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The Effects of Pro-inflammatory Cytokines on the L-type Calcium Current in Mouse Ventricular Myocytes

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Ce mémoire intitulé:

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Présenté par :
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a été évalué par un jury composé des personnes suivantes :

Dr. Lucie Parent, président-rapporteur
Dr. Céline Fiset, directrice de recherche
Dr. Bruce G. Allen, membre du jury

Résumé

L'inflammation: Une réponse adaptative du système immunitaire face à une insulte est aujourd'hui reconnue comme une composante essentielle à presque toutes les maladies infectieuses ou autres stimuli néfastes, tels les dommages tissulaires incluant l'infarctus du myocarde et l'insuffisance cardiaque. Dans le contexte des maladies cardiovasculaires, l'inflammation se caractérise principalement par une activation à long terme du système immunitaire, menant à une faible, mais chronique sécrétion de peptides modulateurs, appelés cytokines pro-inflammatoires. En effet, la littérature a montré à plusieurs reprises que les patients souffrant d'arythmies et de défaillance cardiaque présentent des taux élevés de cytokines pro-inflammatoires tels le facteur de nécrose tissulaire alpha (TNFα), l'interleukine 1β (IL-1β) et l'interleukine 6. De plus, ces patients souffrent souvent d'une baisse de la capacité contractile du myocarde.

Le but de notre étude était donc de déterminer si un lien de cause à effet existe entre ces phénomènes et plus spécifiquement si le TNFα, l'IL-1β et l'IL-6 peuvent affecter les propriétés électriques et contractiles du cœur en modulant le courant Ca^{2+} de type L (I_{CaL}) un courant ionique qui joue un rôle primordial au niveau de la phase plateau du potentiel d'action ainsi qu'au niveau du couplage excitation-contraction. Les possibles méchansimes par lesquels ces cytokines exercent leurs effets seront aussi explorés.

Pour ce faire, des cardiomyocytes ventriculaires de souris nouveau-nées ont été mis en culture et traités 24 heures avec des concentrations pathophysiologiques (30 pg/mL) de TNFα, IL-1β ou IL-6. Des enregistrements de I_{CaL} réalisés par la technique du patch-clamp en configuration cellule entière ont été obtenus par la suite et les résultats montrent que le TNFα n'affecte pas I_{CaL}, même à des concentrations plus élevées (1 ng/mL). En revanche, l'IL-1β réduisait de près de 40% la densité d'I_{CaL}. Afin d'examiner si le TNFα et l'IL-1β pouvaient avoir un effet synergique, les cardiomyocytes ont été traité avec un combinaison des deux cytokines. Toutefois aucun effet synergique sur I_{CaL} n'a été constaté. En outre, l'IL-6 réduisait I_{CaL} significativement, cependant la réduction de 20% était moindre que celle induite par IL-1β.

Afin d'élucider les mécanismes sous-jacents à la réduction de I_{CaL} après un traitement avec IL-1β, l'expression d'ARNm de $Ca_V1.2$, sous-unité α codante pour I_{CaL}, a été mesurée par qPCR et les résultats obtenus montrent aucun changement du niveau d'expression. Plusieurs études ont montré que l'inflammation et le stress oxydatif vont de pair. En effet, l'imagerie confocale nous a permis de constater une augmentation accrue du stress oxydatif induit par IL-1β et malgré un traitement aux antioxydants, la diminution de I_{CaL} n'a pas été prévenue.

Cette étude montre qu'IL-1β et IL-6 réduisent I_{CaL} de façon importante et ce indépendamment d'une régulation transcriptionelle ou du stress oxydatif. De nouvelles données préliminaires suggèrent que I_{CaL} serait réduit suite à l'activation des protéines kinase C mais des études additionelles seront nécessaires afin d'étudier cette avenue. Nos résultats pourraient contribuer à expliquer les troubles du rythme et de contractilité observés chez les patients souffrant de défaillance cardiaque.

Mots-clés : cytokines, inflammation, insuffisance cardiaque, canaux ioniques, arythmies

Abstract

Cytokines are immune system modulators that are secreted in response to an insult. Even though on the short term they play a crucial role in the healing process, the prolonged secretion of pro-inflammatory cytokines, locally or systemically, has many deleterious effects. For almost 20 years reports of alteration in serum cytokine levels have been emerging in patients with various heart failure aetiologies, however it is only recently that the role of inflammation in heart pathologies is being more and more studied. Indeed, several studies have shown that patients suffering from heart failure or arrhythmias have high levels of cytokines. Three particularly of these cytokines in particular are highly present and together they play a central role in the inflammatory response. Tumour Necrosis Factor alpha (TNFα), interleukin 1 beta (IL-1β) and interleukin 6 (IL-6) are secreted chronically by immune cells or the cardiomyocytes themselves and can possibly, as shown by animal studies, induce cardiac remodelling, hypertrophy, apoptosis, fibrosis and generation of highly reactive oxidative species (ROS) among other effects. Furthermore, accumulating evidence suggests that these pro-inflammatory cytokines are not only important mediators of cardiac remodelling that can contribute to worsening of heart failure but they have also been linked to cardiac arrhythmias and prolongation of action potential. Overall, the findings suggests a strong role for pro-inflammatory cytokines in affecting cardiac function and inducing electrical remodelling, thus we hypothesised that high levels of pro-inflammatory cytokines can affect the electrical and subsequently the contractile properties of the heart.

Thus, the aim of this project was to help establish the effects of the above mentioned cytokines on the electrical and contractile properties of cardiac myocytes while exploring the mechanisms by which these cytokines mediate their effect. Using cultured intact mouse neonatal ventricular cardiomyocytes which were treated chronically with various cytokines, at a pathophysiological concentration (30 pg/mL), the specific objective of this study was to measure the direct effect of chronic cytokine treatment on the L-type calcium current (I_{CaL}), an important ionic current responsible for the plateau phase of the

action potential and in the excitation contraction coupling (ECC) and the current l and subsequently, determine via which pathways cytokines are able to affect the calcium current.

Patch-clamp experiments in the whole-cell voltage-clamp configuration were used to measure L-type calcium current and showed that I_{CaL} was not affected by TNFα. Furthermore, no effect at a significantly higher concentration of TNFα (1 ng/mL) could be observed. In contrast, chronic treatment of cardiomyocytes with IL-1β depressed I_{CaL} by up to 40 %. Furthermore, when combining TNFα with IL-1β, two cytokines often reported to act synergistically, no further reduction in I_{CaL} current density compared to IL-1β treatment alone was observed, showing the specificity of IL-1β response. Expression studies using qPCR to quantify the mRNA of $Ca_V1.2$, the underlying alpha subunit channel which encodes for I_{CaL}, were conducted in order to determine if the reduction in current is due to a cytokine mediated change in gene expression. We found that none of the cytokines significantly affected levels of $Ca_V1.2$ mRNA.

A key component of the inflammatory response is the induction of oxidative stress. Indeed, when challenged with cytokines cardiomyocytes exhibited significant increases in ROS level. In an attempt to reverse the depression of I_{CaL} in response to IL-1β, we treated myocytes concurrently with antioxidants and IL-1β. While we observed a significant decrease in intracellular ROS levels, antioxidant therapy failed to restore current density, indicating thus, that ROS produced in response to cytokines does not regulate ion channels. New preliminary data suggests a role for members of the protein kinase C family in regulating the properties of $Ca_V1.2$ in response to cytokines. Nonetheless, exploring this avenue will require substantial experimentation and will be the subject of future work.

Overall our experiments will help provide a better understanding of the role of cytokines in regulating the electric and contractile properties of cardiomyocytes in the setting of inflammatory cardiomyopathies.

Keywords : cytokines, inflammation, heart failure, ion channels, arrhythmia

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List of abbreviations

24 H	Twenty Four Hours
AIDS	Aquired Immunodeficiency Syndrome
β-MHC	Beta Myosin Heavy Chain
ART	Anti-Retroviral Therapy
CaATPase	Calcium ATPase
CICR	Calcium-Induced Calcium-Release
CM-H2DCFDA	2',7' dichlorodihydrofluoresceindiacetate (H2DCFDA)
CRP	C-Reactive Protein
CsCl	Caesium Chloride
CsOH	Caesium Hydroxide
CVD	Cardiovascular Disease
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EAD	Early After Depolarisation
ECC	Excitation-Contraction Coupling
ECG	Electrocardiogram
ELISA	Enzyme-linked Immunosorbent Assay
ERK1/2	Extracellular Signal-regulated Kinases 1 and 2
FBS	Foetal Bovine Serum
gp130	Glycoprotein 130
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
I_{CaL}	L-type Calcium Current
I_{CaT}	T-type Calcium Current
I_{K1}	Inward Rectifying Current
IKKβ	Inhibitor of Nuclear Factor kappa-B Kinase Subunit Beta

I_{Kr}	Delayed Rapid Recifier Potassium Current
I_{Ks}	Delayed Slow Recifier Potassium Current
I_{Kur}	Ultra Rapid Recifier Potassium Current
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
I_{Na}	Sodium Current
iNOS	inducible Nitric Oxide Synthase
IRAK	Interleukin 1 Receptor Associated Kinase
I_{ss}	Sustained Potassium Current
I_{to}	Transient Outward Potassium Current
IV curve	Current Voltage Relationship Curve
JAK	Janus Kinase
LTCC	L-type Calcium Channel
M-199	Medium 199
MAP Kinase	Mitogen-activated Protein Kinase
MEK	Mitogen-activated Protein Kinase Kinase
Mins	Minutes
mRNA	Messenger Ribonucleic Acid
MyD88	Myeloid differentiation primary response gene 88
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
$NaHCO_3$	Sodium Bicarbonate
NCX	Sodium-Calcium Exchanger
NFAT	Nuclear Factor of Activated T-Cells
NMVM	Neonatal Mouse Ventricular Myocytes
NO	Nitric Oxide
pA/pF	Pico-Ampere per pico-Farad
PEG-SOD	Polyethyleneglycol super oxide dismutase
PenG/Strep	Penicillin Streptomycin
PI3K	Phosphatidylinositol 3-kinases

qPCR	Quantitative Polymerase Chain Reaction
RAS	Renin-Angiotensin System
ROS	Reactive Oxygen Species
RyR	Ryanodine Receptor
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SMEM	Suspension Minimum Essential Media
SR	Sarcoplasmic Reticulum
STAT	Signal Transducer and Activator of Transcription
sTNFα	Soluble Tumour Necrosis Factor alpha
TACE	Tumour Necrosis Factor Alpha Converting Enzyme
TEACl	Tetraethylammonium Chloride
TIR	Toll-IL1-Receptors
tmTNFα	Transmembrane Tumour Necrosis Factor alpha
TNFR	Tumour Necrosis Factor Receptor
TNFα	Tumour Necrosis Factor alpha

Dedicated to my family, to which I will always be grateful. A family which has greatly sacrificed for me, offered tremendous support and exceptional opportunities so I can, one day, stand on the shoulders of giants.

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Over the past two years I have gained considerable knowledge in the field of cardiac electrophysiology while also touching up on other important topics and disciplines such as heart failure, inflammation, hormonal regulation and pharmacology. But that is only part of the story. Dr. Céline's remarkable care and investment in her student's training as young scientist has forged part of my personality which will always remain with me and gave me the confidence in myself and in the knowledge that I have acquired so I can tackle the challenges that await me along the road and for that I will be always thankful.

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1 Introduction

Cardiovascular disease (CVD) is one of the leading causes of death in developed countries. In the United States, more than 60 million people are thought to be affected by some form of cardiovascular disease and total deaths due to CVD account for roughly 40% deaths in the population.[1] With a constantly increasing risk in the prevalence of CVD, hospitalisation costs in turn keep increasing, presenting an important burden on governments and society.[2] According to Canadian Institute for Health Information (Ottawa, Ont.: CIHI, 2008) in Canada, the costs of hospitalisation related to circulatory conditions such as angina and heart attacks costs up to 3.3 billion dollars per year, with heart failure and myocardial infarction ranking in the top five most expensive condition in 2005.

Cardiovascular disease, a term commonly used to refer to various diseases affecting the heart and circulatory systems, encompasses a series of different and complex diseases where each can have its own etiology. The list includes hypertension, myocardial infarction, heart failure, arrhythmia and atherosclerosis to name a few, and together they have been the subject of decades of research which has allowed us to gain significant insights into the fundamental aspects of the pathologies down to the molecular levels while opening therapeutic venues that, undoubtedly, have contributed to patient longevity and improvement of quality of life. As different as these diseases might seem to be from one another, the last decade or so has been filled with prominent scientific findings that are now helping us draw a new picture, and shift our vision of how we perceive CVD. Trends and patterns have emerged and indeed, in our search for the molecular mechanisms that underlie complex diseases such as heart failure and atherosclerosis, common cellular or molecular processes which were once thought to be distinct for a type of pathology are now being recognised as a common link between different cardiovascular diseases.[3, 4]

One of the most important biological processes that illustrate this phenomenon is inflammation. In fact inflammation is now not only considered to be a crucially important aspect of atherosclerosis,[5] but it was also shown to be a common factor in diseases such as hypertension, ischemic heart disease and heart failure.[6] In the context of heart disease, inflammation has recently taken on new definitions and today prominent investigators in

the field of inflammation provide new concepts that are believed to be at the core of several CVDs that are characterised by a chronic low-grade inflammation.[4] Under these states, the triggers of inflammation are not necessarily the classical inducers of inflammation such as bacteria, viruses or tissue damage, but instead they verge into a less known area of the field, where inflammation is triggered by a tissue malfunction and homeostatic imbalance which over time can, in an attempt to restore balance, remain in a persistent state or even worsen. This process is thought to play a pivotal role in multiple chronic diseases including atherosclerosis and heart failure.[4]

Perhaps one of the most important and inherent properties of inflammation is the secretion of different modulators, such as pro-inflammatory cytokines, chemokines, vasoactive amines among other effectors, by macrophages and neutrophils.[7] These peptides are thought to be the main effectors and modulators of the signalling cascades that can lead to amelioration, or under chronic conditions worsen the pathology, as seen in several heart diseases.[4] Research using animal and cellular models has extensively used cytokines in order to understand their role, particularly in the heart. Even though several groups have already started teasing out the various pathways that lead to cardiac remodelling including hypertrophy and apoptosis in response to cytokines,[8, 9] an important aspect that remains poorly understood is the effects of these cytokines on one of the fundamental properties of the heart: the 'electricity', which is generated by a myriad of ion channels each with a distinct function, the whole impeccably organised, carefully synchronised and when they play in concert they give rise to a heart rhythm.[10] Unbalancing the finely regulated electrical circuitry of the heart proves to be particularly detrimental considering it will induce various types of arrhythmias that cause an irregular asynchronous heart beat and lead in some cases to sudden cardiac death.

The first part of the introduction of this master thesis will begin by discussing of the role of chronic inflammation in a context of infection and how it can negatively affect cardiac function. This will be followed by a review of inflammation under aseptic conditions such as myocardial infarction, atrial fibrillation, and heart failure. The topics of

the introduction will touch upon clinical data and supporting evidence from animal models, while providing specific details about three of the main cytokines in heart disease TNFα, IL-1β and IL-6 and how they are implicated in heart disease. In the second part of the introduction and the result sections the focus will switch to the fundamental cardiac electrophysiology and then data generated by members of our laboratory, including myself, that demonstrate how pro-inflammatory cytokines are able to affect ion channels will be presented while discussing some on the possible mechanisms involved.

1.1 Inflammation and Heart Disease: Complex Partners

1.1.1 Human Immunodeficiency Virus (HIV) and Heart Disease

HIV is a retrovirus which causes Acquired Immunodeficiency Syndrome (AIDS), a disease which affects millions of lives worldwide. HIV infects immune $CD4^+$ T helper cells, leading to their progressive decline. This severely limits the infected individual from cell-mediated immunity, which leads to an increased susceptibility to infection by opportunistic pathogens along with other complications.[11]

Several years after the discovery of the HIV in 1981 the life expectancy of infected individuals remained extremely poor and topped at most 18 months. Even though they do not present a cure, today's therapies using Antiretroviral Therapy (ART) aimed at limiting viral replication have significantly decreased morbidity and mortality in HIV patients and has prolonged the life expectancy of patients up to decades.[12-14] Indeed, it is estimated that more than half of the people in the United States currently living with HIV will be older than 50 years of age by the year 2015.[15] With the constantly increasing life expectancy of HIV patients, their risk of complications from non-AIDS diseases has also been increasing at significantly higher rates compared to the general aging population.[13] Amongst these diseases, CVD is of particular prevalence in HIV patients. In fact, a study has demonstrated that HIV is an independent risk factor for CVD that has the same magnitude of the standard well established CVD risk factors.[16] Furthermore, several studies have demonstrated that HIV patients have several abnormalities in their ECGs such as a longer QT interval compared to the general population, and an increased risk of mortality from ventricular arrhythmias, sudden cardiac death and other cardiac complications.[17-20] Interestingly, several of the drugs used in ART have been associated with an increase in the duration of the QT interval in patients, which poses as a risk factor for arrhythmia. However, in 2005 Sani MU *et al.* provided substantial evidence demonstrating that HIV itself was directly correlated with a prolongation of the QTc interval, independently of any pharmacological agent.[21] The implications of these findings are considerable, because they strongly suggest that an underlying feature of HIV infection is directly affecting, at least in part, the

electrical properties of the heart. Perhaps unsurprisingly, pro-inflammatory markers and cytokine levels in HIV infected individuals are particularly high: C-reactive protein, D-dimer, IL-6, IL-1β, TNFα to name a few;[22, 23]and even though they are the prime suspects it was still unclear if or how they participated in cardiac pathology and arrhythmia risk in HIV patients.

Significant insight on the effects of these cytokines on the heart came from a mouse model of HIV.[24] These mice showed symptoms that closely resembled the human symptoms of HIV, and interestingly, we showed that they also had a longer QT interval (i.e. a longer repolarisation period, as measured by ECG), which is a prominent risk factor for arrhythmias. These findings demonstrated that in a mouse model of HIV, in the absence of any pharmacological intervention or other disease or confounding variables, HIV alone was able to negatively affect the electrical functions of the heart. Since HIV mice were a transgenic model that expressed one of the HIV viral genes, these mice did not have a 'real' infection per se or any circulating viruses which could directly impact the cardiac function. However, what HIV mice did share with HIV patients was an elevated level of circulating pro-inflammatory cytokines. We therefore hypothesised initially that these pro-inflammatory cytokines were responsible for mediating the adverse effects on the electrical properties of the heart. Indeed, our laboratory subsequently went on to generate evidence in support of this hypothesis which showed that pro-inflammatory cytokines can alter various ion currents in the heart and therefore can potentially be implicated in the generation of arrhythmias. These findings will be discussed further.

1.1.2.1 Inflammation in Myocardial Infarction

As previously mentioned, inflammation is now widely recognised as a key component of several cardiovascular diseases. Whether the cause or triggers are viral, such as HIV, microbial, or tissue damage as seen in acute myocardial infarction (myocardial infarction), unresolved inflammation that becomes chronic and noxious can adversely affect cardiac function. Interestingly, it appears that the degree of inflammation, measured

by markers or cytokines levels from serum or organ biopsies, is positively correlated with the degree of disease. Thus, regardless of the source of inflammation, it is the degree of inflammation itself that is of major importance in CVD.[25] Conditions where inflammation plays a crucial role are numerous; however, a particularly important condition is myocardial infarction, where inflammation seems to be of an extremely critical nature.

Myocardial infarction is a condition where the rupture of an atherosclerotic plaque causes the occlusion of a coronary artery, thus blocking blood flow to areas of the myocardium and resulting in damage, death, or necrosis of cardiac tissue. Ischemic cardiac tissue which is subsequently reperfused initiates a potent inflammatory response that varies in proportion to the duration of ischemia.[26] Perhaps the first report demonstrating the adverse role of inflammation in myocardial infarction came from a canine model of myocardial infarction dating back to the early 70s. When dogs were given corticosteroids after induction of myocardial infarction, infarct size (i.e. the necrotic cardiac area) was significantly reduced. This observation lead the authors to conclude that the pathogenesis of myocardial infarction is, at least in part, mediated by activation of the inflammatory cascade that promotes detrimental outcome on the heart.[27] These findings subsequently lead to administration of corticosteroids in patients with acute myocardial infarction in an attempt to limit damage and reduce infarct zone. The results were catastrophic as patients suffered from increased risk of ventricular tachycardia, and even worse, their infarct zone was further enlarged.[28] The importance of these findings lies in the fact that they lead the scientific community to realise the complexity of the inflammatory response by acknowledging its crucial role in tissue repair and in the reinstatement of cardiac homeostasis. In other words, limiting infarct size is important, however equally important are processes mediated by inflammation such as wound healing and formation of a stable scar that can help limit detrimental outcome. The findings of this study also help focus the research onto looking for specific targets within the inflammatory cascade that could be modulated in order to enhance cardiac outcome.

1.1.2.2 Inflammation in Heart Failure

Despite the advances in the clinical management of myocardial infarction, several complications often arise that worsen the pathology and lead patients into more severe and chronic conditions, notably heart failure (heart failure). The causes of this shift from myocardial infarction to heart failure seem to be a mixture of tissue necrosis and ventricular remodelling. Ventricular remodelling is a complex process used to describe changes in the properties of the myocardium which include thinning of the left ventricular wall, chamber dilation, compensatory hypertrophy that initially acts to maintain stroke volume but eventually decompensates thus, exacerbating the condition and contributing to increasing interstitial fibrosis, oxidative stress and other noxious stimuli.[29] The induction of this detrimental ventricular remodelling is thought to be caused by neurohormonal stimulation which in patients is often associated with chronic β-adrenoceptor stimulation and Renin-Angiotensin-System (RAS) deregulation. Both of these are potent inducers of chronic inflammatory pathways.[30] Indeed accumulating evidence suggest that cytokines, the mediators of inflammation, play a prominent role in cardiac contractility, promoting apoptosis and fibrosis and other adverse remodelling that occurs in heart failure.

1.1.2.3 Atrial Fibrillation

The inducers of inflammation are many, and even if myocardial infarction and heart failure are initially induced by tissue damage, there are other clinically important cardiac conditions where inflammation is present independent of tissue damage or infection where cytokines also seem to promote a worse outcome. A typical example illustrating this would be atrial fibrillation (AF). AF is the most prevalent form of arrhythmia encountered in clinical practice and contributes significantly to mortality and morbidity of patients.[31] AF is characterised by excessively disorganised, rapid impulses and contractions of the atria that

leads to irregular conduction of the cardiac influx to the ventricles posing as a risk for the individual. Despite the prevalence of AF, the mechanisms by which it is brought about and sustained remain poorly understood. Ionic remodelling has been proposed as the main mechanism for AF; however, the processes which ultimately lead to electrical remodelling are not well known.[32, 33] Interestingly, several studies have demonstrated a prominent role for inflammation in AF.[34] In one particular study, when plasma levels of C-reactive protein (CRP), a strong marker of inflammation, were measured in patients suffering from atrial arrhythmia, investigators found a significantly higher level of CRP compared to matched control individuals. Furthermore, patients who suffered chronic AF had higher levels of CRP measured against CRP levels from patients with short term (paroxysmal) AF and an episode of AF within 24 hours also elevated plasma CRP levels significantly higher than other AF patients.[35] These findings suggested a prominent role for inflammation in AF and in the induction of abnormal rhythm and electrical imbalance. Increasing evidence suggests that pro-inflammatory cytokines participate in the induction of electrical remodelling and in the creation of substrates for AF such as increased collagen depositions and fibrosis produced by cytokine-activated cardiac fibroblasts.

