Justus-Liebig-Universität Giessen Klinik für Neurologie

Immune mediated disturbances of bone, connective tissue and vascular metabolism in Complex Regional Pain Syndrome (CRPS) - a new pathogenic mechanism of therapeutic relevance

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ஊழையும் உப்பக்கம் காண்பர் உலைவின்றித்
தாழாது உஞற்று பவர். - திருவள்ளுவர்

One who strive with undismayed, unfaltering mind,
shall leave their opposing fate behind. - ***Thiruvalluvar***

Dedicated To My Loving Grandfather
And Family

Declaration

I declare that the present thesis is my original work and that it has not been previously presented in this or any other university for any degree. I have also abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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(Backialakshmi Dharmalingam)

INDEX

LIST OF ABBREVIATIONS

ACE	Angiotensin-converting-enzyme
ADRB2	Adrenergic beta receptor – 2
ALP	Alkaline phosphatase
BGLAP	Bone gamma carboxyglutamic acid-containing protein
BMP-2	Bone morphogenic protein – 2
cAMP	cyclic Adenosine Monophosphate
CGRP	Calcitonin gene-related peptide
CHRM2	Cholinergic muscarinic receptor – 2
CNS	Central nervous system
Col-1	Collagen – 1
CRP	C-reactive protein
CRPS	Complex regional pain syndrome
DFNS	Deutschen Forschungsverbund Neuropathischer Schmerz
DKK1	Dickkopf – 1
DRG	Dorsal root ganglion
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
GPCR	G-protein coupled receptors
HC	Healthy control
HCMEC	Human cerebral microvascular endothelial cells
HLA	Human leukocyte antigen
IASP	International Association for the Study of Pain
ICAM-1	Intercellular adhesion molecule – 1
IFN	Interferon
IgG	Immunoglobulin G
IL-1β	Interleukin – 1 beta
IL-6	Interleukin – 6
IvIg	Intravenous immunoglobulin
LDH	Lactate dehydrogenase
MCP-1	Monocyte chemoattractant protein – 1
MFI	Mean fluorescence intensity
MSC	Mesenchymal stem cells
NGF	Nerve growth factor
NK1	Neurokinin-1
NMDA	N-methyl-D-aspartate
NSAIDs	Non-steroidal anti-inflammatory drugs
OPN	Osteopontin
PET	Positron emission tomography
PKA	Protein kinase - A

PNS	Peripheral nervous system
QST	Quantitative sensory testing
RA	Rheumatoid arthritis
RF	Radial fracture
RSD	Reflex sympathetic dystrophy
SLE	Systemic lupus erythmatosus
SMP	Sympathetically maintained pain
SP	Substance P
SPECT	Single photon emission computed tomography
SS	Sjögren's syndrome
TGF-β1	Transforming growth factor beta-1
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRPV1	Transient receptor potential vanilloid receptor – 1
VCAM-1	Vascular cell adhesion molecule – 1
VGKC	Voltage gated potassium channels
ZK	Zellen von Knochen (Bone cell)

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1. Complex Regional pain syndrome – Background

1.1.1. Definition

Complex regional pain syndrome (CRPS) formerly known as Sudeck's dystrophy, is a chronic neuropathic condition characterized by spontaneous or stimulus induced pain, swelling, skin color and temperature changes, vasomotor and sudomotor abnormality (sympathetic dysfunction), and impairment of motor function (weakness, tremor, and muscle spasms)(de Mos et al., 2009d). The disorder usually is a painful disabling condition in the distal limb and develops after minor trauma (contusions, sprain, and minor fractures) or surgery and rarely after spinal cord injury, cerebrovascular accidents and cardiac ischaemia (Mitchell et al., 2007b; Swart et al., 2009). Fractures are the most common precipitating events involving the upper extremity more frequently than the lower (Nishida et al., 2009). During cast immobilization, increased pressure and early complaints of tightness are predictive factors for the onset of CRPS (Albazaz et al., 2008). In some cases no clear eliciting event can be identified. The spontaneous pain (skin, subcutaneous tissue, and joints) or hyperalgesia has no correlation to the severity of the trauma and is not limited to the area of the trauma.

1.1.2 Historical background

Initial description about the symptoms relating CRPS probably dates back to 1864, when Silas Weir Mitchell observed a puzzling collection of symptoms in soldiers with injuries of peripheral nerves like constant burning pain in combination with substantial trophic changes. Mitchell named this syndrome "Causalgia" derived from Greek words 'burning' ('NDQVLV' – kausis) and 'pain' ('DOJRV' – algos) (Mitchell et al., 2007b). During the First World War, Rene Leriche successfully treated such syndromes by surgical sympathectomy. This is where the surgeon has presumed the involvement of sympathetic nervous system in this disabling and distressing painful condition. John Bonica (who later founded the International Association for the Study of Pain; IASP) during the 1950s, developed few invasive techniques allowing temporary blockade of the sympathetic nervous system. Through the

efficacy followed by these techniques, Evans coined a new term for this condition, which is known as 'Reflex Sympathetic Dystrophy' (Evans, 1946). At the 24th meeting of the German Society of Surgery, surgeon Paul Sudeck in the year 1864 (Mitchell et al., 1864) gave a lecture on patients with acute inflammatory bone atrophy. Sudeck mentioned certain key symptoms about this syndrome accompanied by inflammation and symptoms might spread beyond the region of initial damage (Mitchell et al., 2007a; Sudeck, 2005). It is to honor his effort, clinically traumatologists temporarily called this disease as Sudeck's dystrophy. The pathogenicity of this pain syndrome is not yet clear but later there was growing evidence for an inflammatory as well as for a sympathetic pathogenesis. Finally, the term RSD was abandoned at a consensus conference held in Orlando, Florida, in 1993 and they formulated a strictly descriptive term called Complex Regional Pain Syndrome (CRPS) and till date this is the official term in the IASP (Stanton-Hicks et al., 1995).

1.1.3 Diagnostic criteria

CRPS is further subdivided into two types: CRPS type I, formerly known as ''reflex sympathetic dystrophy'' (as recommended in 1994 by the International Association for the Study of Pain), and CRPS type II, the new term for ''causalgia'' that always coexists with a documented nerve injury (lesion or tumour). In most cases the upper or lower limb is affected, but also other body parts or different body parts can be involved at the same time (Swart et al., 2009). There were many diagnostic criteria formulated to diagnose the disease condition (van de Beek et al., 2002). To address the problems, the IASP proposed a new diagnostic criterion (**Table 1**) in which the clinical symptoms were listed out including involvement of sympathetic hyperactivity, allodynia and hyperalgesia (Stanton-Hicks et al., 1995). Though the IASP diagnostic criteria explained a new taxonomy it failed to formulate the specificity and internal validity. Most importantly diagnostic criteria should be able to describe a sample and also differentiate it from other patient groups (Bruehl et al., 1999; Harden et al., 1999; Perez et al., 2007). In order to address these variable groups, Bruehl and Harden came up with new criteria, which is commonly denoted as Harden/Bruehl criteria, or The Budapest research criteria (Harden et al., 2007; Harden and Bruehl, 2006). Based on these criteria, the patients will be diagnosed by the physician with CRPS if they fulfill atleast 2 of the 4 categories (**Table 2**).

Table 1: IASP Diagnostic criteria for CRPS (Merskey et al., 1994)

CRPS type I
1. Continuing pain, allodynia or hyperalgesia, edema, changes in skin color, skin blood flow and abnormal sudomotor activity in the pain region
2. Excluded diagnosis that would otherwise account for the degree of pain and dysfunction
CRPS type II
1. Syndrome develops after nerve injury.
2. Spontaneous pain or allodynia / hyperalgesia (not limited to the territory of the injured nerve)
3. Evidence of edema, abnormalities in skin blood flow and sudomotor activity in the region of pain since the inciting event.
4. Excluded diagnosis that would otherwise account for the degree of pain and dysfunction

Table 2: The modified Harden/Bruehl criteria or "The Budapest criteria" (Harden et al., 2007)

For clinical diagnosis, the following criteria must be met

1. Continuing pain, which is disproportionate to any inciting event
2. Must report at least one symptom in three of the four following categories, Fig 1A. Sensory: reports of hypaesthesia and/or allodynia Vasomotor: reports of temperature asymmetry and/or skin color changes and/or skin color asymmetry Sudomotor/edema: reports of edema and/or sweating changes and/or sweating asymmetry Motor/trophic: reports of decreased range of motion and/or motor dysfunction (weakness, tremor, dystonia) and/or trophic changes (hair, nail, skin)
3. Must display at least one sign at time of evaluation in two or more of the following categories Sensory: evidence of hyperalgesia (to pinprick) and/or allodynia (to light touch and/or temperature sensation and/or deep somatic pressure and/or joint movement) Vasomotor: evidence of temperature asymmetry (>1°C) and/or skin color changes and/or

asymmetry. Sudomotor/edema: evidence of edema and/or sweating changes and/or sweating asymmetry Motor/trophic: evidence of decreased range of motion and/or motor dysfunction (weakness, tremor, dystonia) and/or trophic changes (hair, nail, skin)
4. There is no other diagnosis that better explains the signs and symptoms
For research purposes, diagnostic decision rule should be at least one symptom in all four categories and at least one sign (observed at evaluation) in two or more sign categories.

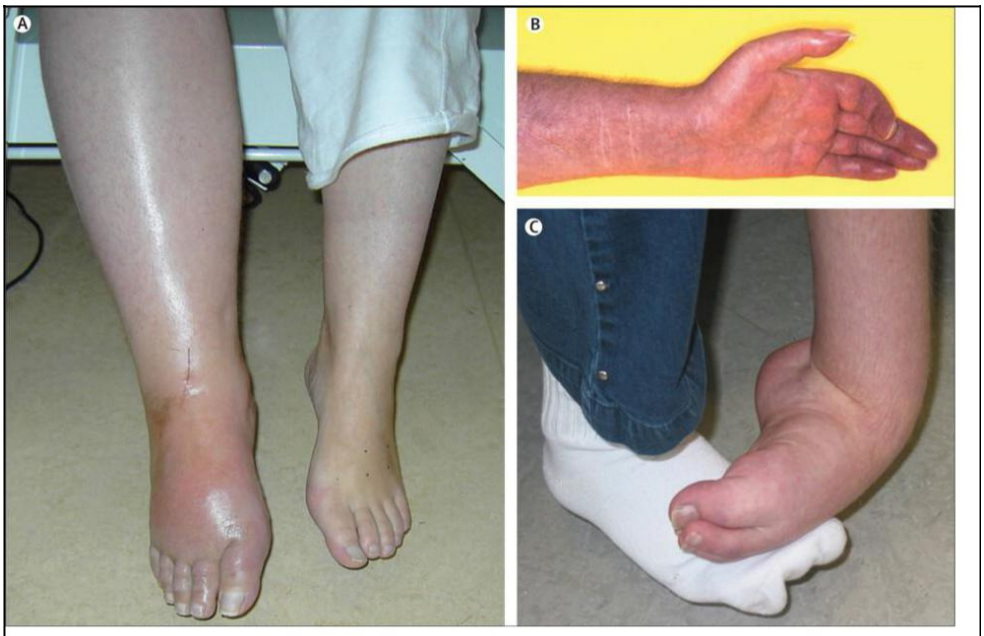

Figure 1A: Acute CRPS, chronic CRPS, and CRPS dystonia *(A) Acute CRPS with hyperaemia, swelling, and glossy skin. (B) Chronic, cold-type CRPS with blue discoloration of the fingers, glossy skin, and increased hair and nail growth. (C) CRPS-related dystonia of the left ankle and foot with plantar flexion and inversion of the ankle, and flexion of the toes; oedema and increased hair growth are also visible. CRPS=complex regional pain syndrome.* ***Source: www.thelancet.com***

1.1.4 Epidemiology

Overall epidemiological screenings were not yet done for CRPS but few studies support the upper limbs are twice as frequently affected as the lower limbs (de Mos et al., 2008; Geertzen

et al., 1998). The epidemiological data of Olmsted County, Minnesota (North America) calculated an incidence rate of 5.46/100,000/year and a prevalence rate of 20.57/100,000 (Sandroni et al., 2003). In the Netherlands, it was found that each year about 26 out of 100,000 people develop CRPS (95% CI: 23.0–29.7). This counts to 4300 new sufferers every year. Its onset ranges from childhood through old age, but most cases were seen between the ages of 50 and 70 years. It is generally believed that CRPS occurs mainly in Caucasian and Japanese people (Allen et al., 1999; de Mos et al., 2009d). Prevalence of CRPS following fractures is between 0.03% and 37% (Atkins et al., 1989, 1990; Bickerstaff and Kanis, 1994; Dijkstra et al., 2003; Field et al., 1992; Raja and Grabow, 2002; Sarangi et al., 1993). Generally females are affected more frequent than males with a ratio approximating 3.5 (Allen et al., 1999; Sumitani et al., 2013). Overall female to male ratio is 2-3:1 and postmenopausal women appeared to be more prevalent to develop CRPS. Most of the patients experienced preceding trauma, in about 40% of the cases fracture or surgery (Beerthuizen et al., 2012). Remarkably, the severity of trauma and degree of CRPS symptoms has no distinct correlation (Stanton-Hicks et al., 1995). CRPS may develop at any age but is very uncommon in children. The age groups with the highest incidence were observed in patients travelling through the 4^{th} to 7^{th} decade of their life (Schwartzman et al., 2009b). Although CRPS seems to occur mainly in adults, the syndrome may also develop in children, where the skin temperature of the involved extremity at onset was more often cooler, the lower limb more frequently affected, and neurological and sympathetic symptoms less pronounced. The approximate amount of pediatric patients is less than <10% of all CRPS patients (Subbarao and Stillwell, 1981; Tan et al., 2008).

1.1.5 Etiology

The main cause or the initial event that leads to this chronic painful condition is not yet known. As known so far, CRPS is a multi-faceted disease and previous studies states that CRPS should be understood as a biopsychosocial disorder, whereby psychological, behavioural, and pathophysiological factors interact in a complex manner (Bruehl and Chung, 2006). Distal radius fracture is the most common fracture type but only few of those patients develop CRPS. There is also evidence for familial occurrence of CRPS (de Rooij et al., 2009). Neurogenic vasodilation is more intensive in CRPS patients than in healthy individuals (Leis et al., 2004). The role of psychological factors, e.g. critical life events or

inadequate coping strategies (i.e. difficulties in dealing with post-trauma consequences) in the development or aggravation of CRPS is controversially discussed. Stressful life events in approximately 80% of patients suffering from CRPS of the upper limb 2 months before or 1 month after the development of CRPS, compared with 20% in a control group were observed (Geertzen et al., 1998). This suggests a predisposition for neurogenic inflammation in CRPS. However, no correlation has been proved so far between the manifestation of CRPS and the polymorphisms in genes encoding for ACE (angiotensin-converting enzyme), a neuropeptide-degrading enzyme (Huhne et al., 2004).

CRPS varies from mild and self-limiting to a chronic disease with a high impact on daily functioning and quality of life (Galer et al., 2000). Chronic patients then experience long-term suffering (Brunner et al., 2008; de Mos et al., 2009d). The syndrome frequently interferes with the ability to work (approximately 62% disability rate), sleep (approximately 96%), mobility (approximately 86%), and self-care (approximately 57%) (Brunner et al., 2008; de Mos et al., 2009d). Remissions and relapses are common. Duman et al., reported that in Turkey almost one-third of patients with CRPS do not return to work (Duman et al., 2007). To obtain a good outcome, early recognition and a multidisciplinary approach to management seems important (Albazaz et al., 2008).

1.1.6 Clinical Manifestation

The symptoms and clinical presentation are multi-dimensional but studies revealed some characteristic features. A striking symptom of CRPS is the presence of distal edema (**Fig. 1B**). Incidence of skin temperature changes at the affected body part is 80% (Birklein et al., 2000). Most of the studies consider a temperature difference of >1°C to be significant. The affected limb is initially warm however it becomes colder in the course of disease (Veldman et al., 1993). The skin temperature decreases over the course of time. The skin color looks red often in the initial stage but turns either pale or livid in chronic stages.

Fifty-five percent of patients with CRPS presented altered sweating of the affected limb with hyperhidrosis rather than hypohidrosis (Birklein et al., 1997). Autonomic disturbances include atrophy of muscles and bones. Most of the patients develop patchy osteoporosis or osteopenia (de Boer et al., 2011; Gierthmuhlen et al., 2012; Sethna et al., 2007). Tropic

changes also include thin and shiny skin, increased hair and nail growth with the nails often brittle, ridged, curved or dull.

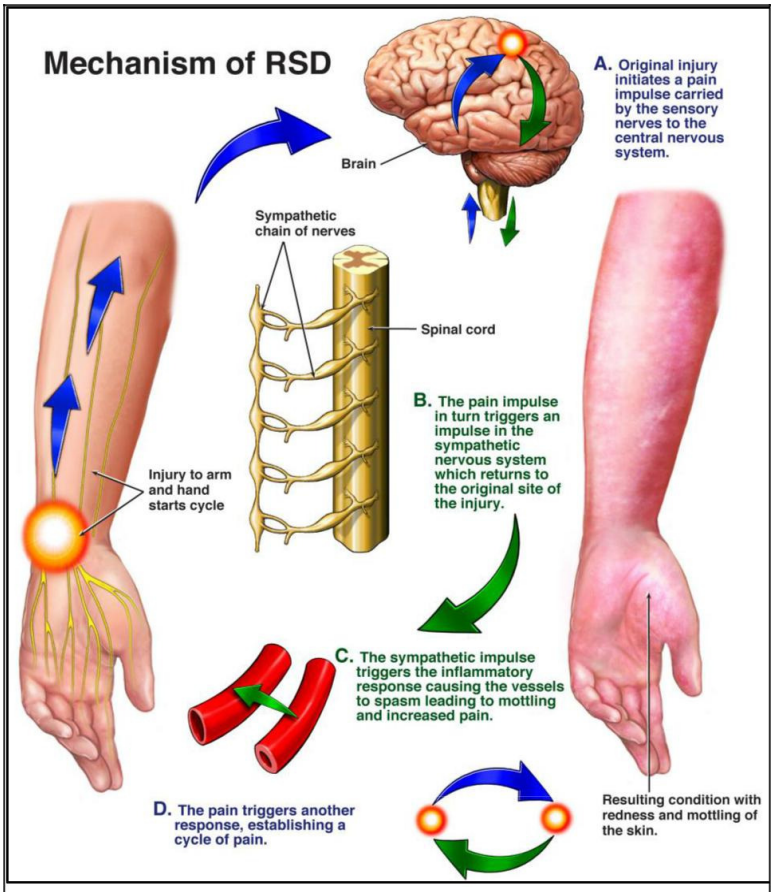

Figure 1B: *A possible mechanism of development of RSD / CRPS.* ***Source: International research Foundation for RSD / CRPS***

1.1.6.1. Sensory disturbances

Sensory disturbances were found in almost 90% of CRPS patients (Birklein et al., 2000; Veldman et al., 1993). The major characteristic of pain in CRPS is not limited to the innervated territory of a single peripheral nerve. The pain can be spontaneous, continuous and episodic in response to physical, noxious or even emotional stimuli (Maihofner et al., 2005; Sieweke et al., 1999). Sensory disturbances are often observed in glove or stocking-like

pattern. Many patients suffer from hyperalgesia and/or allodynia. Adults with CRPS I and CRPS II display symptoms like 'sensory gain' and 'sensory loss' (hypalgesia or hypaesthesia) (Gierthmuhlen et al., 2012). In many cases the pain is more pronounced during night. Spontaneous pain can be evoked by anxiety, exercise, temperature changes and touch even with a light brush. Mostly patients describe the pain as burning, dragging, shooting or stinging (Hassantash et al., 2003; Roganovic and Mandic-Gajic, 2006). Patients with CRPS also display other sensory abnormalities such as allochiria (unilateral tactile stimuli perceived only in the analogous location on the opposite extremity), sensory extinction (bilateral tactile stimuli is perceived in both the limbs) (Acerra and Moseley, 2005; Cohen et al., 2013), dysynchiria (perceiving pain when watching the mirror image of the unaffected limb being stimulated by light touch or pressure so as the affected limb), synchiria (perception of pain to a cold stimulus in both affected and unaffected limb) (Acerra and Moseley, 2005) and referral sensations (referral of somatosensory feelings to areas that are adjacent of cortical map or mislocalization of tactile stimuli) .

1.1.6.2. Motor dysfunction

The majority of patients with CRPS have repeatedly reported motor disturbances such as weakness or limited active range of motion (Forderreuther et al., 2004; Schwartzman and Kerrigan, 1990). Patients often suffer from muscle spasms, myoclonus and dystonia. In many cases the tendon reflexes are exaggerated but in few cases they are diminished (Birklein et al., 2000; Forderreuther et al., 2004; Roganovic and Mandic-Gajic, 2006).

Besides that patients couldn't move their affected extremity, especially complex movements like finger-tapping are severely impaired (Birklein et al., 2000). Some studies have reported neglect-like symptoms (Galer et al., 1995; Galer and Jensen, 1999). Interestingly, some studies claim that patients have varying symptoms such as myoclonic jerks, dystonic muscle contractions, neglect-like symptoms (van der Laan et al., 1998). Few recent studies show that in pediatric CRPS patients also showed a similar pattern of abnormal movements (Agrawal et al., 2009). Moreover, three distinct subtypes or sequential stages were seen in RSD/CRPS. They are a) acute phase where the pain, sensory abnormalities, sudomotor functions are predominate, b) dystrophic phase where the pain and sensory dysfunction become more marked, vasomotor abnormalities persist and significant motor and trophic changes develop and c) an atrophic phase where pain and sensory abnormalities decrease, vasomotor and

trophic changes increase (Bruehl, 2009; Bruehl et al., 2002; de Mos et al., 2009b; Perez et al., 2007).

