suggested that MSCs produce their action via paracrine mechanisms through secreting a number of soluble factors and that these factors can enhance tissue repair without the MSCs themselves. The secreted factors are referred to as secretome, microvesicles, or exosome and can be present in the medium where the MSCs are cultured and this media is called conditioned media.[276] Some studies suggest the ability of MSCs to release factors in response to stimuli like inflammatory stimuli such as IFN-γ and TNF-α where the MSCs responded by releasing anti-inflammatory factors like TGF-β and HGF. LPS and toll-like receptor agonists have been found to stimulate paracrine factors released from MSCs such as VEGF. Also preconditioning of MSCs with IFN-γ and TNF-α *in vitro* prior to cell transplantation may provide a potential strategy for activating and increasing MSC immunosuppressive transplantation.[277]

Since T1DM is mainly an autoimmune disease the immunomodulatory properties of MSCs represent hope as a way to properly control blood glucose level. However, the mechanism by which MSCs can exert their effect is a matter of debate. While MSCs ability to

differentiate into insulin producing cells remains controversial, a lot of information is accumulating regarding MSCs immunomodulatory properties and these capacities are already used in clinical trials.[226] MSCs can modulate immune responses either via a direct effect on T-cells through releasing regulatory cytokines like IL-10 or indirectly by inhibition of dendritic cells maturation. However, the main mechanisms through which MSCs exert their actions have yet to be determined. It has been suggested (and not without controversy) that neither the immunomodulatory nor plasticity of MSCs is able to support pancreatic regeneration.[170]

Many researches have focused on understanding the mechanisms behind the paracrine actions of the MSC-CM. Several protocols have emerged to isolate and concentrate MSC-CM based on using trophic factors produced by these cells. However, the use of MSC-CM as a therapy is still limited; one of the reasons behind this limitation include the possible contamination from animal product, the half-lives of molecules secreted by MSCs and the proper dose needed to produce a functional response *in vivo*.[222]

The culture and collection methods used to prepare the MSC-CM varies, some MSC-CM was prepared using fetal bovine serum or adding other supplement containing complete media while other are prepared without serum. Another parameter is the source of stem cells used, culture duration and whether the cells are cultured under normoxia or hypoxia. However, nearly all of these studies showed successful and promising results yet a standardized method for various MSC-CM formations is still undefined.[225]

Our findings in this study confirm the ability of MSC-CM to block cytokine-induced apoptosis and to restore insulin secretion. This was supported by molecular measurement for the TRAIL and A20 gene expression. After applying the MSC-CM the results indicated

significant down regulation of both genes that may indicate an anti-inflammatory effect for MSC-CM.

Identifying the signaling pathways involved in β-cell apoptosis is important because it could provide insight information regarding the potential pathogenic mechanism that could help in the development of a way to treat T1DM. Some of these signaling pathways have been known such as Fas ligand (FasL) and TNF-α, which have been found to cause apoptosis in mouse β-cells.[278], [79], [279]TRAIL is another member of TNF family it has membrane bound and soluble forms.[280] and acts via type 1 membrane receptor which mediates apoptosis through a cytoplasmic death domain.[281], [280]TRAIL has two receptors (TRAIL-R1 (DR4) and TRAIL-R2 (DR5)) that possess a cytoplasmic death domain and signal apoptosis via activation of caspase cascades.[282], [283]

We have shown in this study that TRAIL-R1 and TRAIL-R2 are expressed in β-cell lines BRIN-BD11 and βTC1.6 and also prove that TRAIL death pathway is functional in these cell lines There are several potential apoptotic pathways that might be functioning in β-cells like the FasL, perforin, and TNF-α. Recently Fas/FasL has been under investigation as an explanation of β-cell death and loss in T1DM.[284] However, it is still unclear which pathway is the main pathway that mediates β-cell destruction in T1DM. This does not exclude the possibility that TRAIL could be functioning via it is death receptor in a manner similar to FasL.[284] In this study using the recombinant murine TRAIL resulted in apoptosis of BRIN-BD11 and βTC1.6 β-cell lines indicating a potential role for TRAIL in developing T1DM.