1.2 From inflammation to cytokines: how do they affect the heart?

The clinical and experimental evidence provided so far in the context of infectious disease (HIV), tissue damage (myocardial infarction) or simply tissue malfunction (some cases of heart failure or AF) strongly supports a common role for inflammation in inducing pathogenesis. It is important to emphasise once again that inflammation itself is not to be portrayed as a toxic condition because its role in tissue repair and homeostasis is of unequivocal importance.[26] However, when inflammation becomes persistent and

uncontrolled it begins to exacerbate the medical condition. Indeed, it is only when the initial phase of inflammation fails to resolve the effects on an insult that the door opens for a new level of chronic inflammation characterised by activation of several signalling cascades that may have a detrimental net outcome.[36] As already mentioned, cytokines are the principal mediators of the inflammatory response. Small peptides normally secreted by various immunoregulatory cells such as macrophages and neutrophils, these cytokines play a crucial role in activating the signalling pathways that are, at least in principle, aimed at restoring tissue homeostasis.[4] The family of cytokines is ever-expanding and in the last few years tens of new members have been discovered.[36] Of note, as modulators of inflammation, cytokines have different roles, while some are pro-inflammatory such as TNFα, IL-1β and IL-6 there are other anti-inflammatory cytokines, IL-10 being one of the most widely recognised. For our purposes pro-inflammatory cytokines will be referred to simply as cytokines, and the terms will be used interchangeably considering the focus of our topic will be exclusively pro-inflammatory cytokines. The cytokine pathways are of extreme complexity. This complexity arises at least in part from their numbers and interconnected signalling, but also from their individually distinct regulation where heterodimerisation, protein maturation, nuclear translocation, and cytokine receptor regulation are only some of the processes that have been describes in cytokinology.[37, 38]

Despite the complexity of cytokine biology there have been constant patterns that have emerged from clinical and experimental studies where a handful of cytokines have always been correlated with the pathogenesis of a disease, notably of cardiac nature. Elevated in both HIV patients and HIV mouse,[39] myocardial infarction, heart failure[6, 25, 40, 41], AF[42, 43], TNFα, IL-1β and IL-6 have become a hallmark in the pathogenesis of inflammation and the role they play in disease has been the focus of years of research. In the next section, detailed molecular structure and signalling cascades of the three aforementioned cytokines will be presented, along with experimental or clinical data when available that demonstrate their role in mediating cardiac disease.

1.2.1 Tumour Necrosis Factor alpha

TNFα is a cytokine which belongs to the TNF superfamily which include about 19 members [44]. TNFα can be produced in various different cells ranging from macrophages, T cells, mast cells, to fibroblasts and cardiomyocytes.[45] The mature and soluble form of TNFα is a 17.35 kDa trimeric protein..[46] Figure 1 illustrates the computer generated 3-dimensional model based on the crystal structure of the human TNFα.

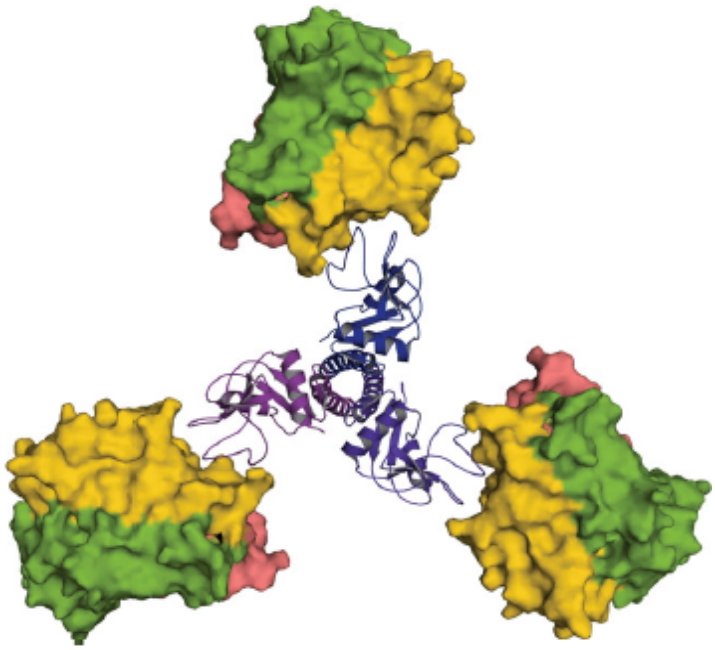


Figure 1. Proposed 3-dimensional structure of TNFα. *Model based on the crystal structure of the human TNFα cytokine. Top view of protein showing three homomonomers of 17 kDa each, that trimerise to form the mature homotrimer soluble form of the TNFα protein.*[47]

The mature and soluble sTNFα (i.e. circulating) form is initially produced as a precursor protein composed of a trimer of 26 kDa homomonomers embedded in the membrane known as transmembrane TNFα or tmTNFα. Following cleavage by the TNFα-Converting-Enzyme (TACE), tmTNFα is released into the circulation as the sTNFα.[45]Even though both soluble and transmembrane forms of TNFα possess a biological activity the production of either forms is strictly controlled and will depend on several different parameters such as the nature of inducing stimulus, metabolic state of the cell, TACE activity and TACE inhibitors such as metalloproteinases.[47] Both sTNFα and tmTNFα are capable of binding two

receptors, TNFR1 and TNFR2. These two receptors differ mainly in their affinity for ligands.In humans the sTNF binds with higher affinity to TNFR1 (dissociation constant of [K_d] ~20 pM) compared to TNFR2 ([Kd] ~400 pM) while tmTNFα binds TNFR2 with higher affinity.[48] Other differences between these two receptors include cellular expression profiles, cytoplasmic tail structures and signalling mechanisms. However it is widely accepted that most of the effects of sTNFα are attributable to TNFR1 signalling.[45] TNFα signalling is of high complexity due to the differences that exists between the two receptors and the various signalling cascades associated downstream of each receptor but also because the tmTNF can act as a reverse receptor and actually serve as a negative feedback on the TNFα activated cascades such as of NF-κB and apoptosis, thus acting similarly to a TNFα antagonist.[49] The TNFα cytokine biology is summarised in the schematic diagram of figure 2.

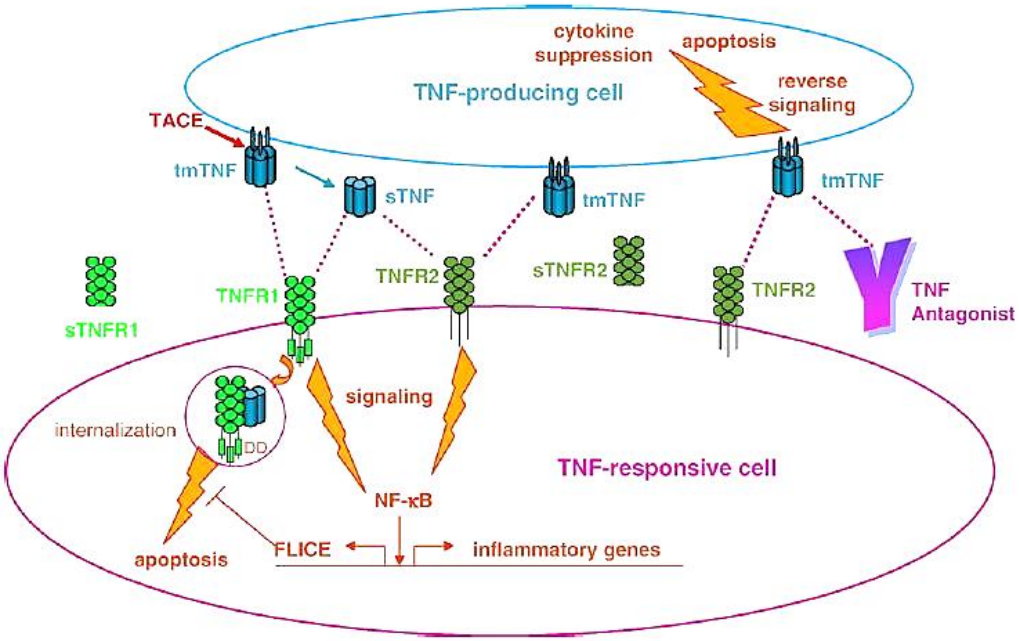


Figure 2. ***Summary diagram of TNFα biology***. *TNFα producing cells produce a tmTNF initially which can bind to TNFR2 and induce signalling cascades such as activation of NF- κB. The sTNFα, after TACE cleavage is released from the cell and will bind preferentially to TNFR1. Induction of apoptosis via sTNFα requires internalisation of the ligand-receptor complex, which can be inhibited by FLICE (Fadd-like IL-1β-converting enzyme) or alternatively by reverse signalling via tmTNF or antagonist antibodies such as infliximab or adalimumab. (Adapted from [45])*

Since the discovery of an association between TNFα and heart failure in 1990,[50] innumerable reports have emerged in an attempt to elucidate its role in the pathogenesis of cardiac disease.[51] Clinically, increasing levels of TNFα in patients have been associated with the deterioration heart function according to of the New York Heart Association (NYHA) classification of heart failure. Furthermore, studies have also shown that TNFα and notably TNFR1, due to its much longer half-life compared to the 30 minutes of sTNFα, are strong predictors of mortality in the population.[52] TNFα has been since regarded as a central cytokine in the inflammatory response and it has been associated with a list of devastating and pleiotropic effects on the myocardium based on several experimental studies. Early reports using cultured myocytes attributed a pro-hypertrophic role for TNFα.[53] Indeed when cardiomyocytes were cultured for several days in TNFα-containing media, cellular capacitance, leucine incorporation and levels of reactive oxidative species were all significantly increased. Additionally, TNFα was shown to significantly reduce contractility in cardiomyocytes.[54] Furthermore, when canine hearts were infused with TNFα, the result was an impairment of left ventricular function. Experimental data suggested that TNFα up-regulated matrix metalloproteinases expression which in turn increased Nitric Oxide (NO). NO has a negative inotropic effect but can also uncouple β-adrenoceptors from adenyl cyclase thus, contribute further to reduction in contraction. Comparable results were obtained when TNFα was overexpressed in the murine heart.[55] The effects of TNFα over-expression were a severe ventricular and atrial remodelling, which ultimately leads to a dilated cardiomyopathy, reduction of ejection fraction and an electrical remodelling which increases susceptibility to arrhythmia.[55, 56]

Globally, the overwhelming evidence in support of a deleterious role for TNFα, whether it is in heart disease or other inflammatory disorders such as rheumatoid arthritis, has led to the development of therapies aimed at antagonising the effects of TNFα. Even though many of the drugs prescribed to heart failure patients, such as angiotensin-converting-enzyme inhibitors or β-blockers might have some anti-inflammatory effect,[57] the need for a specific and potent treatment was increasing.[25] The therapies subsequently developed which are aimed at neutralizing TNFα made largely use of antibodies. Currently

there are three antibodies used in clinical practice (adalimumab, human monoclonal; infliximab, a chimeric human/mouse monoclonal antibody; etanercept, a soluble receptor construct) and others being developed such as certolizumab and golimumab and other less or not used pharmacological agents that directly suppress or modify TNFα production.[58] The mechanisms by which TNFα antagonists actually neutralise TNFα are not well known but they implicate at least a direct binding to TNFα and blockade of the receptor binding sites.[45]

Despite the significant success of TNF antagonist therapy in inflammatory conditions such as Crohn's disease or rheumatoid arthritis the efficacy of anti-TNF therapy in heart failure proved to be more than just poor and in fact contributed to increased mortality in some cases.[59] Many explanations have been forwarded by the scientific community in order to explain these results and it would appear that the adverse effects of antagonising TNFα would possibly be linked to the high specificity of antibodies that reduce TNFα concentration below a threshold required for tissue repair. Additionally, some antibodies might bind the tmTNFα in cardiomyocytes that are actively producing TNFα thus inducing cardiomyocyte apoptosis.[25] Alternatively some have regarded the TNF blockade therapy as ineffective due to redundancy in the inflammatory cascade where a myriad of other cytokines, such as IL-6, remain at elevated levels and mediate the pathogenesis of disease independently of TNFα.[60]

1.2.2 Interleukin 1 beta

Along with TNFα, IL-1β is one of the key pro-inflammatory cytokines that are at the top of the inflammatory cascade. Since its discovery in 1976, several studies have subsequently focused on understanding and characterising the fundamental properties of IL-1β at a genomic and protein level.[61-63] The IL-1β protein is a 17.5 kDa 153 amino acid peptide which is produced initially as a 35 kDa precursor that has no biological activity.[64] Maturation of the precursor form involves cleavage by Caspase-1, originally termed interleukin-1β-converting-enzyme, and release of the soluble and mature form of IL-1β, which is known to have an extremely wide repertoire of activities in several different

cellular systems including induction of more IL-1β.[65] Figure 3 illustrates a typical IL-1β activation cascade.

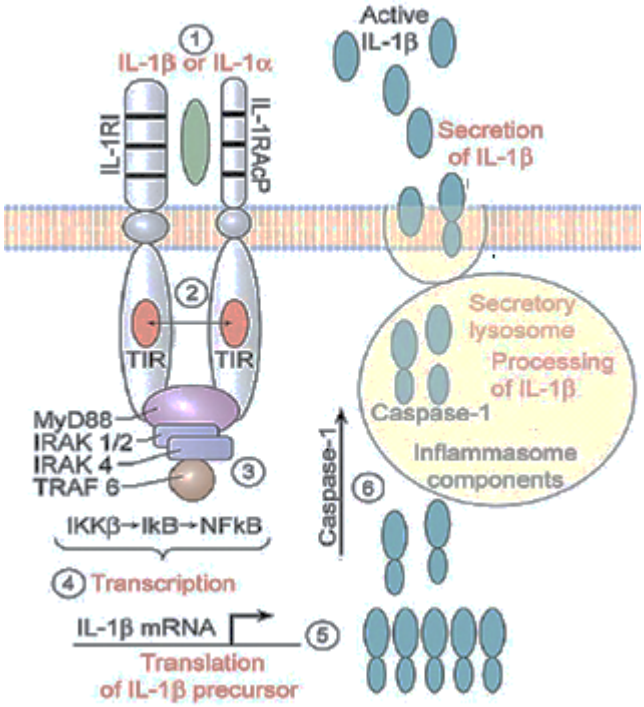


Figure 3. Diagram of a Typical IL-1β Production Cascade. *(1) IL-1β or IL-1α bind to the same receptor IL-1RI and facilitates recruitment of its accessory protein IL-1RAcP. Intracellular Toll-IL1-Receptors (TIR) approach from each other (2) and recruit Myeloid differentiation primary response 88 (MyD88), phosphorylate Interleukin 1-Recepetor-Associated-Kinase (IRAK) along with IKKβ which activates NF-κB (3) leading to activation of transcription of IL-1β gene or other genes (4). Once IL-1β transcripts are translated into the precursor form of IL-1β (5) caspase-1 produced elsewhere cleaves IL-1β into its mature form which is secreted as shown in (6). Modified from* [67].

Steps 1 to 4 in figure 3 are the basic signalling cascades that are activated after IL-1β binding to the IL-1RI (structure shown in figure 4)[66] and are common to several other cascades with different downstream targets: in this particular case the production of additional IL-1β in response to IL-1β binding is illustrated. However, binding of IL-1β increases the transcription of hundreds of genes within 30 minutes and this effect can last several hours.[65]

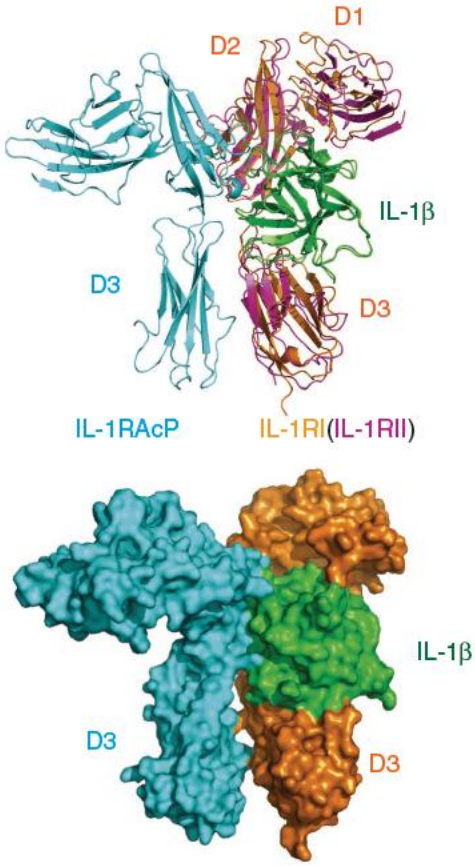


Figure 4. IL-1β, IL-1R1 and IL-1RAcP Structure and Interaction. *Molecular structure and surface representation of IL-1β bound to its main receptor IL-1RI and accessory receptor IL-1RAcP. Modified from* [66].

Decades of research into the downstream targets activated by IL-1β have led us to a great understanding of the cytokine biology related to the interleukin 1 family which comprises 11 members to date.[64, 67] Several prominent researchers including Dr. Charles A. Dinarello, considered to be the founding father of cytokines, in particular the interleukin 1 family, have provided crucial information that significantly broadened our understanding of the IL-1 mechanisms which have ultimately led to the development of anti-IL-1β based therapies for treatment of several inflammatory diseases. The regulation of IL-1β proves to be distinct from other cytokines and is very tightly controlled. In fact, several negative feedback mechanisms aimed at terminating the IL-1β response have evolved in the IL-1 biological system and they include the presence of a decoy receptor which has no intracellular tails and thus mediates no response because it fails to recruit MyD88, and a naturally occurring receptor antagonist that is a potent negative regulator of the IL-1 cascades.[67] Indeed, the anti-IL-1 therapies developed

include the administration of IL-1Ra (interleukin 1 receptor antagonist), the natural antagonist of the receptor, which was proven to be effective in several diseases and became a standard therapy in autoinflammatory disorders.[68, 69] Of note, autoinflammatory diseases are not to be confused with autoimmune diseases. The former refers to a dysfunction of the mediators of innate immunity i.e. macrophages and neutrophils which become chronically activated and secrete cytokines locally or systemically whereby anti-cytokine therapy is often effective in reducing adverse outcomes. Conversely, in autoimmunity the principal cause of disease is a malfunction in T cells and the instigators of inflammation become T cells and B cells themselves. Treatment of autoimmune disease requires at least immunosuppression therapy and administration of anti-cytokines is considered trivial.[70]

In the heart, the pathogenesis of IL-1β has also been extensively researched ever since it was noted that serum IL-1β are elevated in patients suffering from heart disease such as myocardial infarction, heart failure, or dilated cardiomyopathy.[71-74] However the mechanistic insight only came from in *in vitro* or animal models which examined the direct effects of IL-1β on the myocardium. In one report using human cardiac tissues, treatment with IL-1β for a few hours resulted in a significant decrease in contractility.[75] Additional studies demonstrated that IL-1β might also have negative inotropic and chronotropic effects most likely due to changes it induces in calcium homeostasis and calcium handling.[75] Cardiomyocyte hypertrophy was also observed in several animal studies which were in some cases attributed to nitric oxide signalling (NO) via the inducible Nitric Oxide Synthase (iNOS) while in some other cases it involved instead p38 MAP kinase signalling. Last but not least in vitro studies have also suggested that IL-1β might also be involved in apoptosis and altered expression of α and βMHC expression.[76]

Altogether, these observations strongly suggest that IL-1β may recapitulate several of the adverse effects and phenotypes typically seen in heart failure. As a result the need to develop therapies aimed at antagonising IL-1β was appreciable and became a goal for clinical medicine. In fact anti-IL-1 treatments was one of the first cytokine specific treatments that emerged, and it was not until later that anti-TNFα therapies were

developed.[70] Treatments aimed at reducing IL-1β proved to be efficacious in several chronic and autoinflammatory conditions such as gout, type-2 diabetes, smoldering multiple myeloma, post-myocardial infarction heart failure and osteoarthritis.[77] Interestingly, patients suffering from type-2 diabetes, today considered an autoimmune (and inflammatory) condition, have also an increased risk of cardiac disease such as myocardial infarction. Diabetic patients who suffered from myocardial infarction have an even higher risk of a subsequent myocardial infarction, which suggests a positive feedback loop where the increases in inflammation, and IL-1β in particular, may contribute greatly to worsening cardiac pathologies. Thus anti-IL-1 therapy might be profitable for diabetic patients on a twofold basis: reduce inflammation to ameliorate diabetes and decrease the diabetes-mediated risk of heart disease. Therapies aimed at antagonising IL-1β make use of the naturally occurring IL-1 receptor antagonist. IL-1Ra is a form of IL-1β that binds to same receptor IL-1RI but illicit no response (figure 5). Thus, it appeared to be the ideal treatment because even though a recombinant form, it is a naturally occurring protein that will bind with high affinity to the same receptor of IL-1β thus blocking access to IL-1β and, most importantly, without causing side effects.[77] For example in rheumatoid arthritis patients, administration of anakinra (IL-1Ra) resulted in comparable improvement of symptoms compared to other therapies but with fewer side effects.[70] Additionally, in type 2 diabetes patients, inflammation induced by free fatty acids, leptin and IL-1β itself cause a toxicity to liver β-cells that could also be reduced by administration of IL-1Ra thus contributing to the possible reinstatement of their function[78]. However, in heart disease patients there have been very few studies examining

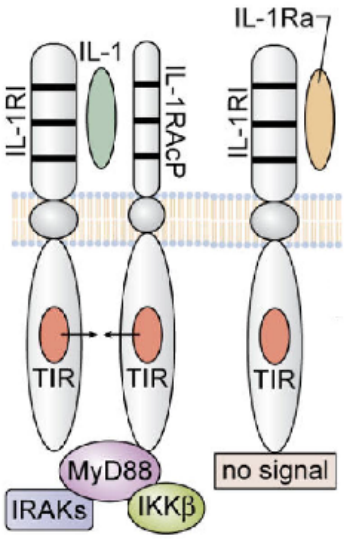


Figure 5. IL-1Ra Signaling. *IL-1Ra binds to IL-1RI similarly to IL-1β however fails to recruit IL-1RAcP and thus occupies the receptor but produces no response. The recombinant IL-1Ra anakinra produces the same effects as the naturally occurring IL-1Ra. Modified from [67].*

the role of anti-IL-1 therapy. One prominent study was amongst patients with STEmyocardial infarction (ST Segment Elevated Myocardial Infarction), a condition with a high mortality risk. When a daily dose of anakinra was given along with the therapy after angioplasty, echocardiographic and blood tests results taken 14 days post-surgery showed a significant reduction in ventricular remodelling and in plasma CRP levels. Furthermore, after 18 months, none of the patients who received anakinra developed heart failure whereas 60% of placebo patients went into a stage IV heart failure.[79] Thus it appeared that anakinra was able to significantly improve cardiac outcome after myocardial infarction by reducing adverse remodelling, however it remains to be investigated whether anti-IL-1β therapy is effective in patients with non-myocardial infarction chronic heart failure.