1.1.7. Pathophysiological Mechanisms

CRPS is a disorder with varying clinical symptoms and disease severity. Many factors were thought to contribute to CRPS. The major factors include:

I. Neurogenic inflammation, central sensitization and neuropeptides in pain
II. Sympathetic–afferent coupling
III. Hypoxia and free radical formation
IV. Cortical re-organization
V. Immunological disturbances
VI. Genetic predisposition

1.1.7.1. Neurogenic Inflammation and central sensitization

CRPS patients exhibit all signs of inflammation such as swelling, pain, redness, infiltrate in joint, muscle, skin and heat. Systemic inflammation seems to be more pertinent in CRPS rather than local inflammatory responses. Neurogenic inflammation regularly accompanies excitation of primary afferent nociceptors causing plasma extravasation and vasodilatation. Compared to controls bradykinin was four times higher in venous blood of CRPS patients (Albazaz et al., 2008; de Mos et al., 2009d; Nishida et al., 2009). Few studies detected Langerhans cells and infiltration of lymphocytes in skin biopsies (Calder et al., 1998; Kozin et al., 1976). ACE inhibitors are shown to influence the neuroinflammatory mechanisms in CRPS by their interaction with the catabolism of SP and bradykinin (de Mos et al., 2009a).

The increase in serum/plasma CGRP was associated with acetylcholine-induced sweating in CRPS patients (Schlereth et al., 2006). Apart from this, the role of CGRP and SP in neurogenic inflammation has been investigated in rat tibial fracture casting model (Guo et al., 2004). At the time of cast removal, upregulation of SP, CGRP and expression of other

neuropeptides was detected in skin, sciatic nerve and in DRG (Blair et al., 1998; Weber et al., 2001; Wei et al., 2009). After tibia fracture, the SP-induced protein extravasation was primarily due to the SP-receptor or the neurokinin-1 (NK1) receptor on endothelial cells and keratinocytes, whereas decreased enzymatic degradation of SP did not make a major contribution (Kingery et al., 2003; Wei et al., 2009). SP was also shown to play a major role in activation and proliferation of keratinocytes thereby causing epidermal thickening in rat tibia fracture cast model (Wei et al., 2012). Skin biopsy sample from CRPS I patient also showed a similar pattern of epidermal thickening and elevated levels of NK1 receptor expression on keratinocytes (Kingery, 2010).

Calcitonin gene-related peptide (CGRP) and substance P (SP) are considered to be the most important inflammatory mediators. Both SP and CGRP are systemically elevated in CRPS patients, which is mainly responsible for vasodilation and protein extravasation respectively. Substance P and CGRP do not directly participate in the pain events. Nevertheless, according to the tibia fracture model of CRPS I (Guo et al., 2004; Guo et al., 2012; Wei et al., 2012; Wei et al., 2009) and sciatic nerve transsection model of CRPS II (Kingery et al., 2003), they play a role in spontaneous pain behavior and nociceptor sensitization. A network of inflammatory mediators acts together and heightens the pain responses. Those include interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)- D and nerve growth factor (NGF) (Guo et al., 2012; Li et al., 2010; Li et al., 2009a; Li et al., 2009b; Sabsovich et al., 2008a; Sabsovich et al., 2008b). These agents can further upregulate the neuropeptide production resulting in additional inflammation and nociceptive sensitization. Additionally, skin mast cells produces other mediators such as tryptase and other proteases, histamine, prostaglandins, cytokines and neuropeptides (Leung and Cahill, 2010; Lewin and Mendell, 1993; Schafers and Sorkin, 2008). Amputated-limb CRPS patients have confirmed the presence of nerve degeneration and evidence of vascular inflammation (Coderre and Bennett, 2008). Continuous nociceptive input, nerve injury, and primary afferent depolarization induce neuropeptide release within the dorsal horn, where they can mediate central sensitization through interaction with Neurokinin 1 (NK-1) and N-methyl-D-aspartate (NMDA) receptors (de Mos et al., 2009d; Latremoliere and Woolf, 2009; Seybold, 2009). In long-standing CRPS, there is significant posterior horn cell loss and activation of both microglia and astrocytes most prominently at the corresponding level of the original injury (Del Valle et al., 2009). Spinal neuronal sensitization comprises a state of hyperexcitability and disinhibition,

causing a decreased stimulation threshold. Eventually, normally non-painful stimuli become painful (de Mos et al., 2009d).

1.1.7.2. Sympathetic–afferent coupling

The central nervous system interacts with the peripheral system via neural and chemical channels and has direct control over the autonomic system, thereby giving rise to the clinical irregularity of peripheral vasomotor and sudomotor changes without peripheral neural damage. Impaired balance exists in CRPS-affected limbs between vascular regulation systems responsible for vasoconstriction and vasodilation (Dayan et al., 2008). The results of sympathetic or autonomic hyperactivity are increased sweating, trophic changes, and vasoconstriction- related coldness of the affected limb. In Sympathetically Maintained Pain (SMP), painful sensations are provoked by sympathetic outflow through sympathetic–afferent coupling in which adrenergic receptors are expressed on primary afferent nerve endings. Increased sympathetic outflow (hyperactivity) may be one mechanism, whilst abnormal sensitivity of adrenergic receptors for normal sympathetic outflow may be another (de Mos et al., 2009d).

The sympathetic skin reflexes in CRPS patients are increased. The underlying cause may be sprouting of new sympathetic nerves centrally in the dorsal horn or peripherally in the upper dermis (de Mos et al., 2009d). The density of D-adrenoreceptors is enlarged in hyperalgesic skin from CRPS-affected limbs, while the sympathetic innervation of sweat glands and vasculature is abnormal (Albrecht et al., 2006). In contrast, temporarily diminished sympathetic stimulation has been suggested as an underlying cause of the adrenergic receptor upregulation and sensitization in CRPS patients (de Mos et al., 2009d). Sweating and trophic disturbances are not the most predominant features of CRPS but can also be explained as neuropeptide effects. Hence, SMP and sympathetic dysregulation can be a part of the CRPS symptom complex (de Mos et al., 2009d).

1.1.7.3. Hypoxia and free radical formation

Free radicals are involved in the pathophysiology of CRPS-I. Presence of hypoxia in CRPS is endorsed by several observations (de Mos et al., 2009d). Extreme vasoconstriction was

thought to cause it, either resulting from a local imbalance between endothelial factors, or sympathetically thriven (Schattschneider et al., 2006). Impaired nutritive blood flow could be a reason for sympathetic dysfunction and causes hypoxia. The actual presence of local hypoxia leading to reactive oxygen species formation (known enhancers of pain and of neuroinflammatory responses) has been demonstrated in clinical studies in CRPS patients (de Mos et al., 2009c). Higher amounts of malondialdehyde, lactic dehydrogenase, and cellular antioxidants (peroxidase, superoxide dismutase, and uric acid) are present in the serum, and especially in the saliva (Coderre and Bennett, 2008). These data confirms various indirect evidence of oxidative stress in CRPS-I patients and strengthen the justification for the use of antioxidants and free radical scavengers in the treatment and prevention of CRPS-I. This is proven by the positive outcomes of randomised clinical trials in human CRPS, wherein scavengers, such as dimethylsulfoxide are effective in the early treatment (Albazaz et al., 2008).

1.1.7.4. Cortical re-organization

Continuous nociceptive contribution by hypoxia, inflammation, or sympathetic stimulation may lead to sensitization and alterations in cortical organization of sensory and motor units. Some CRPS patients experience referred sensations or body perception disturbances that are in line with the studies that demonstrate altered brain activation patterns (Maihofner et al., 2005) and sensory mapping (de Mos et al., 2009d). One major finding was that watching a mirror image of the stimulated unaffected limb evokes pain in the affected limb when the stimulated area corresponded to the area of allodynia (Swart et al., 2009).

In CRPS patients, changes in motor cortex representation, mislocalisations of noxious stimuli, changes in size and organization of the somatosensory map, and body perception disturbances are some features of cortical reorganization (Raja, 2009; Swart et al., 2009). Some patients exhibit abnormal or shifted brain activity to motor tasks, imagined motor tasks and show that the extent of observed functional re-organizations is linked to the properties of CRPS pain (Gieteling et al., 2008). A direct correlation has been exhibited between the extent of cortical re-organization and the level of pain perceived in CRPS. The reduction of pain in CRPS patients was associated with the changes on the somatotopic map (McCabe and Blake, 2008).

1.1.7.5. Immunological disturbances

Proinflammatory cytokines are linked to chronic pain, inflammation and also CRPS (Milligan and Watkins, 2009). Both in CRPS patients and CRPS animal models the skin is the primary source of the pro-inflammatory cytokines. Extensive studies are being done to measure the levels of pro-inflammatory mediators in the skin blister fluids of patients with CRPS. It has been showed that samples from the affected limb had higher concentrations of TNF-D, IL-1E compared to the unaffected side (Groeneweg et al., 2008b; Groeneweg et al., 2006; Heijmans-Antonissen et al., 2006; Huygen et al., 2002). Although numerous studies suggest that the keratinocytes and mast cells are the primary candidates none of the studies proved the exact cellular source of these cytokines (Li et al., 2010; Li et al., 2012; Li et al., 2009b; Wei et al., 2012). A recent study showed that among 148 patients, 36% of patients had increased levels of numerous cytokines such as Interferon-J, IL-1E, IL-2, IL-6, IL-7, IL-8, IL-10 (Alexander et al., 2012; Wesseldijk et al., 2008a, b). In some patients, the CSF (cerebrospinal fluid) had up-regulated proinflammatory cytokines and other mediators such as glutamate and Nitric Oxide (NO) (Alexander et al., 2005).

1.1.7.6. Genetic predisposition

Genetic determinants may play a role in the predisposition of CRPS such as familial case reports of CRPS/RSD/algodystrophy (de Rooij et al., 2009; Shirani et al., 2010). These patients often reported to have early onset of disease, spreading of disease to multiple sites or dystonia. In few cases, the affected siblings show identical HLA profiles. A Dutch study showed that HLA-DQ1 was significantly more frequently observed in 52 CRPS patients compared to 295 regional controls. Few studies showed that HLA-DR13, HLA DR-DQ genotypes were seen in majority of patients in a cohort study. But, a clear pattern explaining the inheritance is yet to be determined (Kemler et al., 1999; van de Beek et al., 2000; van Hilten et al., 2000).

Primarily warm CRPS has been associated with a polymorphism in one of the TNF-D promoter genes and homozygosity for this allele increased the involvement of more than one extremity. The ACE I/D polymorphism was increased in a small population of 14 Japanese CRPS patients (Kimura et al., 1998) but not in the European study of 60 patients (de Mos et

al., 2009d; Huhne et al., 2004). These studies shows that the deletion/deletion of ACE gene was overrepresented in CRPS I patients. Genetic disorders of mitochondrial DNA may also be associated with clinical manifestation of CRPS according to the study done in a Genetics clinic with approximately 500 children (Higashimoto et al., 2008). Polymorphism distribution of various cytokine, neurotransmitter and adrenoreceptor genes were also observed in a study that was held in northern German patients who did and did not develop CRPS I after distal radial fracture surgery. These patients reported a significant association of polymorphism in the D1a - adrenoreceptor gene (Herlyn et al., 2010). Although, exact relations between HLA features and CRPS are not yet clear but the current data suggest a possible genetic predisposition of CRPS.

1.2. CRPS and Autoimmunity

1.2.1. Autoimmunity

Autoimmunity is a condition in which the organism fails to recognize between the 'self' and 'non-self' antigens and elicits an immune response against its own body tissues. Antibodies are immunoglobulins produced by the immune system against foreign bodies such as bacteria, virus, parasites etc., whereas autoantibodies represent immune responses against the body's self-components.

1.2.2. Autoantibodies against cell surface receptors

Autoantibodies can bind to cell surface receptors and can cause diseases either by stimulating a receptor or blocking its stimulation by its natural ligand. Few examples are: Myasthenia gravis in which autoantibodies alter the acetylcholine receptor reorganization on the post-synaptic muscle membrane and block acetylcholine neurotransmission functionally and these autoantibodies are responsible for fatal weakness (Vincent, 2002). Some subsets of epilepsy patients harbor serum autoantibodies to either glutamate/AMPA receptor or double-stranded DNA (dsDNA), which enter the blood-brain barrier and might kill neurons in brain fluids after hemispherotomy (Ganor et al., 2004; Ganor et al., 2005; Levite, 2002). In Graves' disease, the autoantibodies are produced against Thyroid-Stimulating-Hormone receptor

(TSH) in the thyroid cells and stimulate excessive production of thyroid hormone. Recently, it has been shown autoantibodies to B-cells stimulate cellular activation of EBV (Epstein-Barr virus), which is a human herpes virus that is the causative agent of infectious mononucleosis. Moreover, autoantibodies (autoAbs) against anti-M3 muscarinic acetylcholine receptor (M3R) were identified in sera of around 50% of patients with Sjögren's syndromes (SS), and several B cell epitopes have been identified including the 1st, 2nd and 3rd loop of Muscarinic 3 receptor (Sumida et al., 2012).

1.2.3. Autoantibodies against nervous system

Paraneoplastic neurological syndromes (PNS) are nervous system dysfunctions in cancer patients and have antineuronal antibodies and anti-synaptophysin autoantibodies (Blaes and Tschernatsch, 2008; Tschernatsch et al., 2008). These autoantibodies are the result of a cross-reactive immune process directed against tumor and nervous system antigens. Autoantibodies against surface-binding receptor or ion channels are thought to be pathogenic in the paraneoplastic syndrome (Blaes, 2012). PNS has highly specific autoantibodies against onconeuronal antigens. It has been reported that these pathogenic receptor autoantibodies can cause limbic encephalitis (Blaes and Tschernatsch, 2010). Autoantibodies against CNS structures were observed in children with OMS (Opsoclonus-myoclonus syndrome) and their parents exhibiting some unspecific autoantibodies could a possible explanation for the genetic susceptibility of OMS in pediatric patients (Blaes et al., 2007a). Besides autoantibodies against intracellular proteins (anti-Hu, alpha-enolase, and KHSRP), specific binding of autoantibodies to the surface of neuroblastoma cells and cerebellar granular neurons have been found (Blaes et al., 2005; Blaes et al., 1998; Kirsten et al., 2007; Tschernatsch et al., 2007). Autoantibodies against Myelin Oligodendrocyte Glycoprotein (MOG) were described in patients with inflammatory demyelinating diseases of the CNS, including multiple sclerosis (Tsuburaya et al., 2014). KIR4.1 specific autoantibodies are potassium channel antibodies and seen in children with acquired demyelinating disease (Kraus et al., 2014).

Autoimmune encephalitis is a new category of treatment-responsive encephalitis associated with antibodies to neuronal cell membrane antigens, including voltage-gated potassium channel (VGKC), N-methyl-D-aspartic acid receptor (NMDAR), alpha-amino-3-hydroxy-5-

methyl-4-isoxazolepropionic acid receptor (AMPAR), gamma-aminobutyric acid (GABA) B receptor and other antigens that have not yet been characterized. Among the different forms of this encephalitis, anti-NMDAR antibodies have been studied extensively in the recent years and some studies show that the autoantibody bind to extracellular conformational epitope in the NR1/NR 2 heteromers of the NMDAR. This disorder often affects young women with ovarian teratoma as a paraneoplastic syndrome (PNS) but may also affect women and men without an underlying tumor (Iizuka, 2008; Iizuka and Hara, 2009; Iizuka and Sakai, 2008; Iizuka et al., 2010). Autoantibodies targeted against voltage-gated potassium channel (VGKC)-associated proteins have been identified in limbic encephalitis (LE) and acquired neuromyotonia (aNMT) (Park et al., 2014).

1.2.4. G-Protein Coupled Receptors (GPCRs)

GPCRs, heptahelical, seven transmembrane (7TM) receptors, are a family of integral membrane proteins which possess seven membrane-spanning domains and are linked to a guanine nucleotide-binding protein (heterotrimeric G protein) (Peeters et al., 2011). Adrenergic, chemokine, olfactory, muscarinic cholinergic, opioid, neurotransmitter and rhodopsin receptors also belong to the family of GPCRs. These GPCRs are activated by an external signal such as ligands or other signal mediators. After binding of a ligand to its respective receptor, a conformational change takes place in the receptor leading to the activation of a G-Protein. GPCRs include receptors for sensory signal mediators such as adenosine, bradykinin, endothelin, neuropeptide Y, GABA (J-aminobutyric acid), opioid peptides, growth factors, vasopressin, tachykinins, chemokines, lipid mediators of inflammation includes prostaglandins and platelet-activating-factor, peptide hormones (eg., calcitonin, acetylcholine for muscarinic effect, serotonin) and biogenic amines (dopamine, epinephrine, norepinephrine, histamine, glutamate, neurokinin, oxytocin) . Activation of GPCRs with their ligand causes desensitization (either homologous desensitization in which the activated GPCR is down-regulated or heterologous in which the activated GPCS down-regulates another GPCR). GPCR downregulation mostly ends up in the phosphorylation of the intracellular or cytoplasmic receptor by protein kinases. Phosphorylation by cAMP-dependent protein kinases (Protein kinase A or PKA) is activated via adenylate cyclase and cyclic AMP (cAMP). In β2-adrenoreceptors, this kind of phosphorylation results in switching of coupling from the G_0 to G_i class of G-protein. An example of this type is the dopamine

receptor D2 activation via β2-adrenoreceptors. GRKs or G-protein coupled receptor kinases are protein kinases phosphorylate only active GPCRs. The phosphorylation of this kind may result either in translocation or E-arrestin linking. Physiological roles of GPCRs include regulation of immune system activity and inflammation, cell density sensing, autonomic nervous system transmission, behavioral and mood regulation **Fig. 2**.

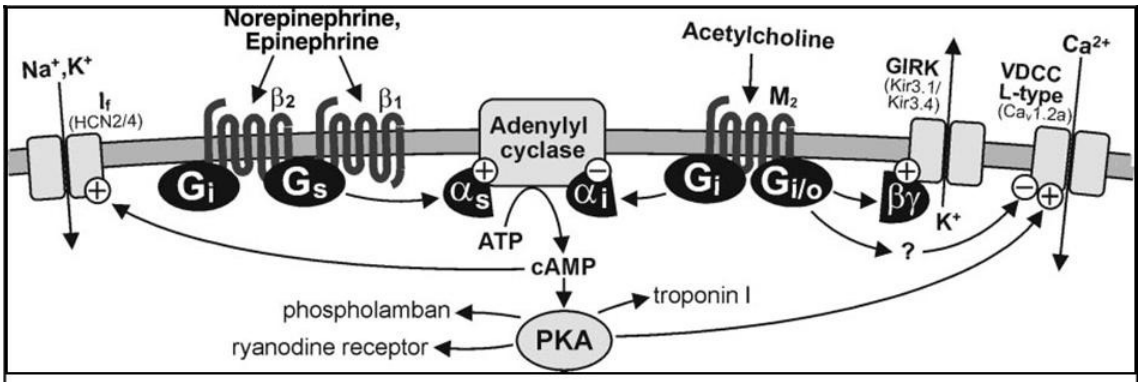

Figure 2: Role of heterotrimeric G proteins in mediating autonomic control by sympathetic and parasympathetic system. *β_1/β_2, β_1- and β_2-adrenergic receptor; M_2, muscarinic receptor; I_f, pacemaker channel; GIRK, G protein-regulated inward rectifier potassium channel;VDCC, voltage-dependent calcium channel; PKA, protein kinase A*

Source : Physiology Review, vol 5, 2005, www.prv.org.

1.2.5. Functional autoantibodies against ADRB2 & CHRM2 in CRPS

The neuroautoimmune responses are determined by how infiltrating leukocytes react to autoantibodies, which bind to autoantigens located on the surfaces of neuronal and glial cell targets. In this case, the initiation of CRPS is due to the development of autoantibodies develop against the β2AR and M2R neurotransmitter receptors by breakdown of immunologic self-tolerance (Cooper and Clark, 2013). Autoimmune etiology in CRPS patients is a new pathophysiological concept in chronic pain. (Blaes et al., 2007b). Blaes *et al.* showed through competitive binding assay that sera from patients with CRPS showed autoantibodies against structures of autonomic nervous system (Blaes et al., 2004). Our group and others also demonstrated an increased prevalence of antibodies to parvovirus B19 in CRPS patients (Goebel, 2001; van de Vusse et al., 2001). Additionally, patients with

autoimmune autonomic neuropathy also reported autoantibodies to ganglionic acetylcholine receptors (Vernino et al., 2000).

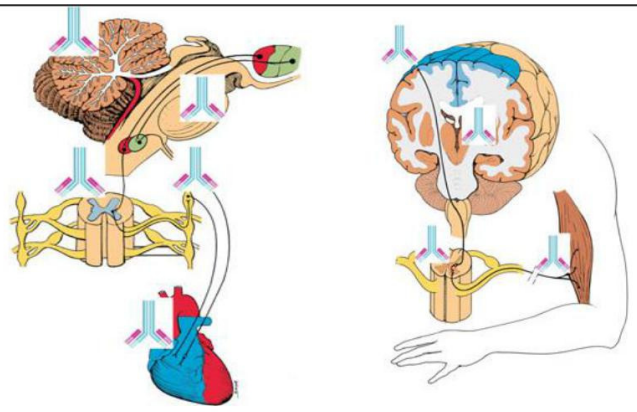

Figure 3: Possible mechanism of action and potential autoantibody binding sites in CRPS patients for Beta-2-adrenergic (2AR) and Muscarinic-2 acetylcholine receptor M2R autoantibodies. (A) Autoantibody-binding site are available for 2AR are located in the CNS, heart, cerebellum, sympathetic autonomic nervous system, astrocytes and microglia. ***(B)*** Generation of pain in skeletal muscles and possible mechanism of action and potential autoantibody binding sites in CRPS patients for Muscarinic-2 acetylcholine receptor (M2R) autoantibodies. Autoantibody-binding site are available for M2R in the parasympathetic autonomic nervous system, heart, pyramidal motor pathway to skeletal muscles, motor cortex, peripheral nerves and thalamus *(Cooper and Clark, 2013)*.