FasL and TNF-α could initiate apoptosis via recruitment of a common adaptor protein FADD or TRADD/FADD. Then FADD activates caspase 8 to form death inducing signaling complex (DISC), which leads to stimulation of caspase cascades by activating caspase 3

as well as triggering the mitochondrial damage via the cleavage of BID (a death agonist member of the Bcl-2 family) leading to cell death. Although the stimulation events start the different signal cascades, all of them feed into a final common apoptotic pathway that ends by the death of the cell.[285, 286] However, there is a debate regarding the role of FADD in TRAIL receptor activation. Some initial papers showed that TRAIL stimulates apoptosis through FADD-independent pathways, whilst a recent study showed that FADD is needed for TRAIL-R1 and TRAIL-R2 mediated apoptosis.[287] Therefore, it is possible that TRAIL could use the common FADD-dependent pathway just like other ligands and an FADD-independent signal transduction pathway through which TRAIL directly stimulates caspase-3.[285] It is well known that TRAIL can induce apoptosis in a wide range of transformed cells, Although most normal tissue expresses TRAIL-R1 and TRAIL-R2.[288], this could be due to the presence of decoy receptors TRAIL-R3 and –R4 as these receptors do not have intracellular domain and they are considered to inhibit TRAIL-induced apoptosis.[289, 285]Clear enhancement of TRAIL expression was found in infiltrating cells of islets from people with T1DM.[191]. These finding indicated the possibility of TRAIL pathway involvement in β-cells death in T1DM due to the infiltration of cytotoxic T-cells.[191]However, the actual biological function of TRAIL and its receptor is still unclear.

Our findings in this study suggests the involvement of TRAIL death pathway in β-cell apoptosis. Blocking the action of TRAIL via the use of TRAIL antibody inhibited cytokine-induced apoptosis raising the possibility of a TRAIL-dependent pathway mechanism of action in these cells. However, the use of MSC-CM blocks TRAIL and cytokine-induced apoptosis. This finding made us ask the question of how the MSC-CM could prevent apoptosis.

MSCs immunomodulatory properties have been explored to treat autoimmune disease. Recent clinical trial data suggests a powerful immune modulatory effect of the MSCs to treat graft versus host disease.[208]An additional study showed that using a transwell culture system with a semipermeable membrane to separate MSCs from leukocytes did not stop the ability of MSCs to suppress inflammation, indicating the presence of soluble factors that may play an important role.[290]Among the many candidates that are found to be present in the MSC-CM is IL-10.[163] IL-10 was first identified by Mosmann and colleague.[291]There are four major T-cells sources of IL-10, these include T-helper 2 (Th2), T-regulatory 1 (Tr1), Th1, and Th17.[292]There are other types of cells that are also able to secrete IL-10 like monocytes and some subset of DCs. It is indeed difficult to decide which cells are the most important producer of IL-10.

IL-10 mediates its action via two receptors: IL-10R1 and IL-10R2. The binding of IL-10 to it's receptor will activate the JAK1 and Tyk2 respectively which in turn will activate STAT3 that will translocate to the nucleus where it binds to STAT binding element that will promote the transcription of various genes.[237] including the suppressor of cytokines signal 3 (SOCS 3) which in turn will exert negative effects on many cytokines genes.[238]Several studies on IL-10 knockout mice demonstrate the ability to limit autoimmune pathology.[256]However, the molecular mechanism by which IL-10 modulates the secretion of pro-inflammatory cytokines is still unclear.[257]Studies indicate that STAT3 activation is necessary, but not sufficient for the anti-inflammatory ability of IL-10. Some research suggested that IL-10 treatment resulted in diminished NF-κB activation in response to many stimuli.[258]IL-10 has the ability to inhibit NF-κB translocation to the nucleus and prevent DNA binding, which could be specific for p65 subunit of NF-κB that has a cardinal role in inflammatory and immune response. This results in induction of p50 monomers and homodimers which act as transcriptional repressor[259]The suppression of NF-κB by IL-10

could explain the large number of stimuli following treatment with IL-10. Suppression of NF-κB would also prevent dendritic cell maturation and diminish antigen-presenting cell function[260]In the present study treating the β-cells with a mixture of IL-10 and cytokines protected the cells from IFN-γ and TNF-α induced apoptosis but not from IL-1β. This may suggest that the apoptotic pathway of each individual cytokine is not the same.