1.2.3 Interleukin 6

The third and last cytokine that will be discussed is interleukin 6. Along with TNFα and IL-1β, IL-6 has long been considered to be at the top of the inflammatory cascade; however the role it has in the pathogenesis of cardiac disease is much less understood. Several clinical studies have pointed out a strong association between IL-6, severity of heart failure and mortality risk. Additionally, IL-6 has been proposed by some to be a stronger predictor of prognosis than CRP.[80] In fact in one particular study, IL-6 levels were shown to be predictive of all causes of mortality in elderly males, thus highlighting its possible role in low grade chronic diseases that are often found in the ageing population.[80] There have been however some confounding data on a clinical and basic science level regarding the role of IL-6 in the heart. One reason might be the difficulties encountered in measuring IL-6 in

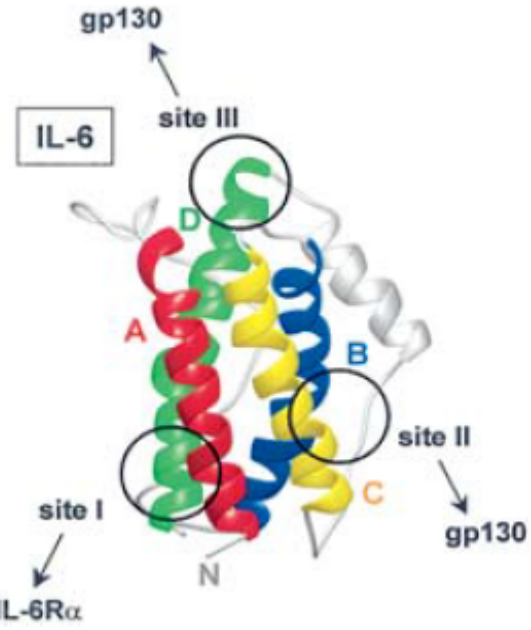


Figure 6. 3-Dimensional Structure of IL-6. *IL-6 structure shows 4 α helices A, B, C and D along with 3 receptor binding sites encircled. Modified from* [81].

serum of patients due to its extremely short half-life or sex differences as shown by the study conducted on British women, where contrary to men, there was no association between IL-6 and coronary heart disease.[6]

IL-6 is synthesised as a precursor 212 amino acid peptide, which is subsequently cleaved to form the mature 185 amino acid protein. Figure 6 shows the structure of IL-6, which comprise four long α-helices (A, B, C and D) that are arranged in an up-up-down-down topology.[81] IL-6 can be produced by a number of cells including a range of inflammatory cells but also cardiomyocytes and fibroblasts. The signalling of IL-6 is mediated by IL-6R and another common receptor glycoprotein 130 (gp130), a transmembrane protein crucial for mediating intracellular signalling.[81] Figure 7 summarises IL-6 signalling: once bound to its receptor, IL-6R dimerises with gp130 leading to an

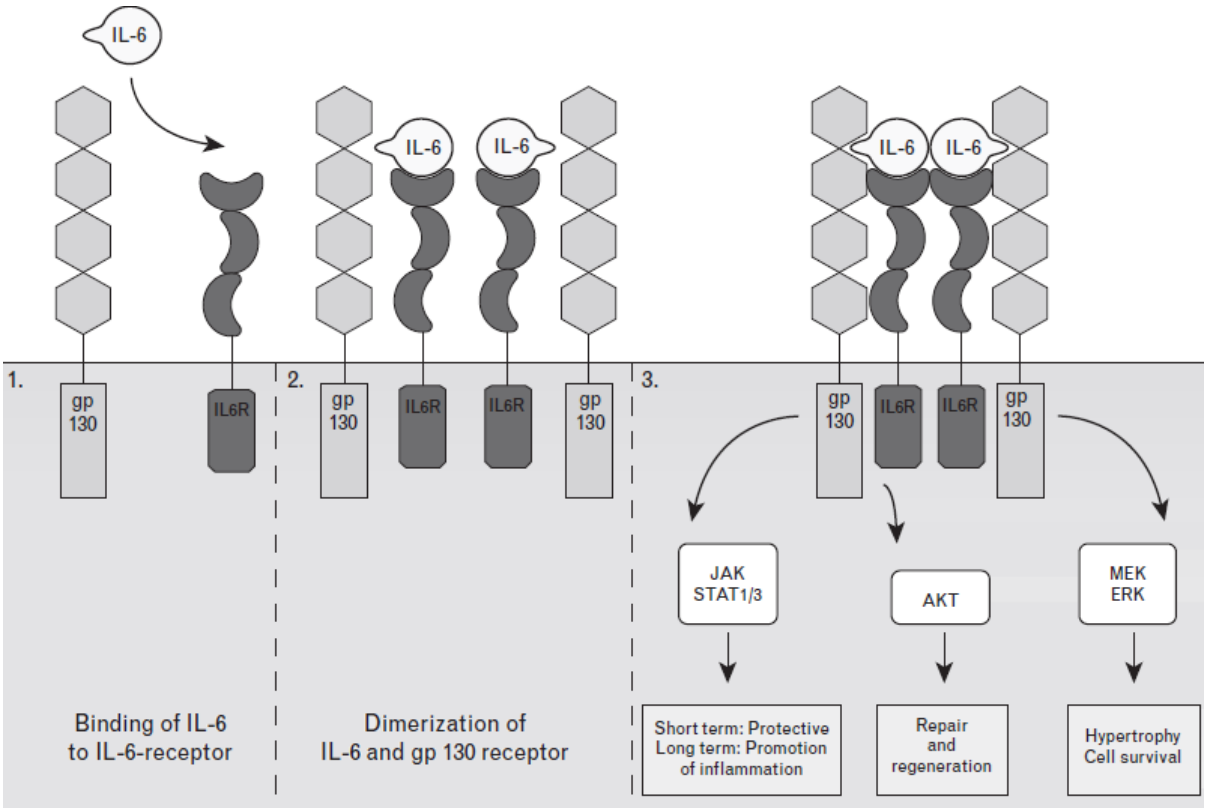


Figure 7. IL-6R signalling pathways. *IL-6 binds to IL-6R (1) which subsequently dimerises with gp130 (2) allowing the recruitment and activation of downstream cascades such as JAK/STAT, AKT and MEK/ERK. Modified from* [6].

activation of JAK/STAT 1/3 which may regulate various aspects of cell survival growth or differentiation. Additionally PI3K/AKT, MEK and ERK1/2 pathways are also activated.[6]

As previously mentioned, clinically, IL-6 has almost always emerged as an important marker for heart disease and predictor of mortality. However, in animal studies the image is less clear and several studies have yielded mixed results regarding the specific effects of IL-6. It is established that increased STAT3 signalling is essential after myocardial infarction where it might play a cardioprotective role while it also permits a preconditioning before myocardial infarction. Conversely, diminishing STAT3 abolishes all of these positive effects.[82] However, IL-6 activates these pathways via gp130 yet the negative outcomes in patients are clearly correlated with IL-6 serum levels thus adding ambiguity in regards to the specific role of IL-6. Nonetheless, a prominent study examining the role of gp130 concluded that chronic or long term activation of JAK/STAT3 downstream of gp130 is actually detrimental whereas only early and short term activation proves to be cardioprotective. Indeed, by using transgenic mice for a mutant form of gp130 which does not terminate the STAT3 signalling cascade, myocardial infarction induced in these mice resulted in a severe ventricular remodelling and higher mortality compared to control mice.[83] It was only when this signalling cascade was blocked, that the adverse effects seen in transgenic were reduced. Thus it appears that the chronic secretion of IL-6 in an attempt to restore homeostasis will result in a deregulation of the STAT3 signalling pathways leading to its constant activation, a phenomenon associated with detrimental outcome.

1.3 Cytokines and Ion Channels

1.3.1 Cardiac Action Potential and Modulation by Cytokines

The inflammatory response in general and cytokines in particular are of crucial importance when it comes to disease. Indeed the role of inflammation in mediating the pathogenesis of chronic heart disease could not be emphasised enough. We have so far

discussed its role in several clinically relevant cardiac pathologies such as heart failure. However, the implications of cytokines extend far beyond the scope of this work, and even though we have gained significant insight on the specific role of a myriad of cytokines in heart disease, the findings discussed previously are far from complete and several venues remain to be explored in order to better understand how cytokines affect the heart.

A fundamental property of cardiac tissue is its ability to conduct current and contract. This is made possible by a complex system of tightly regulated ion channels coupled to a contractile machinery. When they both function in harmony, the result is the initiation of an electric influx that propagates in an organised and predictable way across the heart allowing subsequent contraction. Deregulating ion channels can have profound effects, in fact if the electrical flux was to be perturbed this could lead to a chaotic dispersion of the electrical activity which would trigger irregular and asynchronous heart beats termed arrhythmia, a life threatening condition.

The effects of inflammation in increasing adverse risk and worsening of cardiac disease have now been attributed to several processes such as cardiac remodelling, however it is still not well understood how inflammation, and pro-inflammatory cytokines in particular, could modulate cardiac ion channels. Several clinical observations have allowed us hypothesis that cytokines can alter ion channel properties, thus adding another level of complexity to their list of adverse effects in cardiac pathologies. Indeed, the QT interval measured on the ECG, which is an index of ventricular repolarisation, is lengthened in HIV patients, independently of anti-retroviral therapy drugs.[21] An increased QT interval presents itself as a risk factor for lethal arrhythmias termed *torsades de pointes* which can lead to sudden cardiac arrest[84] and the fact that an ECG is a measure of electrical activity provides irrefutable evidence that ion channel activity is being altered. Furthermore, in patients suffering from arrhythmias, high levels of cytokines, notably TNFα, have been observed.[85] Thus, overall these observations suggest a causative link between cytokines, modulation of ion channels and pre-disposition to arrhythmias. The mechanisms by which cytokines are able to affect ion channels are yet to be elucidated. But before exploring the effects of the cytokine-mediated regulation of ion channels, a brief introduction to cardiac

electrophysiology will be provided in order to outline the electrical activity of the heart and its origins.

A fundamental property of several different types of cardiac cells, known as cardiomyocytes, whether derived from the atria or ventricle, is their ability to display an electrical activity termed action potential in response to an electrical stimulus. Even though cardiac myocytes originating from different parts of the heart have different action potential properties, they all share a common set of essential mechanisms which allow the induction of an AP. The root of the electrical activity lies in ion transport. As with any eukaryotic cell with a bilayer plasma membrane, diffusion of electrically charged ion across the membrane is nearly impossible. Cardiomyocytes however, have evolved a complex set of ion channels, which are membrane-bound proteins that allow passive transport (or active in some instances) of ions across the membrane. These ion channels are not simple openings that allow the ions to go in and out of the cell, but they tightly regulate ion flow in an ion-specific and voltage-dependent manner. This means that for each of the main ions in the circulation (e.g. Na^+, K^+, Ca^{2+} or others), there are specific ion channels that will conduct, with a high degree of selectivity, only one particular ion. For our purposes, we will only focus on three types of ion channels: the ventricular voltage-gated, sodium, potassium and calcium channels. These families of ion channels play the largest role in shaping the action potential. The ionic mechanisms responsible for generating an action potential were first described in the giant squid axon by the Hodgkin-Huxley model in 1952.[86] Significant research and development into field of electrophysiology came afterwards with the invention of the patch-clamp technique, a method that allows the direct quantification of current or, ion flow, across these channels.[87]

The ventricular action potential is composed of several different phases, where each phase is the result of an activation and inactivation of a set of ion channels. The left panel of Figure 8 shows a diagram of a human ventricular action potential with the several ion channels that are responsible for its generation. Adjacent to it, is the mouse action potential that has a different configuration due to the different expression profile of ion channels. In the introduction we will focus on the mouse ion currents particularly the sodium current

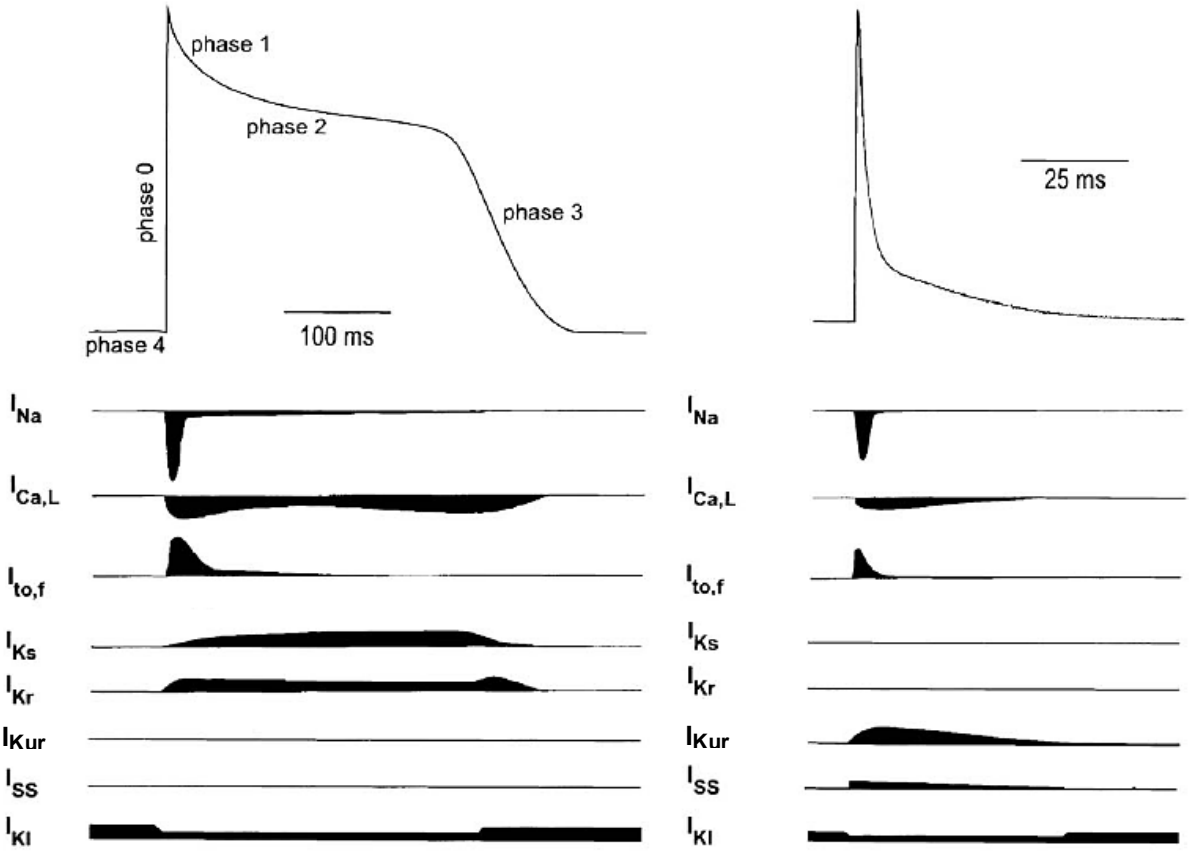


Figure 8. Human and Mouse Ventricular Action Potentials. *Human ventricular action potential (AP) (left) compared to mouse AP (right). Note the speed of the mouse AP and the difference in the ion current profile, where I_{Ks} and I_{Kr} are absent and repolarization is mediated by I_{Kur} and I_{ss} in the mouse ventricle. Additionally, I_{CaL} is significantly smaller compared to human counterpart. Altogether these differences could explain the speed of the mouse AP and the absence of a plateau phase. Modified from* [88].

(I_{Na}), potassium currents (I_{to}, I_{Kur}, I_{ss}, I_{K1}) and the L-type calcium current (I_{CaL}). See Abbreviation List for the full names of these ion currents. Importantly, in adult mouse ventricular myocytes, repolarisation is mediated by I_{to}, I_{Kur}, I_{K1} in addition, I_{ss} a steady-state K^+ current might also participate.[88-91]

The human ventricular action potential comprises five phases. As previously mentioned, Figure 8 illustrates these phases on the left panel along with the respective currents underlying each phase. The right panel, on the other hand, displays the mouse action potential with its underlying currents. While at rest, cardiomyocytes are negatively charged compared to the extracellular milieu. The voltage difference across the membrane of a ventricular cardiomyocyte, measured by an electrode grounded to the extracellular environment, is about -90 mV. At this voltage, only a few channels are active, such as the inward rectifying potassium channel Kir 2.1 which encodes for I_{K1}, a current that play a crucial role in maintaining resting membrane potential.[92] When sensing a strong depolarisation (i.e. an electrical impulse that will shift the resting membrane potential enough towards a more positive value) this triggers the fast depolarisation phase, termed phase 0, where the sodium channels $Na_V1.5$ sense the depolarisation and quickly activate to open allowing an influx of positively charged Na^+ ions. This will shift the membrane potential up to +30 mV. Subsequently, in phase 1, also known as the early repolarisation phase, a sharp but light drop in membrane potential occurs due to a fast K^+ efflux mediated by the potassium K_V 4.2/4.3 and 1.4 which encode the I_{to} current. Meanwhile, the calcium current $Ca_V1.2$ which encodes I_{CaL} is activated allowing an influx of Ca^{2+} into the cell, triggering contraction via in a phenomenon termed calcium-induced calcium release (CICR)[93, 94] which will be discussed further. Activation of an inward positively charged current I_{CaL}, along with repolarising potassium current I_{Kr} and I_{Ks} which extrudes the positive K^+ out of the cell in an attempt to revert to the resting membrane potential, results in quasi-balance of charges and a visible plateau phase, the longest phase of the action potential known as phase 2. Phase 3, the repolarising phase, follows afterwards when the calcium channels inactivate and additional K^+ is driven out of the cell via I_{Kr}, I_{Ks} and at late

phase 3, I_{K1}. This will bring the cell back to its initial resting membrane potential of -90 mV also known as phase 4.[92] In adult mouse ventricular myocytes, the rapidly activating I_{to} and I_{Kur} largely contribute to repolarisation. Consequently, the activation window of I_{CaL} is significantly reduced compared to the human counterpart thus there is no plateau phase. This explains the triangular shape and short duration of the mouse action potential.

At the cellular level, the 5 phases of the action potential are perpetually repeated, in the exact manner in the healthy ventricle. However, cardiomyocytes do not act individually and when looking at the whole heart they become an entity comprised of an intricate and organised network of cells where the electrical influx is capable of progressing in an organised fashion and triggering a contraction accordingly. As shown in figure 9, the electrical influx begins from the spontaneously action potential firing sino-atrial node, then crosses the atria, converges towards the atrio-ventricular node, goes down the his bundles and Purkinje fibres and into the ventricles.[10] This electrical activity that begins at the sino-atria node, progressively advances, permits contraction of the muscle and finally terminates, actually corresponds to thousands of myocytes firing their action potentials and terminating it an extreme synchronicity. The total electrical activity of the heart, or the sum of action potentials at a given time and location, is made visible by the ECG with the different phases it displays. The different complexes of the ECG corresponding with each cardiac electrical activity are also shown on figure 9.

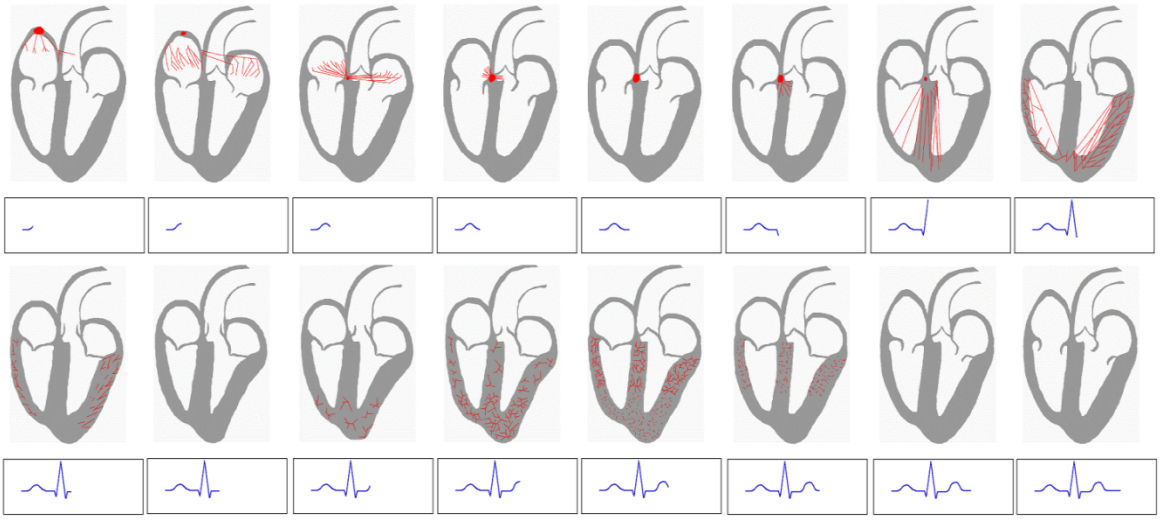


Figure 9. Cardiac Electrical Activity and ECG. *Cardiac electrical activity representing the influx in red that starts from the sino-atrial node, propagates (depolarization) across the atria and into the ventricles then dissipate (cardiac repolarization and relaxation in last 6 steps, measured by QT interval) thus completing one heartbeat. ECG traces of each phase are drawn below each diagram. Arrow indicates intended reading direction. Modified and licensed from the Wikimedia Commons.*

The cardiac action potential needs to be finely regulated in order to preserve its proper duration, however under disease conditions, several ion channels are affected which might alter the properties of the action potential. For instance reduction in I_{to} is now regarded as a hallmark of the failing human myocardium which is associated with an increase incidence of severe ventricular arrhythmia.[95, 96] Precisely, the direct effect of reduced potassium current is a prolongation of repolarisation and action potential duration, which increases the probability of a re-activation of calcium channels, thus allowing another depolarisation before the action potential has actually been terminated. This phenomenon termed early afterdepolarisation (EAD) can cause a spacial and temporal instability in repolarisation which predisposes the individual to *torsades de pointes* and subsequently triggered activity or ventricular fibrillation, an often lethal condition.[84, 97]

Electrical instability is often observed in heart failure and is thought to be one of the main causes of sudden cardiac death in heart failure patients.[84] Heart failure is recognised as a complex and multifactorial condition where a broad range of noxious stimuli negatively impact the heart, leading to remodelling and an impairment of cardiac function. Inflammation has been recognised as one of the players involved in shifting the heart towards a pathological state and the role of cytokines in mediating several of these deleterious changes have been discussed previously. However, it remained unclear whether cytokines could directly affect the electrical properties of the heart or modulate ion channels before the structural remodelling actually occurred. The first evidence that pointed us towards hypothesising that pro-inflammatory cytokines could modulate ion channels was the observation that HIV patients had an increase in QT interval that was not due to the HIV drug therapy.[21] However, direct evidence came from experimental data obtained from a mouse model of HIV.[24] By expressing one of the HIV genes, *nef,* these mice showed similar symptoms to human HIV patients furthermore, they also had elevated levels of circulating pro-inflammatory cytokines.[22, 23] In fact, the symptoms were so similar that when an ECG was performed, a significant prolongation in the QT interval was also observed.[39]

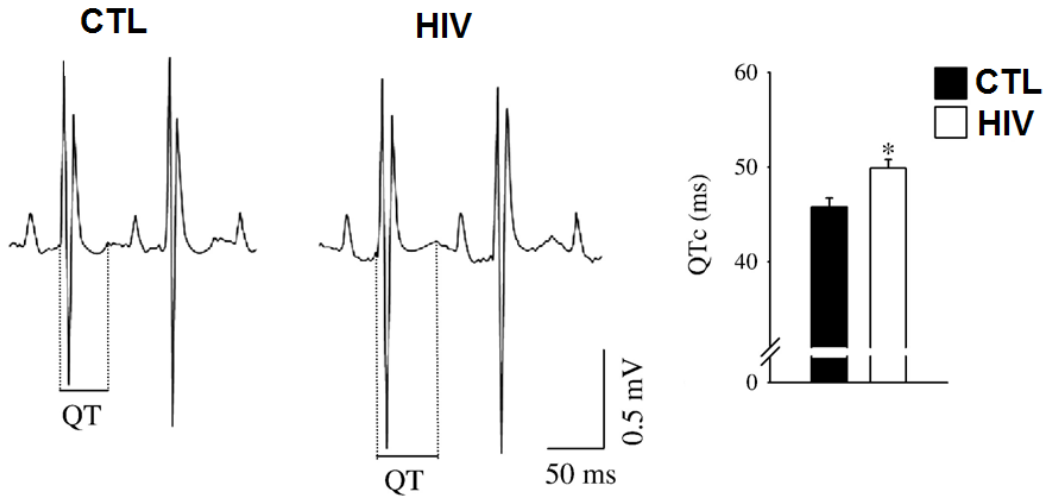


Figure 10. ECG of Control and HIV Mice. *Representative examples of ECG recording (left) and mean data (right) obtained from control and HIV mice which exhibit a significant (*) increase in QT interval. Modified from [39].*

Figure 10 shows ECG recording from these mice with a prolonged QT interval which indicated that these mice likely suffered from a delay in repolarisation. When the hearts of these mice were isolated, ventricular cardiomyocytes dispersed and currents recorded from individual myocytes we observed at first a significant increase in action

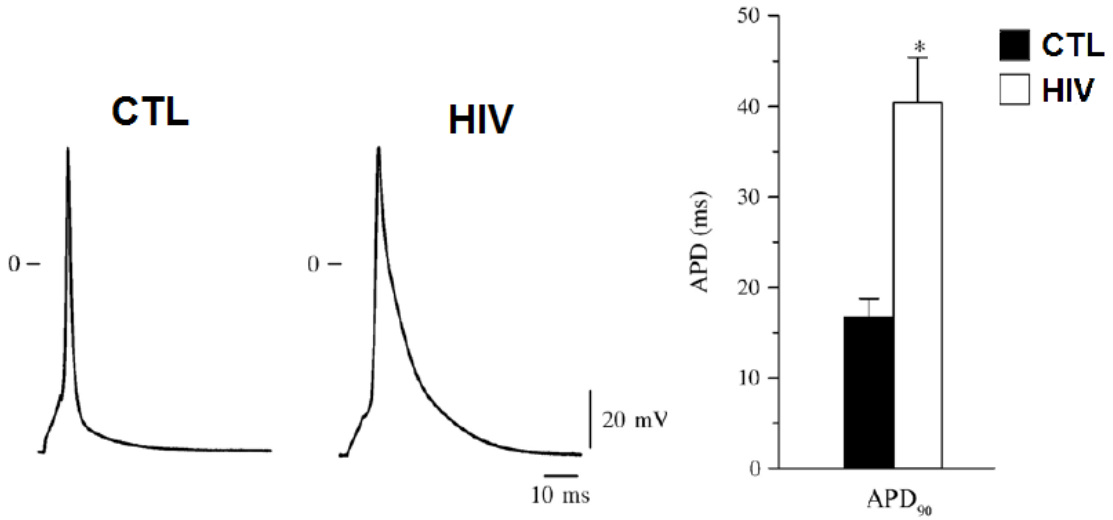


Figure 11. Ventricular Myocyte Action Potential from Control and HIV Mice.
Action potentials were recorded from myocytes isolated from either control or HIV mice. Note the nearly two-fold increase action potential duration (APD) in HIV cardiomyocytes. Modified from [39].

potential duration, which explained the prolonged QT interval, as shown in figure 12. Furthermore, the patch-clamp technique allowed us to gain additional insight into the underlying mechanisms of this QT interval and action potential prolongation. Indeed our data showed that all of the potassium currents involved in repolarisation have been reduced in HIV mice. Figure 12 shows that I_{peak} the total outward potassium current, which comprises of I_{to}, I_{Kur} and $I_{ss,}$ was reduced and this reduction was attributable to a reduction in all of three currents I_{to}, I_{Kur} and I_{ss}. Overall the data indicated that HIV mice which suffered from high levels of cytokines [98] showed severe signs of delayed repolarisation and prolongation of action potential duration. These adverse changes in cardiac function predispose individuals to arrhythmia and might increase risk of sudden cardiac death.