Surface-binding autoantibodies against an inducible autonomic nervous system autoantigen are found in 30–40% of CRPS patients (Kohr et al., 2009). In another study by Kohr et al., functionally active autoantibodies against β2 adrenergic receptor and M2 muscarinic receptor were present in CRPS patients' serum (Kohr et al., 2011). A recent study determined that passive transfer of serum IgG from CRPS patients resulted in behavioral and motor impairment in mice (Goebel and Blaes, 2013; Goebel et al., 2011). In CRPS IgG-transfer-trauma model, mice developed clinical and laboratory features similar to that of the human disease condition (Tekus et al., 2014). Potential autoantibody binding sites for β2AR and M2R in CRPS patients is shown in **Fig. 3** (Cooper and Clark, 2013).

1.2.6. Vascular and bone-related disturbances in CRPS

CRPS patients repeatedly report vascular disturbances as mentioned above. Molecular level understanding of the clinical progression that leads to severe vascular disturbances is still lacking. As mentioned above, most of the CRPS patients have peripheral nerve injury and a pattern of vascular leakage could be expected in these patients. Vascular disturbances are

commonly found in CRPS patients. Vascular leakage is a crucial step by which the autoantibodies gain access to the neuroautoantigens of CRPS patients. Our group found autoantibodies against neuronal epitopes in CRPS patients (Kohr et al., 2009). Regulation of these surface receptors may be controlled by the accumulation or activation by the autoantibodies and likely to initiate inflammatory responses (Cooper and Clark, 2013). There are many hypotheses, which states that vascular lesions can be a main reason behind many neuroinflammatory diseases (Mayer et al., 2010). The questions that remains unanswered was do CRPS patients have autoantibodies against vascular structures (endothelial cells) and how far they contribute to the pathophysiology of this painful disease.

Bone related issues are obvious in CRPS patients. The mechanism that causes osteoporosis in CRPS patients is poorly understood. Numerous studies have shown that β-Adrenergic receptors (βAR) play an important role in regulating bone remodeling. The expression of β-Adrenergic receptor kinase-like activity and β-arrestin was observed in osteoblasts (Bliziotes et al., 1996). Among the βAR, hypothalamic β1 and β2-adrenergic system stimulates bone formation while the β2AR in bone tissue activate osteoclastogenesis and increase bone resorption (Zofkova and Matucha, 2014). Various studies supports impaired wound healing and exacerbating inflammation via a novel crosstalk between adrenergic and toll-like receptor pathways in BM-MSCs (Bone-marrow derived mesenchymal stem cells) and (neonatal keratinocytes) NHKs (Dasu et al., 2014). Gianfagna *et al.* did a study in European children and showed that the Neuromodin U (NMU) plays an important role in regulating the bone strength and this regulation is mediated through ADRB2 gene (Gianfagna et al., 2013). Additionally, studies show that in BMMs (bone marrow macrophages) and RAW 264.7 cells Reactive Oxygen species (ROS) acts as a regulatory molecule and induces osteoclastogenesis via E-Adrenergic receptor signaling (Kondo et al., 2013). Numerous studies have shown that patients with hip fractures were treated successfully with beta-blockers (such as propranolol hydrochloride) (Zofkova and Matucha, 2014). Some studies show that CRPS patients have elevated levels of osteocyte activity (Boyce et al., 2006; Bucay et al., 1998). While some studies revealed that OPN (osteopontin) plays a major role in sympathetic tone regulation of bone mass takes place through the modulation of β2AR/cAMP signaling system (Nagao et al., 2011). Model of regulation of β2AR signaling by OPN is shown in **Fig. 4.** Other controversial discussions are available which says OPG (Osteoprotegerin) might play a protective role and can be used as a biomarker for impaired bone metabolism in CRPS (Boyce and Xing, 2007; Kramer et al., 2014).

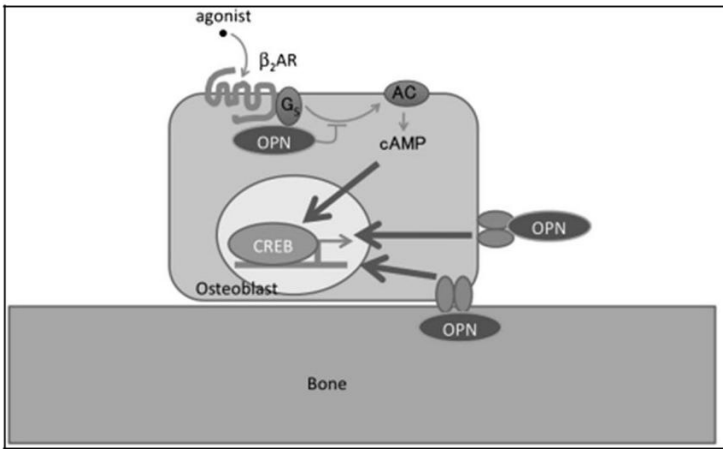

Figure 4: Model of regulation of β2AR signaling by OPN in osteoblasts. OPN regulates β2AR signaling in osteoblasts by interacting with G_S to reduce the time course of cAMP production and CRE-dependent transcription activity to ultimately reduce bone mass. (Nagao et al., 2011).

Chapter 1.3: CRPS - Therapeutic Approaches

1.3.1. Pharmacological therapeutic approaches

Pharmacological treatments are mainly to provide pain relief and to accelerate physical rehabilitation. Unfortunately, in RCTs, oral, topical, and intravenous medications targeting the sympathetic nervous system and D-adrenergic receptors (clonidine) have not been proven effective.

Opiods, anticonvulsants and antidepressants are most commonly used in other neuropathic pain conditions but have not been extensively studied in CRPS. Immunomodulating agents such as infliximab and thalidomide were successful in few CRPS studies (Bernateck et al., 2007). Intriguingly, potent anti-inflammatory agents such as corticosteroids have shown to be beneficial for CRPS in RCTs (de Mos et al., 2009d; Kalita et al., 2006; van Rijn et al., 2009). Use of Gabapentin in patients with CRPS showed positive results (Mellick, 1995; Mellick and Mellick, 1995; van de Vusse et al., 2004).

Glucocorticoids use in CRPS patients have shown positive effect as they help in inhibiting the expression of neuropeptides, pro-inflammatory cytokines such as TNF-D, IL-1β etc., and inflammatory mediators like prostaglandins (Bernateck et al., 2007; Guo et al., 2006; Huygen et al., 2004; Kingery et al., 2001a; Kingery et al., 2001b; Piedimonte et al., 1991). To reduce limb sensitivity and swelling, low-dose local anaesthetics (ropivacaine and levo-bupivacaine) are used with corticosteroids (methyl prednisolone, triamcinalone, and beclomethasone) or clonidine in nerves blocks (peripheral, plexus, or neuraxis). Apart from this, supporting

studies showed that free radical scavengers like fatty cream with 50% DMSO in RCTs showed improvement in inflammatory signs and pain (Zuurmond et al., 1996). At present, it is not possible to determine the effect of sympathetic blockade (with local anaesthetic) on long-term pain relief (Cepeda et al., 2005). The effect of sympathetic blockade is unclear, with some studies showing brief benefit and others showing no beneficial effects and most important is to consider about the small group of patients (Albazaz et al., 2008; de Mos et al., 2009d).

Intravenous Immunoglobulin (IvIg) is used to treat immunodeficiency and autoimmune disorders. Recently, IvIg has been successfully used to treat chronic CRPS (Goebel et al., 2010; Goebel et al., 2005). In another study a young patient who developed CRPS secondary to sciatic compression was treated with high dose of IvIg (2g/kg) and recovered completely within 10 days after infusions (Medlin et al., 2013). An open study was made to determine the immunoglobulin maintenance in treating long-standing CRPS (Goebel et al., 2013). Ketamine, magnesium or tadalafil have been shown to be effective in treating CRPS (Birklein and Sommer, 2010; Collins et al., 2009; Groeneweg et al., 2008a; Schwartzman et al., 2009a). Other therapeutic approaches such as using inhibitors of osteoclasts activity were also shown to be convincing for the treatment of CRPS mediated bone disturbances. Bisphosphonates (clodronate, alendronate, and ibandronate) have been studied in controlled trials and showed the potentiality to reduce pain associated with bone loss in patients with CRPS I (Albazaz et al., 2008; Brunner et al., 2009). Use of calcitonin showed positive effects on pain but it failed to show positive effects on CRPS-mediated osteoporosis (Forouzanfar et al., 2002; Gobelet et al., 1992; Perez et al., 2001).

1.3.2. Non-pharmacological therapeutic approaches

Non-pharmocological therapies are often physiotherapy, exercise and other activities in which the patients are actively involved and aimed at improving or restoring the affected limb. A recent systematic review showed that spinal cord stimulation was effective in reducing the chronic neuropathic pain of CRPS type I (Simpson et al., 2009). Transcutaneous electric nerve stimulation (TENS) is another kind of anelgetic therapy which showed reduction of pain in patients with CRPS (Robaina et al., 1989).

A potential beneficial effect was demonstrated in studies with physiotherapy and cognitive behavioral therapy. No relation between psychological factors and CRPS were shown in majority of studies (de Mos et al., 2009d). Due to lack of high-quality methodological studies the actual association between psychological factors and CRPS remains controversial. The severe chronic pain and disability of CRPS may cause psychological distress. Experts emphasize the need for psychological and behavioral therapy as part of optimal treatment programs for CRPS patients (Bruehl and Chung, 2006; de Mos et al., 2009d). Co-morbidities, such as depression and anxiety, should be treated concurrently. Extreme fear for pain can lead to disuse of the affected extremity, thereby making a feasible contributor to the disease course of CRPS (de Mos et al., 2009d).

2. AIM OF THE THESIS

Complex regional pain syndrome (CRPS) is a painful condition in which patients are profoundly affected with decreased ability to participate in normal activities of daily living. An increased interest in CRPS has driven the understanding of the pathophysiology of this painful disease. Studies have shown a possible involvement of immune system and development of autoimmune harmony in CRPS. Our group has shown that these patients exhibit autoantibodies against β2-adrenergic and M2 muscarinic receptors. However, the role of functionally active autoantibodies is yet to be illustrated. Therefore, the main objectives of this study were:

1. To investigate the contribution of autoantibodies in development of the local trophic changes in the affected limb.
2. To identify the mechanism of functional autoantibodies in mediating the inflammation in vascular and bone cells.
3. To determine the possible therapeutic role of Intravenous Immunoglobulin (IvIg) as a new treatment option for CRPS.

3. MATERIALS AND METHODS

3.1. PATIENT SAMPLES

Serum was obtained from 13 clinically defined CRPS patients according to the IASP diagnostic criteria (Bruehl et al., 1999; Harden et al., 2007) after informed consent and approval from the ethical committee of the Johannes-Gutenberg-University Mainz. Sera of 11 sex and age matched healthy controls (HC) and 7 radial fracture (RF) patients (who did not develop CRPS) served as controls. None of the healthy controls or radial fracture patients had a history of chronic neuropathic pain. **Table 3** shows the detailed clinical and immunological data of the CRPS patients.

3.2. MATERIALS

3.2.1. Chemicals, acids and bases

Compounds	Company
Acetic acid	Merck KGaA, Darmstadt, Germany
Ammonium Persulphate (APS)	Carl Roth, Karlsruhe, Germany
Agarose	Bioline GmbH, Luckenwalde, Germany
Atropine	Sigma-AldrichChemieGmbH,Taufkirchen, Germany
Bovine Serum Albumin (BSA)	Sigma-AldrichChemieGmbH,Taufkirchen, Germany
Bromophenol Blue	Neolab, Heidelberg, Germany
Disodium hydrogen phosphate (Na_2HPO_4)	Merck KGaA, Darmstadt, Germany
Distilled water (Ecostrain®)	Braun, Melsungen, Germany
Dimethylsulfoxide (DMSO)	Carl Roth, Karlsruhe, Germany
DNase	Qiagen, Hilden, Germany
EDTA	Carl Roth, Karlsruhe, Germany
Ethanol 100%	Sigma-AldrichChemieGmbH,Taufkirchen, Germany
FBS	PAA Laboratories, Pasching, Austria

Glycerol	Carl Roth, Karlsruhe, Germany
Glycin	Merck KGaA, Darmstadt, Germany
Isopropanol	Merck KGaA, Darmstadt, Germany
Methanol	Merck KGaA, Darmstadt, Germany
2-Mercaptoethanol	Sigma-AldrichChemieGmbH,Taufkirchen, Germany
NP40	US Biologicals, Swampscott, MA
Paraformaldehyde (PFA)	Sigma-AldrichChemieGmbH,Taufkirchen, Germany
Potassium chloride (KCL)	Merck KGaA, Darmstadt, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck KGaA, Darmstadt, Germany
10x PBS for cell culture (DPBS)	Lonza, Köln, Germany
2-Propanol	Sigma-AldrichChemieGmbH,Taufkirchen, Germany
RNAse free water	Millipore corporation, MA, USA
Rotiphorese Gel (30% acrylamide mix)	Carl Roth, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth, Karlsruhe, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Merck KGaA, Darmstadt, Germany
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck KGaA, Darmstadt, Germany
Sodium azide (NaN_3)	Merck KGaA, Darmstadt, Germany
Sodium Dodecyl Sulfate (SDS)	Neolab, Heidelberg, Germany
Trishydroxymethyl aminomethane (Tris)	Carl Roth, Karlsruhe, Germany
Tryphan blue	Carl Roth, Karlsruhe, Germany
Tris acetate-EDTA buffer (TAE) 10x	Carl Roth, Karlsruhe, Germany
Tris HCl	Carl Roth, Karlsruhe, Germany
Trypsin (2.5 g/l)	Gibco, Invitrogen, Carlsbad, USA
Tween 20	Merck KGaA, Darmstadt, Germany
TEMED	Carl Roth, Karlsruhe, Germany
Intravenous Immunoglobulin (IvIg) Gamunex® 10%	TalecrisbiotherapeuticsGmbH,Frankfurt, Germany
Bovine serum Albumin fraction V	Merck KGaA, Darmstadt, Germany
Non-fat dry milk powder	Cell Signaling Technology, MA, USA

Cell lysis buffer (10x)	Cell Signaling Technology, MA, USA

3.2.2. Laboratory consumables

Consumables	Company
Cellstar® 6 Well and 24 well Cell Culture Plate	GreinerBioOne, Frickenhausen, Germany
Cellstar® Plastikpipettes (5 ml, 10 ml)	GreinerBioOne, Frickenhausen, Germany
Cellstar®U-shapewithLid,TC-Plate,96 well, sterile	GreinerBioOne, Frickenhausen, Germany
Cellstar® Flat bottom withLid, TC-Plate, 96 well, sterile	GreinerBioOne, Frickenhausen, Germany
Cellstar® 75 cm^2 Cell cultur flasks	GreinerBioOne, Frickenhausen, Germany
Cellstar® 125 cm^2 Cell cultur flasks	GreinerBioOne, Frickenhausen, Germany
Cell scrapper	GreinerBioOne, Frickenhausen, Germany
Cryobox 136x136x130 mm	Ratiolab GmbH, Dreieich, Germany
Cryo TubeTM vials (1,8 mL; 4,5 mL)	Sarstedt AG & Co, Nümbrecht, Germany
Polysterene disposable cuvettes for Bradford assay	Ratiolab GmbH, Dreieich, Germany
FACS-Tubes 0,5 mL 38 x 6,5 mm PS	Sarstedt AG & Co, Nümbrecht, Germany
Falcon5mLPolystyreneRound-Bottom Tube	Becton Dickinson, Heidelberg, Germany
Falcon tubes (15 ml, 50 ml)	Becton Dickinson, Heidelberg, Germany
Glass Pasteur pipettes 150 mm	Brand, Wertheim, Germany
Ministart single use filter (0,2 µm, 0,45 µm)	SartoriusStedimBiotechGmbH,Göttingen, Germany
Nitra-Tex® powder free gloves	B. Braun Melsungen AG, Germany
Parafilm	Pechiney Plastic packaging, Menasha, WI
Pipette tips withoutfilter (10 µL, 100 µL, 1000 µL)	Sarstedt AG & Co, Nümbrecht, Germany
Eppendorf tubes 1,5 mL, 2 mL	EppendorfVertriebDeutschlandGmbH, Wesseling-Berzdorf, Germany
Eppendorf tubes1,5mL, 2mL (PCR	Nerbe Plus GmbH, Winsen (Luhe), Germany

clean- pyrogen & DNase free)	
Servapor® dialysis tubing (6 mm, 25 mm)	Serva Electrophoresis GmbH, Heidelberg, Germany
S-Monovette® 7,5 mL Z (Serum-Tubes)	Sarstedt AG & Co, Nümbrecht, Germany
Sterile PCR- clean pyrogen & DNase free with filter (10, 100, 200, 1000 µl)	Nerbe Plus GmbH, Winsen (Luhe), Germany
Tissue culture dishes steril 35,0 / 10 mm	GreinerBioOne, Frickenhausen, Germany
UV-Spectroscopic cuvettes (RNA quantification)	BioRad, München, Germany
Falcon® Plastic pipettes 25 ml, 10 ml, 5 ml	Becton Dickinson, Heidelberg, Germany
Glasswares (different sorts)	Fisherbrand; IDL; Schott&Gen; Simax
Syringe 25 ml for BSA	B. Braun Melsungen AG, Germany
Syringe5ml,10mlforserumfiltration	B. Braun Melsungen AG, Germany
Nitrocellulose membrane	GEHealthcare,Amersham™HybondECL, Buckinghamshire, UK
PCR Tube-strips	Applied Biosystems, Darmstadt, Germany
PCR Cap-strips	Applied Biosystems, Darmstadt, Germany
Dialysis clips	Ratiolab GmbH, Dreieich, Germany
Safety-Multifly®–Set, sterile, pyrogenfree (Cannulae)	Sarstedt AG & Co, Nümbrecht, Germany
Grid inserts for Cryobox	Ratiolab GmbH, Dreieich, Germany

3.2.3. Laboratory instruments

Instruments	Company
ELISA-Reader Multiscan EX	Thermoelectroncorporation,Langenselbold, Germany
Fusion FX7 chemiluminescence system	Peqlab Biotechnologie GmbH, Erlangen, Germany
Neubauer improved Haemocytometer	Brand, Wertheim, Germany
SmartSpecTM Plus Spectrophotometer	BioRad, München, Germany
Trans-Blot® SD Semi-dry transfer cell	BioRad, München, Germany

Nanophotometer	Implen GmbH, München, Germany
StepOne® Real-Time PCR system	Applied Biosystems, Darmstadt, Germany
Light Microscope for cell culture	Carl Zeiss Microscopy GmbH, Oberkochen Germany
BD FACS Calibur	BD Biosciences, Heidelberg, Germany
Hettich centrifuge (cooling)	Hettich GmbH, Kirchlengen, Germany
Magnetic stirrer	IKA£ Werke GmbH, Staufen, Germany
Centrifugetype2-6Easiashaker Medgenix diagnostics	Sigma-AldrichChemieGmbH,Taufkirchen, Germany
Centrifuge Universal 32 R (cell culture)	Hettich GmbH, Kirchlengen, Germany
Nuaire™ Biological Safety Cabinets Class II type A/B3 (Sterilbank)	INTEGRABiosciences GmbH, Fernwald, Germany
Arpege 75, Liquid nitrogen tank	AirLiquideMedicalGmbH,Düsseldorf, Germany
Nalgene™ Cryo 1°C Freezing container	Nalgene®, Germany
pH-Meter	Mettler Toledo GmbH, Giessen, Germany
Pipette boy	INTEGRABiosciences GmbH, Fernwald, Germany
Power pack	Peqlab Biotechnologie GmbH, Erlangen, Germany
ProSpec (Nephelometer)	Dade BehringMarburg GmbH, Marburg, Germany
Pump P-1 (Pump for IgG purification)	GE Healthcare Europe GmbH, Freiburg, Germany
Refrigerators and freezers	Different companies
Rotamax 120 (Shaker)	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
Swivel platform	Peqlab Biotechnologie GmbH, Erlangen, Germany
Sanyo Incu-safe incubator for cell culture	Ewald InnovationstechnikGmbH,Bad Nenndorf, Germany
Table top centrifuge EBA 20	Hettich GmbH, Kirchlengen, Germany

Table top centrifuge micro 120	Hettich GmbH, Kirchlengen, Germany
Vortexer vortex-Genie2	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
Weighing balance	SartoriusStedimBiotechGmbH,Göttingen, Germany
Water bath	Memmert GmbH + Co.KG, Germany
HiTrap¥proteinGHP(1mLund5mL Protein G columns)	GEHealthcareEuropeGmbH,Freiburg, Germany
Fluorescence microscope	Carl Zeiss Microscopy GmbH, Oberkochen Germany
Gel tank for western blotting	BioRad, München, Germany
Combs for western blotting	BioRad, München, Germany
Gel casting trays and frames	BioRad, München, Germany
BEP 2000 Advance (ELISA-Reader)	DadeBehringMarburgGmbH,Marburg, Germany

3.2.4. DNA and Protein ladders

Markers	**Company**
PageRuler™ Plus Prestained Protein Ladder	Fermentas, Invitrogen, Carlsbad, USA
Spectra™ Multicolor Broad Range Protein Ladder	Thermo Fischer Scientific, Rockford, USA
Fluorescent Long Range DNA Ladder	Jena Bioscience, Jena, Germany