6.2 Conclusions

In conclusion, our finding confirms that the *in vitro* induction of pancreatic β-cells and primary islets with individual pro-inflammatory cytokines was enough to produce apoptosis in these cells whether they are grown under normoxic or hypoxic culture conditions. The presence of serum did not affect the results as most concentrations that had been chosen for subsequent experiments remained the same. However, the addition of MSC-CM blocked cytokine-induced apoptosis and downregulated the genetic expression of A20 and TRAIL. Based on our present findings, cytokine-induced apoptosis is mediated through the TRAIL-dependent pathway. The MSC-CM was able to block the TRAIL and cytokine-induced apoptosis. The analysis of MSC-CM content revealed a high concentration of IL-10 but, treating our cells with a mixture of IL-10 and cytokines protected the cells against IFN-γ and TNF-α-induced apoptosis, but not from IL-1β or LPS despite the fact that our MSC-CM blocked all the cytokine-induced apoptosis tested in this study.

6.3 Future directions

Given this, more experiments are needed to understand the role of the TRAIL death pathway in the induction of β-cell apoptosis that should include studies on primary islets. If we are able to understand the regulation and control of the TRAIL pathway this may help in the treatment and prevention of β-cells apoptosis. The finding in this study gave

promising results regarding the ability of MSC-CM in the treatment of T1DM, and it is already used in some disease conditions. Therefore, further analysis of MSC-CM content is required in order to fully understand the activity of secreted factors founds in MSC-CM like Leptin, Eotaxin, SCF and TGF-β which are noted to be present in a high amount based on the cytokines array done by one of my colleagues (appendix 1). Testing, the ability of MSC-CM *in vivo* is essential to determine its capacity and to garner real therapeutic benefit from these promising preliminary results in the pancreatic β-cell.

In addition, testing different methods for MSC culture, various ways to prepare MSC-CM and comparing the outcome from different types of conditioned media would help in identifying the best way to prepare and use the MSC-CM under normoxia and hypoxia condition.

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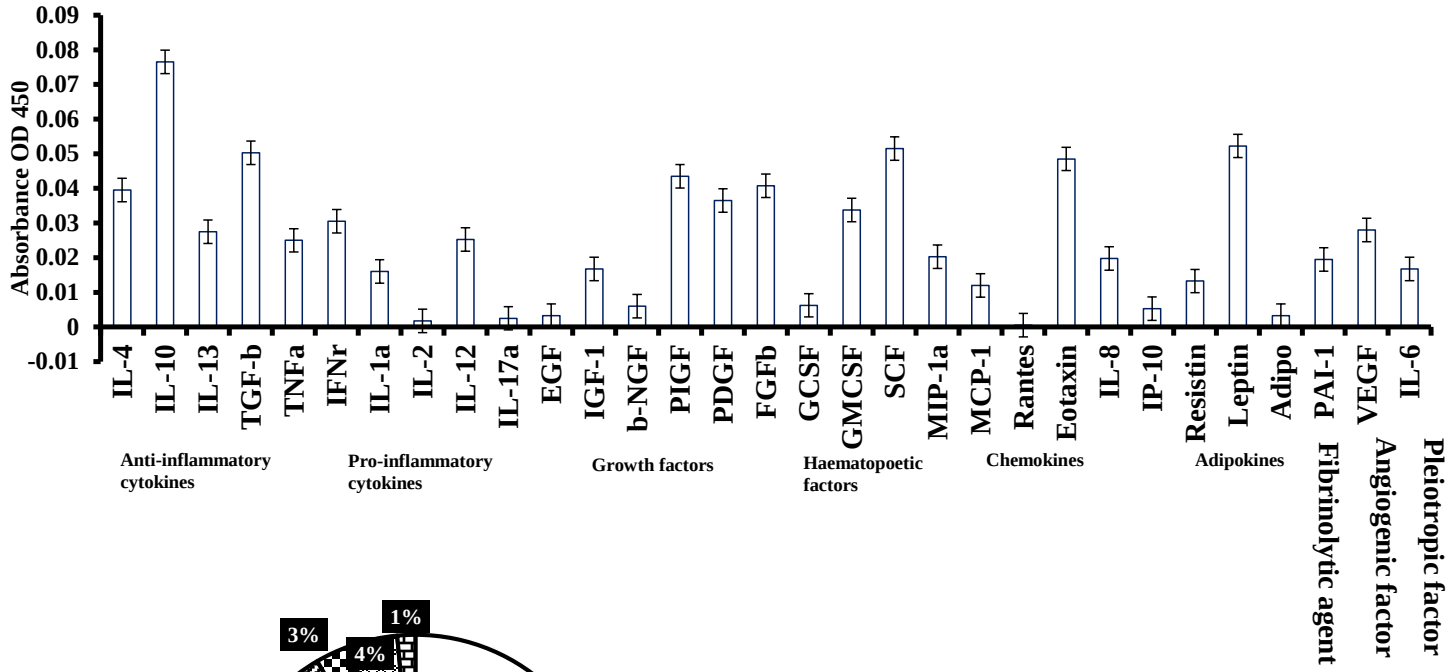
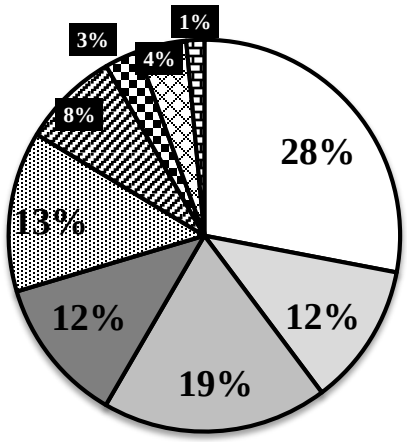
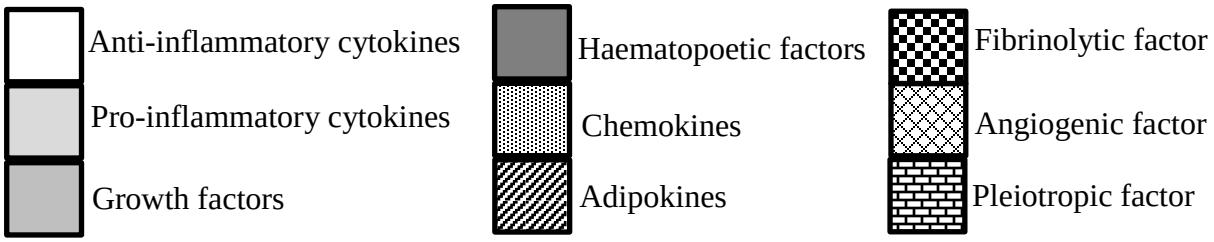
APPENDX -1