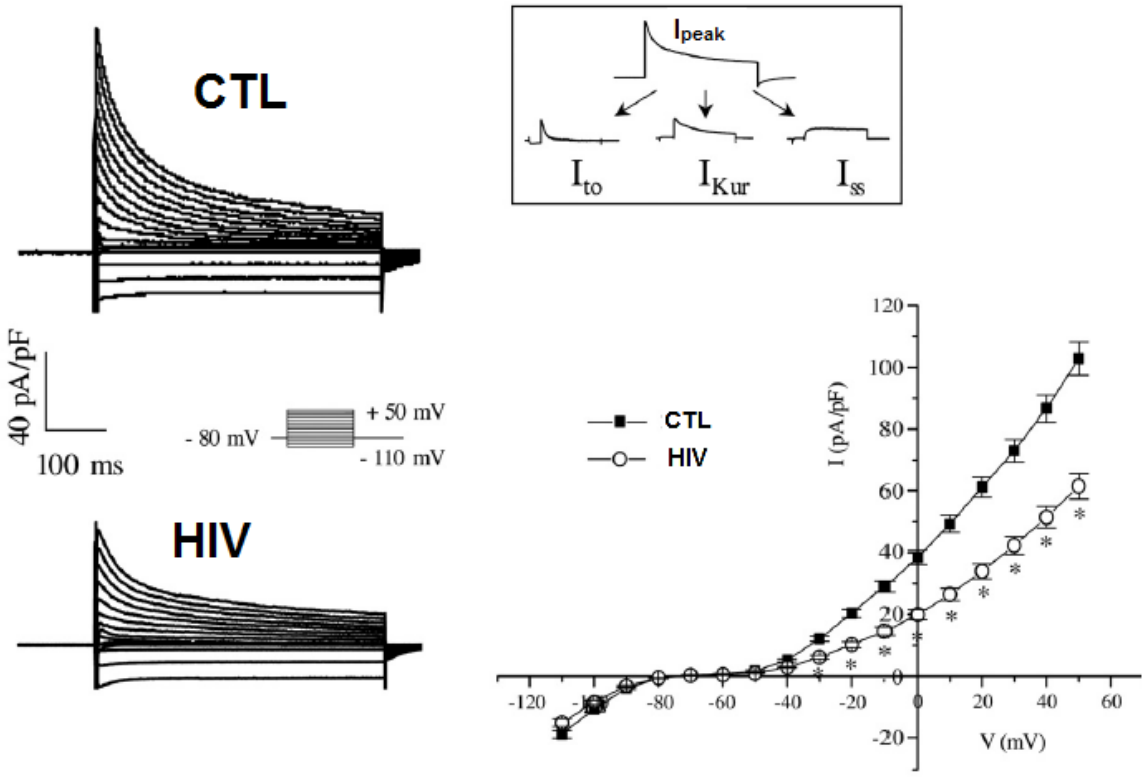


Figure 12. Total K^+ Currents in Control and HIV Mice. *Total K^+ currents or I_{peak} were recorded from control and HIV cardiomyocytes. Data shows a significant (*) reduction in repolarising outward K^+ current which was attributed to a reduction in I_{to}, I_{Kur} and I_{ss}. Modified from* [39].

Interestingly, the adverse effects of HIV on cardiac electrophysiological parameters were not just limited to potassium currents but also extended to the sodium current I_{Na} which is the current responsible for the phase 0 of the action potential, or the fast depolarising phase which plays a crucial role in excitability of cardiomyocytes and is one of the main components of cardiac electrical conduction.[98] The first evidence of an alteration in sodium channels in HIV mice was observed on the ECG, which showed a significant increase in

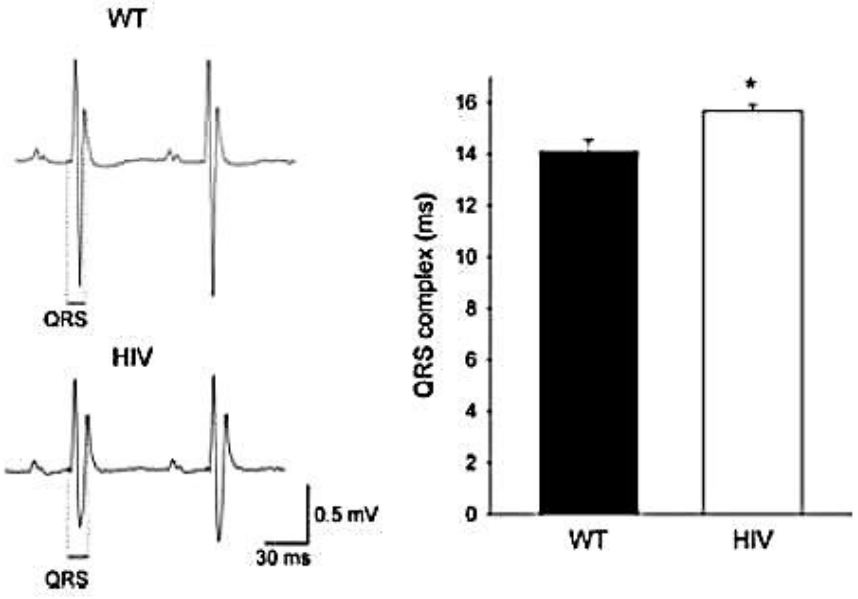


Figure 13. The QRS Complex in HIV Mice. *ECG recording obtained from wild type (WT) and transgenic HIV mice that shows an increase in QRS complex duration. Mean data is shown on the right panel. Modified from* [98].

duration of the QRS complex. The QRS complex is an indicator of ventricular depolarisation which is mediated by the voltage-gated sodium channels that open and allow a rush of Na^+ into the cardiomyocytes. Indeed, the progressive electrical activation, shown in figure 10, actually corresponds to the depolarisation induced by I_{Na} which propagates across the cardiac tissue from cell to cell and when it reaches the ventricles, it is observed as the QRS complex on an ECG. Figure 13 shows an ECG recording obtained from an HIV mouse displaying a significantly increase in the QRS complex duration. This prolongation was most likely attributable to a decrease in the sodium current density. Indeed, at the cellular level, the observed changes in the ECG were explained by action potential measurements that showed not only a diminished upstroke of phase 0 of the action potential but also a decrease in the upstroke velocity, which overall can decrease conduction and action potential propagation in the heart thus contributing to the development of arrhythmia. Furthermore, these action potential parameter modifications were explained by patch-clamp experiments which quantified the sodium current recorded from both control and HIV ventricular myocytes. The data indicated that HIV mice suffered from a significant reduction in sodium current density, as shown in figure 15. The implications of reduced sodium (and potassium) currents in HIV mice are considerable. When free roaming HIV-mice were monitored by telemetry, several episodes

of rhythm disturbances were observed whereas none occurred in the wild-type mice,[98] thus suggesting a significantly increased risk for arrhythmia. Importantly, HIV mice had no evidence of myocardial remodelling or impaired cardiac function. Echography results showed no signs of hypertrophy, preserved ejection fraction and fractional shortening[39]. Thus, HIV which elevated cytokine levels, appeared to directly affect ion channels independently of structural remodelling.

The mechanisms by which the sodium current, and potassium to this extent, were reduced in HIV mice remained unknown until then, and despite all the evidence that

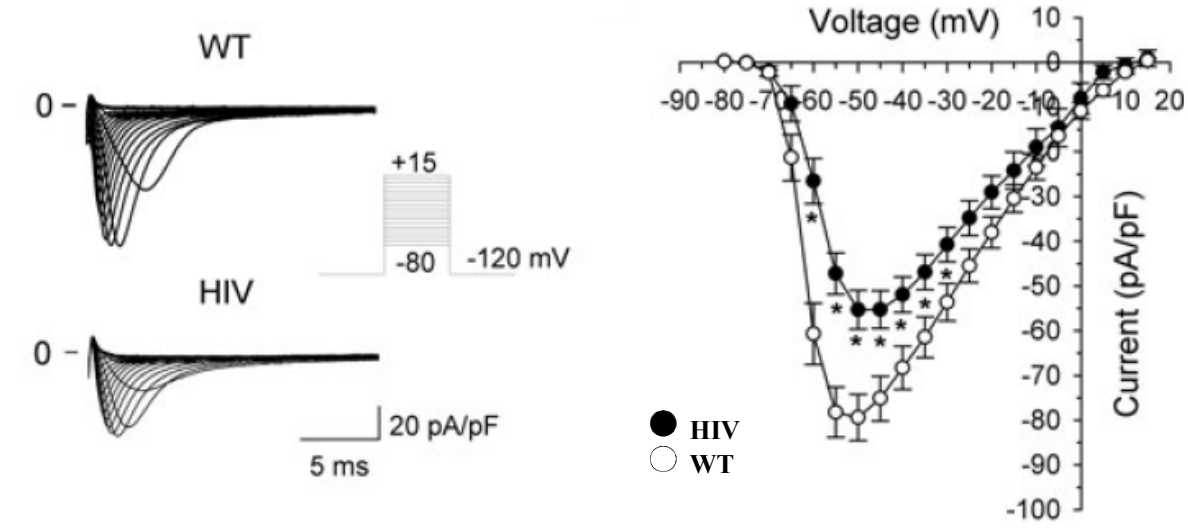


Figure 14. Ventricular Sodium Currents from Control and HIV Mice. *Typical examples of sodium current recording from wild-type and HIV mice are shown on the left while mean data curve on the right. Modified from* [98].

suggested a prominent role for cytokines in mediating these effects on ion channels, it was not of complete certainty whether the observed effects were directly or indirectly linked to cytokines. Considering HIV is a complex disease which might generate systemic feedback from other organs such as the kidneys a finer approach was needed in order to answer our problematic. Subsequently, several different experiments were devised and additional experimentation was performed. The overall result strongly suggested that cytokines are

able to directly affect cardiac ion currents. Indeed, when wild-type mice were treated with a pathophysiologically relevant dose of TNFα for 6 weeks, these mice showed a significant change in their action potential profile.[99] These changes were caused by a significant decrease of total K^+ current I_{peak} due to a decrease in I_{to} and I_{Kur}. Figure 15 shows examples of recordings and mean data for the two main repolarising currents in mouse I_{to} and I_{Kur}.

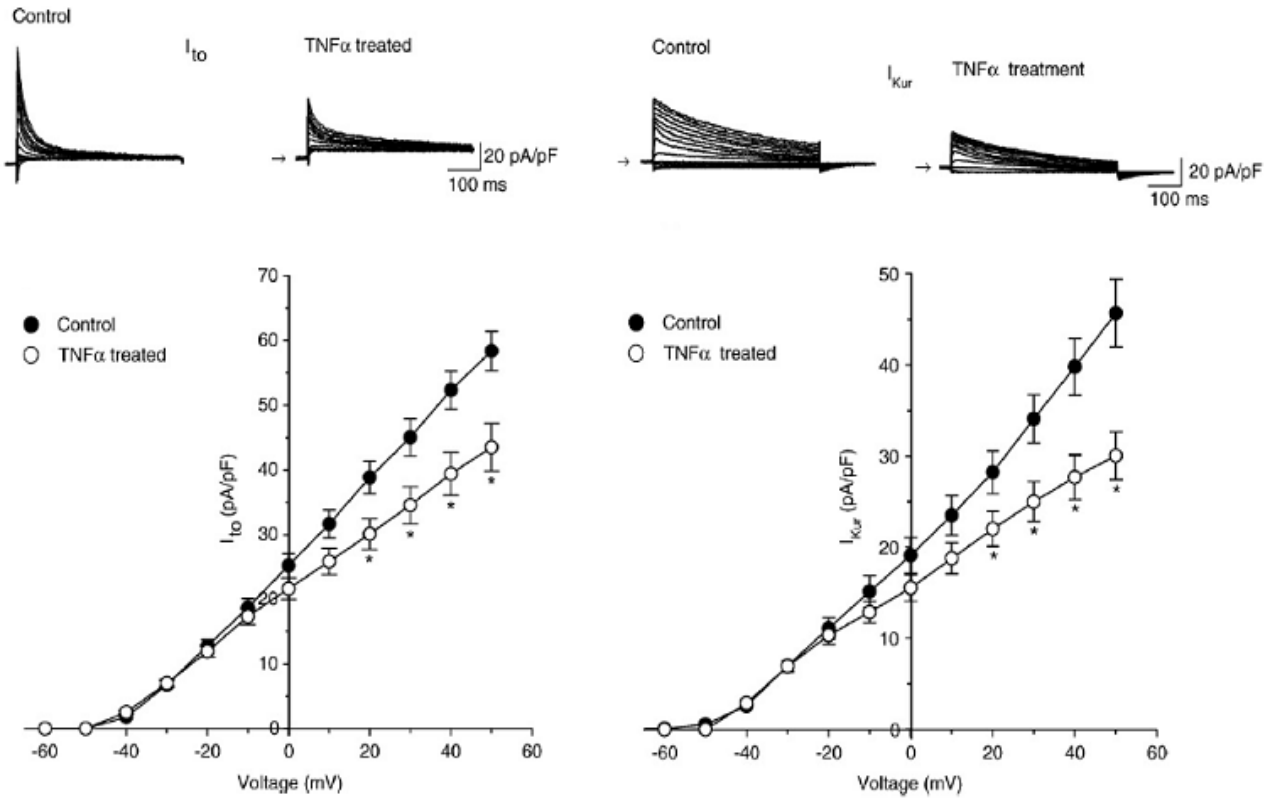


Figure 15. Ventricular Potassium Currents in TNFα Treated Mice. *Typical examples and mean data for I_{to} (left panel) and I_{Kur} (right panel) showing significant reductions in both current in mice treated with TNFα. Modified from* [99].

Overall, this study showed that by simply treating mice chronically with TNFα, several of the adverse effects on cardiac currents seen in HIV mice were recapitulated. This indicated that TNFα alone could modify the repolarsing K^+ currents and reshape the action potential. The findings could, in part, explain the prolonged QT interval in both HIV mice

and HIV patients. Furthermore, it appears that cytokines, when circulating at pathophysiological levels, do not necessarily induce ventricular remodelling, considering HIV-mice showed normal ejection fraction, fractional shortening and heart morphology. TNFα-treated mice also exhibited no apparent signs of cardiac hypertrophy. Thus TNFα, at pathophysiological concentrations, appears to modulate ion currents independently of hypertrophy or cardiac remodelling. Cytokine-mediated reductions in K^+ currents were also independent of ion channel mRNA regulation, post-transcriptional (protein) regulation and changes in current kinetics as shown by our studies.[39, 39, 98, 99]

1.3.2 Excitation-Contraction Coupling

As mentioned previously, by using multiple approaches we were able to demonstrate that cytokines are able to modulate directly ion currents. Indeed, TNFα was able induce several adverse changes in cardiac electrical properties similarly to those observed in HIV-mice. However, TNFα is not the only cytokine elevated in HIV mice in particular, and heart disease in general. In fact, Bioplex assays on plasma samples from HIV mice revealed elevated levels of the three main cytokines which we discussed earlier, TNFα, IL-1β and IL-6.[39] Although we obtained some insight on the effects of TNFα, it was still not known if IL-1β and IL-6 could alter other ion currents and contribute to the pathogenesis of heart disease and, if so, what were the underlying mechanisms. Sodium and potassium currents were reduced following chronic exposure to pathophysiological levels of cytokines, yet it remained unknown if the L-type calcium current (I_{CaL}) was affected. The particular importance of I_{CaL} lies in the fact that it plays a crucial role at the plateau phase of the AP but is also a key player in excitation-contraction coupling.[100] Hence, if this current were to be modulated the repercussions could be of electrical nature (i.e. a change in action potential configuration which increase the risk of arrhythmia) but also of contractile nature (e.g. depression of myocardial contractility). But before we discuss the effects of cytokines on I_{CaL}, excitation-contraction coupling (ECC) will be introduced while emphasising on the importance of I_{CaL} in the heart. The L-type calcium channel (LTCC) is

encoded by *CACNA1C,* the α_{1c} gene ($Ca_V1.2$) which is the main functional component of the channel and is considered to be the main isoform in the heart due to its prominent role in ECC.[94] $Ca_V1.3$ and Ca_V 3.1/3.2 are the other calcium channels in the heart however they do not seem to be involved in ventricular ECC and they perfrom other functions, which will not be further discussed in this work. The $Ca_V1.2$ ion channel is comprised of four homologous domains (I–IV), each containing six transmembrane segments (S1–S6), S4 being the voltage sensor and the loops between S5 and S6 form the main pore of the channel.[101-103] Figure 16 illustrates the proposed transmembrane topology of LTCC. The channel has also auxiliary subunits such as β and δ which might be implicated it modulating kinetics or gating properties and channel trafficking to the membrane.[104-106]

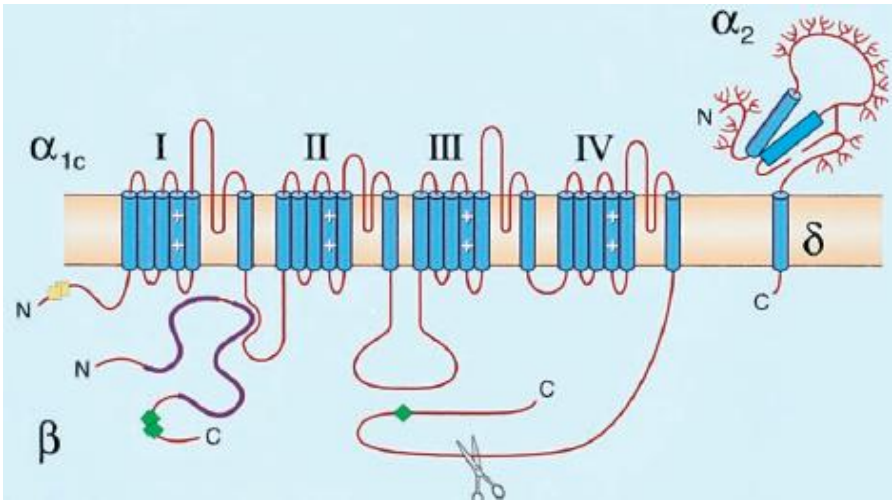


Figure 16. Transmembrane Topology of the L-type Calcium Channel. *Showed are the four domains with six transmembrane segments where S4 is the voltage sensor. When the four domains are folded the S5-S6 loops form the channel pore which conducts calcium ions. Modified from* [101].

The LTCC are located at the invaginations of the sarcolemma termed T-tubules and face the sarcoplasmic reticulum (SR), as shown in figure 18. The presence of LTCC at this location is not random but serves for a functional role. Indeed a certain population of LTCC are found to be opposing the ryanodine receptors (RyR) also known as calcium release channels.[107] This positioning is thought to be the crux of calcium-induced calcium-release (CICR). RyRs are also arranged in organized arrays of ~200 RyR at the junctions between the SR and sarcolemma and beneath the LTCC. The whole arrangement is often referred to

as couplon and there are about 10-25 LTCC for each 100 RyR.[94] CICR implicates many players at the couplon region. In fact, depolarisation of the cell, via the sodium channels, triggers activation of the LTCC leading to an influx of calcium into the cell whereby only a few calcium ions (2-4) can activate a RyR to release calcium from the SR stores, massive calcium release from the SR occurs when several RyR are activated in a couplon. This often leads to a near depletion of SR Ca^{2+} and an increase in intracellular calcium concentration, which favours calcium binding to troponin C on the myofilaments and induces contraction.[108] Termination of the contraction necessitates rapid removal of calcium from the intracellular space. This is achieved by the sarcoplasmic reticulum calcium ATPase (SERCA) which actively pumps Ca^{2+} back into the SR. In addition, sodium calcium exchanger (NCX) also participates in removal of calcium by exchanging 2 intracellular Ca^{2+} for 3 Na^{+}. Finally, the calcium ATPase at the sarcolemma actively extrudes Ca^{2+} out of the cell. Figure 17 summarises ECC and CICR.