3.2.5. Buffers

Buffers	**Components**	**Volume**
1X SDS-PAGE Running Buffer	Carl Roth, Karlsruhe, Germany (Rotiphorese® 10X SDS-PAGE) H_2O	100ml 900ml
10x PBS (1 Liter)	137mM NaCl	80g

	2 mM KH_2PO_4 2.7 mM KCl 10 mM Na_2HPO_4 H_2O pH	2.4 g 2 g 14.4 g 1000 ml 7.4
10x TBS (1 Liter)	Tris NaCl H_2O pH	24.2 g 87.7 g 1000 ml 7.2 to 7.6
1x TBS-Tween (TBST) (1 Liter) (Stored at RT)	1x TBS 0.1% Tween®20	100 ml 1 ml
Lysis Buffer (250 ml)	NaCl Tris EDTA Glycerol NP40 NaN_3 pH	2.19 g 0.61 g 0.07 g 25 ml 2.5 ml 0.025 g 7.4
6xSDS-PAGELoadingBuffer (Laemmli buffer)	60 mM Tris-HCl 2% SDS 0.01% Bromophenol blue 10% Glycerol ddH_2O ß-Mercaptoethanol	36 ml (pH 6.8) 60 ml 60 mg 60 ml 144 ml 65 µl/ml
SDS-PAGETransferbuffer(1 Liter)	10x Running buffer Methanol ddH_2O	100 ml 200 ml 700 ml
5%Non-fatdrymilk(blocking buffer)	Milk powder TBST	5 g 100 ml
Blocking buffer (5% BSA)	Bovine Serum Albumin fraction V TBST	5 g 100 ml
4% Paraformaldehyde (250 ml)	Paraformaldehyde	4 g
0.1 M Glycine Buffer (500 ml) –	Glycine	3.75 g

Washing buffer	H_2O pH	500 ml 9.0
0.1 M Glycine Buffer (100 ml) – Elution buffer	Glycine H_2O pH	0.75 g 100 ml 2.7
1 M Tris HCl	pH	8.5
FACS Buffer (500 ml)	10x PBS 10% FBS 10% NaN_3 H_2O	50 ml 5 ml 5 ml 500 ml
10x Trypsin EDTA	10x Trypsin ddH_2O	5 ml 45 ml
10%Ammonium Persulfate (APS)	APS ddH_2O	1 g 10 ml
10% Sodiumdodecylsulfate (SDS)	SDS ddH_2O	1 g 10 ml
1xCelllysisBuffer(Forprotein extraction of primary osteoblasts)	20 mM Tris HCl 150 mM NaCl 1 mM Na_2EDTA 1 mM EDTA 1% Triton 2.5 mM sodium pyrophosphate 1 mM ß-glycerophosphate 1 mM Na3VO4 1 µg/ml leupeptin	

3.2.6. Molecular biology KITS and Assay reagents

Kits	Manufacturer	Artikle Nr.	Method
peqGOLDTotal RNA Isolation Kit	PeqlabBiotechnologieGmbH, Erlangen, Germany	12-6834-02	RNA Isolation
BCA Protein Assay	Pierce®ThermoScientific,IL,	23225	Protein

Kit	USA		quantification
QuantiTect® Reverse Transcription Kit	Qiagen GmbH, Hilden, Germany	205314	Reverse Transcription
Quanti Fast™ SYBRGreenPCR kit	Qiagen GmbH, Hilden, Germany	204156	PolymeraseChain Reaction (PCR)
iTaq™ Universal SYBR£ Green qPCR Master Mix	Bio-Rad, CA, USA	172-5124	PolymeraseChain Reaction (PCR)
Cell Proliferation reagent WST-1	Roche Diagnostics GmbH, Mannheim, Germany	11644807 001	Proliferation Assay
Cytotoxicity detection kit (LDH)	Roche Diagnostics GmbH, Mannheim, Germany	11644793 001	Cytotoxicity assay
Annexin-V-Fluos Apoptosis Staining kit	Roche Diagnostics GmbH, Mannheim, Germany	11988549 001	Apoptosis staining
Lipofectamine£ RNAiMAX reagent	Lifetechnologies GmbH, Darmstadt, Germany	13778-075	siRNAmediated transfection

3.2.7. Primary antibodies

Name	Host	Reactivity	Mol. Weight	Method	Cat. No.	Company
Anti-ADRB2	Rabbit	H M	46 kDa	FACS, WB	ab61778	Abcam plc, Cambridge, UK
Anti-CHRM2	Mouse	H M P R	64 kDa	FACS, WB	ab2805	Abcam plc, Cambridge, UK
Anti-Akt	Rabbit	H M R MK	60 kDa	WB	9272	Cell Signaling Technology, MA, USA

Anti-pAkt (Ser473)	Rabbit	H M R MK	60 kDa	WB	4060s	Cell Signaling Technology, MA, USA
Anti-Erk 1/2	Rabbit	H M R MK	44/42 kDa	WB	9102	Cell Signaling Technology, MA, USA
Anti-pErk 1/2	Rabbit	H M R Mk Pg Sc Hm B Z	44/42 kDa	WB	4370s	Cell Signaling Technology, MA, USA
Anti-Total P38 MAPK	Rabbit	H M R Mk GP (C)	43 kDa	WB	9212s	Cell Signaling Technology, MA, USA
Anti-pP38 MAPK (Thr180/ Tyr182)	Rabbit	H M R Mk	43 kDa	WB	9211s	Cell Signaling Technology, MA, USA
Anti-Stat1	Rabbit	H M R MK	84, 91 kDa	WB	9172	Cell Signaling Technology, MA, USA
Anti-pStat1	Rabbit	H M R	84, 91 kDa	WB	9171s	Cell Signaling Technology, MA, USA
Anti-GAPDH	Mouse	Ca H M R	38 kDa	WB	MAB374	Chemicon/ Millipore, Temecula, CA
Anti-Total SAPK/JNK	Rabbit	H M R Hm Z B Sc	46, 54 kDa	WB	9252	Cell Signaling Technology, MA, USA
Anti-Collagen 1D1	Sheep	H	140 kDa	WB	Af6220	R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany

3.2.8. Secondary antibodies

Antibody	Host	Cat. No.	Company
Anti-Rabbit HRP	Goat	sc-2004	Santa Cruz Biotech, CA, USA
Anti-Mouse HRP	Donkey	sc-2318	Santa Cruz Biotech, CA, USA
Anti-Goat HRP	Donkey	sc-2020	Santa Cruz Biotech, CA, USA
Anti-Sheep HRP	Donkey	sc-2473	Santa Cruz Biotech, CA, USA
Anti-Human IgG/FITC	Human	F 0202	DAKOCytomation, Glostrup, Denmark

3.2.9. Softwares

Softwares	Company
Fusion X7 software	PEQLAB, Erlangen, Germany
Fusion BioID software	PEQLAB, Erlangen, Germany
GraphPad Prism version 4.00	GraphPadSoftware,SanDiego,California, USA
Microsoftword, powerpoint,Excel 2010	Microsoft corporation
Cell Quest software	BDCellQuestProsoftwareonMac®OSfor flow cytometry
BEP 2000 SW V.1.23.4 (ELISA reader)	Thermo Fischer Scientific, Rockford, USA
StepOne Software v2.1	AppliedBiosystemsbyLifetechnologies GmbH, Darmstadt, Germany
Primer 3.0 online tool	http://primer3.ut.ee
Primer Blast NCBI online tool	http://www.ncbi.nlm.nih.gov/tools/primer-blast/

3.2.10. Primer sequences

Primer name	Primer sequence	Nucleotide ID
Hu BMP-2 Fw	5´- CCTCAGCAGAGCTTCAGGTT -3´	NM_001200.2
Hu BMP-2 Rv	5´- AATTCGGTGATGGAAACTGC -3´	

Hu TGFE-1 Fw	5´- GTACCTGAACCCGTGTTGCT -3´	NM_000660.5
Hu TGFE-1 Rv	5´- CACGTGCTGCTCCACTTTTA -3´	
Hu MHC-I Fw	5´- CAGGACACTGAGCTTGTGGA -3´	NM_005514.6
Hu MHC-I Rv	5´- TCTTCTCCAGAAGGCACCAC -3´	
Hu MHC-II Fw	5´- CTGGAACAGCCAGAAGGAAG -3´	NM_002124.3
Hu MHC-II Rv	5´- ACCTCGTAGTTGTGTCTGCAC -3´	
Hu ICAM-1 Fw	5´- CAAGGCCTCAGTCAGTGTGA -3´	NM_000201.2
Hu ICAM-1 Rv	5´- GTGTCTCCTGGCTCTGGTTC -3´	
Hu VCAM-1 Fw	5'- ACACTTGCTGCTGGTGATTC -3'	NM_001078.3
Hu VCAM-1 Rv	5'- ACACTTGCTGCTGGTGATTC -3'	
Hu MCP-1 Fw	5'- ACACTTGCTGCTGGTGATTC -3'	NM_002982.3
Hu MCP-1 Rv	5'- ACACTTGCTGCTGGTGATTC -3'	
Hu GAPDH Fw	5´- AATCCCATCACCATCTTCCA -3´	NM_001256799.
Hu GAPDH Rv	5´- TGGACTCCACGACGTACTCA -3´	1
Ms GAPDH Fw	5´- ACTCCACTCACGGCAAATTC -3´	NM_008084.3
Ms GAPDH Rv	5´- CTCCTGGAAGATGGTGATGG -3´	
Hu ADRB2 Fw	5´- CTGGTGCGAGTTTTGGACTT -3´	NM_000024.5
Hu ADRB2 Rv	5´- ATGGCAAAGTAGCGATCCAC -3´	
Hu CHRM2 Fw	5´- GTCAAGCGGACCACAAAAAT -3´	NM_001006632.
Hu CHRM2 Rv	5´- CCAGAAGAGAATGGCTGGAG -3´	1
CHO GAPDH Fw	5´- AAGAGGGTCATCATCTCCGC -3´	NM_001244854.
CHO GAPDH Rv	5´- TGCTGACAATCTTGAGGGAGT -3´	2
Hu RANK Fw	5´- ATCTGGCCAAGAGGAGCAAG -3´	NM_033012.3
Hu RANK Rv	5´- TTGGAGATCTTGGCCCAACC -3´	
Hu CTR Fw	5´- GCAATGCTTTCACTCCTGAGAAA -3´	NM_001164737.
Hu CTR Rv	5´- ACACGAAAATCCCCAGGGAA -3´	1
Hu CTSK Fw	5´- TTCCGCAATCCCGATGGAATA -3´	NM_000396.3
Hu CTSK Rv	5´- GCAAAGCTCACCACAGGTAG -3´	
Hu Col 1 Fw	5´- ACGTCCTGGTGAAGTTGGTC -3´	NM_000088.3
Hu Col 1 Rv	5´- ACCAGGGAAGCCTCTCTCTC -3´	
Hu DKK 1 Fw	5´- AGCGTTGTTACTGTGGAGAAG -3´	NM_012242.2
Hu DKK 1 Rv	5´- GTGTGAAGCCTAGAAGAATTACTG -3´	

Hu ALP Fw	5´- CCTCCTCGGAAGACACTCTG -3´	NM_000478.4
Hu ALP Rv	5´- AGACTGCGCCTGGTAGTTGT -3´	
Hu TRAP Fw	5´- GATCCTGGGTGCAGACTTCA -3´	NM_001611.3
Hu TRAP Rv	5´- GCGCTTGGAGATCTTAGAGT -3´	
Hu Integrin β3 Fw	5´- TCGAGTTCCCAGTGAGTGAG -3´	NM_000212.2
Hu Integrin β3 Rv	5´- GACAGGTCCATCAAGTAGTAG -3´	
Hu Osteocalcin Fw	5´- GGCGCTACCTGTATCAATGG -3´	NM_199173.4
Hu Osteocalcin Rv	5´- TCAGCCAACTCGTCACAGTC -3´	

3.2.11. Cell culture medium and solutions

CHO-WT(wild type)	DMEM/HAM's F-12 medium (with 1% L-glutamine 15 mM HEPES 1.2 g/L NaHCO3)	Basal medium 10% fetalbovine serum (FBS) 1% Penicillin/ Streptomycin	500 ml 50 ml 5 ml	PAN Biotech GmbH, Aidenbach, Germany
hβ2AR-CHO		0.2 mg/ml G418	200 µl/10 ml	Invitrogen GmbH, Darmstadt, Germany
hM2R-CHO				
HCMEC	Microvascular Endothelial cell growth medium classic mix	Basal medium Supplement mixture Antibiotics solution	500 ml 50 ml 5 ml	Cellovations ,PELO Biotech GmbH, Planegg, Germany

HEK293 (Fibroblasts)	RPMI 1640	Basal medium 10% fetal bovine serum (FBS) 1% Penicillin/ Streptomycin	500 ml 50 ml 5 ml	PAN Biotech GmbH, Aidenbach, Germany
A10	DMEM(with 4.5 g/L glucose and NEAA (non-essential aminoacids)	Basal medium 20% fetal bovine serum (FBS)	500 ml 100 ml	Gibco£ GmbH, Darmstadt, Germany
Mesenchymal Stem Cells (MSCs)	F-12K medium	Basal medium 20% fetal calf serum (FCS) 1% Penicillin/ Streptomycin	500 ml 100 ml 5 ml	PAA Laboratories GmbH, Pasching, Austria
MSCs to Primary Osteoblast differentiation	DMEMlowglucose (1g/L)withL-Glutamine	Basal medium 10% fetal calf serum (FCS) Dexamethasone Ascorbicacid-2-phosphate Glycerolphosphates Penicillin Streptomycin	500 ml 50 ml 0.1 µM 0.05 mM 10 mM 100 U/ml 100 g/ml	PAA Laboratories GmbH, Pasching, Austria

3.2.12. Cell lines

Cell line name	Origin	Source
CHO-WT (wild type)	Chinese hamster ovary	Kindly provided by Dr. Mohr, Bonn, Germany
hβ2AR-CHO	Chinese hamsterovary, transfected with hβ2AR	Kindly provided by Dr. Gerd Wallukat, Berlin, Germany

hM2R-CHO	Chinese Hamster ovary, transfected with hM2R	KindlyprovidedbyDr.Mohr, Bonn, Germany
HCMEC	Human Cerebral MicrovascularEndothelial cell line	AG Blaes, Neurology - Giessen
HEK293	Human fibroblasts cell line	AG Blaes, Neurology - Giessen
U937	Human monocyte cell line	AG Blaes, Neurology - Giessen
A10	Rat smooth muscle cell line	AG Blaes, Neurology - Giessen
Primary Osteoblast ZK37	Trauma patient ID ZK37	Laboratory of experimental trauma surgery, Giessen
Primary Osteoblast ZK48	Trauma patient ID ZK48	Laboratory of experimental trauma surgery, Giessen
Primary Osteoblast ZK56	Trauma patient ID ZK56	Laboratory of experimental trauma surgery, Giessen
Primary Osteoblast ZK58	Trauma patient ID ZK58	Laboratory of experimental trauma surgery, Giessen
Primary Osteoblast ZK59	Trauma patient ID ZK59	Laboratory of experimental trauma surgery, Giessen
SAOS-2	Human Osteosarcomacell line	Laboratory of experimental trauma surgery, Giessen

3.3 METHODS

3.3.1. Expt. set 1: Determination of pathogenicity of CRPS IgG in immortalized cells

3.3.1.1. IgG Purification by affinity chromatography

IgG purification was performed using a HiTrap Protein-G column (HiTrap Protein-G HP). Sera from the CRPS patients, HC and RF are diluted to 1:5 in 0.1 M Glycine buffer (pH 9.0). For eluting the bound IgG from the Protein-G column, elution buffer is used (0.1 M Glycine buffer pH 2.9). The eluted IgG is dialysed against phosphate-buffered saline (PBS, pH 7.4). IgG-concentration is determined by nephelometry and stored in aliquots at -20 °C at a stock

concentration of 3 g/l until used. The working concentration for all the experiments was constantly maintained as 12.5 µg/ml.

3.3.1.2. Cell culture methods

Chinese hamster ovary (CHO) cells stably transfected with human muscarinic-2 receptor (hM2R) or human β2-adrenergic receptors (hβ2AR) were cultured in DMEM/HAMs F-12 medium. The medium for transfected cells was supplemented with 63mg/L of Geneticin G418. Dr. Gerd Wallukat, Berlin, Germany and Prof. Dr. Mohr, Bonn, Germany generously supplied the hβ2AR-CHO and hM2R-CHO cells respectively.

Human cerebral microvascular endothelial cells (HCMEC) were grown in Human Microvascular Endothelial Cell Growth medium. This microvascular endothelial medium was enriched with 5% fetal calf serum together with supplements and antibiotics provided by the manufacturers.

Human embryonic kidney (HEK293) cells and monocyte cell line U937 were cultured using RPMI 1640 nutrient medium. The medium was additionally supplemented with 10% FBS and 1% Penicillin/Streptomycin.

Rat smooth muscle (A10) cells were cultured using DMEM (with 4.5 g glucose, Non-essential amino acids without sodium pyruvate and Glutamine) and suplemented with 20% FBS without antibiotic. All cells were grown under standard cell culture conditions (37 $^{\circ}$C, 5% CO_2).

3.3.1.3. Expression study by Immunoblotting

Stably transfected CHO cells overexpressing the β2-adrenergic receptor and M2-muscarinic receptor, HCMEC, U937, A10 and HEK293 cells were plated in 6-well plates at a concentration of $1x10^5$ cells/ml and examined in western blotting for the expression of Human Adrenergic β2 receptor (ADRB2) and Muscarinic M2 receptor (CHRM2). Cells were lysed using the lysis buffer (Cell signaling technology, Danvers, MA, USA) and the amount of protein was estimated by Bradford assay and normalized. Equal amount of protein (60 µg) were loaded and separated by 10% SDS-PAGE, transferred (Trans Blot, Semi dry Transfer

cell, BioRad) to a nitrocellulose membrane (GE Healthcare, Amersham™ Hybond ECL, Buckinghamshire, UK) and blocked with 5% BSA for 1hour. The membranes were incubated overnight at 4°C with the primary antibodies Anti- β2 Adrenergic receptor antibody; Anti-Muscarinic Acetylcholine Receptor 2 antibody (31-1D1) (1:500; Abcam, Cambridge, UK). Membranes were then incubated for 1 h with goat anti-rabbit and donkey anti-mouse secondary antibodies (1:1000, Santa Cruz Biotechnology, CA, USA) and developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo scientific, Pierce Biotechnology, Rockford, IL, USA) in Fusion FX7 chemiluminescence system (PEQLAB, Erlangen, Germany). GAPDH (Santa Cruz Biotechnology, CA, USA) was used as a loading control.

3.3.1.4. ADRB2 and CHRM2 expression study by flow cytometry

Stably transfected CHO cells overexpressing the β2-adrenergic receptor and M2-muscarinic receptor, HCMEC, U937, A10 and HEK293 cells were plated in 6-well plates at a concentration of $1x10^5$ cells/ml and examined in flow cytometry for the expression of Human Adrenergic β2 receptor (ADRB2) and Muscarinic M2 receptor (CHRM2). Cells were washed with 1x PBS and harvested using cell scrapper. Then the cells are plated in 96-well U bottom plate. The plate is washed twice by centrifugation at 4000 rpm for 4 mins using 100 µl FACS buffer. Then, plates containing cells were incubated with Anti-ADRB2 and Anti-CHRM2 antibodies for 30 mins at 4°C. After the incubation, cells were washed twice by centrifugation at 12000 rpm for 4 mins with 100 µl FACS buffer. The cells were then incubated with respective secondary anibodies conjugated with FITC and incubated at 4°C for 30 mins. Again the cells are washed by centrifugation using 100 µl FACS buffer and the cells are collected in FACS tubes and analysed for relative expression of receptors by flow cytometry (FACS Calibur, Beckton Dickinson Biosciences, Heidelberg, Germany) using Cell Quest software. The cells are gated with M1, M2 gates and the cells whichever were positive for the receptor falls on the M2 gate. The percentage (%) of positivity is determined from the histogram statistics table.

3.3.1.5. CHRM2 siRNA transfection of HEK293 cells

HEK293 cells were selected to act as negative control cell line for our experimental hypothesis. However, the expression data from western blot revealed the endogenous expression of CHRM2 (M2-muscarinic receptor), and few of our CRPS IgG showed unspecific binding to the HEK293 cells. Therefore, we knocked down the CHRM2 receptor in HEK293 cells using CHRM2 siRNA (Hs_CHRM2_6, Qiagen GmbH, Hilden, Germany). The lyophilized siRNA was resuspended with 250 µl RNase-free water (5 nmol scale) as mentioned by the manufacturers to get 20 µM stock solution. HEK293 cells were seeded at a concentration of $1x10^6$ cells in 6-well plates. The cells were maintained at 37°C in the CO_2 incubator until they became 70% confluent. Lipofectamine® RNAiMAX Reagent (Cat. No. #13778-075) and Opti - MEM® I reduced serum medium (Cat. No. #31985-062) was purchased from Invitrogen™ by Life technologies, Van Allen Way Carlsbad, CA. 250 µl of Opti-MEM® I reduced serum medium was added to dilute 8 µl of Lipofectamine® RNAiMAX Reagent and 4 µl of CHRM2 siRNA. Then the diluted CHRM2 siRNA was added to diluted Lipofectamine® RNAiMAX Reagent (1:1 ratio). This mixture is vortexed and incubated for 20 mins at room temperature to form siRNA-reagent complex. Then 500 µl of this siRNA-reagent complex is added to the cells and maintained in an antibiotic free medium for 8 h at 37°C in the CO_2 incubator. After 8 h, the antibiotic free medium is replaced with normal HEK293 nutrient medium mentioned earlier and the transfection efficiency is analyzed at 24, 48 and 72 h after transfection by western blotting. CHRM2 siRNA HEK293 were used as comparative negative control cell to efficiently differentiate the binding of CRPS IgG to the HCMEC cells.