Cytokines array and distribution of different types of cytokines/ results from Marwan Mohammad Merkhan







APPENDX -2

Table (1). The maximum expression of TRAIL and A20 gene noted in BRIN BD11 and βTC1.6 cell lines after treating them with cytokines in a time dependent manner (media with serum).

	Cytokines	BRIN BD11 cells		βTC1.6	
		TRAIL maximum expression	A20 maximum expression	TRAIL maximum expression	A20 maximum expression
NORMOXIA	IFN-γ	24 hours	2 hours	24 hours	24 hours
	TNF-α	4 hours	1 hours	24 hours	24 hours
	IL-1β	24 hours	2 hours	24 hours	24 hours
	LPS	24 hours	4 hours	24 hours	24 hours
HYPOXIA					
	IFN-γ	24 hours	4 hours	24 hours	24 hours
	TNF-α	24 hours	1 hours	24 hours	24 hours
	IL-1β	24 hours	4 hours	24 hours	24 hours
	LPS	24 hours	4 hours	24 hours	24 hours

Table (2). The maximum expression of TRAIL and A20 gene noted in BRIN BD11 and βTC1.6 cell lines after treating them with cytokines in a time dependent manner (media without serum).

	Cytokines	BRIN BD11 cells		βTC1.6	
		TRAIL maximum expression	A20 maximum expression	TRAIL maximum expression	A20 maximum expression
NORMOXIA	IFN-γ	24 hours	15 minute	24 hours	24 hours
	TNF-α	24 hours	2 hours	24 hours	24 hours
	IL-1β	15 minute	1 hours	24 hours	24 hours
	LPS	4 hours	4 hours	24 hours	24 hours
HYPOXIA					
	IFN-γ	24 hours	4 hours	24 hours	24 hours
	TNF-α	4 hours	24 hours	24 hours	24 hours
	IL-1β	2 hours	1 hours	24 hours	24 hours
	LPS	24 hours	24 hours	24 hours	24 hours