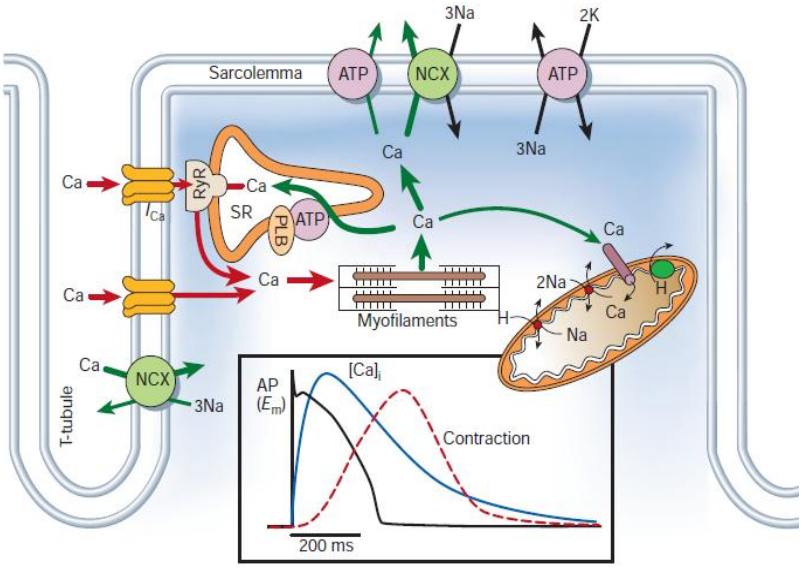


Figure 17. Excitation-contraction Coupling in the Heart. *After a depolarisation, LTCC are activated which drives Ca^{2+} into cell and triggers subsequent calcium release from the SR via RyR in a phenomenon termed CICR. Ca^{2+} binds to myofilaments an induces contraction which is terminated by removal of Ca^{2+} from cytosol mainly via SERCA (ATP) which actively pumps Ca^{2+} back into the SR along with NCX and Ca^{2+} ATPase at the sarcolemma. Modified from [94].*

As with many cellular proteins and processes in heart, ECC in general and LTCCs in particular, are affected by adrenergic receptor signalling. β-adrenergic receptors (β-AR) are

G-protein coupled receptors that are of crucial importance in the heart. Their main function is to induce positive inotropy and lusitropy, in other words increase contraction force and heart rate, after catecholamine binding. β-AR are coupled to heterotrimeric G proteins that stimulate (via G_s) the activity of adenylyl cyclase.[101] Activation of adenylyl cyclase leads to an increase in cyclic AMP (cAMP) levels. This favours binding of cAMP to the regulatory domain of protein kinase A (PKA) thus, dissociating the regulatory and catalytic subunits of the protein. PKA is a serine-threonine kinase and once the catalytic subunit has been dissociated after cAMP binding, it will phosphorylate multiples sites on the LTCC and cause a robust increase in I_{CaL} density.[109] The effects of β-AR/cAMP/PKA pathway on LTCC and ECC have been extensively elaborated and studies have shown that multiple proteins involved in ECC, such as ryanodine receptors (RyR2) and phospholamban, are targets for PKA phosphorylation.[110]

1.3.3 Objective of this Work

ECC is a finely regulated process involving many calcium handling proteins that are subject to phosphorylation and other regulatory modifications in order to insure proper contraction and to maintain control of intracellular calcium concentrations. A key player in ECC is the LTCC, which is responsible for the CICR, one of the first steps in the ECC cascade. Thus modulating the LTCC can have profound effects on ECC and on contraction overall. Indeed, if the properties of LTCC were to be altered, CICR could be impaired or enhanced, which is detrimental in either case. As previously mentioned, Ca^{2+} enters the cell via the LTCC and triggers a large release of Ca^{2+} from the SR. Importantly, on a cellular level, the density of the calcium current, I_{CaL}, will actually correlate with the amount of subsequent Ca^{2+} released from the SR. Thus, if I_{CaL} were to be reduced, the action potential duration would be shortened, and contractility would be reduced or compromised.[84] Indeed, it has been observed that in patients with severe heart failure who suffer for a significant depression in contractility I_{CaL} was considerably reduced. Conversely, if I_{CaL} or the calcium influx were to be augmented, the effects will be an increase in in action potential duration

and an alteration in calcium handling which might prove to be detrimental as well.[111-114] In patients with heart failure it was also observed that in response to a β-adrenergic stimulation, I_{CaL} failed to increase. This means that in cardiac disease there also seems to be an uncoupling of β-adrenergic signalling from the contractile response.[112] Interestingly, in an early study dating from 1989, when supernatants of activated immune cells were added to the culture media of neonatal cardiomyocytes, induction of β-adrenergic stimulation by isoproterenol, a β-receptor agonist, failed to increase myocytes contractility. These effects on contractility were attributed to TNFα and IL-1β; however, it was not investigated whether these effects were caused by alterations in ionic currents of the cardiomyocytes.[115] Recently, it has been shown that knockdown of the L-type Ca^{2+} channel α_{1c} subunit, significantly reduced I_{CaL} but exacerbated hypertrophy and worsened cardiac function in mice subjected to physical activity or isoproterenol stimulation.[116]

Overall, these observations highlight the importance of LTCC in mediating proper cardiac contractility and shaping the action potential. Hence, modulating I_{CaL} could prove to be detrimental, as shown by human subjects of heart failure and animal models. Nonetheless, it is still not well understood how, under disease conditions, LTCC can be modulated. Some studies, along with our previous work suggest that cytokines can directly affect cardiac ion channels thus altering their function, modifying the action potential configuration and increase the susceptibility to arrhythmia and adverse outcomes. Nonetheless, it was not investigated whether cytokines could also affect the LTCC which would further add to their pathogenesis in heart disease by extending their effects to modulation of action potential and reducing contractility. Therefore, the objective of this master's project was to elucidate the effects of the three main pro-inflammatory cytokines in heart disease discussed earlier: TNFα, IL-1β and IL-6 on the cardiac L-type calcium current, while exploring the mechanism by which these cytokines mediate their effects. We used an *in vitro* approach of cultured neonatal ventricular myocytes treated chronically (24 H) with clinically relevant concentrations of cytokines. The calcium current was measured by the patch-clamp technique in voltage-clamp mode. We also investigated some of the potential mechanisms by which cytokines might mediate their effects, notably we explored

the gene regulation of the LTCC and the effects of reactive oxygen species (ROS) on calcium current densities.

2 Methods

2.1 Isolation of Neonatal Mouse Ventricular Myocyte

Often in cardiovascular research investigators need to study processes on a cellular level by using *in vitro* approaches which can yield significant insight on a particular research topic. When studying cardiomyocytes, cultures of either adult or neonatal derived myocytes can be used, with each having their own advantages and disadvantages. The neonatal cultures offer several advantages such as division and replication of cells. Considering neonatal cardiomyocytes are not terminally differentiated and day 1 pups hearts can contain up to 55% dividing cardiomyoblasts. This is one of the reasons why culturing cells from pup hearts older than 48 H yields a poorer quality preparation.[117] In addition, neonatal cultures offer the advantage of yielding tens of millions of viable cardiomyocytes which under normal culture condition retain their phenotype up to weeks and do not dedifferentiate, unlike adult cells which begin losing their T-tubules in as early as 24 H of culture. In the early 1980s Dr. Paul Simpson and colleagues established the neonatal rat culture as a model for studying the cardiac hypertrophic response and since, this model has seen a tremendous expansion in its utilisation and has been the subject of numerous other applications. With the technological developments allowing genetic manipulation of mice, the rat neonatal cardiomyocytes isolation technique was adapted to mice which allowed researchers to focus on their particular target of interest in lines of which either had a transgene, an over-expression or knock-out of the target and all in a controlled environment which was independent of systemic feedback, haemodynamic or hormonal effects. We have chosen the cultured neonatal mouse cardiomyocyte as a model because it offers great flexibility. Indeed, for our purposes this model allows several different cytokines to be tested in much shorter times compared to *in vivo* approaches. For instance, in our previous study examining the effect of chronic TNFα on K^+ currents, mice were treated twice a week for a total of six weeks with recombinant TNFα, whereas in this project in order to administer chronic treatments of cytokines to cultured cardiomyocytes,

we only had to treat the myocytes for 24 to 48 H. In addition, our laboratory has extensive experience with mice models, as well as the techniques and materials that go along with them. This allows more efficient experimentation and for the results of this project to be reconciled with our previous findings.

The neonatal ventricular myocyte was used as previously published.[89] As mentioned earlier, the preparation of neonatal cardiomyocytes cultures require 0-2 days old pups for optimal yield. Personally, I have found cells derived from pups born and used in the preparation in the same day gave much higher cell count and cell quality, at least in terms of membrane quality, a critical factor for patch-clamp experiments. Under sterile conditions, the pups were decapitated, hearts rapidly excised and put in SMEM solution containing $NaHCO_3$, DL-carnitine and $MgSO_4.7H_20$, pH 7.35. The atria were then discarded and the ventricles sliced in order to remove excess blood. A minimum of 15 pups was needed for a good preparation. Fewer pups reduced cell viability and quality seemed rather poor, whereas using significantly more pups also had its challenges since the hearts needed to be rapidly excised an digested without further delay. Having 30-40 hearts proved to be challenging considering the hearts had to remain in the suspension media several minutes which also affected cell viability. Once all the hearts were excised and excess blood removed they were transferred into the enzymatic solution containing the previous solution supplemented with bovine serum albumin, taurine and collagenase. In most cases, experimenters also add trypsin to the digesting solution, which allows faster and more efficient digestion of the hearts. However, we completely avoid trypsin since we use the cells for electrophysiology purposes where cellular membrane quality needs to be preserved, this is achieved by only using collagenase, particularly Yakult (Tokyo, Japan), a gentle means of cell dispersion collagenase. Importantly, since trypsin is a potent protease, it is avoided since it could possibly cleave and modify many surface proteins, notably ion channels, thereby possibly compromising our results. These effects of trypsin have been documented in other cell types.[118]

Once the ventricles were placed into the digestion solution, they were minced and digested for a total of 45-60 minutes. The enzymatic solution was collected every 5 minutes and replaced by fresh one. The collects, which contained cardiac cells, were added to an inactivating solution containing (M-199, 30% foetal bovine serum (FBS), 1.5% insulin (100 U/ml) and 1% PenG/Strep (10000 U/ml). The inactivating solution with its high FBS concentration halts the digestion by inhibiting the collagenase. Once the digestion was completed and all the cardiac cells transferred into the inactivating solution, the tube was centrifuged for 5-7 minutes at 800 rpm in order to pellet the cells. Next, the cells were resuspended in an M-199 solution containing 15% FBS, 1.5% insulin (100 U/ml), and 1% PenG/Strep (10000 U/ml). FBS delivers essential nutrients and growth factors for cardiomyocytes and allows their adherence to the plates, however it also promotes proliferation of non-cardiomyocytes notably fibroblasts and endothelial cells. Therefore, fibroblasts were reduced in the preparation by preplating the whole cell suspension for 20-30 minutes. Since non-cardiomyocytes adhere rather quickly to the plates, after the waiting time, the supernatant was collected and it contained up to 90% cardiomyocytes. It is very common for researchers to use bromodeoxyuridine, a synthetic nucleoside analogue of thymidine which will incorporate into the DNA sequence of cells or arabinosylcytosine another DNA synthesis blocker which together will limit proliferation of fibroblasts.[119] However, since we are using the cardiomyocytes 24 H after initial isolation preplating is sufficient to significantly limit fibroblast count. The last step in the preparation required the suspended cardiomyocyte-enriched media to be plated on coverslips in petri dishes incubated for 24 H at 37°C with 5% CO_2 along with the appropriate treatments administered at the time of plating.

The main treatments employed in these studies consisted of recombinant TNFα, IL-1β or IL-6, or other treatments such as antioxidants that were also used by direct administration into the culture media. The cytokines were diluted in water, aliquoted and stored at -20°C in accordance with manufacturer's instructions. The final concentration of all three cytokines was 30 pg/mL. Unlike most studies that use cytokines at a concentration that is tens to thousands of folds higher, our concentration is drastically lower than most

reports. We employed clinically relevant concentrations that were observed in heart failure patients and the HIV-mouse model.[39-41] Concentration-dependent effects will be discussed later.

2.2 Electrophysiology: The Patch-Clamp Technique

The patch-clamp technique is the main electrophysiology technique used to measure ion flow via ion channels and across biological membranes. The technique was developed in the beginning of the late 1970s by Erwin Neher and Bert Sakmann who subsequently, in 1991, shared the Nobel Prize in Physiology or Medicine for their discovery of the fundamental role of ion channels and how they can shape action potentials.[120, 121] In order to successfully record ionic currents, the patch-clamp technique requires several pieces of equipment and devices along with a set of technical skills. The basic principle of the technique is illustrated in figure 18. A slide of cardiomyocytes is placed in perfused bath on the plate of an inverted microscope. A glass pipette or microelectrode is then moved across the bath and towards the cell of

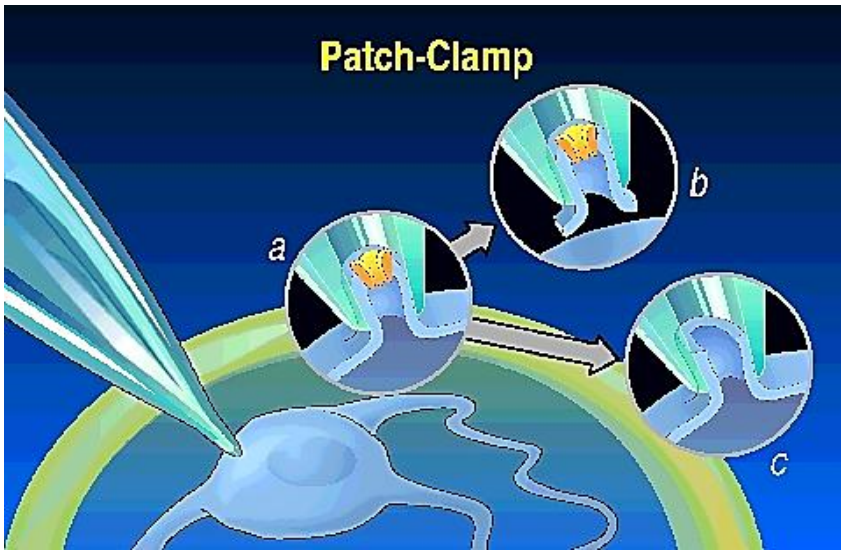


Figure 18. The patch-clamp technique. *A micropipette is oriented over the cell lowered until it touches the surface. Gentle application of pressure will suck a part of the membrane into the pipette (a). Next, this section of the plasma membrane containing one or two ion channels can be completely excised (b) or the total flux across all ion channels of the cell can be recorded using the whole cell configuration (c) where the pipette contents diffuse into the cytosol via a rupture of cellular membrane. Source: www.ipmc.cnrs.fr retrieved 01/05/12.*

interest. The pipette will be lowered very gently until it touches the surface of the cell, subsequently a light negative pressure will be applied in order to increase the suction and form a tight seal between the tip of the pipette and the cell membrane, known as the giga-ohm seal (resistance $>10^9$ Ω), a seal of extremely high resistance. This high resistance will electrically isolate this section of the membrane from the rest of the cell. Additional negative pressure can then be applied in order to rupture the membrane and gain access to the cytosol of the cell. This is known as the whole-cell configuration (figure 18 c) as it allows one to record activity of all the ion channels in the cell at once. Conversely, detaching parts of the membrane or remaining in a cell-attached mode, where one or two ion channels are under the pipette, will allow recording of individual ion channel activity (figure 18 a and b).[120]

We used the whole-cell configuration to record global currents and to gain access to all the ion channels of the cardiomyocytes. In order to record one type of currents, such as L-type calcium current in our case, one should isolated the calcium current from all the others (sodium, potassium etc.). Several methods have been derived in order to isolate ion currents. One of the main techniques to achieve this takes advantage of the voltage properties of each different ion channel. For example, ventricular myocyte L-type calcium currents are maximal arounf 0 mV whereas sodium currents peak at around -45 mV. Thus, a short prepulse of -45 mV can be applied before recording calcium currents. This will serve to inactivate the sodium channels, thus omitting their contribution from the recording. Modifying the perfusion and pipette solutions is also critical. In our case, the solution in which the cardiomyocytes are perfused contains neither sodium nor potassium, hence, there will be virtually no contribution of these channels to our current recordings due to ion channel selectivity and permeability. Lastly, some investigators use pharmacological agents in order to block specific ion channels, such as tetrodotoxin to block sodium channels or 4-aminopyridine to block the 4-aminopyridine sensitive potassium channels such as Kv4.2/4.3 or Kv1.5.[90]

In our experiments, the borosilicate glass pipettes had a resistance of 2-4 MΩ and were filled with and internal solution consisting of (in mM): 100 Aspartic Acid, 70 CsOH, 40 CsCl, 2 $MgCl_2$, 4 MgATP, 10 EGTA, 10 HEPES (pH adjusted to 7.2 with CsOH). The bath solution, or external, in which cardiomyocytes were perfused contained (in mM): 145 TEACl, 10 CsCl, 2 $CaCl_2$, 0.5 $MgCl_2$, 5 HEPES, 5.5 glucose (pH adjusted to 7.4 with CsOH) and perfused at 36 ± 1 °C. The data were corrected offline for a -10 mV liquid junction potential that was calculated using the pClamp 10.2 software. The internal solution, which slowly diffuses into the opened cell, is electronically connected via an electrode to an amplifier which serves to impose and record current. A digitiser links the amplifier to the computer and transforms the electrical measurements and changes in voltage or current into computerised data. [122]

The electrophysiology experiments were done as previously published.[122, 123] The neonatal ventricular myocytes were held at -50 mV before the recordings. Since a holding potential of -50 mV inactivates sodium and T-type calcium currents a prepulse was not necessary. The recording protocol consisted of a series of step voltages from ranging from -50 mV to +70 mV, in 10 mV increments, each step lasting 250 ms. The pClamp 10.2 suite (Molecular Devices, Foster City, USA) was used to perform calcium current recording protocols, record and analyse data. In addition, the recordings were low-pass filtered at 1 kHz with a 4-pole Bessel analogue filter and digitized at 4-10 kHz. Because in the whole-cell configuration the amount of current is proportional to the cell size, i.e. larger cells will yield larger currents and vice versa, all currents were normalized to cell capacitance (corresponds to the electrical capacity of a membrane to store electric charge that represents total membrane surface area) and expressed in terms of pico-Ampere per pico-Farad (pA/pF). The amplitude of each current trace, obtained at a given voltage step (from -50 to +70 mV), was quantified, normalised to cell capacitance then plotted on a graph where the x-axis corresponded to the imposed voltage and the y-axis the resultant measured current. This plot is called the I-V curve, or whole-cell current-voltage relationship curve, and is considered the standard means of expressing current density across a broad range of voltages which will indicate how the channels behave globally at each voltage.

A successful preparation yields 6 to 10 35-mm petri dishes depending on how many hearts were used. The patch-clamp experiments were then performed on these cells where typically 7-10 cells could be recorded per day. For each experimental condition the IV curve was generated from a minimum of three different preparations. The total number of patch-clamped cells was almost the same between each series of cell preparations. Every IV curve contained its own original set of recordings, in other words, none of the data was reused or pooled from one IV curve to another.

2.3 Quantitative Polymerase Chain Reaction (qPCR)

The development of the polymerase chain reaction, largely attributed to Kary Mullis in 1983, revolutionised the field of molecular biology, and in essence allowed us 20 years later to sequence the human genome.[124] The technique was revolutionary in a sense that it permitted rapid amplification of DNA sequences using a couple of primers that would anneal on a DNA template, extend the template then the newly synthesised product would be separated and the two DNA strands would serve as two new templates for another set of primers. This process would be repeated several times until significant amount of DNA was generated, a technique which transitioned researchers into a new era of molecular biology which facilitated gene cloning, sequencing and overtime gave rise to the field of functional genomics. The basic principle of PCR is quite simple. A double stranded DNA is put in a mixture with primers, oligonucleotides and a DNA-dependent DNA polymerase usually the thermostable DNA polymerase derived from the thermophilic bacterium *Thermus aquaticus* which is stable up to 95°C.[125] First, the DNA strands are separated by heat, the primers would then anneal to specific regions of the DNA sequences. The polymerase would use the primers as a start point to extend the DNA templates and synthesise a new strand of DNA by incorporating the supplied oligonucleotides at a lower temperature. The reaction is then repeated several times until sufficient DNA is produced. Of note, since two strands of DNA are separated at each reaction in order to yield two double stranded DNA,

the amplification of DNA product thus assumes an exponential function. Thus, about $1x10^9$ copies of the initial DNA strands could be generated in 30 steps

Several years later a quantitative PCR technique was developed which allowed for the amplification reaction to be followed step by step and the quantity of DNA to be accurately quantified. Several qPCR techniques exist, we will consider the one which makes use of SYBR green, a fluorescent die which binds double stranded DNA non-specifically, thus will fluoresce proportionately to the quantity of DNA. For our purposes we used qPCR to quantify gene expression of ion channels. First, total RNA was isolated from the neonatal cell cultures with an RNeasy Fibrous Tissue kit (Qiagen) including a treatment with DNaseI to prevent contamination by genomic DNA and according to the manufacturer's protocol. cDNA was then synthesized with SuperScriptIII first-strand (InVitrogen) and primers specific for $Ca_V1.2$. The qPCR was subsequently performed with Platinum SYBR Green qPCR Supermix (InVitrogen) using the real-time PCR system (MX3005P qPCR system, Stratagene). qPCR results for $Ca_V1.2$ were normalised to the housekeeping gene 18S ribosomal RNA and adjusted with an internal calibrator which was conserved in every plate in order to insure reproducibility. All of the qPCR were performed with n=3 for each condition. A single n corresponded to a total mRNA extracted from 4 petri dishes, thus for a single n, 15-20 pups were needed.

2.4 Reactive Oxygen Species Assay

Reactive oxygen species (ROS) is at general term which refers to a broad range of reactive molecules and free radicals derived from oxygen which include potent oxidants such as H_2O_2 and O_2^- that can oxidise several other molecules and transform them into oxidising agents such as HO^- or HO_2. It is still not fully understood how ROS can affect the cells and organisms. ROS signalling pathways and their secondary effects are still the subject of investigations. Recent work has demonstrated that ROS may be implicated in

causing direct DNA damage, inducing apoptosis, activation gene transcription and several other signalling cascades. [126]

As a first step in studying oxidative stress, intracellular ROS levels have to be quantified and for that purpose several techniques have been devised.[127] In general, all the techniques make use of molecules that once oxidised emit fluorescence. There are several molecules that are commercially available, but if one was to quantify intracellular ROS, ideally the molecules needs to be cell permeable, exhibit high retention and fluorescence in response to ROS and also not to cause severe toxicity to the cell. A cell permeable ROS probe which has been extensively used is the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA).[128] However, for the current study we used 5-(and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), which is a chloromethyl derivative of H_2DCFDA that diffuses much less out of cell thus, exhibits higher intracellular retention and allows better analysis of fluorescence.[129] As already mentioned, CM-H_2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases. One the product has been de-esterified,

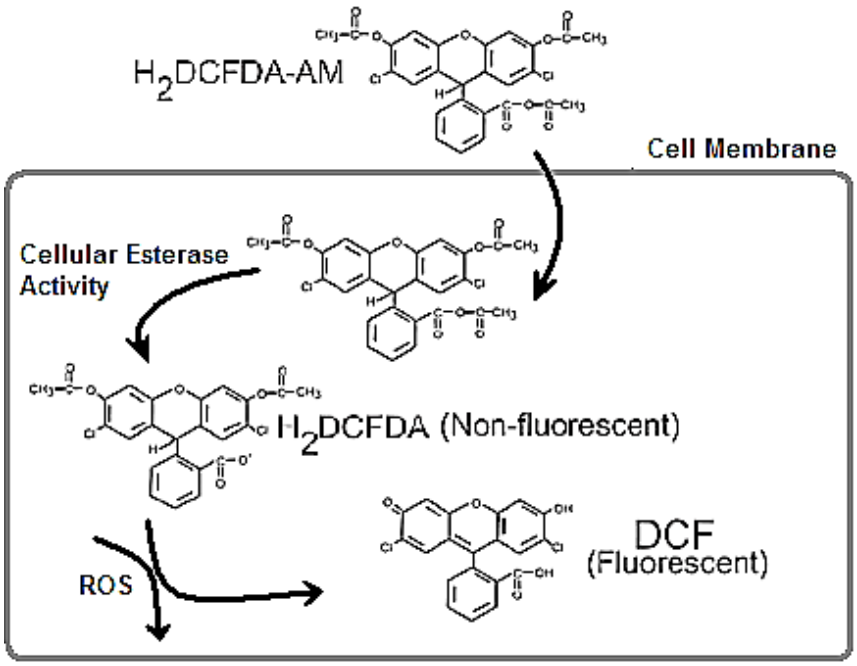


Figure 19. The Reactive Oxidative Species Fluorescent Molecular Probe. *ROS probe H_2DCFDA readily diffuses into the cells where the intrinsic esterase activity cleaves the ester groups on the compound rendering it fluorescent upon oxidation by ROS.*

oxidation by ROS occurs which yields a fluorescent molecule that is retained inside the cell. Figure 20 summarises the basic functional principle of the probe.

As with any other fluorescent compound, a consistent and empirically optimised protocol must be used in order to reduce variability of results. Loading cells for ROS assays is relatively simple however there are many aspects of the technique to which one should pay particular attention.

The first step of the assay was to dissolve the ready-packaged probe (50 μg) in DMSO and then load the petri dish containing the cells with a final concentration of 5 μM, with total DMSO concentration < 0.1%. The petri was then placed back in the incubator for 30 minutes. Subsequently, the media was removed and cells gently washed three times with a 37°C M199 cell culture media supplemented with insulin, and free of phenol red. The petri dish was then placed on top of a Carl Zeiss LSM 710 confocal microscope in a temperature and CO_2 controlled chamber (37°C/5%). A few minutes afterwards, the probe was excited at 480 nm and emitted light collected from 520 nm. The fluorescence was quantified using the LSM software where the sum of intensities of all individual pixels of a cell was divided by the cell area in order to give a fluorescence density.