3.3.1.6. Autoantibody binding assay by flow cytometry

Stably transfected CHO cells expressing the β2-adrenergic receptor and M2-muscarinic receptor (hβ2AR-CHO and hM2R-CHO), HCMEC, U937, A10, HEK293 and CHRM2 siRNA HEK293 cells were examined. Purified IgG (1g/L) was pre-absorbed with wild type CHO cells for 24 h in FACS buffer to avoid any unspecific binding. Flow cytometry was used to detect surface binding antibodies (Kohr et al., 2011; Kohr et al., 2009). Cells were plated into 96-well-plates at a final concentration of $1x10^5$ cells/ml and incubated with the

pre-absorbed IgG (final concentration (12.5 µg/ml)) for 30 min at 4°C. Cells were then washed with FACS-buffer and incubated with fluorescein isothiocyanate (FITC) labeled anti-human IgG secondary antibody (1:75, DAKO), in FACS-buffer for 30 min at 4°C. Cells again were washed and analyzed by flow cytometry (FACS Calibur) using cell quest software. Antibodies binding to the cell surface are expressed as mean fluorescence intensity (MFI).

3.3.1.7. Cytotoxicity (Lactate Dehydrogenase, LDH) assay

Possible cytotoxicity effects of autoantibodies against endothelial (HCMEC), HEK293, U937 and A10 cells were measured after incubation of the cells with CRPS, radial fracture or control-IgG using LDH cytotoxicity assay according to the manufacturer's instructions (Roche applied science, Indianapolis, IN). Cells were seeded in optically clear flat-bottomed 96 well plates in the concentration of $1x10^5$ cells/ml. 100 Pl of this suspension is filled in the 96 well plates and kept in culture for 24 h for the cells to adhere in the flat bottom plate. Then, old medium was discarded and 200 µl containing IgG (CRPS, RF, HC final concentration 12.5 µg/ml) were added and incubated for another 24 h at standard cell culture conditions. Assay medium was used as background control, 100 Pl cell suspension and 100 Pl assay medium as low control. To determine the maximum amount of LDH enzyme activity, 2% Triton-X 100 was used as high control. After 24 h, the plate was centrifuged at 250xg for 10 mins. From this, 100 µl supernatant was transferred into new 96-well plates and 100 µl assay reagent was added and incubated for 30 mins at room temperature in dark. The amount of color produced due to the release of LDH enzyme was read in 490 nm in an ELISA reader. All the assays were carried out in triplicates, average absorbance values of the triplicates were calculated and background control subtracted. Cytotoxicity (%) was calculated as follows:

% Cytotoxicity = [(Experimental value – low control value)/ (High control value – low control value)] x 100

3.3.1.8. Proliferation (WST-1) assay

Immortalized cells such as HCMEC, HEK293, U937 and A10 cells cells were seeded in 96 well plates in a final volume of 100 Pl/well ($1x10^5$ cells/ml), maintained in culture until the

cells tightly adhere to the bottom of the plates. Then the old medium was removed and the cells were incubated for 24 h with fresh medium containing CRPS, RF or HC-IgG (12.5 µg/ml). Then 10 Pl of WST-1 reagent (Cell proliferation reagent WST-1, Roche applied science, Indianapolis, IN) was added in each well and the assay was carried out as mentioned in the instructions provided by the manufacturer. Plates were shaken thoroughly for 1 min on a shaker and incubated for 4 h at standard cell culture conditions. The color developed from the soluble formazan dye cleaved from the tetrazolium salt WST-1 was measured at an absorbance of 420-480 nm in an ELISA reader. 100 Pl of assay medium was taken as blank control for measuring the background absorbance. All experiments were carried out in triplicates. To calculate proliferation, mean absorbance was calculated and background absorbance (medium alone) subtracted from each value. The percentage of specific proliferation was quantified using the following calculation:

% **Proliferation = (experimental release / spontaneous release) x 100**

3.3.1.9. Real-Time PCR

HCMEC cells were plated in 24-well plate at a concentration of $1x10^5$ cells/ml. Cells were then incubated at different time points (48 and 72 h) with HC, CRPS and RF IgG (12.5 µg/ml) and maintained in culture. After 24 h, cells were lysed using lysis buffer provided in the total mRNA kit (peqGOLD total RNA kit, Peqlab) and total mRNA was extracted according to the instructions given by the manufacturers. Quality of samples (A260/A280 ratio) was controlled using Nanophotometer (Implen) and quantification was done using the same. 1 µg of mRNA was reverse transcribed using the Quantitect reverse transcription cDNA synthesis kit (Qiagen) according to the manufacturer's instructions. qRT-PCR reactions were carried out using SYBR Green Master mix (BIORAD) with 5 ng of cDNA. Inflammatory mediator gene expression was carried out using StepOne Real-Time PCR system (Applied biosciences) according to a standardized protocol. Primers were designed Primer-BLAST and the list of primers used for the study is shown in the section **3.2.10** (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

3.3.1.10. Secondary messenger pathways

The expression study in HCMEC cells on treatment with different IgG showed upregulation of various inflammatory cytokines in the CRPS IgG treated group. In order to study the signaling mechanism activated by the functional autoantibodies, the HCMEC cells were plated in 6-well plate and grown as mentioned above. When the cells were adhered and monolayer was formed, they were serum starved for 12 h and treated with HC and CRPS IgG for 2, 10 and 30 mins. Cells were lysed using the lysis buffer (Cell signaling technology, Danvers, MA, USA) and quantified the protein by BCA assay and normalized. Equal amount of protein (100 µg) were loaded and separated by 10% SDS-PAGE, transferred (Trans Blot, Semi dry Transfer cell, BioRad) to a nitrocellulose membrane (GE Healthcare, Amersham™ Hybond ECL, Buckinghamshire, UK) and blocked with 5% BSA for 1 h. The membranes were incubated overnight at 4 °C with the primary antibody Anti-P38 (MAPK pathway), Anti-ERK 1/2, Anti-STAT1, ANTI-AKT (Dilutions and manufacturers are mentioned in table 7). Membranes were washed with 1x TBST and incubated for 1 h with goat anti-rabbit and donkey anti-mouse secondary antibody (1:1000, Santa Cruz Biotechnology, CA, USA). After 1 h, the membranes were washed again ith 1x TBST and developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo scientific, Pierce Biotechnology, Rockford, IL, USA) in Fusion FX7 chemiluminescence system (PEQLAB, Erlangen, Germany). GAPDH (1:5000, Santa Cruz Biotechnology, CA, USA) was used as a loading control.

3.3.2. Expt. set 2: Pharmacological antagonists assay in endothelial HCMEC cells

As we are interested to find out the mechanism of functional autoantibodies in vascular and bone disturbances we continued our study with endothelial HCMEC cells and primary osteoblasts in detail.

3.3.2.1. Autoantibody binding assay with βAR and M2R antagonists

HCMEC endothelial cells were cultured at a concentration of $1x10^6$ cells/ml. At 80% confluency, the cells were serum starved for 1 h. Later, it was incubated with βAR antagonist (1 µM of (±)-Propranolol hydrochloride, Sigma-Aldrich, Saint Louis, Missouri, USA) and/or

M2R antagonist (1 µM of Atropine, Sigma-Aldrich, Saint Louis, Missouri, USA) in a serum-free medium for 30 mins at standard cell culture conditions. The cells preincubated with antagonists were washed with ice-cold PBS to completely remove the medium containing antagonists. Then the HCMEC cells were processed for extracellular flow cytometry procedure as described in 3.3.1.6. Later the cells were analyzed in BD FACS Calibur to determine the antagonistic activity of the CRPS IgG antibodies.

3.3.2.2. LDH assay with βAR and M2R antagonists

The same procedure mentioned in 3.3.1.7 was repeated in HCMEC cells preincubated with βAR antagonist (1 µM of (±)-Propranolol hydrochloride) and/or M2R antagonist (1 µM of Atropine) to check the antagonistic cytotoxicity effects of CRPS IgG after blocking the cells with respective blockers.

3.3.2.3. WST-1 assay with βAR and M2R antagonists

To measure the proliferation rate the protocol 3.3.1.8 was repeated in HCMEC cells preincubated with βAR antagonist (1 µM of (±)-Propranolol hydrochloride) and/or M2R antagonist (1 µM of Atropine). To evaluate whether the antagonists could improve the proliferative effects in the HCMEC cells after stimulating with CRPS IgG.

3.3.3. Expt. set 3: Detection of pathogenicity of CRPS IgG in primary osteoblasts

3.3.3.1. Cultivation and establishment of primary osteoblasts

The isolation, cultivation and differentiation of osteoblasts from human bone debris has been established in the Laboratory of Experimental trauma surgery, Department of Trauma surgery, Justus-Liebig Universität, Giessen and is carried by the following method: the incidental drill dust is obtained from the patients (ZK37, ZK48, ZK56, ZK58, ZK59) having femur or tibia fracture and undergoing osteosynthesis, and is kept in F-12K medium containing 20% fetal calf serum (FCS), 100 U/ml penicillin and 100 g/ml streptomycin. The growing cells, which correspond to many characteristics of mesenchymal stem cells, are differentiated by the addition of osteogenic differentiation medium (DMEM low glucose with

L-glutamine, 10% FCS, 0.1 µM dexamethasone, 0.05 mM Ascorbic acid-2-phosphate, 10 mM-glycerolphosphates, 100 U/ml penicillin and 100 g/ml streptomycin). After 3 weeks of incubation in differentiation medium, the cells start showing the typical characteristics of osteoblasts (for example, synthesis of collagen-1, mineralization and expression of osteoblast markers).

3.3.3.2. Autoantibodies binding to primary osteoblasts by flow cytometry

Primary osteoblasts ZK37, ZK48, ZK56, ZK58 and ZK59 were cultured in 75 m^2 cell culture flasks as described in 3.3.3.1. Cells were then processed for extracellular flow cytometry staining procedure as described in 3.3.1.6. Later the cells were analyzed in BD FACS Calibur to determine the binding efficiency of the CRPS IgG antibodies to the primary osteoblasts.

3.3.2.2. LDH assay in primary osteoblasts

The primary osteoblasts from different trauma patients (ZK37, ZK48, ZK56, ZK58, ZK59) were cultured in 24-well microtiter plates and were treated with HC, CRPS and RF IgG (12.5 µg/ml) to determine the amount of cytotoxicity. The procedure for cytotoxixity assay was followed as mentioned in 3.3.1.7.

3.3.2.3. WST-1 assay in primary osteoblasts

The primary osteoblasts from different trauma patients (ZK37, ZK48, ZK56, ZK58 and ZK59) were cultured in 24-well microtiter plates and were treated with HC, CRPS and RF IgG (12.5 µg/ml) to determine the proliferation rate. The procedure for WST-1 proliferation assay was followed as mentioned in 3.3.1.8.

3.3.2.4. Immunofluorescence in primary osteoblasts

Annexin-V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS). It is used to stain the apoptotic cells, which has altered PS at the outer leaflet of the plasma membrane. This staining method also has propidium iodide (PI), which helps to stain the DNA, and helps in excluding the necrotic cells and quantifying the

Annexin-V stained positive apoptotic cell cluster. Primary osteoblasts from different trauma patients ZK37, ZK48, ZK58 were cultured in 24-well plates as described above. When the cells were 80% confluent, they were treated with HC, CRPS and RF IgG (12.5 µg/ml) and incuabated for 24 h at standard cell culture conditions. After incubation was over, cells were washed twice with PBS to completely remove any active debris in the cells. The cells are then fixed with 4% paraformaldehyde (PFA) for 20 mins at 4°C. The cells were then washed with 1x PBS and then stained with Annexin-V-FLUOS working reagent solution and left in 4°C for 30 mins. Finally the cells were washed with 1x PBS and observed under fluroscence microscope. The cells that were positive for apoptosis could be seen in blebs and were stained with Annexin-V and the cells that were stained for both Annexin-V and PI are necrotic cells. The plate was imaged and the cells that are positive for apoptosis are counted using grid counting method.

3.3.2.5. RT-PCR in primary osteoblasts

Primary osteoblasts from ZK37, ZK48 and ZK58 were plated in 24-well plate and grown in standard cell culture conditions until the cells are completely differentiated to osteoblasts. Cells were then incubated for 24 h with HC, CRPS and RF IgG (12.5µg/ml) and maintained in culture. After 24 h, cells were lysed using lysis buffer provided in the total mRNA kit (peqGOLD total RNA kit, Peqlab) and total mRNA was extracted according to the instructions given by the manufacturers. Quality of samples (A260/A280 ratio) was controlled using Nanophotometer (Implen). 1 µg of mRNA was reverse transcribed using the Quantitect reverse transcription cDNA synthesis kit (Qiagen) according to the manufacturer's instructions. qRT-PCR reactions were carried out using SYBR Green Master mix (BIORAD) with 5 ng of cDNA. Osteoblast and osteoclast specific markers gene expression was carried out using StepOne Real-Time PCR system (Applied biosciences) according to a standardized protocol. Primers were designed Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers used for the study is shown in section **3.2.10**.

3.3.2.6. Immunoblotting in primary osteoblasts

Primary osteoblast from ZK58 was plated in 6-well plate and grown as mentioned above. When the cells are completely differentiated to osteoblasts were treated with HC, CRPS and

RF IgG for 24 h. Later, cells were lysed using the lysis buffer (Cell signaling technology, Danvers, MA, USA) and quantified the protein by BCA assay and normalized. Equal amount of protein (90 µg) were loaded and separated by 10% SDS-PAGE, transferred (Trans Blot, Semi dry Transfer cell, BioRad) to a nitrocellulose membrane (GE Healthcare, Amersham™ Hybond ECL, Buckinghamshire, UK) and blocked with 5% BSA for 1 h. The membranes were incubated overnight at 4 °C with the primary antibody Anti-collagen1-D1 (1:1000, R&D systems). Membranes were washed with 1x TBST and incubated for 1 h with goat anti-rabbit and donkey anti-sheep secondary antibody (1:1000, Santa Cruz Biotechnology, CA, USA). After one hour the membranes were washed again with 1x TBST and developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo scientific, Pierce Biotechnology, Rockford, IL, USA) in Fusion FX7 chemiluminescence system (PEQLAB, Erlangen, Germany). GAPDH (1:5000, Santa Cruz Biotechnology, CA, USA) was used as a loading control.

3.3.4. Expt. set 4: Intravenous Immunoglobulin (IvIg) study

3.3.4.1. Intravenous Immunoglobulin (IvIg)

IvIg commercially available as Gamunex® 10% was bought from the Talecris biotherapeutics company and dialysed against phosphate-buffered saline (PBS, pH 7.4) to remove the glycine. 25 mg/ml final concentration of IvIg was used throughout the experiment.

3.3.4.2. LDH Assay

Possible cytotoxicity effects of autoantibodies against endothelial cells (HCMEC), A10 and HEK293 were measured after incubation of the cells with HC, CRPS IgG, CRPS IgG + IvIg and IvIg alone using LDH-assay kit. The experiment to determine the cytotoxicity after incubation with IvIg was carried out as mentioned in 3.3.1.7.

3.3.4.3. WST-1 Assay

HEK293 and HCMEC were seeded in 96 well plates in a final volume of 100 µl/well ($1x10^5$ cells/ml), left to adhere for 24 h and then incubated for 24 h with medium containing CRPS

IgG, CRPS IgG + IvIg, IvIg alone and HC-IgG (final concentration 1 mg/ml). Then the proliferation (WST-1) assay was carried out in the same way as mentioned in 3.3.1.8.

3.3.5. Statistical analysis

Surface binding of antibodies, cytotoxicity and proliferation assays which has multiple groups were analyzed using One-Way ANOVA and a post-test was done using Bonferroni post-test or Dunn's test to compare all pairs of columns after validating the data for normal distribution by Kolmogorov-Smirnov test. Surface binding of antibodies that has only two groups were analyzed by unpaired t-test or Mann-Whitney U test based on their distribution. All data are presented as Mean ± SD. $p < 0.05$ was considered statistically significant. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Positivity for binding, cytotoxicity and proliferation were calculated by the formula (Mean + (Standard deviation X 2.5)) of the healthy controls and a line is drawn in graphs.

4. RESULTS

4.1. *Expt. set 1:* IgG from complex regional pain syndrome (CRPS) patients binds effectively to various cell lines expressing ADRB2 and CHRM2 receptors

4.1.1. Patient details

Serum was obtained from 13 clinically defined CRPS patients according to the IASP diagnostic criteria (Bruehl et al., 1999; Harden et al., 2007) after informed consent and approval from the ethical committee of the Johannes-Gutenberg-University Mainz. Sera of 11 sex and age matched healthy controls (HC) and 7 radial fracture (RF) patients (who did not develop CRPS) served as controls. None of the healthy controls or radial fracture patients had a history of hereditary or chronic neuropathic pain. **Table 3** shows the detailed clinical and immunological data of the CRPS patients.

Table 3: Clinical and epidemiological data of CRPS patients

Age	49.0 ± 16.0	
Sex	7 female / 6 male	
CRPS I / CRPS II	10/3	
Warm / cold CRPS	11/2	
Pain score	Median 4.5	
Spontaneous pain in rest	5	(13)
Hyperalgesia	5	(10†)
Affected limb (upper / lower)	10/3	(13)
Surgery	12	(13)
Trauma	10	(13)
Motor impairment*	4	(9†)
Sensory impairment**	5	(9†)
Trophic changes	9	(13)
Time to serum analysis***		
Minimum	4	
Maximum	152	

CRPS = Complex Regional Pain Syndrome.

* Any paresis in the affected limb, not related to nerve lesion.

** Any sensory disturbances, not related to nerve lesion.

*** Time between onset of symptoms and obtaining of serum in weeks.

† Data not available from all patients

4.1.2. Determination of ADRB2 and CHRM2 receptor expression

Expression study was done to determine cells taken for the study expresses ADRB2 and CHRM2 receptors. Relative gene expression of ADRB2 and CHRM2 receptors in CHO wild type (WT) and CHO cells overexpressing ADRB2 (hβ2AR-CHO) and CHRM2 (hCHRM2) receptors, HCMEC, HEK293 and A10 were analyzed in Quatitative PCR (qPCR, Fig. 5A and 5B) and in western blotting (Fig. 5C and 5E). Unexpectedly, the negative control cell line HEK293 also expresses the target receptors. This may be due to the endogenously expressing CHRM2 in HEK293 cells. To avoid this and to make them proper negative control cell line, HEK293 was knocked down with CHRM2 siRNA and the knockdown efficiency is measured by western blotting and the result for expression of hM2R is shown (Fig. 5D).

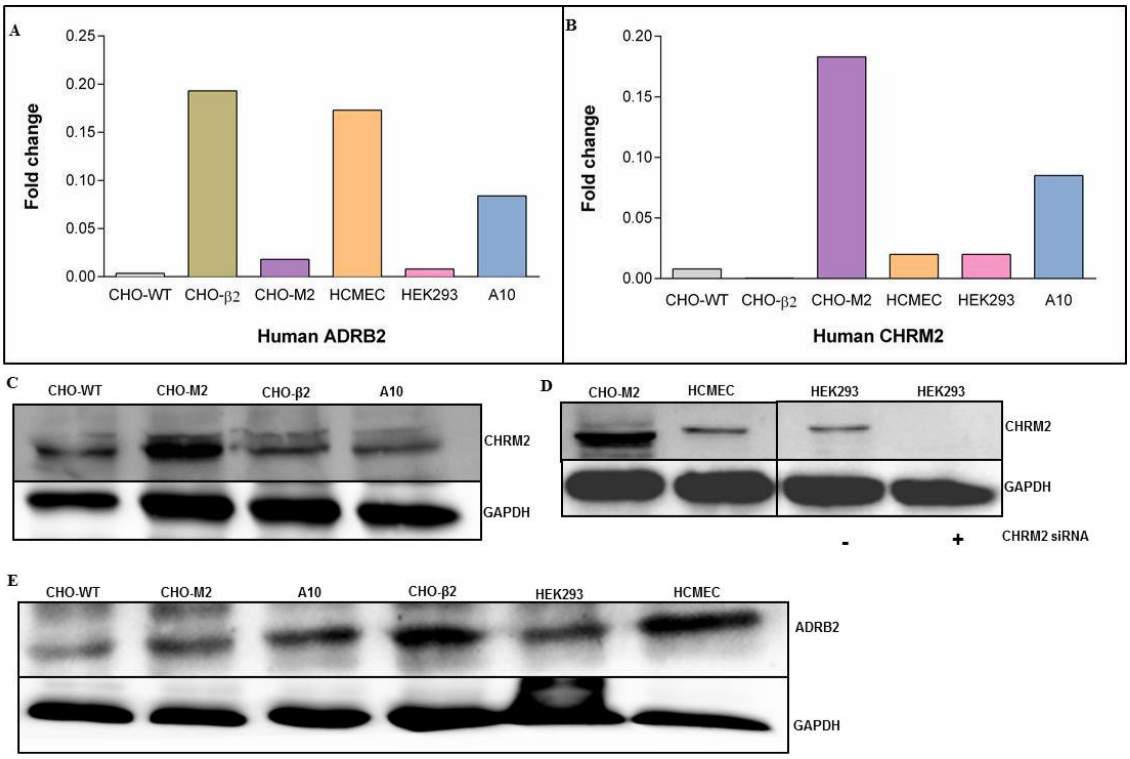

Figure 5: Expression of ADRB2 and CHRM2 receptors in CHO (WT), hβ2AR-CHO, hCHRM2-CHO, HCMEC, HEK293 and A10 cells. (A) Relative gene expression of ADRB2 in all the cell lines. (B) Relative gene expression of CHRM2 in all cell lines. (C) Expression of CHRM2 by western blotting and (D) HEK293 cells showing knockdown of CHRM2. (E) Expression of ADRB2 in all cell lines by western blotting where GAPDH served as housekeeping control gene.