Since H_2DCFDA is not ratiometric probes and is susceptible to oxidation by various different agents to different extents and fluorescence is affected by the intracellular antioxidant levels amongst other variables, the fluorescence is therefore not directly proportional to the amount of ROS. This means that the absolute ROS concentration cannot be obtained by measuring fluorescence. The approach thus serves more as a qualitative measurement than quantitative. In order to limit variability, conditions were strictly controlled and another approach of estimating ROS levels was devised. The approach basically renders every cell its own control. At t=0 an initial image was taken, then the cell was subjected to a constant ROS stimulation via the microscope laser, just before the fluorescence signal saturates which was t=5 min. The fluorescence of each time point was then be measured and expressed as an F_0/F_1 ratio. This served to normalise for basal fluorescent intensities which might vary from cell to cell. Additionally, by plotting the

intensity of the fluorescent signal from all the time points between t=0 and t=5 vs. time one could get a curve that shows a basal fluorescence level which increases then plateaus. Overall, cells that have been subjected to treatments such as peroxide or ROS-inducing drugs will show a much steeper slope to saturation and the F_0/F_1 ratio will be usually higher. Since this protocol uses a laser to induce ROS, in cells where the fluorescence slope is steeper and the plateau level higher this would indicate that either ROS was significantly increased or anti-oxidant abilities of the cell have been compromised which in either case is detrimental. All the experiments were performed on cultured neonatal ventricular cardiomyocytes, thus entire fields containing tens of cells were imaged and analysed. Three different petri dishes which were derived from at least two different preparations were used for each condition tested. The fluorescence was quantified using the computer software Zen (Zeiss, Germany). Fluorescence signal was normalised to cell area and expressed as density. Lastly, it is important to note that this fluorescence approach to measure ROS using H_2DCFDA is not ideal and should be always complemented with another approach in order to validate and allow proper interpretation of the findings. Our findings were always complemented by electrophysiological measurements.

2.5 Statistical Analysis

All results are expressed as mean ± standard error of the mean (SEM). Statistical significance was considered for p-values < 0.05. For electrophysiological recordings, different mean data IV curves were compared using unpaired Student's T-test or 1-way ANOVA. In the ROS experiments fluorescence densities of all analysed cells were averaged at t=F0 and t=F1 and a paired Student's T-test was used for statistical analysis.

3 Results

3.1 Effect of TNFα on L-type Calcium Current

The first cytokine that was investigated was TNFα. Since we have previously shown that TNFα is elevated in HIV mice, and that chronically treating wild type mice with TNFα significantly reduced several potassium currents, we hypothesised that TNFα could also reduce the cardiac L-type current. This would explain the depression in contractility observed in some studies investigating TNFα[54, 75] and additionally contribute further to the worsening of electrical remodelling by affecting I_{CaL} on top of the K^+ and Na^+ currents.

Figure 20 shows the patch-clamp data obtained after neonatal cardiomyocytes were treated for 24 H with 30 pg/mL of TNFα. The data shows no reduction in calcium current at all. Thus, unlike our previous findings which showed that TNFα significantly reduced potassium currents.[99] This cytokines appeared to have no effect on the L-type calcium current in neonatal ventricular myocytes. Since no significant differences in I_{CaL} were noted after TNFα treatment, we tried in a complementary approach to increase the concentration of TNFα 33 times higher up to 1 ng/mL, which is not a clinically relevant concentration but is within the range of what has been reported in the literature.[74] Figure 21 shows the mean data for I_{CaL} recorded from CTL and TNFα (1 ng/mL for 24 H) treated cells where the mean IV curve shows no significant decrease in current density.

Since no differences in calcium current density were observed at supraphysiological concentrations of TNFα, we concluded that TNFα does not affect I_{CaL} in neonatal ventricular myocytes, and from here we focused onto the second pro-inflammatory cytokines of our investigation, interleukin 1β.

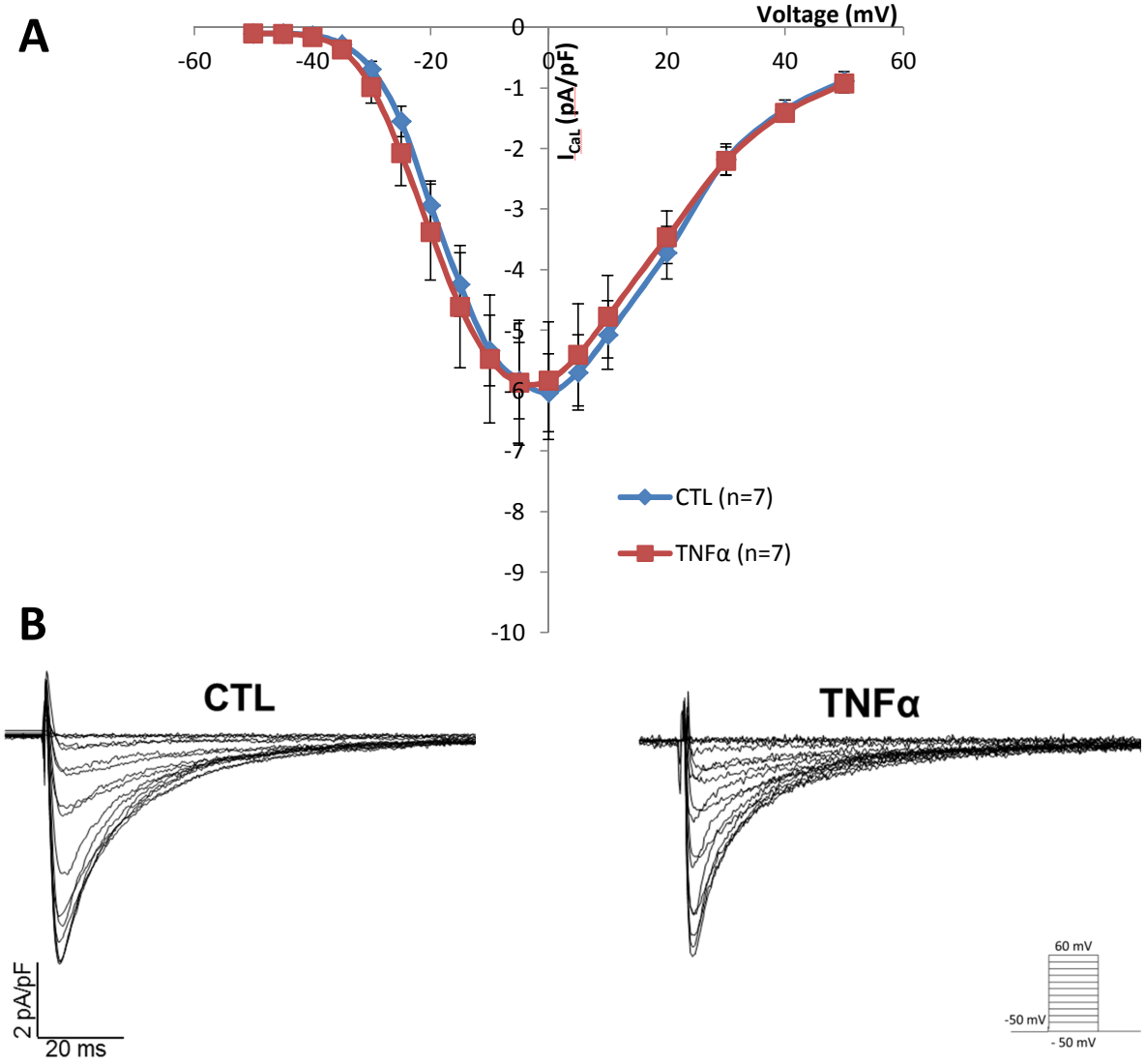


Figure 20. Mean IV curve for I_{CaL} in TNFα treated cardiomyocytes. *Panel A shows mean IV curve for I_{CaL} recorded in control (CTL) and TNFα 30 pg/mL for 24H treated neonatal ventricular mouse myocytes. At 0 mV I_{CaL} density expressed in pA/pF: CTL: -6.0±0.6, n=7 and, TNFα: -5.8±1.0, n=7, p>0.05. Current recordings were derived from at least 3 different experiments. Representative examples of raw data recordings showing the calcium current in both CTL and TNFα treated cells are presented in panel B.*

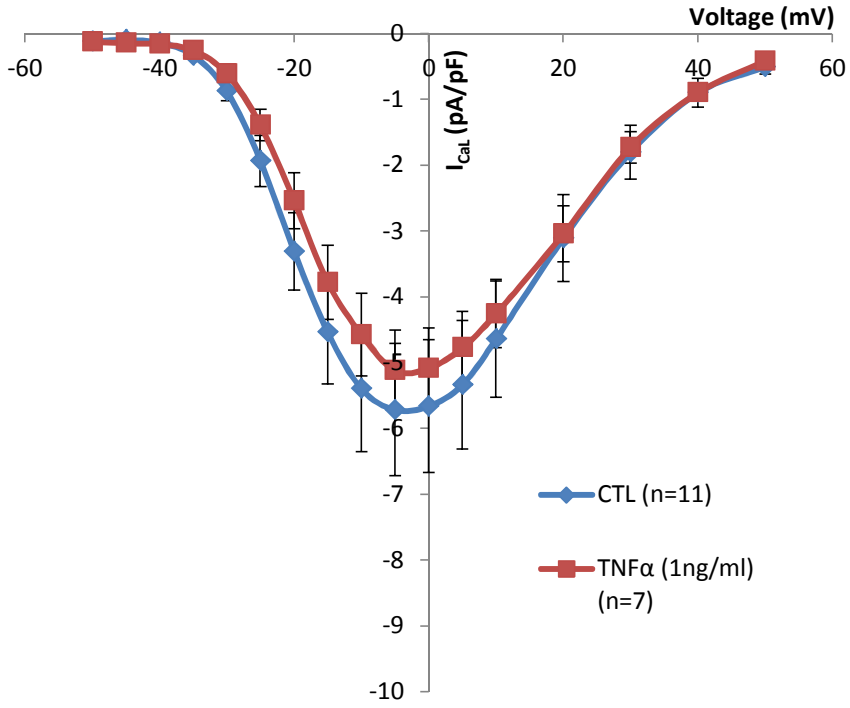


Figure 21. Mean IV curve for I_{CaL} in cardiomyocytes treated with 1 ng/mL TNFα. *Mean IV curve for I_{CaL} recorded in control (CTL) and TNFα treated (TNFα) at 1 ng/mL for 24 H in neonatal mouse ventricular myocytes. I_{CaL} density (pA/pF) at 0 mV: CTL: -5.7±1.0, n=11 and, TNFα: -5.1±0.61, n=7, p=NS. Current recordings were derived from at least 3 different experiments.*

3.2 Effects of IL-1β on the L-type Calcium Current

Using the same approach as in the TNFα-treated cells we subsequently tested the effects of another cytokine, IL-1β on I_{CaL}. Figure 22 shows the mean IV curve I_{CaL} recorded from control and IL-1β-treated mouse ventricular myocytes. The data indicates a 36% decrease in the density.

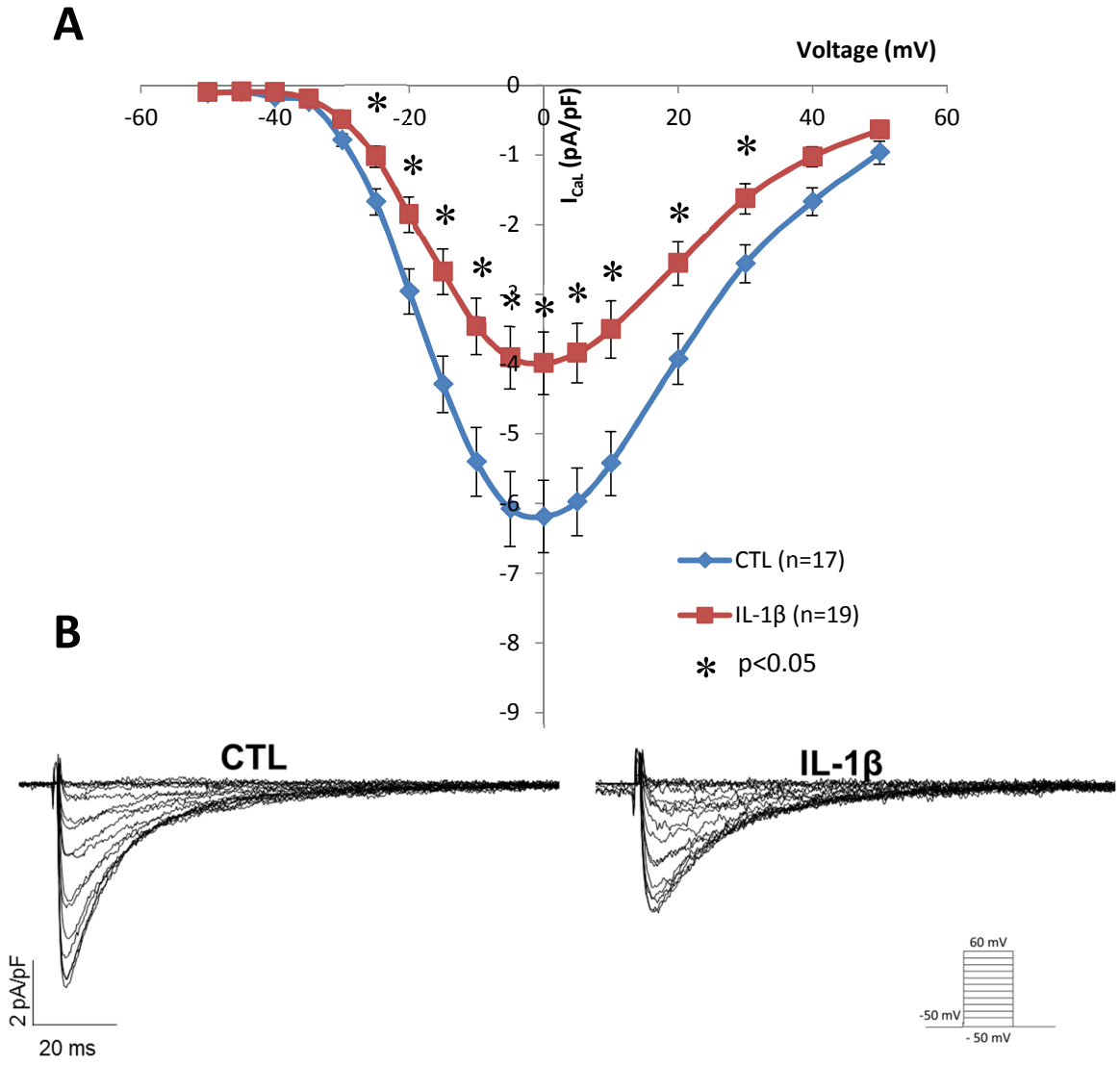


Figure 22. Mean IV curve for I_{CaL} in IL-1β treated cardiomyocytes. *Panel A shows mean IV curve for I_{CaL} recorded from control (CTL) and IL-1β treated (IL-1β), (at 30 pg/mL) neonatal ventricular myocytes. I_{CaL} current density (pA/pF) of at 0 mV: CTL: -6.2±0.5, n=17 and, IL-1β: -4.0±0.5, n=19 p<0.05. Current recordings were derived from at least 3 different experiments. Panel B shows typical raw data recordings obtained from both conditions.*

3.3 Effects of Combined TNFα and IL-1β on the L-Type Calcium Current

The fact that TNFα did not affect I_{CaL} whereas a significant reduction in current density was seen after a 24 H treatment with IL-1β highlighted the distinct effect of each cytokine in mediating its effect. In fact, many studies have reported that TNFα and IL-1β could often be recruited together as part of the inflammatory response and can synergistically mediate their response. Notably, this has been reported in the context of cardiac contractility, where presence of both cytokines had a synergistic effect in depressing contractility of cardiac tissue.[75, 130, 131] Thus, using the same protocol as previously, we treated neonatal mouse ventricular myocytes with 30 pg/mL of TNFα and IL-1β. The cytokines were administered concurrently in order to investigate any synergistical effect they might have on I_{CaL}. Figure 23 shows mean IV curve traces which indicate a decrease in I_{CaL} similar to the one observed when NMVM were treated with only IL-1β thus demonstrating that TNFα and IL-1β have no synergistic effect on I_{CaL}. Total reduction in I_{CaL} compared to the control cells were of 34%, which is a similar reduction compared to IL-1β-treated NMVM.

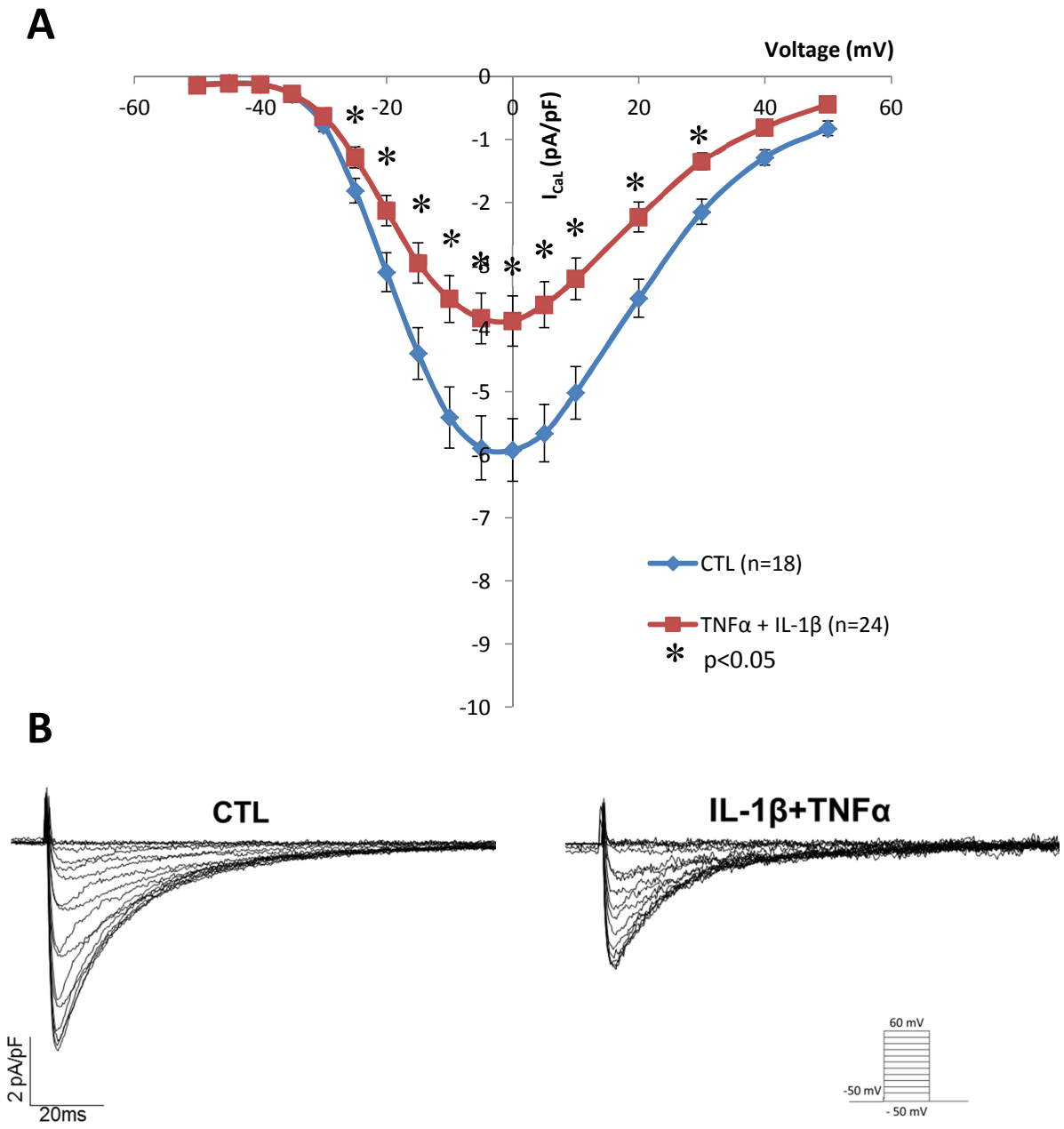


Figure 23. Mean IV curve for I_{CaL} in TNFα + IL-1β treated cardiomyocytes. *Panel A shows mean IV curve for I_{CaL} recorded from of control (CTL) and TNFα + IL-1β treated (30 pg/mL for 24 H), NMVM. I_{CaL} current density (pA/pF) at 0 mV: CTL: -5.9±0.5, n=18 and, TNFα + IL-1β: -3.9±0.4, n=24 p<0.05. Current recording were obtained from at least 3 different experiments. Panel B shows typical raw data recordings obtained from both conditions.*

3.4 Effects of IL-6 on the L-type Calcium Current

The last of the three cytokines examined was IL-6. NMVM were treated for 24 H with 30 pg/mL of IL-6 then patched in order to record I_{CaL}. As seen on the mean IV curves of figure 24 IL-6 significantly decreased I_{CaL} by 21%.

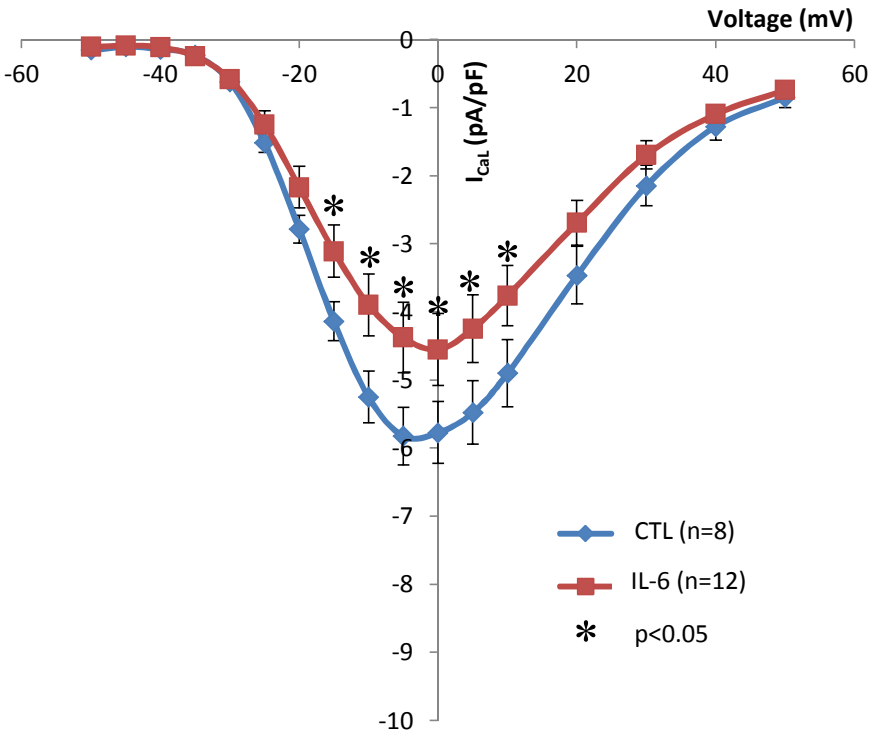


Figure 24. Mean IV curve for I_{CaL} IL-6 treated cardiomyocytes. *Mean IV curve for I_{CaL} recorded in control (CTL) and treated (IL-6) neonatal ventricular myocytes. I_{CaL} density at 0 mV, CTL: -5.8±0.5, n=8 and, IL-6: -4.6±0.5, n=12 p<0.05. Current recording were obtained from at least 3 different experiments.*

Despite the significant decrease in I_{CaL} after a 24 H IL-6 treatment, the effects of IL-6, at least at the present concentration, were not as potent as those of IL-1β. Therefore, the

following experimentation was focused primarily on IL-1β and in particular the mechanisms by which IL-1β depressed I_{CaL} by 36%.

3.5 Effects of TNFα and IL-1β on the L-type calcium Channel Gene Expression

In order to elucidate the mechanisms by which IL-1β mediates its effect on I_{CaL}, the mRNA abundance of the alpha subunit $Ca_V1.2$, which encodes for I_{CaL} was assayed using qPCR. Indeed, if IL-1β decreased I_{CaL} by a transcriptional mechanism, it would have been reasonable to find a decrease in the mRNA levels of $Ca_V1.2$.

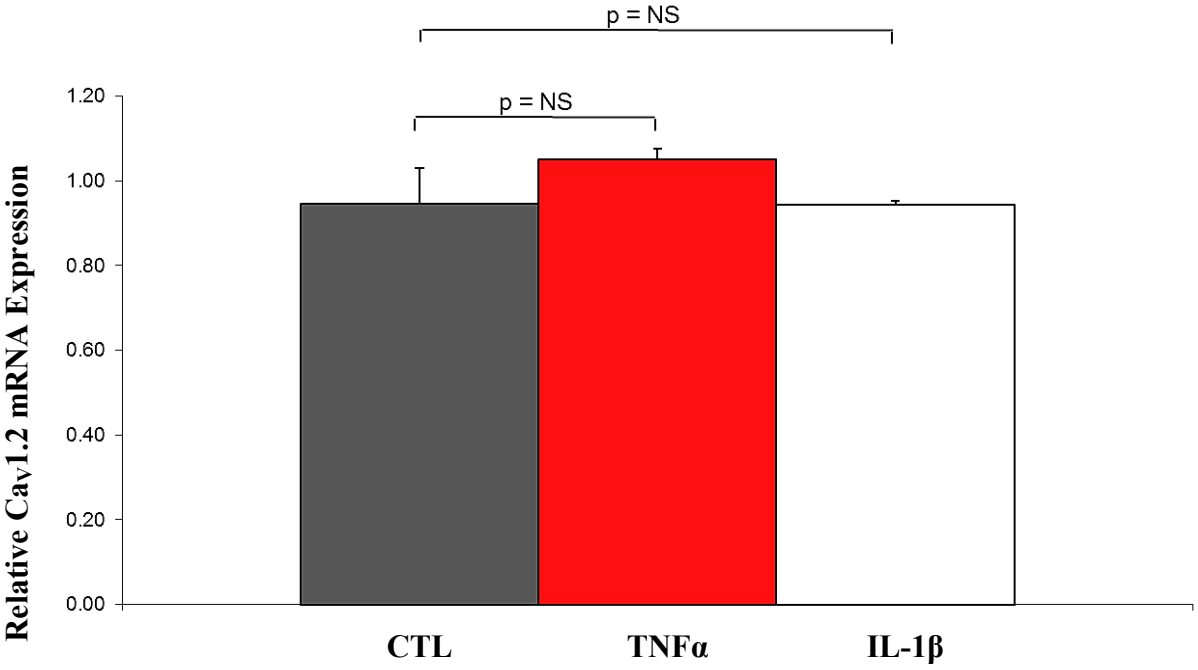


Figure 25. Effects of Cytokines on mRNA Expression of $Ca_V1.2$. *Quantitative PCR showing mRNA levels for $Ca_V1.2$ under different conditions: control (CTL), TNFα and IL-1β. (n=3 for each condition) Each n was obtained from NMVM isolated from 15-20 hearts and cultured 24 H under aforementioned conditions. Each sample was analysed in triplicate.*

Moreover, in our previous study examining the effects of TNFα on K^+ currents, we showed that TNFα significantly decreased K^+ currents without altering the mRNA expression levels of either of the corresponding K^+ channel α-subunits.[99] Thus we investigated the effects of

TNFα on mRNA of $Ca_V1.2$ even though I_{CaL} was not affected. As shown by figure 25, the $Ca_V1.2$ mRNA was unchanged by TNFα or IL-1β. Based on these and findings we concluded that cytokines, at pathophysiological concentrations, decrease ion current densities without affecting the expression of the main α subunit of ion channels. Interestingly, the TNFα treated mice did not only have an unchanged mRNA abundance of K^+ channel, but at the protein level none of the K^+ ion channels were decreased.[99] We therefore hypothesised that these cytokines alter ionic current densities by modulating intracellular signalling cascades regulation ion channel function.

3.6 Cytokines and Oxidative Stress

Several studies have shown that pro-inflammatory cytokines are potent inducers of oxidative stress in heart disease and various cell types.[53, 132, 133] Thus, we tested whether IL-1β, the cytokine which depressed I_{CaL} the most, could induce ROS in a manner that would affect the calcium current. Figure 26 shows mean fluorescence data for the ROS assay under control and cytokine treated conditions. Overall the data showed that IL-1β is capable of inducing oxidative stress. After stimulation with the laser, ROS levels were higher in control cells compaired to IL-1β-treated cells. This suggests that IL-1β increases ROS or dimishes the cellular levels of antioxidants. Typical microscopy examples showing NMVM after laser stimulation are displayed below, along with mean data.

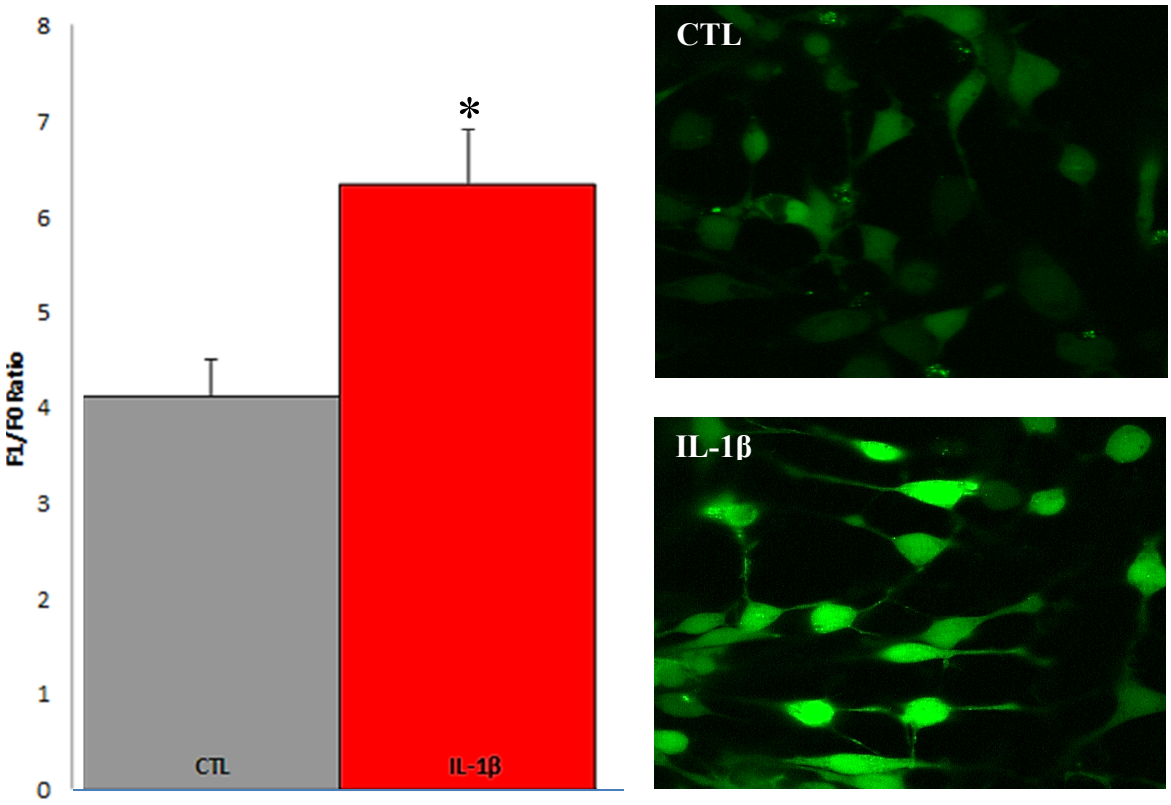


Figure 26. Confocal Analysis of IL-1β-Induced Oxidative Stress. *ROS assay showing mean H_2DCFDA fluorescence intensity of CTL and IL-1β treated NMVM. Pictures on the right represent typical confocal microscopy at the F_1 time, i.e. after laser induced ROS production. (* $p<0.05$ vs. CTL). A minimum of 2 experiments were conducted where at least 25 cells per condition were analysed to generate mean data.*

Of note, TNFα also significantly increased ROS production. The ROS production seemed to be even more important when TNFα and IL-1β were combined in the treatment of NMVM. Subsequently, we tested whether antioxidants could reduce the ROS production. To accomplish this we used apocyanin, a NADPH oxidase inhibitor, and polyethylene

glycol linked to superoxide dismutase (PEG-SOD) a naturally occurring antioxidant enzyme which catalyses the reaction of transforming O_2^- into H_2O_2, a far less potent oxidising agent. Cytokines were administered concurrently with antioxidants to NMVM. Figure 27 shows the effect of apocyanin on IL-1β induced ROS production.

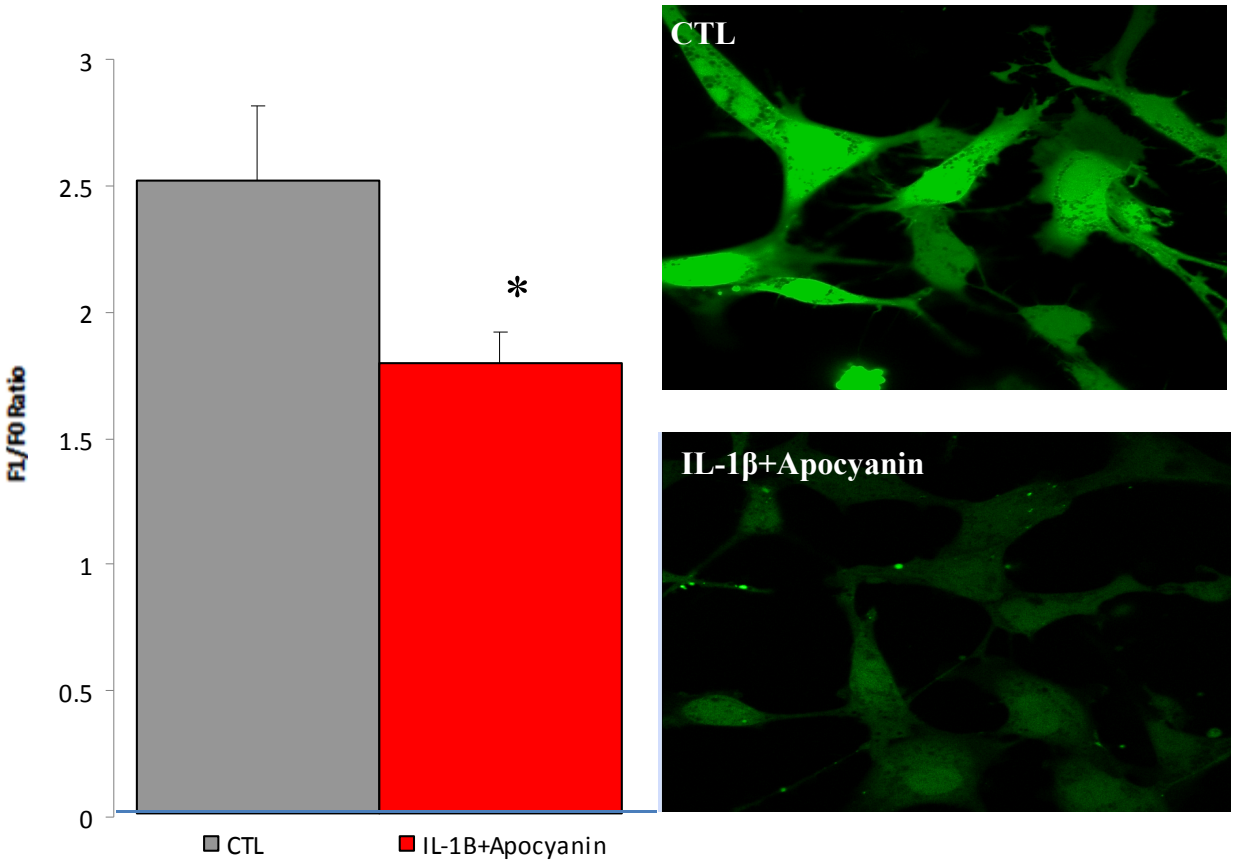


Figure 27. Effects of apocyanin on ROS levels. *ROS assay showing mean H_2DCFDA fluorescence intensity of CTL and IL-1β+Apocyanin treated NMVM. Pictures on the right represent typical confocal microscopy at the F1 time, i.e. after laser induced ROS production.* $p<0.05$. A minimum of 2 experiments were conducted where at least 25 cells per condition were analysed to generate mean data.*

We then tried to obverse whether the decrease in I_{CaL} density was reversed by antioxidants. Despite the success of antioxidants at reducing ROS levels, when NMVM were patch-clamped in the presence of IL-1β and apocyanin or PEG-SOD, the depression in I_{CaL} density persisted and was not reversed at all. Figures 28 and 29 show mean IV curve data for IL-1β and IL-1β+antioxidant-treated cells.

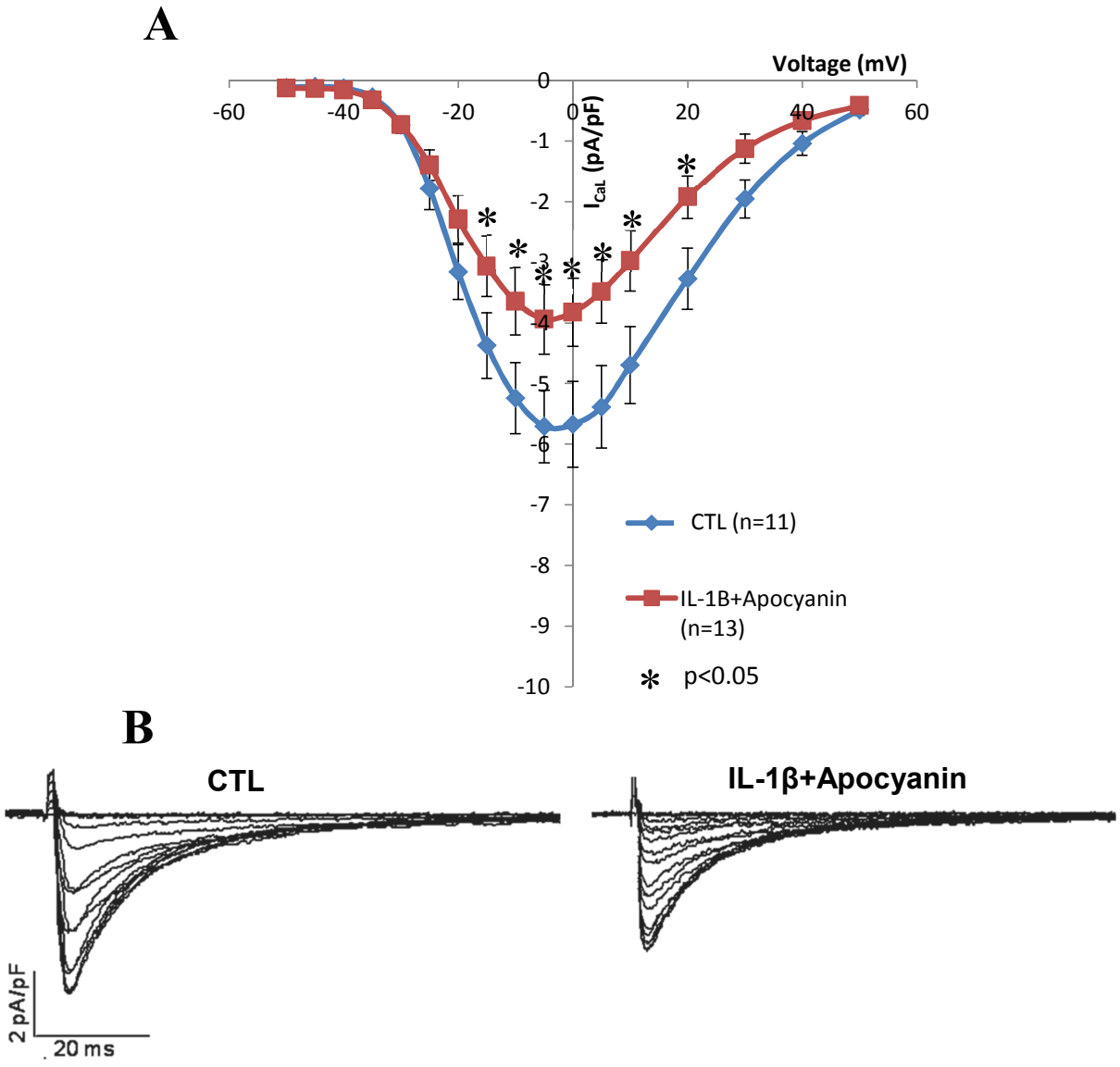
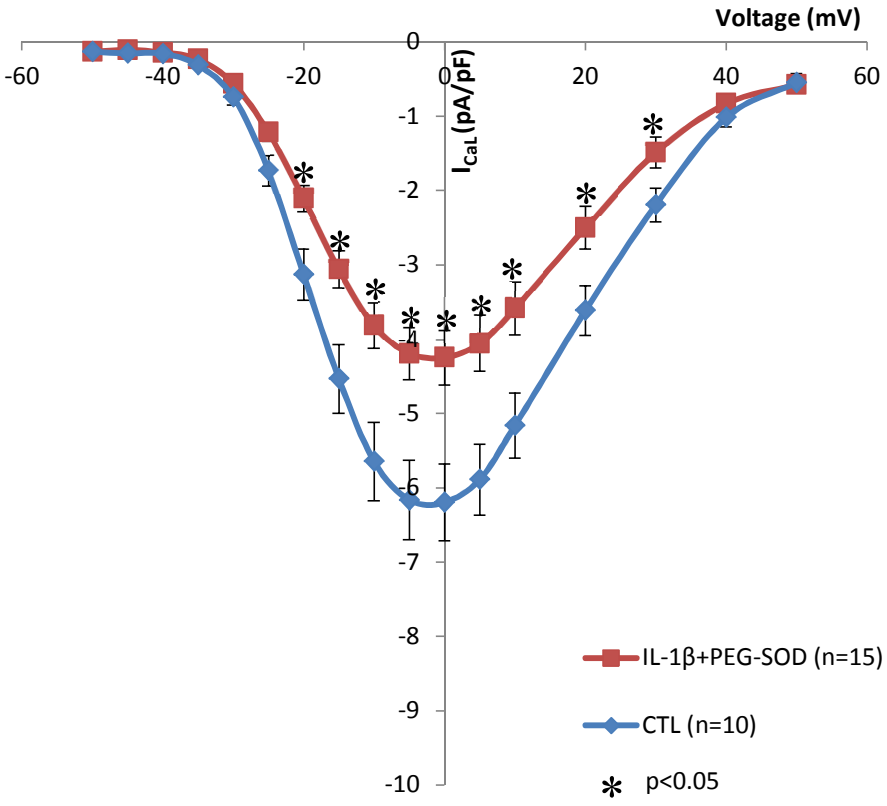


Figure 28. Mean IV curve for I_{CaL} in IL-1β+Apocyanin-treated cardiomyocytes. *Panel A shows mean IV curve for I_{CaL} recorded from control (CTL) and treated (IL-1β + apocyanin) neonatal ventricular myocytes. Current recording were obtained from at least 3 different experiments. Panel B shows typical raw data recordings obtained from both condition.*

A



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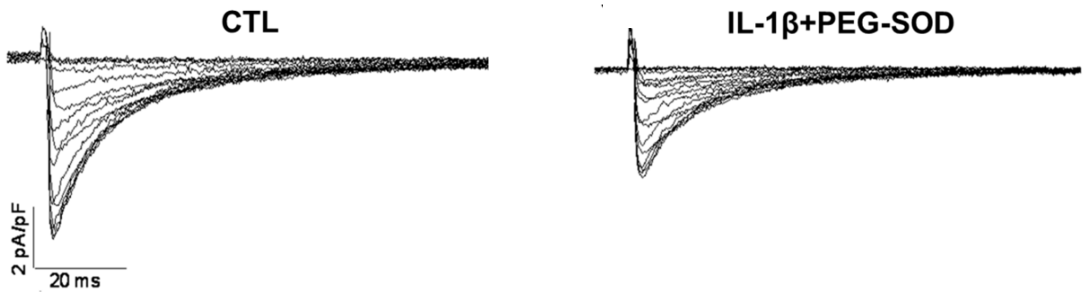

Figure 29. Mean IV curve for I_{CaL} in IL-1β+PEG-SOD treated cardiomyocytes. *Panel A shows mean IV curve for I_{CaL} recorded from control (CTL) and treated (IL-1β + PEG-SOD) neonatal ventricular myocytes. Current recording were obtained from at least 3 different experiments. Panel B shows typical raw data recordings obtained from both condition.*

Overall the data clearly indicated that the calcium current density could not be recovered by treating cells with antioxidants aimed at reducing NADPH oxidase activity and superoxide anion concentration.

3.7 Effects of IL-1β on Cellular Hypertrophy and I_{CaL} in Summary

An important aspect of this study relates to the cytokine concentration that was used. We chose to use pathophysiologically relevant concentrations but we also showed that at these concentrations the effects of cytokines are distinct from what has been reported in the literature where much higher doses were used. Indeed, several studies have shown that cytokines are able to induce cardiomyocyte hypertrophy.[53, 119, 134] However, as previously mentioned the "ng/mL" range of cytokine concentrations are several fold higher than the clinical pathophysiological concentration seen in heart failure or several other cardiac diseases. Figure 30 shows the effect of IL-1β concentration on cellular capacitance. At 1 ng/mL, IL-1β significantly increased cell capacitance suggesting an increase in cell size.

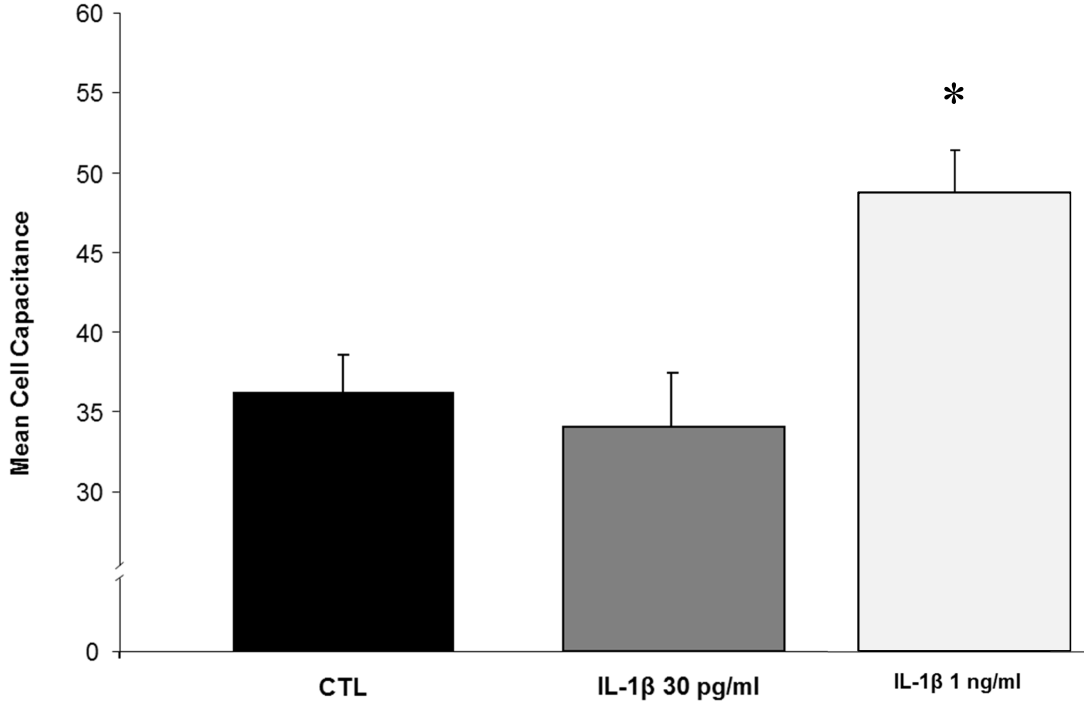

Figure 30. Effects of IL-1β on Cardiomyocyte Hypertrophy. *NMVM mean cellular capacitance under three different conditions. Control (CTL n=20), IL-1β at a concentration of 30 pg/mL (n=22) and 1 ng/mL (n=12). * p<0.05 vs CTL.*

Cellular capacitance is an electrical estimation of cellular surface. Figure 30 clearly shows that when 1 ng/mL of IL-1β was used to treat the NMVM cellular capacitance significantly increase which suggested that cellular hypertrophy has occurred. At higher concentration of IL-1β the decrease in I_{CaL} tended to be slightly larger as shown by figure 31 which also summarises the effects of various treatments on the density of I_{CaL}.