4.1.3. Binding of CRPS IgG to CHO cells overexpressing ADRB2 & CHRM2

Surface binding of autoantibodies to CHO cells overexpressing adrenergic, muscarinic receptors. CRPS IgG showed higher binding to the hβ2AR-CHO compared to RF and HC samples (CRPS 19.05 ± 5.94 vs HC 11.35 ± 2.58 vs RF 7.32 ± 4.41, $p < 0.01$, Fig. 6A). There was also higher binding of CRPS IgG to hM2R-CHO (CRPS 19.19 ± 2.35 vs HC 13.35 ± 1.67 vs RF 8.12 ± 1.68, each $p < 0.001$, Fig. 6B). Among all seven RF samples only one sample showed positive binding to the hβ2AR (Fig. 6A). None of the HC or RF IgG showed autoantibodies to the M2 receptors ($p < 0.001$, Fig. 6B).

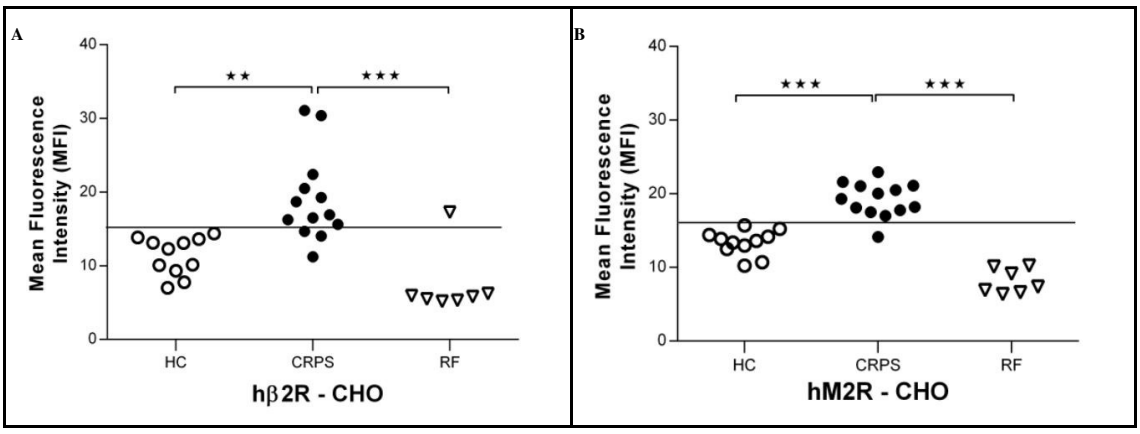

Figure 6: Determination of surface binding of HC, CRPS and RF IgG to CHO cells overexpressing ADRB2 and CHRM2 receptors by flow cytometry. (A) CRPS IgG shows higher binding to the CHO cells overexpressing ADRB2 receptor (hβ2AR-CHO), ANOVA, $p<0.01$. (B) In CHO-cells overexpressing CHRM2 receptors (hM2R-CHO), CRPS IgG binds strongly to the surface receptors with a higher statistical significant difference. ANOVA, $p<0.001$. (HC n= 11; CRPS n= 13; RF n= 7) Cut-off marked as horizontal line, was determined using the formula mean of the controls + 2.5x standard deviation. HC - healthy control; CRPS - complex regional pain syndrome; RF - radial fracture.

4.1.4. Autoantibody detection by FACS with antagonists

β2 and M2 receptor specific antagonists pre-incubated hβ2AR-CHO and hM2R-CHO cells antagonizes CRPS IgG binding to the ADRB2 and CHRM2 receptors. Binding of CRPS IgG to hβ2AR-CHO and hM2R-CHO cells were significantly reduced on blocking the hβ2AR with βAR antagonist, 1 µM of (±)-Propranolol hydrochloride and/or hM2R with M2R antagonist, 1 µM of Atropine (CRPS 19.05 ± 5.937 vs CRPS+1 µM (±)-Propranolol 11.75 ±

6.738, p < 0.01, Fig. 7A and CRPS 19.19 ± 2.353 vs CRPS+1 µM Atropine 10.65 ± 5.172, p < 0.001, Fig. 7B).

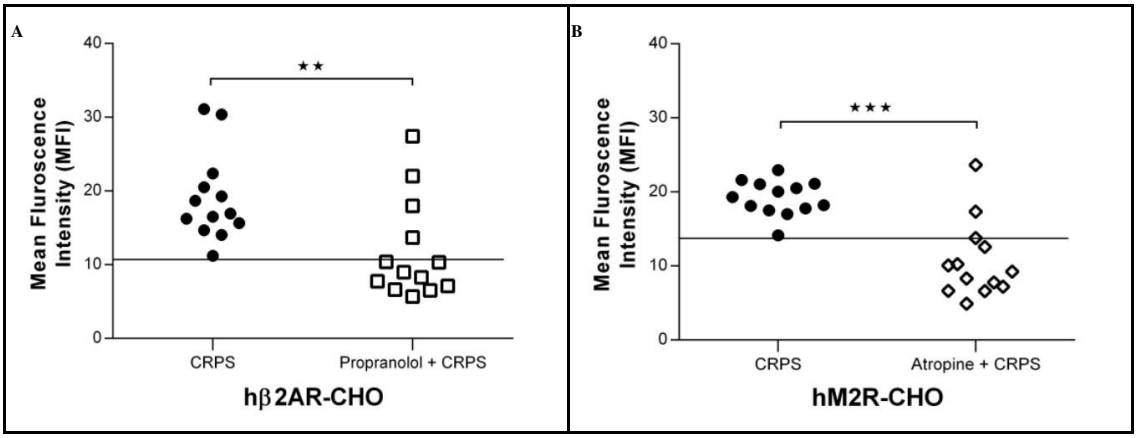

Figure 7: hβ2AR-CHO and hM2R-CHO cells pre-incubated with βAR antagonist, 1 µM of (±)-Propranolol hydrochloride or the M2R antagonist, 1 µM of Atropine, significantly reduced the surface binding of autoantibodies. (A) 1 µM (±)-Propranolol HCl pre-incubated hβ2AR-CHO cells blocked the binding of CRPS IgG to the CHO cells overexpressing ADRB2 receptor (hβ2AR-CHO); ANOVA, p < 0.01. (B) 1 µM Atropine incubated hM2R-CHO also blocked the binding efficiency of CRPS IgG to CHO cells overexpressing CHRM2 receptor (hM2R-CHO); ANOVA, p < 0.001. (HC n= 11; CRPS n= 13; RF n= 7) Cut-off marked as horizontal line, was determined using the formula mean of the controls + 2.5x standard deviation.

4.1.5. In vitro flow cytometry assay in immortalized cells

In vitro flow cytometry assay shows CRPS IgG binds effectively to the endothelial, smooth muscle, fibroblast and osteosarcoma cell lines but not to the CHRM2 knockdown HEK293 cells. CRPS IgG showed higher binding to the endothelial HCMEC cells (CRPS 22.59 ± 5.08 vs HC 14.28 ± 3.57 vs RF 17.66 ± 2.42, p < 0.01, Fig. 8A). In our negative control cell line HEK293, three CRPS patients showed positive binding but not statistically significant comparing to healthy control but to RF (CRPS 36.62 ± 34.35 vs HC 20.12 ± 12.03, n.s., CRPS 36.62 ± 34.35 vs RF 7.90 ± 3.12, p < 0.01, Fig. 8B). This unspecific binding of CRPS IgG to HEK293 cells may be due to the endogenously expressing CHRM2 in HEK293. Therefore, endogenously expressed muscarinic receptors in HEK293 cells were knockdown with CHRM2 siRNA. CHRM2 siRNA HEK293 cells resulted in blocking of unspecific binding of the CRPS IgG and no statistically significant difference was observed between the groups (CRPS 11.29 ± 3.84 vs HC 11.50 ± 2.67 vs RF

10.66 ± 3.21, $p > 0.05$, n.s., Fig. 8B). Besides this CRPS IgG also showed higher binding to the smooth muscle cells, A10 (CRPS 33.73 ± 19.75 vs HC 14.78 ± 4.70, $p < 0.05$ and CRPS 33.73 ± 19.75 vs RF 32.23 ± 14.03, $p > 0.05$, n.s., Fig. 8C) and Osteosarcoma, SAOS-2 cell lines (CRPS 9.17 ± 2.68 vs HC 6.32 ± 1.73, $p < 0.05$ and CRPS 9.17 ± 2.68 vs RF 7.70 ± 1.40, $p > 0.05$, n.s., Fig. 8D).

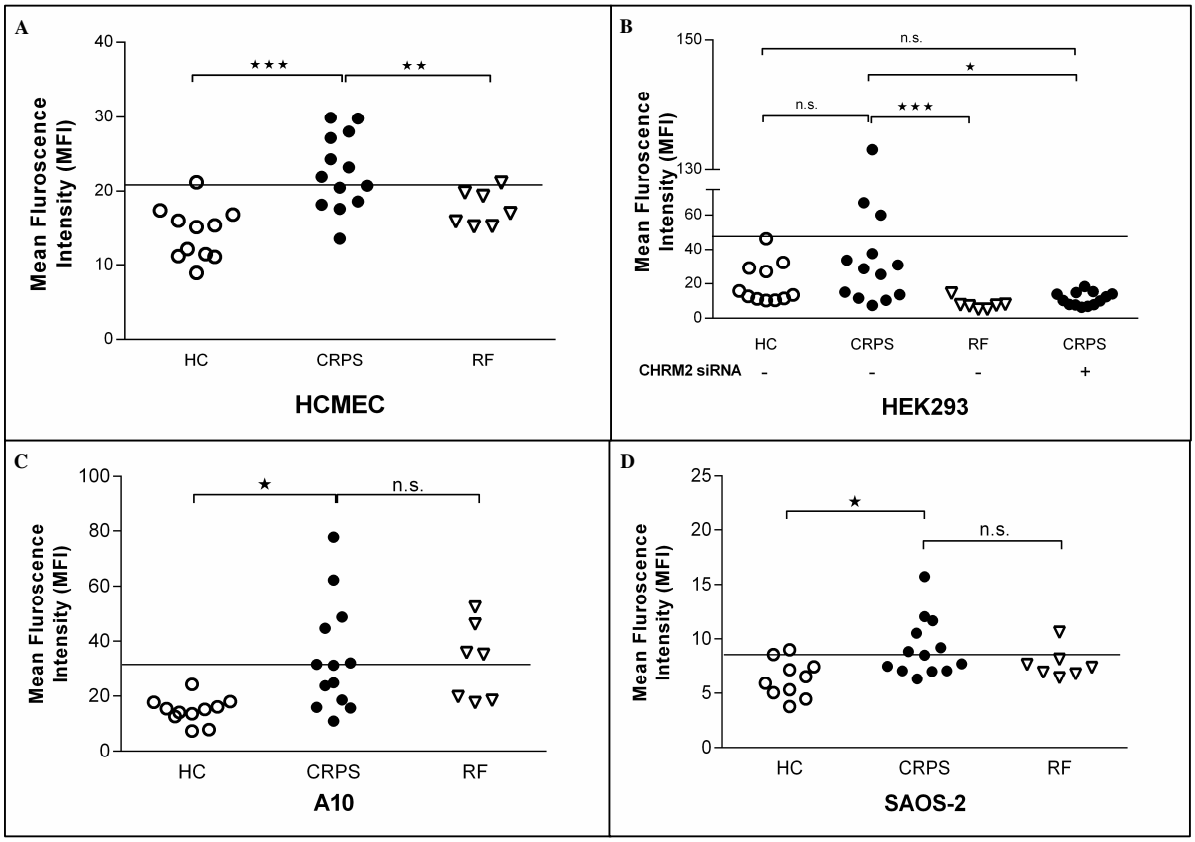

Figure 8: Binding of functionally active autoantibodies against ADRB2 and CHRM2 receptors in (A) Endothelial HCMEC (B) HEK293 with and without knockdown of CHRM2 receptor (C) A10 smooth muscle and (D) SAOS-2 cells. To all the above-mentioned cells, CRPS IgG shows increased binding compared to the healthy control (HC) and radial fracture (RF) patients. ANOVA, $p < 0.001$ in HCMEC and $p < 0.05$ in A10 and SAOS-2 cells. To avoid unspecific binding of CHRM2 receptors in HEK293, cells were knockdown using siRNA. When knockdown, the unspecific binding has been ruled out as shown above in (B). (HC n= 11; CRPS n= 13; RF n= 7) Cut-off marked as horizontal line, was determined using the formula mean of the controls + 2.5x standard deviation.

4.1.6. Cytotoxicity assay in immortalized cells

CRPS IgG causes higher cytotoxicity in HCMEC, A10 and SAOS-2 cells but not in the negative control HEK293 cell line. The cytotoxic effect of HC, CRPS and RF IgG on

HCMEC cells were analyzed in HCMEC, HEK293, A10 and SAOS-2 cell lines. After incubating HCMEC cells with different IgG, CRPS IgG showed higher cytotoxic effects compared to both HC and RF IgG (CRPS 14.90 ± 5.645 vs HC 1.494 ± 3.871, $p < 0.01$; CRPS 14.90 ± 5.645 vs RF -12.81 ± 2.716, $p < 0.001$, Fig. 9A). A10 Smooth muscle cell and negative control cell line HEK293 did not show any statistically significant cytotoxic effects between the HC and CRPS IgG ($p > 0.05$, n.s.) but with RF IgG ($p < 0.05$, Fig. 9B and 9C respectively). SAOS-2 cells also showed higher cytotoxic effects between the groups (CRPS 21.70 ± 10.12 vs HC 1.904 ± 8.655, $p < 0.05$; CRPS 21.70 ± 10.12 vs RF 44.87 ± 15.52, $p < 0.05$, Fig. 9D).

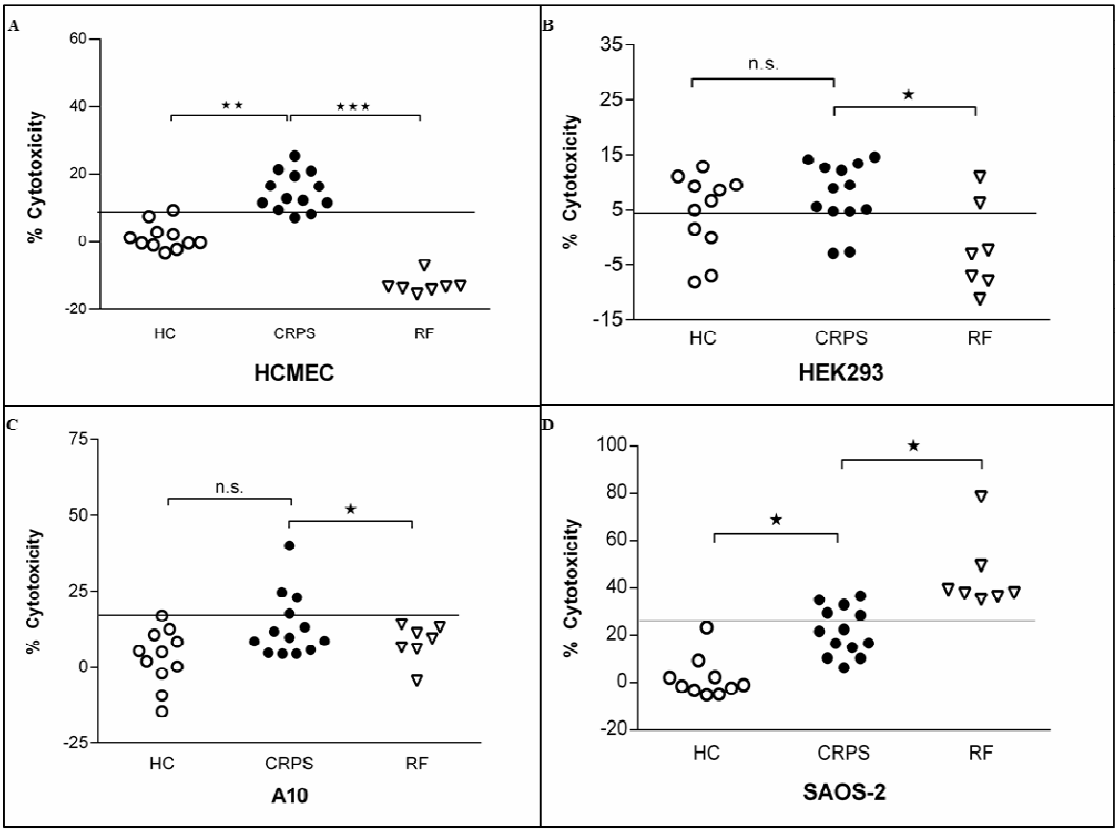

Figure 9: CRPS IgG mediated cytotoxicity in HCMEC, HEK293, A10 and SAOS-2 cells. (A) CRPS IgG stimulated HCMEC cells showed severe cytotoxic effects of about 80%, $p < 0.01$ compared to the HC and RF controls. (B) Negative control cell line HEK293 showed equal cytotoxicity in both HC and CRPS groups, $p > 0.05$, n.s., leaving a significant difference between the CRPS and RF groups, $p < 0.05$. (C) Equal cytotoxic effects were observed in A10 cells between the groups and (D) CRPS IgG stimulation resulted in higher cytotoxic effect in SAOS-2 cells compared to HC, $p < 0.05$ but RF IgG had still more compared to the CRPS group, $p < 0.05$. (HC n= 11; CRPS n= 13; RF n= 7) Cut-off marked as horizontal line, was determined using the formula mean of the controls + 2.5x standard deviation.

4.1.7. Proliferation assay in immortalized cells.

CRPS IgG severely reduces proliferation of endothelial, smooth muscle and SAOS-2 cells. HCMEC, A10, SAOS-2 and HEK293 cells were stimulated with HC, RF, and CRPS IgG for 24 h. And 1 h incubation with WST-1 proliferation reagent revealed that HCMEC cells treated with CRPS IgG has less proliferation rate compared to the HC (CRPS 11.55 ± 3.14 vs HC 16.74 ± 0.546, $p < 0.001$, Fig. 6A; CRPS 11.55 ± 3.14 vs RF 16.94 ± 0.40, $p < 0.001$), without statistical difference between HC and RF samples ($p > 0.05$, n.s., Fig. 10A). Similar effects were also observed in A10 and SAOS-2 cells.

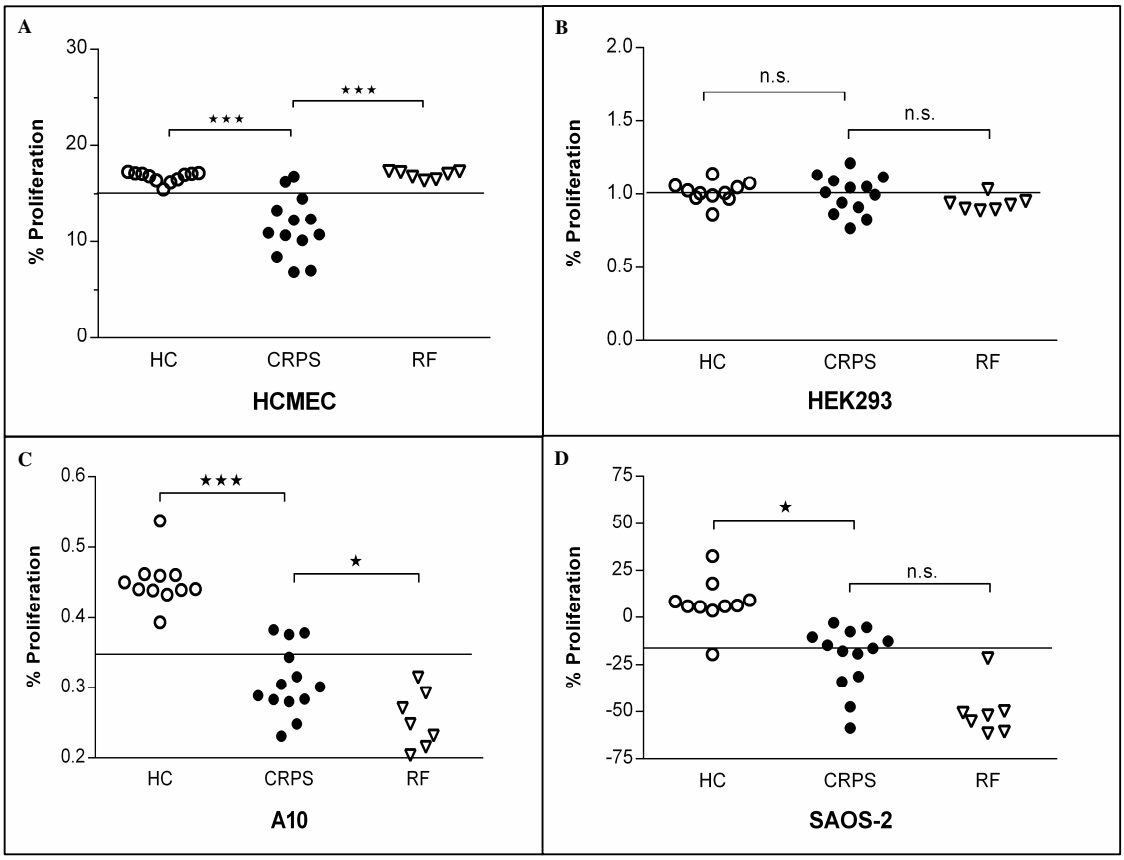

Figure 10: CRPS IgG mediated proliferation effects in endothelial, smooth muscle, SAOS-2 and HEK293 cells. (A) CRPS IgG stimulated HCMEC cells showed severe decrease in proliferation compared to the HC and RF controls, $p < 0.001$ each. (B) Negative control cell line HEK293 showed equal cytotoxicity in all three groups, $p < 0.05$, n.s.(C) Proliferation was drastically reduced in CRPS IgG stimulated A10 cells compared to the HC, $p < 0.001$, leaving the RF IgG stimulated group with even lesser proliferation rates. (D) SAOS-2 cells stimulated with various IgG showed significant decrease in CRPS IgG stimulated group compared to HC, $p < 0.05$ with no significant difference between the RF IgG and CRPS group, $p < 0.05$. (HC n= 11; CRPS n= 13; RF n= 7) Cut-off marked as horizontal line, was determined using the formula mean of the controls + 2.5x standard deviation.

In A10 cells, the CRPS IgG stimulated group had very less proliferating cells compared to that of the HC and RF IgG stimulated groups (CRPS 0.30 ± 0.05 vs HC 0.450 ± 0.03, $p < 0.001$; CRPS 0.30 ± 0.05 vs RF 0.25 ± 0.04, $p < 0.05$, Fig. 10C). SAOS-2 cells stimulated with CRPS IgG also has lesser proliferation rate comparing to the other two groups (CRPS -21.40 ± 16.82 vs HC 7.619 ± 12.94, $p < 0.05$; CRPS -21.40 ± 16.82 vs RF -50.24 ± 13.46, $p > 0.05$, n.s., Fig. 10D). HEK293 negative control cells had no statistically significant difference in proliferation between the different IgG stimulated groups ($p > 0.05$, n.s., Fig. 10B).

4.1.8. β2 and M2 receptor specific antagonists study

β2 and M2 receptor specific antagonists pre-incubated HCMEC endothelial cells antagonizes CRPS IgG binding to the target receptors. To understand whether the blocking of ADRB2 and CHRM2 receptors with their respective antagonists would antagonize the binding efficiency thereby decrease the cytotoxic effects and recover the proliferation rate caused by the CRPS IgG in the endothelial cells, endothelial cells were pre-incubated with 1 µM (±)-Propranolol HCl or 1 µM Atropine and stimulated with CRPS IgG and analyzed for functional assays.

CRPS IgG showed significant decrease in binding to surface receptors in βAR and M2R antagonists pre-incubated HCMEC cells (CRPS 22.59 ± 5.08 vs CRPS + 1 µM (±)-Propranolol HCl 15.66 ± 4.64, $p < 0.01$, vs CRPS + 1 µM Atropine 12.97 ± 3.91, $p < 0.001$, Fig. 11A).

Cytotoxicity caused by CRPS IgG was not prevented when the HCMEC endothelial cells were preincubated with βAR and M2R antagonists. Although there is a reduction in the cytotoxic effects in the βAR and M2R antagonists pre-incubated HCMEC cells, we couldn't find a statistically significant difference between the groups (CRPS 14.90 ± 5.64 vs CRPS+1 µM (±)-Propranolol HCl 8.84 ± 7.66 vs CRPS+1 µM Atropine 9.88 ± 7.24, n.s., Fig. 11B).

On treating CRPS IgG with the HCMEC cells that were pretreated with βAR and M2R antagonists, the proliferating property of the cells increased to a statistically significant level (CRPS 11.55 ± 3.14 vs CRPS+1 µM (±)-Propranolol HCl 13.95 ± 2.02, $p < 0.01$ and CRPS 11.55 ± 3.14 vs CRPS+1 µM Atropine 14.58 ± 1.74, $p < 0.001$, Fig. 11C).

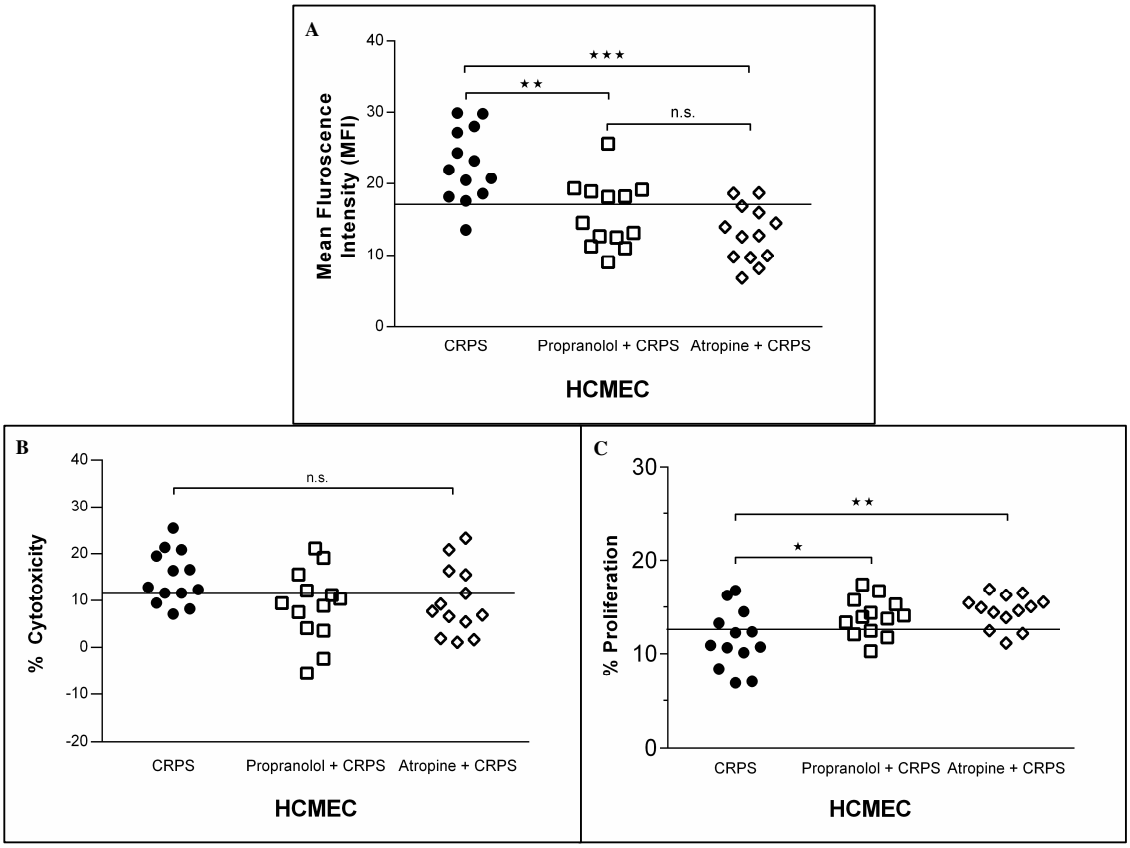

Figure 11: Antagonizing effects of CRPS IgG by pre-incubating the HCMEC cells with βAR and M2R antagonists (1 µM (±)-Propranolol HCl and 1 µM Atropine respectively). (A) CRPS IgG showed significant decrease in binding to surface receptors in βAR ($p < 0.01$) and M2R antagonists ($p < 0.001$) pre-incubated HCMEC cells. (B) Cytotoxicity caused by CRPS IgG was not prevented when the HCMEC endothelial cells were preincubated with βAR and M2R antagonists, $p > 0.05$, n.s. (C) The proliferating property was recovered (almost equal to the HC IgG treated group) on treating CRPS IgG with the HCMEC cells that were pretreated with βAR, $p < 0.05$ and M2R antagonists, $p < 0.01$. (HC n= 11; CRPS n= 13; RF n= 7). Cut-off marked as horizontal line, was determined using the formula mean of the controls + 2.5x standard deviation.

4.1.9. Gene expression of pro-inflammatory cytokines

Expressions of pro-inflammatory cytokines were elevated in CRPS IgG stimulated endothelial HCMEC cells. To investigate the study in gene level, HCMEC endothelial cells were taken to study in detail as the vascular disturbances and inflammation in CRPS patients are yet to be understood. HCMEC cells were stimulated with HC, CRPS and RF IgG for 24 h and the relative gene expression of endothelial specific pro-inflammatory cytokines such as ICAM-1 (CD54), VCAM-1, MCP-1, TGF-E1 and BMP-2 were analyzed. CRPS IgG stimulated HCMEC cells showed higher expression of pro-inflammatory cytokines such as ICAM-1 ($p < 0.001$, Fig. 12A), VCAM-1 ($p < 0.05$, Fig. 12B) and MCP-1 ($p < 0.01$, Fig.

12C). Simultaneously, CRPS IgG treated HCMEC cells showed significant decrease in the expression of proliferation and differentiation related genes such as TGF-E1 ($p < 0.001$, Fig. 12D) and BMP-2 ($p < 0.05$, Fig. 12E). All data normalized to GAPDH, blank is set to 1.

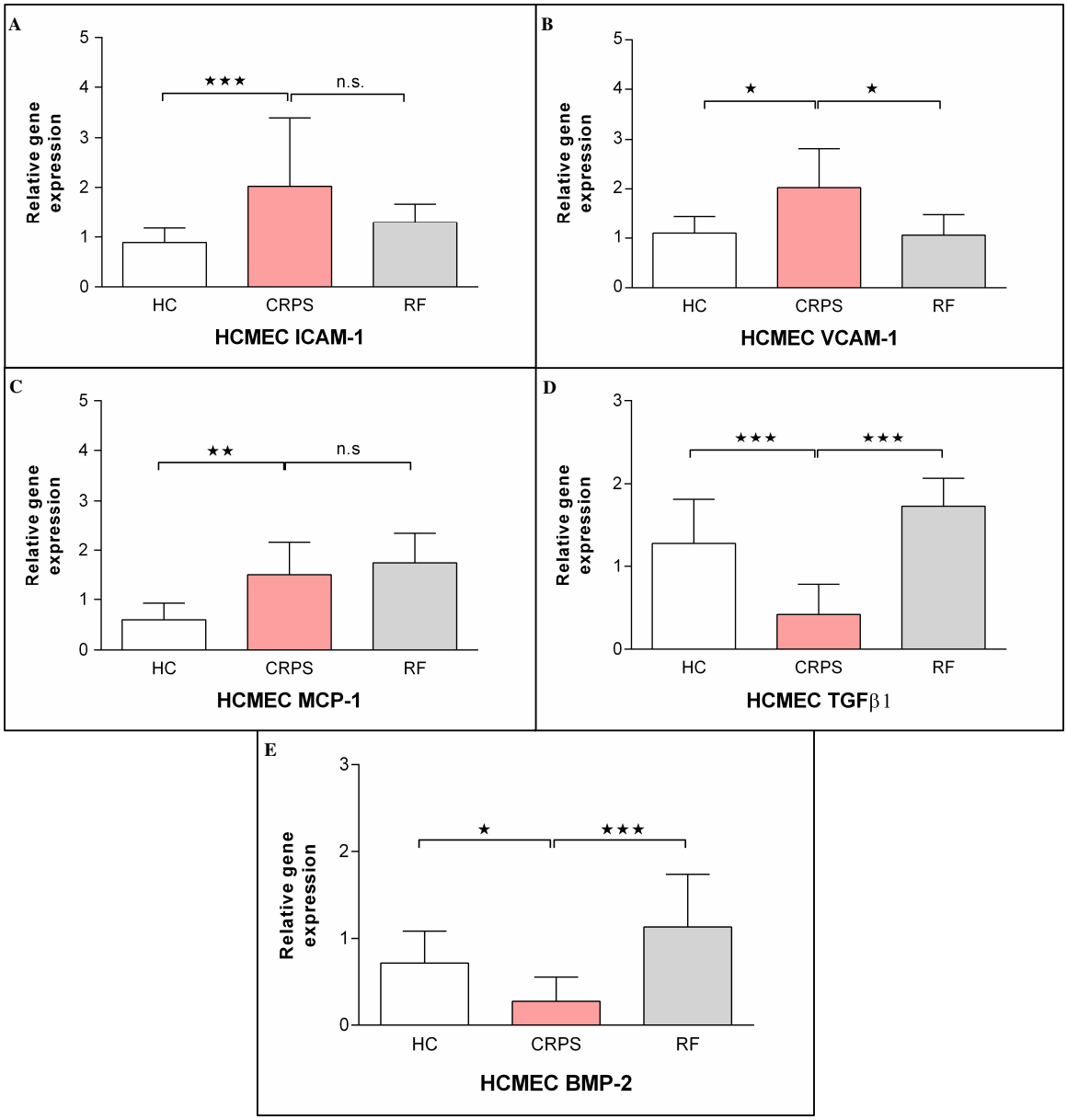

Figure 12: Expression of endothelial specific pro-inflammatory cytokines and differentiation related genes in HCMEC cells after stimulating with HC, CRPS and RF IgG. CRPS IgG stimulated group had higher expression of pro-inflammatory cytokines (A) Intercellular adhesion molecule – 1, ICAM-1/CD54, $p < 0.001$ (B) Vascular cell adhesion molecule – 1, VCAM-1, $p < 0.05$ (C) Monocyte chemoattractant protein – 1, MCP-1, $p < 0.01$. CRPS IgG stimulated group had decreased expression of proliferation and cell differentiation related genes (D) Transforming growth factor - E1, TGF-E1, $p < 0.001$ (E) Bone morphogenic protein – 2, BMP-2, $p < 0.05$. (HC n= 11; CRPS n= 13; RF n= 7, all data normalized to GAPDH, blank is set to 1).

4.1.7. Secondary messenger pathways

Regulation of secondary messengers in HCMEC cells after treating with HC and CRPS IgG. In order to analyze the effects of CRPS IgG on inflammation mediating MAPK signaling pathways in HCMEC endothelial cells, we treated them with HC and CRPS IgG. Interestingly, CRPS IgG treated HCMEC cells promoted a marked 3-4 fold increase in ERK1/2 activation that was not observed in samples from healthy controls (HC), phospho ERK1/2 (pERK1/2, $p < 0.001$, Fig. 13A, 13B and 13C). Similar elevated levels of pP38 (3-fold increase), the major MAPK family member were also observed in CRPS IgG treated HCMEC cells, phospho P38 (pP38, $p < 0.001$, Fig. 14A, 14B and 14C). In order to confirm the inflammatory effects caused by the CRPS IgG in the HCMEC cells, we also screened the levels of pSTAT-1, a well-known pro-inflammatory mediator and we observed a 4-5 fold increase that was not observed in HC IgG treated HCMEC cells, phospho STAT-1 (pSTAT-1/ Y701, $p < 0.001$, Fig. 15A, 15B and 15C). Additionally, CRPS IgG treated HCMEC cells showed significant down-regulation in proliferation and differentiation protein phospho AKT (pAKT, $p < 0.001$, Fig. 16A, 16B and 16C). All data were quantified using BIO-ID software (the graph is placed adjacent to the blots) and normalized to their respective total proteins such as total STAT-1, total P38, total AKT and total ERK 1/2, blank is set to 100.

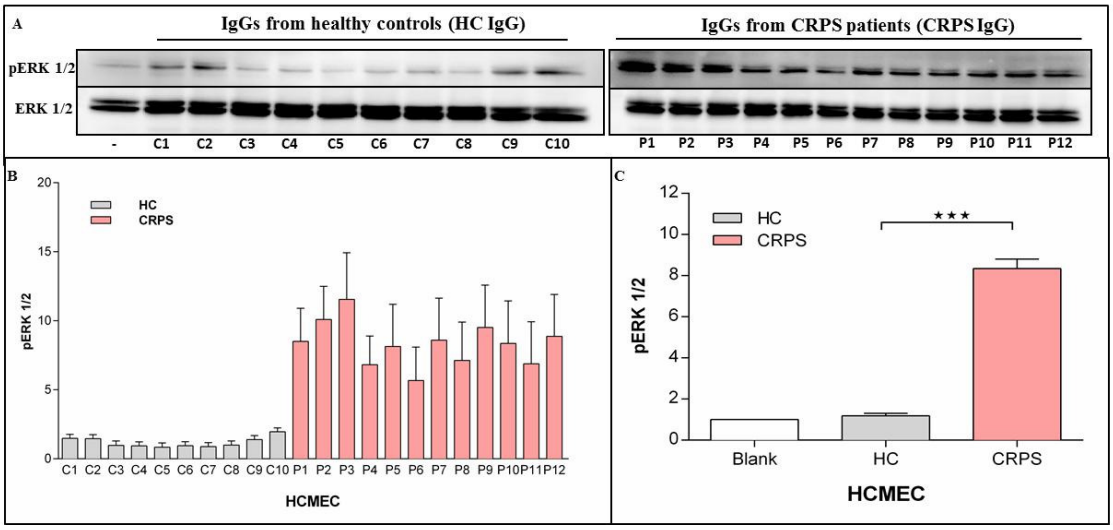

Figure 13: CRPS IgG are potent stimulators of ERK 1/2 pathway in HCMEC cells. A) HCMEC cells were incubated for 5 mins with IgGs (12.5 µg/ml) obtained from different HC and CRPS patients and ERK 1/2 activation were assessed using specific anti-phospho ERK antibodies (pERK 1/2). Blots were reprobed for total cellular ERK 1/2 levels to normalize the results. B) Data are represented as fold-stimulation of ERK activity over basal conditions (without any IgG stimulation, considered as 100), and are represented as mean ± SE of 3 independent experiments. C) ERK stimulation by CRPS patients IgG was significantly higher ($p < 0.001$) when compared to the healthy controls (Unpaired t-test). Representative blots are shown in the panels. (HC n= 10; CRPS n= 12).

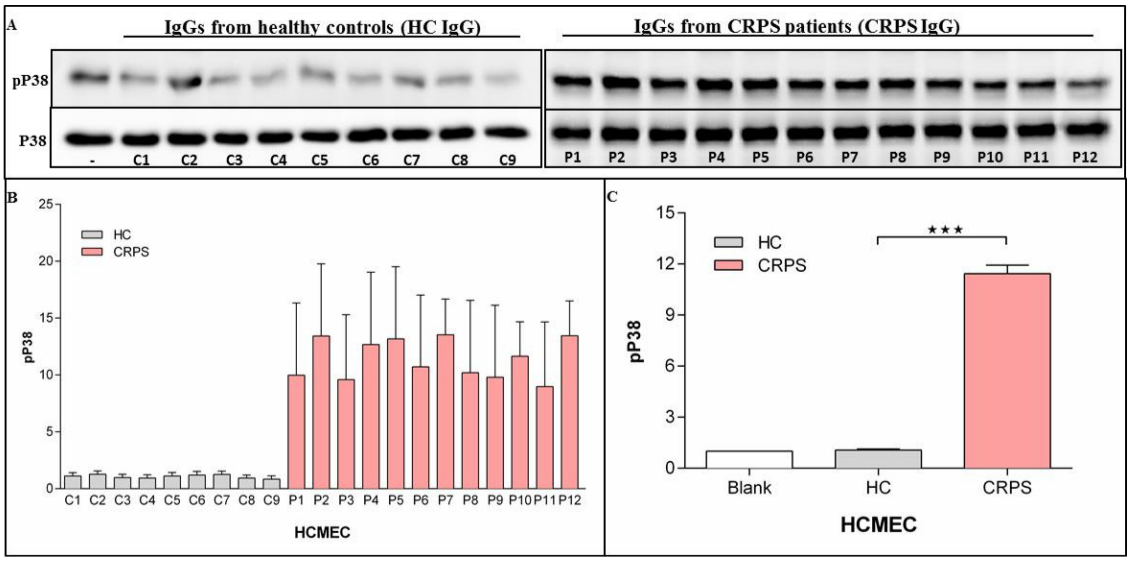

Figure 14: CRPS IgG are effective activators of phospho P38 MAPK pathway proteins in HCMEC endothelial cells. A) HCMEC cells were incubated for 5 mins with IgGs (12.5 µg/ml) obtained from different HC and CRPS patients and pP38 activation was assessed using specific anti-phospho P38 antibodies (pP38). Blots were reprobed for total cellular P38 levels to normalize the results. B) Data are represented as fold-stimulation of P38 activity over basal conditions (without any IgG stimulation, considered as 100), and are represented as mean ±

SE of 3 independent experiments. C) P38 stimulation by CRPS patients IgG was significantly higher ($p < 0.001$) when compared to the healthy controls (Unpaired t-test). (HC n= 9; CRPS n= 12)

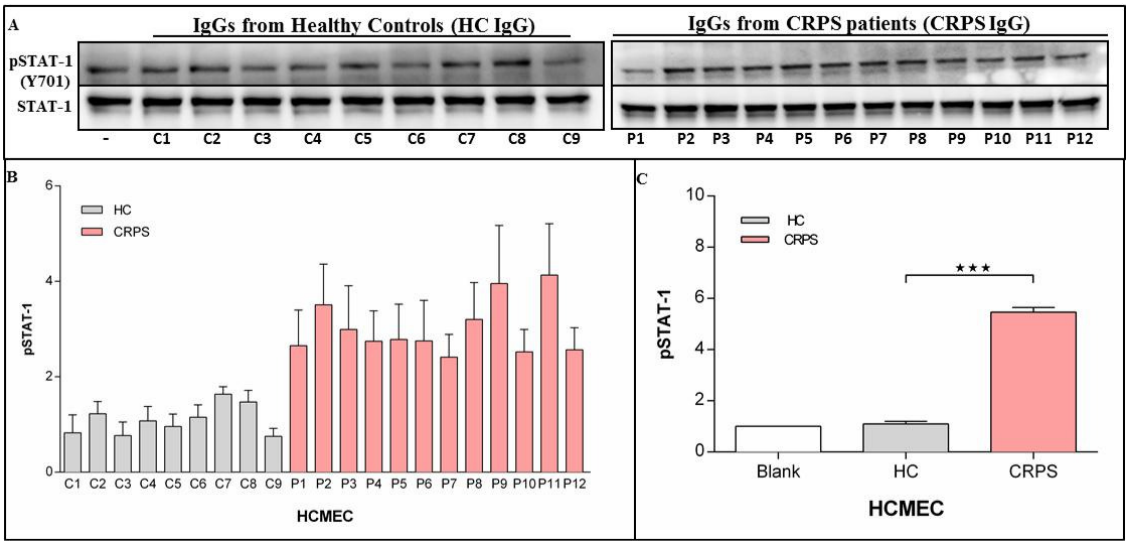

Figure 15: CRPS IgG are effective activators of pSTAT-1 (a pro-inflammatory mediating protein) in HCMEC cells. A) HCMEC cells were incubated for 5 mins with IgGs (12.5 µg/ml) obtained from different HC and CRPS patients and pSTAT-1 activation were assessed using specific anti-phospho STAT-1 antibodies (pSTAT-1). Blots were reprobed for total cellular STAT-1 levels to normalize the results. B) Data are represented as fold-stimulation of STAT activity over basal conditions (without any IgG stimulation, considered as 100), and are represented as mean ± SE of 3 independent experiments. C) ERK stimulation by CRPS patients IgG was significantly higher ($p < 0.001$) when compared to the healthy controls (Unpaired t-test). (HC n= 9; CRPS n= 12).