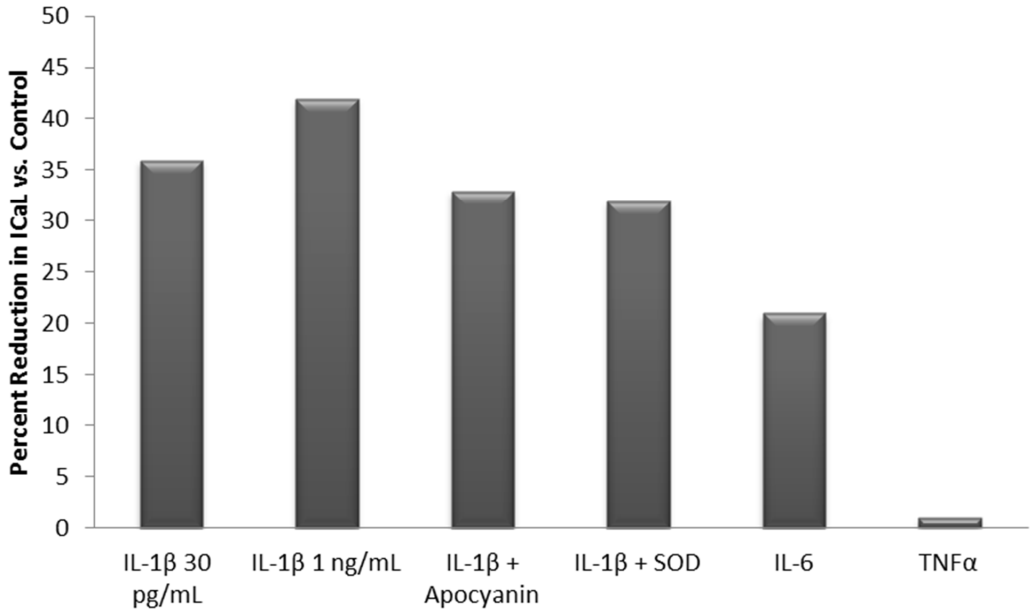

Figure 31. Summary Graph Showing Percent Reduction in I_{CaL} at 0 mV. *Calcium current was recorded from NMVM under varying conditions as previously discussed, reduction in current density at 0 mV was calculated from mean IV curves n=7-19 for each data set.*

3.8 Effect of IL-1β on T-Type Calcium Currents

The T-type calcium current is highly expressed during early stages of development and then gets down-regulated in the adult heart at which time its expression becomes restricted to the conduction system.[135] However reports have suggested that under some pathological conditions, T-type calcium channels could be re-expressed in the ventricle where they would provide an additional Ca^{2+} influx into the cell which would contribute to electrical instability, increasing arrhythmia risk and induce the expression of different genes implicated in exacerbating heart disease condition.[135-137] In NMVM, T-type calcium channels are highly expressed compared to the adult heart. We have investigated whether IL-1β could increase the T-type current (I_{CaT}), as seen under several cardiac pathologies such as hypertrophy.[135, 137, 138] Figure 32 summarises the I_{CaT} IV curves recorded from CTL and IL-1β-treated cells and shows that unlike other cardiac disease, IL-1β actually contributed to a decrease in I_{CaT} density. Overall, we concluded that at 30 pg/mL, IL-1β decreased L and T-type calcium currents, independently of hypertrophy.

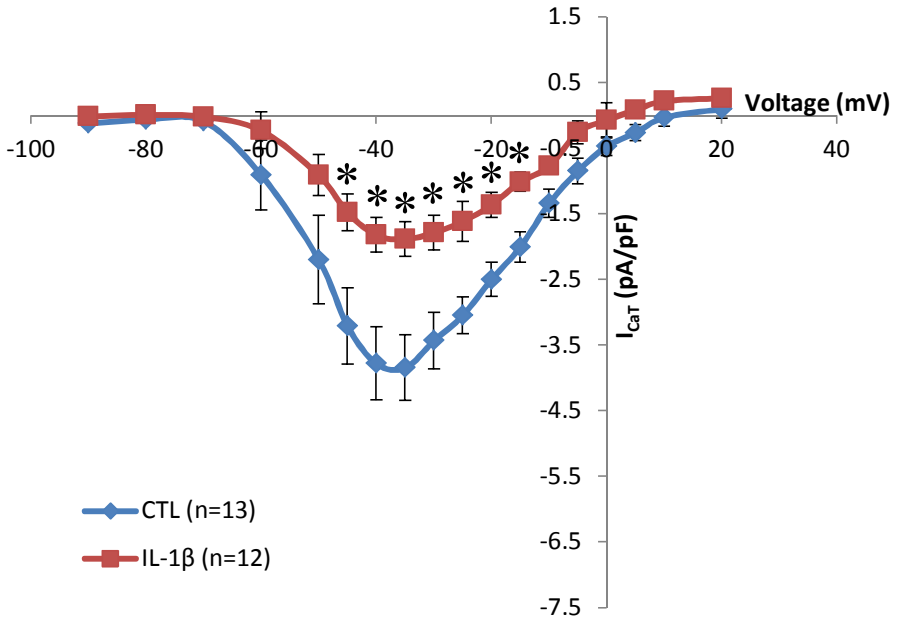

Figure 32. Mean IV curve for I_{CaT} recorded from NMVM treated with IL-1β. *Figure shows the T-type calcium currents recorded from control (CTL) and IL-1β treated (IL-1β) NMVM were actually reduced. *p<0.05 vs. CTL. Current recording were obtained from at least 3 different experiments.*

4 Discussion

4.1 Summary of Findings and Relevance

As previously mentioned and demonstrated by a significant amount of literature, cytokines play a crucial role in the development of several cardiac diseases.[25, 139] Early observations in patients undergoing septic shock showed that in these patients cardiac performance was severely compromised,[74, 115] which strongly suggested a role for immuno-modulatory cells and cytokines in mediating these effects. However cytokines levels were also altered in serum from patients with severe heart failure[50]or other cardiac diseases such as myocardial infarction.[73] These basic clinical observations lead the scientific community to investigate the cellular and molecular mechanisms underlying cytokine production, regulation, and signalling. Collectively, the findings we have gained until today have allowed us to considerably clarify the role of cytokines in heart disease, amongst others diseases. Additionally, the findings have also led to the production of several therapeutic applications aimed at modulating cytokines.

In the introduction, we have discussed the effects of the three main pro-inflammatory cytokines at the top of the inflammatory cascade which are also elevated in heart disease. Experimental evidence from animal models and clinical observations suggest that these cytokines might be implicated in inducing adverse cardiac remodelling such as hypertrophy and fibrosis, induction of ROS, altering contractile machinery and calcium homeostasis, depressing contractility and dampening the β-adrenergic response.[6, 54, 55, 75, 115, 131, 134, 139] Nonetheless, in heart failure patients where cytokine levels are elevated, it has been well established that arrhythmias are highly prevalent and contribute to increasing risk of sudden cardiac death,[97]which suggests that along with the structural remodelling there is a prominent electrical remodelling. Importantly, it is still not well known whether this electrical remodelling is secondary to structural remodelling or whether cytokines are also

able to directly induce changes in cardiac electrical properties thus directly contributing to an increased risk of arrhythmia. In an attempt to answer these questions, our lab has devised a complete research axis which focuses on the role of cytokines in modulating the electrical properties of the heart. In this project, and along with previous work done in our lab, we have provided new insights on how cytokines can modulate cardiac ion currents. In brief, we have previously shown that HIV mice, which exhibit high levels of cytokines, suffered from a delayed repolarisation attributed to a significant reduction of the repolarising K^+ current. In addition, the depolarising Na^+ currents were also reduced.[39, 98] Thus, the net effect was an alteration in conduction and repolarisation, which pose as two risk factors for arrhythmias. Interestingly, all these effects involved no changes in cardiac function or morphology, in other words, ejection fraction and fractional shortening were preserved and there were no signs of hypertrophy or cardiac remodelling. This provided us with the first evidence that might implicate cytokines in directly inducing electrical remodelling. In an attempt to provide additional proof, and distinguish the specific roles of each cytokines, we then proceeded to treat wild-type mice with pathophysiological doses of TNFα for six weeks. The electrophysiological data not only revealed a significant decrease in K^+ currents but the action potentials from TNFα-treated mice showed a decrease in the amplitude of phase 0, which suggests that Na^+ currents were also decreased in TNFα treated mice. Overall, it appeared that TNFα was able to induce several of the adverse cardiac electrical remodelling seen in HIV mice, thus providing substantial proof that cytokines, or at least TNFα, directly affects ion currents without altering the structure of the myocardium. Interestingly, the TNFα effects on K^+ currents were also independent of mRNA and protein changes of all the underlying K^+ channels suggesting that direct modulation of channel properties are likely to be the cause of current reduction.[99] Of note, in a study using transgenic mice with cardiac specific over-expression of TNFα, reductions in K^+ were also observed; however, the results were attributed to a reduction in potassium channel protein levels of $K_V4.2/4.3$ and $K_V1.5$.[56] In addition, these transgenic mice had a severe cardiac remodelling including hypertrophy, fibrosis and contractile dysfunction amongst other effects all secondary to TNFα overexpression.[55] Even though interesting, the

results obtained by over-expressing TNFα, portrayed this cytokine as the "holy grail" of cardiac disease which increases mortality rates and mediates, by itself, nearly all the pathologies seen in heart failure. This is highly unlikely. It is more probable, that the adverse remodelling effects observed in this transgenic mouse are actually secondary to the excessively high concentration of TNFα which saturates the signalling cascades, rendering its effects non-specific and thus diverges from normal pathophysiology and from clinical relevance.

In this project we have continued to explore the effects of cytokine-mediated regulation or modulation of ion currents. By using an *in vitro* model of neonatal mouse ventricular myocytes treated with cytokines we investigated the effects of the three main cytokines, TNFα, IL-1β and IL-6, on the L-type calcium current, which plays a fundamental role in excitation-contraction coupling and in mediating the plateau phase of the action potential. The first results we obtained demonstrated that (unlike our previously results on K^+ currents), TNFα had no effect on the calcium current. In fact, even when increasing the cytokine's concentration by 33 folds, I_{CaL} was still unchanged. This finding demonstrates the specificity of TNFα in affecting ion channels. Furthermore, no hypertrophy was observed in these cultured myocytes, in accordance with our previous results from TNFα treated mice. Thus, at pathophysiologically relevant concentrations, TNFα induces no hypertrophy. This is in clear contrast to the transgenic mouse model overexpressing TNFα.[99] It would have been interesting to confirm whether the K^+ currents in TNFα treated neonatal ventricular myocytes were reduced similarly to the TNFα-treated adult mice. However, neonatal mouse ventricular myocytes express very little K^+ currents and are thus not an appropriate model for studying potassium currents.[89, 140] The sodium current is however highly expressed in neonatal mouse ventricular myocytes and to look at how it is affected by cytokines will be an avenue we might explore in future studies.

Subsequently, we began exploring the effects of IL-1β, a cytokine which was not explored in our previous studies. When neonatal mouse ventricular myocytes were treated for 24H with IL-1β and then patched-clamped, the data revealed that I_{CaL} was reduced by

36%. This robust effect was also specific to IL-1β, because when we administered a treatment to neonatal mouse ventricular myocytes combining TNFα and IL-1β in order to investigate any synergistical effects, which have been often reported for these two cytokines[75, 131], we observed a reduction in I_{CaL} that was similar to the one obtained when neonatal mouse ventricular myocytes were treated with IL-1β alone. This finding also confirmed that TNFα exerts no effect on I_{CaL}. Interestingly, the effects of IL-1β on I_{CaL} did not seem to increase in a concentration-dependent manner. Figure 30 and 31 clearly show that when we increased the concentration of IL-1β from the clinically relevant concentration up to 1 ng/mL the calcium current density was reduced by 40%, compared to a 36% reduction at 30 pg/mL IL-1β. However, significant hypertrophy occurred as measured by mean cellular capacitance. The ability of IL-1β to induce hypertrophy has been previously documented and it has been attributed to NO and NFAT signalling.[119, 134] This finding highlights several important aspects. Firstly, I_{CaL} was reduced independently of hypertrophy, and was not further decreased by hypertrophy, which suggests that in response to chronic low grade IL-1β, as seen in patients with heart failure, the L-type calcium current is likely to be directly affected by IL-1β and not as a result of structural remodelling. Second, by using pathophysiological concentrations of IL-1β, we avoid the non-specific effects on I_{CaL} that might be secondary to hypertrophy or other saturated signalling cascades induced by high doses of IL-1β. A typical example where such non-specific effects exist is the TNFα transgenic mouse model that recapitulated a myriad of cardiac complication simply due to TNFα being overproduced in the heart. Furthermore, under hypertrophic conditions, T-type calcium channel expression has been shown to be increased and, unlike L-type calcium currents, the T-type calcium currents are activated at more negative voltages and do not normally play a functional role in the adult ventricle since their expression is restricted to the conduction system. However T-type calcium channels can become re-expressed in the ventricle as foetal genes under disease conditions, notably hypertrophy, and are thought to worsen the pathology by creating an additional calcium influx which might cause electrical instability and induce abnormal automaticity.[136, 137] In neonatal mouse ventricular myocytes T-type channels are naturally expressed. However,

figure 32 shows that, unlike pro-hypertrophic factors which have been shown to increase T-type currents, IL-1β actually significantly decreased the density of I_{CaT}. This adds further evidence to the distinct effects of IL-1β at pathophysiological concentrations.

Lastly, the effect of IL-6 on I_{CaL} was also examined. Our data shows a mild but significant 21% decrease in peak current density. This moderate effect might be due to the lesser amount of IL-6 present in the media. Since IL-6 has a slightly larger molecular weight (~21 kD) compared to IL-1β (17.5 kD), 30 pg/mL of IL-6 would therefore contain less protein for a same concentration of IL-1β. However this would need to be confirmed by increasing the concentration of IL-6 and noting the changes in I_{CaL} density, or performing a dose-response curve. Nonetheless since IL-1β exerted the most severe effect on I_{CaL} we chose to focus our further investigations on the mechanisms by which IL-1β is able to depress I_{CaL}. Additionally, considering IL-6 signals through completely different pathways, the mechanisms underlying the IL-6 response on I_{CaL} will be the subject of future investigation.

4.2 Potential Mechanisms of Cytokine Mediated Effects on Calcium Channels

We first investigated the effects of IL-1β on I_{CaL}. Briefly, what we have found is that the reduction in I_{CaL} caused by chronic exposure to IL-1β is not attributable to a gene regulation nor it is caused by elevation is ROS levels.

Indeed, qPCR data revealed that following IL-1β treatment (and TNFα) mRNA of $Ca_V1.2$, the underlying α subunit which encodes for I_{CaL}, was unchanged. This finding was in accordance with our previous study where TNFα treated mice that also showed no change in mRNA (or protein) levels of K^+ channels in response to TNFα. Thus it was expected that the protein levels of $Ca_V1.2$ will not be changed, and that the decrease in current density is more likely to be caused by intracellular regulation, such as phosphorylation, which can affect single-channel properties. It is also possible that IL-1β exerts its effects on β-subunits of the L-type calcium channel without influencing the expression or regulation of the α-subunit.

As previously mentioned, our results are in contrast with the TNFα transgenic mouse model where TNFα concentrations are several fold higher than our conditions and where protein levels of several ion channels, notably potassium channels, were decreased. Thus, it appears at pathologically relevant concentrations, pro-inflammatory cytokines do not regulate ion currents via transcriptional or translational mechanism but rather involve intracellular signalling that influence ion channel properties or activity.

Several studies have suggested that pro-inflammatory cytokines are potent inducers of oxidative stress.[34, 141, 142] Some studies even suggested that ROS produced by cytokines can reduce ionic currents.[143] For instance, the oxidation of sulfhydryl groups of $Na_V1.5$ was seen to decrease peak current density without affecting the current kinetics.[144] We therefore investigated whether ROS could contribute to the decrease in I_{CaL} by a mechanism where ROS would oxidise sulfhydryl groups and depress I_{CaL} density. The first step was to measure ROS levels, using a ROS-sensitive fluorescent probe. Our results showed a significant increase in oxidative stress after IL-1β treatment. In an attempt to halt the ROS production induced by IL-1β we subsequently treated cells concurrently with IL-1β and two different antioxidants, apocyanin or PEG-SOD. These antioxidants either directly inhibit NADPH oxidase a main source of ROS, or quench O_2^- a potent oxidising agent, by transforming it into peroxide, a far less reactive species. Apocyanin, the NADPH inhibitor, decreased ROS levels; however, our electrophysiology data clearly demonstrated apocyanin failed to reverse the decrease in I_{CaL}. Interestingly, TNFα was also able to induce ROS, and when neonatal mouse ventricular myocytes were treated with both IL-1β and TNFα there was a synergistical increase in ROS production. Thus, oxidative stress is not implicated in reducing the density of I_{CaL}. In addition, TNFα does exert a biological activity in the neonatal mouse ventricular myocytes, and lack of effect on I_{CaL} is not attributable to a lack of TNFα receptors in these cells.

Lastly, even though the kinetics of I_{CaL} were not changed after IL-1β treatment, we examined whether protein kinases were implicated in the observed effects in particular,

protein kinase C (PKC). There are multiple potential PKC phosphorylation sites on the L-type calcium channel (lozenges on figure 16). Preliminary data obtained showed that when neonatal mouse ventricular myocytes were treated with the PKC activator PMA (phorbol 12-myristate 13-acetate) for 30 mins I_{CaL} density was significantly reduced to the same extent as observed with IL-1β. No change in in I_{CaL} kinetics or the IV properties were observed either. Furthermore, when PMA was incubated for 24 H, which serves to down-regulate PKC,[144, 145] the density of I_{CaL} increased, however additional experimentation will be required in order to reach statistical significance.

4.3 Neonatal Cardiomyocytes as an *in vitro* Model

Cell culture has been a tool of unequivocal importance in research. Stable cell lines which replicate and maintain their phenotype over long periods of time have provided significant insights into thousands of cellular mechanisms. They are tailored to the investigator's needs and are often used as a ground work for subsequent research. Stable cell lines are extensively used in biotechnology, vaccine development and drug screening.[146, 147] In contrast, primary cultures such as the neonatal mouse ventricular myocytes are obtained from freshly isolated hearts and can only be maintained in culture for short periods of time considering as these replicate slightly, if at all. However, they offer the advantage of being molecularly 'closer' to the *in vivo* model than stable cell lines. The neonatal cardiomyocyte culture protocol was first described by Harary and Farley in 1963 and over the last 50 years several investigators have modified or updated the technique.[148] For instance in the early 1980s the neonatal cardiomyocytes cultures served as model to study cardiac hypertrophy.[119] However the use of neonatal cardiomyocyte cultures extends far beyond that and indeed this *in vitro* model offer many other advantages. A preparation yields millions of viable cardiomyocytes that can be treated with various pharmacological agents, transfected, used for electrophysiological or molecular purposes which will be analysed in contexts independent of systemic feedback. Thus, they offer an approach focused on the precise and isolated cellular mechanisms. Additionally, compared

to adult cardiomyocytes which begin de-differentiating after 24 H of culture, neonatal mouse ventricular myocytes can be maintained up to weeks in culture without losing their phenotype and overall, they do resemble adult cardiomyocytes on several levels including intracellular signalling cascades and expression of some ion channels. We have used the neonatal mouse ventricular myocytes in order to investigate the chronic effects of cytokines on I_{CaL}. The model has allowed us to explore the role of three cytokines in parallel under chronic conditions, which are at 24 H and more, compared to 6 weeks *in vivo*. Therefore, neonatal mouse ventricular myocytes offer an exclusive advantage in terms of flexibility, time, and allowed us to control the experimental conditions and exclude the systemic or indirect that might arise in performing such studies *in vivo.*

In regard to our research interests there are several differences between adult and neonatal cardiomyocytes that one should keep in mind. In terms of electrophysiology the calcium and sodium channels are robustly expressed and are regulated in a similar fashion to the adult cardiomyocytes,[149] however neonatal mouse ventricular myocytes are not well suited for studies related to K^+ currents, as these currents are very small or totally absent from these cells.[140] Additionally, as previously shown, ECC requires organised structures and a close proximity of LTCC to RyR in order to induce CICR. Since neonatal mouse ventricular myocytes have not developed T-tubules,[149] the basis of ECC is therefore very different from the adult heart. Even though many of the calcium handling proteins (RyR, SERCA, PLC, NCX etc.) are expressed and functional their role in ECC or calcium handling differs from the adult cardiomyocyte; however exploring their regulation could still be insightful. Interestingly, ECC seems to develop as the neonatal mouse ventricular myocytes are kept longer in culture. Some authors have reported that after 7 days of culture despite spontaneous contractions, a mechanism similar to CICR develops in neonatal mouse ventricular myocytes, however significant differences from the adult cardiomyocyte in terms calcium handling persist.[150] Overall, as with any other model, neonatal mouse ventricular myocytes have their own respective advantages and disadvantages. We have used them to gain insight, independently of systemic effects, on the regulation calcium current by cytokines. The properties and regulation of I_{CaL} in neonatal mouse ventricular

myocytes are on many levels similar to their adult counterpart including response to angiotensin II (Mathieu S *et. al* unpublished data), endothelin-1, and β-adrenergic agonists [149, 151, 152] thus, the findings provide a ground work for subsequent investigations, and the observations are highly likely to be recapitulated in *in vivo* models, where they can be further developed.

4.4 Perspectives and Future Investigation

We have shown that IL-1β can significantly decrease the L-type calcium current independently of gene regulation or oxidative stress. Currently we are investigating the regulatory pathways that involve protein kinases, in particular PKC. The PKC family has been shown to interact with L-type calcium channel.[101] Even though studies have shown mixed effects regarding the exact modulation of LTCC by PKC, significant research has shown that different PKC isoforms may have opposing results on I_{CaL}.[101] Our preliminary data suggest that PMA mimics the effect of IL-1β. Thus a rapid activation of PKC decreases calcium current density in similar fashion to IL-1β. Nonetheless, it remains to be investigated whether IL-1β actually mediates its effects via activation of PKC, and if proven to be true, to which of the isoforms is the effect attributable. Furthermore, early studies have reported a decreased response to β-adrenergic stimulation in IL-1β treated cardiomyocytes, due to decreased cAMP levels.[115] A decrease in PKA activity, which is known to enhance I_{CaL} density, might be reduced and consequently this would reduce I_{CaL} density. This would have a net outcome of depressing contractility and affecting cardiac excitability.

An important question that remains to be answered would be "what is the physiological consequence of a decrease in I_{CaL}?" Will it just be a decrease in contractility? Research has shown that reductions in I_{CaL} can affect calcium transients by lowering SR calcium release. In an adaptive response, RyR sensitivity is significantly increased, which might lead to increase SR leak, known as sparks, which poses a risk factor for spontaneous depolarisations.[116, 131] In an attempt to restore Ca^{2+} homeostasis, calcium handling proteins

can also adapt their expression patterns thus adding further complexity to the underlying mechanisms. Likewise, many signalling mechanisms depending on calcium influx from LTCC could be modified.

Overall, there are several paths to be investigated. It is possible to focus on the molecular determinants of ion channel regulation by cytokines in neonatal mouse ventricular myocytes. However exploration of all the ECC mechanism will require ultimately a model that possesses typical adult ECC dynamics.

Conclusion

Our studies regarding the regulation of ion channels by cytokines have revealed several important findings. We have demonstrated that long term exposure to pro-inflammatory cytokines is able to directly modulate several voltage-depend ion currents, which in every case so far, resulted in a decrease of current. Overall our data suggest that TNFα, IL-1β and IL-6, the cytokines at the top of the inflammatory cascade, which are elevated in patients suffering from various heart diseases, affect the repolarising K^+ currents, the depolarising Na^+ current and the calcium currents. Specifically, TNFα decreases K^+ and Na^+ currents but not Ca^{2+}. On the other hand, IL-1β and IL-6 decrease the L-type calcium current. However, further experimentation will be required in order to assess the effects of these three cytokines on K^+, Na^+ and Ca^{2+} currents in order to develop a bigger picture of how each cytokine might affect a particular current.

The net effects of the decreases in K^+, Na^+ and Ca^{2+} currents in response to cytokines that we observed over the course of this project could prove to be lethal. Indeed, by affecting conduction, prolonging repolarisation, and decreasing I_{CaL}, the risk of arrhythmia is for the part significantly increased. Over time, if these currents remained decreased, cardiac function will undoubtedly worsen, as seen in patients with heart failure.

Importantly, we also demonstrated that chronic low grade inflammation where cytokine levels are maintained in the lower ranges but for longer periods of time cytokines do not necessarily induce structural remodelling, in contrast to many studies which used extremely high doses of cytokines (ranging from 1 to 500 ng/mL). Additionally, the cytokines' effects on ion currents were for the most part independent of gene and protein expression levels and are thought to implicate intracellular regulatory mechanisms which alter ion channel properties. These findings could contribute to explain the rhythm disturbances, arrhythmia susceptibility and decrease in contractility observed in patients

suffering from heart disease where pro-inflammatory cytokines levels are chronically elevated.

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