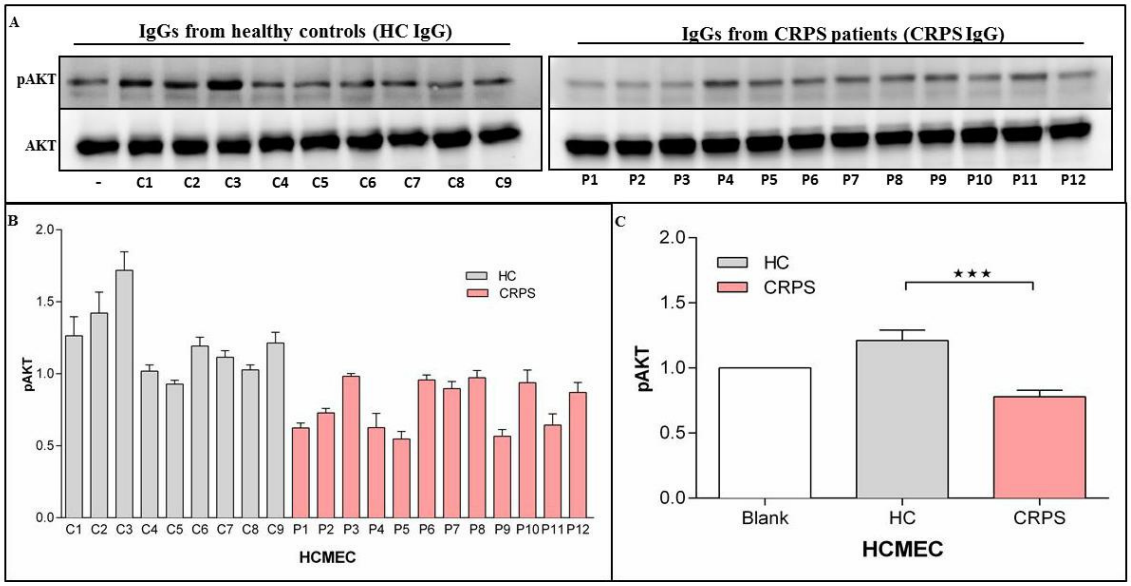

Figure 16: CRPS IgG are effective activators of pAKT (cell differentiation and survival mediator) in HCMEC cells. A) HCMEC cells were incubated for 5 mins with IgGs (12.5 µg/ml) obtained from different HC and CRPS patients and pAKT activation was assessed using specific anti-phospho AKT antibodies (pAKT). Blots were reprobed for total cellular AKT levels to normalize the results. B) Data are represented as fold-stimulation of AKT activity over basal conditions (without any IgG stimulation, considered as 100), and are represented as mean ± SE of 3 independent experiments. C) ERK stimulation by CRPS patients IgG was significantly higher ($p < 0.001$) when compared to the healthy controls (Unpaired t-test). (HC n= 9; CRPS n= 12).

4.2. *Expt. set 2:* Autoantibodies from CRPS IgG bind to some primary osteoblasts.

4.2.1. Autoantibody detection by FACS in primary osteoblasts

Primary osteoblasts differentiated from MSCs of different trauma surgery patients were incubated with HC, CRPS IgG and analyzed in flow cytometry for the surface binding autoantibodies. Primary osteoblasts such as ZK22, ZK34 and ZK38 showed no statistical significant difference in binding between the HC and CRPS IgG stimulated groups ($p > 0.05$, n.s., Fig 17A, 17B and 17C respectively). CRPS IgG binds efficiently to the ZK37, ZK48 and ZK58 primary osteoblasts surface receptors ($p < 0.05$, n.s., Fig 17D, 17E and 17F respectively). (HC n= 10; CRPS n= 10).

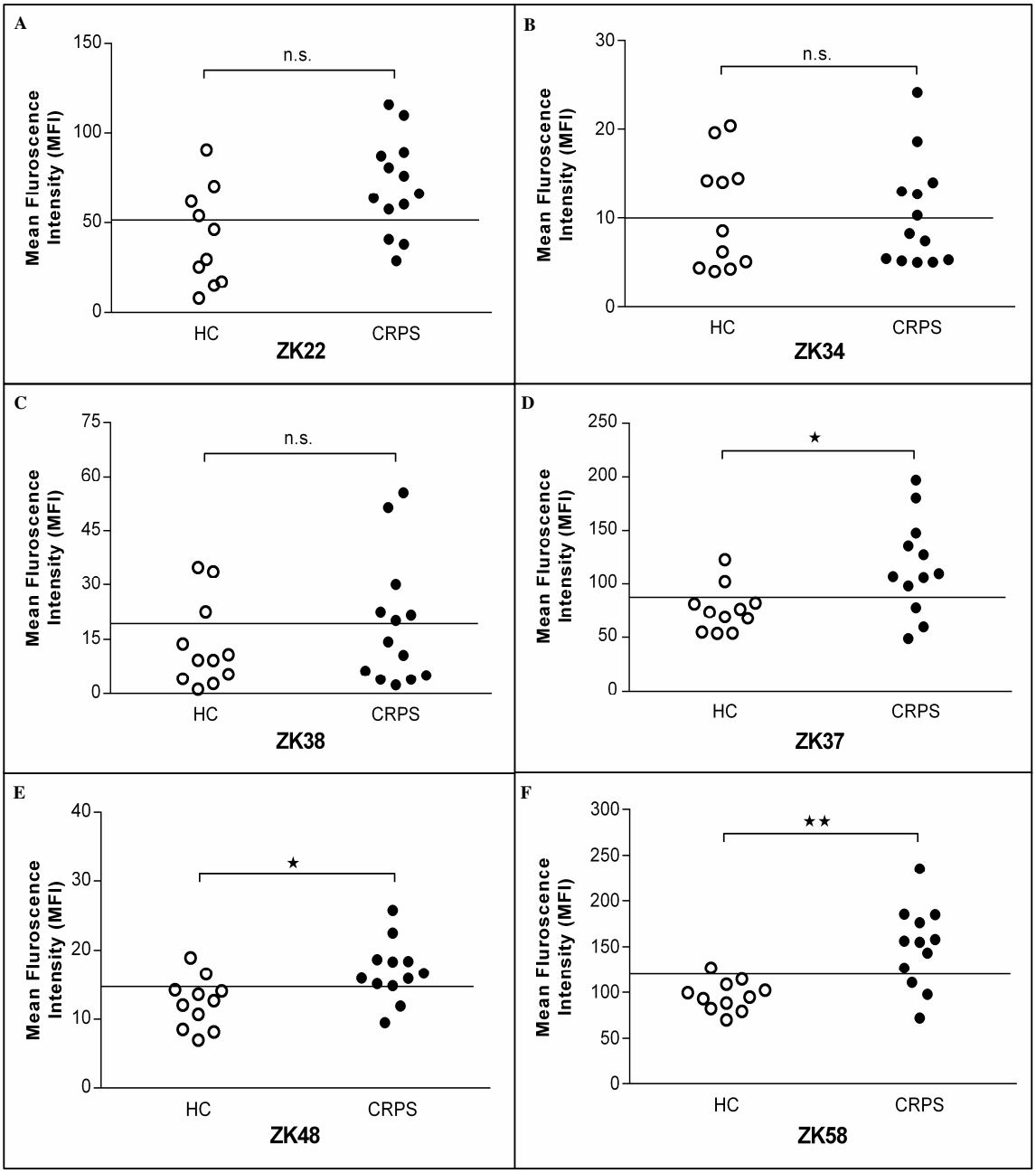

Figure 17: Determination of surface binding autoantibodies in primary osteoblasts differentiated from mesenchymal stem cells (MSCs). (A, B, C) ZK22, ZK34, ZK38 patient's derived osteoblasts did not show statistically significant difference between the groups in binding of functionally active autoantibodies, $p > 0.05$, n.s. (D, E, F) Whereas CRPS IgG stimulated group showed increased amount of functionally active autoantibodies in ZK37, ZK48 and ZK58 osteoblasts, $p < 0.05$. (HC n= 11; CRPS n= 13) Horizontal line is the mean of the samples within the groups.

4.2.2. Functional assays in primary osteoblasts

Primary osteoblasts from three patients (ZK37, ZK48, and ZK58) who showed positivity for surface autoantibody binding were taken to carry out the further cell based functional assays and gene level expression studies. Cell based assays such as cytotoxicity and proliferation assays were carried out in all these three different osteoblasts. All three primary osteoblasts were stained for Annexin-V and propidium iodide in immunofluorescence to understand in detail whether the cytotoxic effects are consequences of apoptosis or necrosis. Later, osteoblasts specific markers expressions were analyzed in qPCR. Finally, immunoblotting was done for quantification of collagen-1, an osteoblast specific marker in these primary osteoblasts.

4.2.2.1. Cytotoxicity assay

To determine whether the patient's cells which were positive for binding leads to higher cytotoxicity, Lactate Dehydrogenase (LDH) release assay was carried out in all the three patient osteoblast cells. The cytotoxic effect of HC, CRPS and RF IgG on ZK37, ZK48 and ZK58 osteoblasts were analyzed. After incubating ZK37, ZK48 and ZK58 cells with different HC, CRPS and RF IgG, CRPS IgG caused higher cytotoxic effects in ZK37 (HC vs CRPS, $p < 0.05$ and CRPS vs RF $p > 0.05$, n.s., Fig 18A) and also in ZK58 (HC vs CRPS, $p < 0.001$ and CRPS vs RF $p > 0.05$, n.s., Fig. 18C). On the other hand, ZK48 did not show any statistically significant difference in their cytotoxicity. All the three groups in ZK48 showed equal cytotoxic effects (HC vs CRPS vs RF, $p > 0.05$, n.s., Fig 18B). (HC n= 10; CRPS n= 10; RF n= 7)

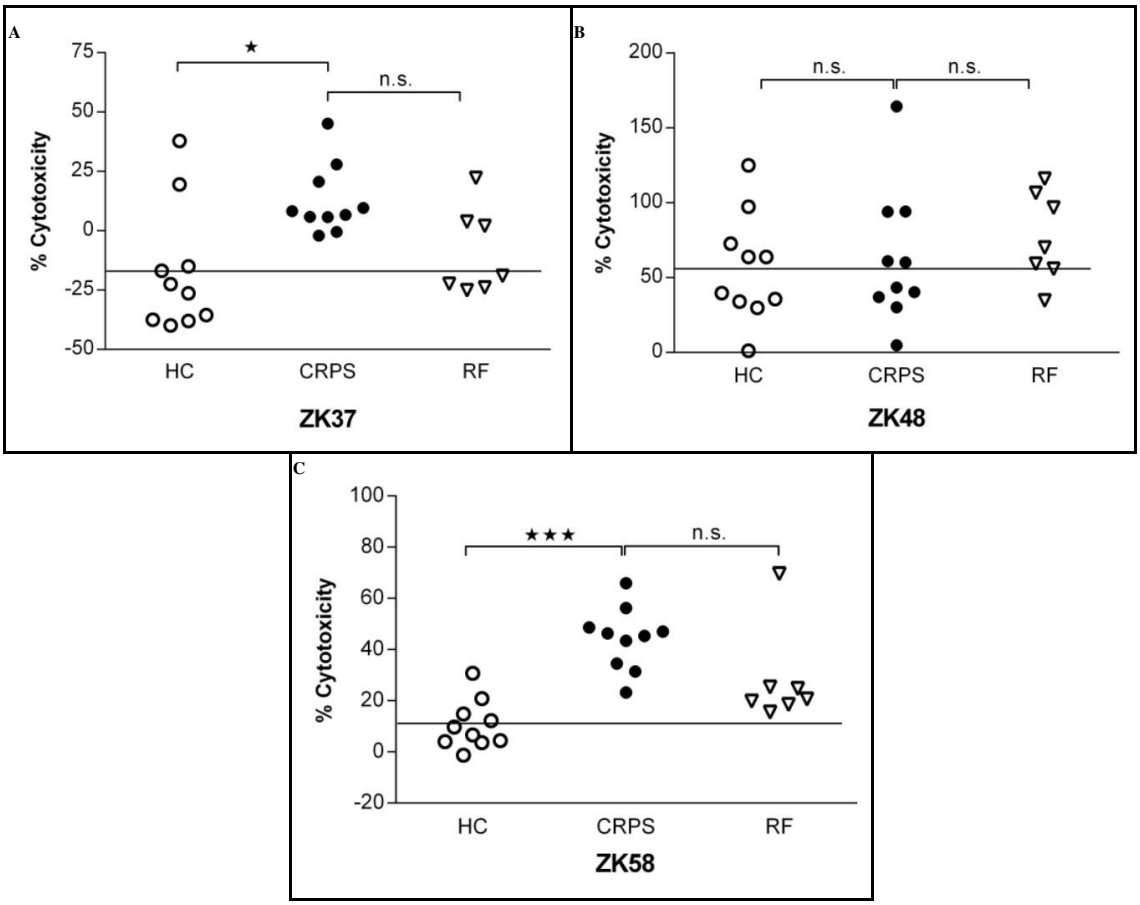

Figure 18: Cytotoxicity assay of primary osteoblasts after incubating different IgG for 24 h. (A) ZK37 osteoblast cells after stimulation showed higher cytotoxicity in CRPS IgG stimulated group, $p < 0.05$ showing no difference between CRPS vs RF group, $p > 0.05$, n.s. (B) All the three different IgGs caused equal cytotoxicity in ZK48 cells, HC vs CRPS vs RF, $p > 0.05$, n.s. (C) ZK58 cells had higher cytotoxicity in CRPS IgG treated cells, $p < 0.001$ with no significant difference in RF group, $p > 0.05$, n.s. (HC n= 10; CRPS n= 10; RF n= 7) Cut-off marked as horizontal line, was determined using the formula mean of the controls + 2.5x standard deviation.

4.2.2.2. Proliferation assay

The proliferation rate of primary osteoblasts (ZK37, ZK48 and ZK58) after stimulation of HC, CRPS and RF IgG for 24 h was analyzed. After incubating ZK37, ZK48 and ZK58 cells with different HC, CRPS and RF IgG, CRPS IgG treated cells had very less proliferation rate in all three different osteoblasts. ZK37 showed decreased proliferation property in CRPS IgG stimulated group comparing to other two groups (HC vs CRPS, $p < 0.05$ and CRPS vs RF $p >$

0.05, n.s., Fig 19A). CRPS IgG treated ZK48 also had very less proliferating effect (HC vs CRPS, $p < 0.001$ and CRPS vs RF $p > 0.05$, n.s., Fig 19B). In the ZK58 cells the proliferating rate was drastically decreased in both CRPS and RF IgG treated groups (HC vs CRPS, $p < 0.001$ and CRPS vs RF $p < 0.001$, Fig 19C). (HC n= 10; CRPS n= 10; RF n= 7).

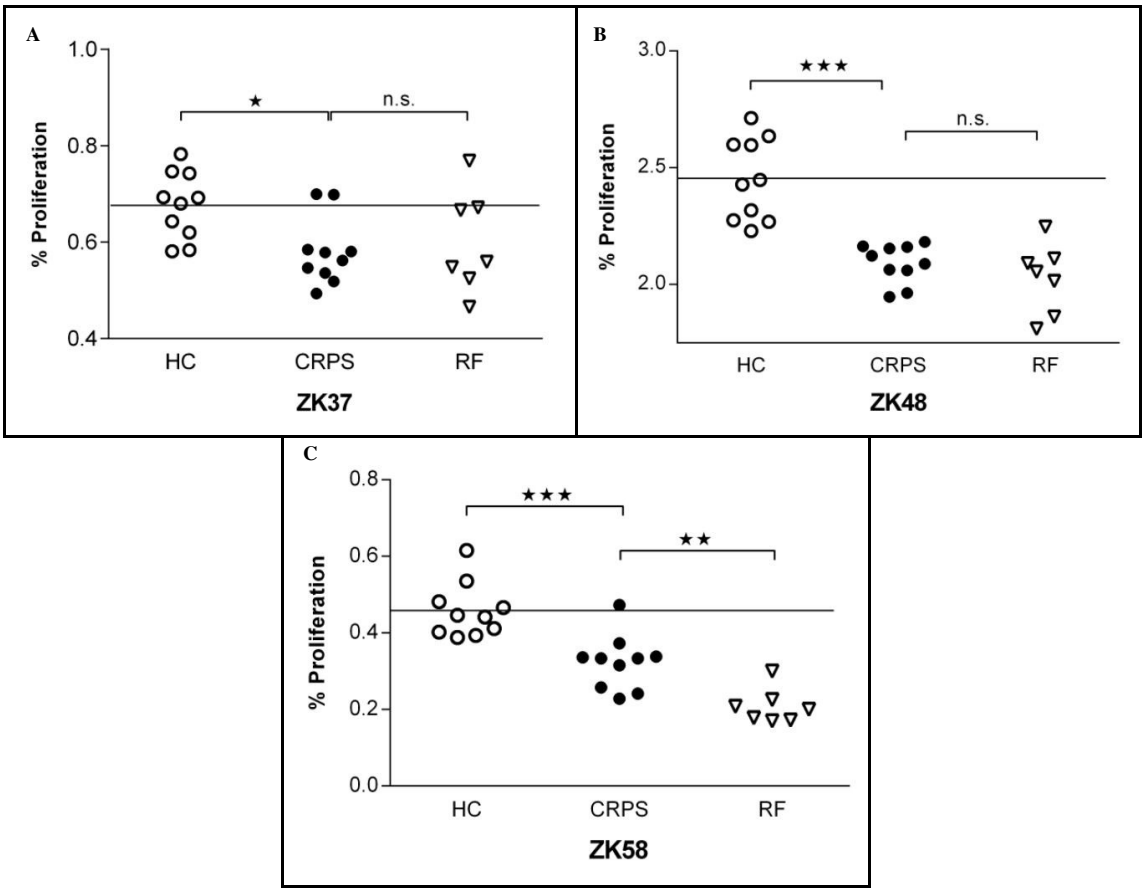

Figure 19: Proliferation assay in primary osteoblasts. In all the three different osteoblasts, CRPS IgG mediated a significant decrease in proliferating property. (A) ZK37, $p < 0.05$ (B) ZK48, $p < 0.001$ and (C) ZK58, $p < 0.001$. (HC n= 10; CRPS n= 10; RF n= 7) Cut-off marked as horizontal line, was determined using the formula mean of the controls + 2.5x standard deviation.

4.2.3. Immunofluorescence staining of primary osteoblasts

Primary osteoblasts showed increased necrosis by stimulation of CRPS IgG. The cytotoxic effects caused by the CRPS IgG were studied further in immunofluorescence to

understand whether the cytotoxicity is due to apoptosis or necrosis. For this reason, the primary osteoblasts were incubated with HC, CRPS and RF IgG for 24 h and stained with Annexin-V FLUOS staining kit. Annexin-V-FLUOS binds in a Ca^{2+}- dependent manner to negatively charged phospholipid surfaces, and shows high specificity for phosphatidylserine (PS). Therefore, it stains apoptotic and necrotic cells. Since necrotic cells also expose PS because of lost membrane integrity, propidium iodide is utilized as a DNA stain to distinguish necrotic cells from Annexin-V-labeled cell clusters.

Cells with no treatment were considered as blank (Fig 20A), 1 µM digitonin (Fig 20B) and staurosporine (Fig 20C) were used as apoptotic and necrotic positive controls respectively. When the cells were stimulated with HC, CRPS and RF IgG for 24 h in all the three different (ZK37, ZK48 and ZK58) osteoblasts, CRPS IgG stimulated cells showed completely compromised cytoplasm and allowing the propidium iodide to stain the leaky nucleus. Quantification of number of cells positive for propidium iodide was done by randomly choosing three fields in each image of every sample and the average was taken and plotted in bar graph. In ZK37, comparing to HC and RF, the CRPS IgG mediated a very high necrotic effect. The quantification graph is shown, ($p < 0.001$, Fig 20D, E, F, G). ZK48 also shows higher number of necrotic cells in CRPS IgG stimulated cells, ($p < 0.05$, Fig 20H, I, J, K). ZK58 showed compromised cytoplasm in both CRPS and RF IgG treated groups comparing to the HC, ($p < 0.05$, Fig 20L, M, N, O). (HC n= 10; CRPS n= 10; RF n= 7).

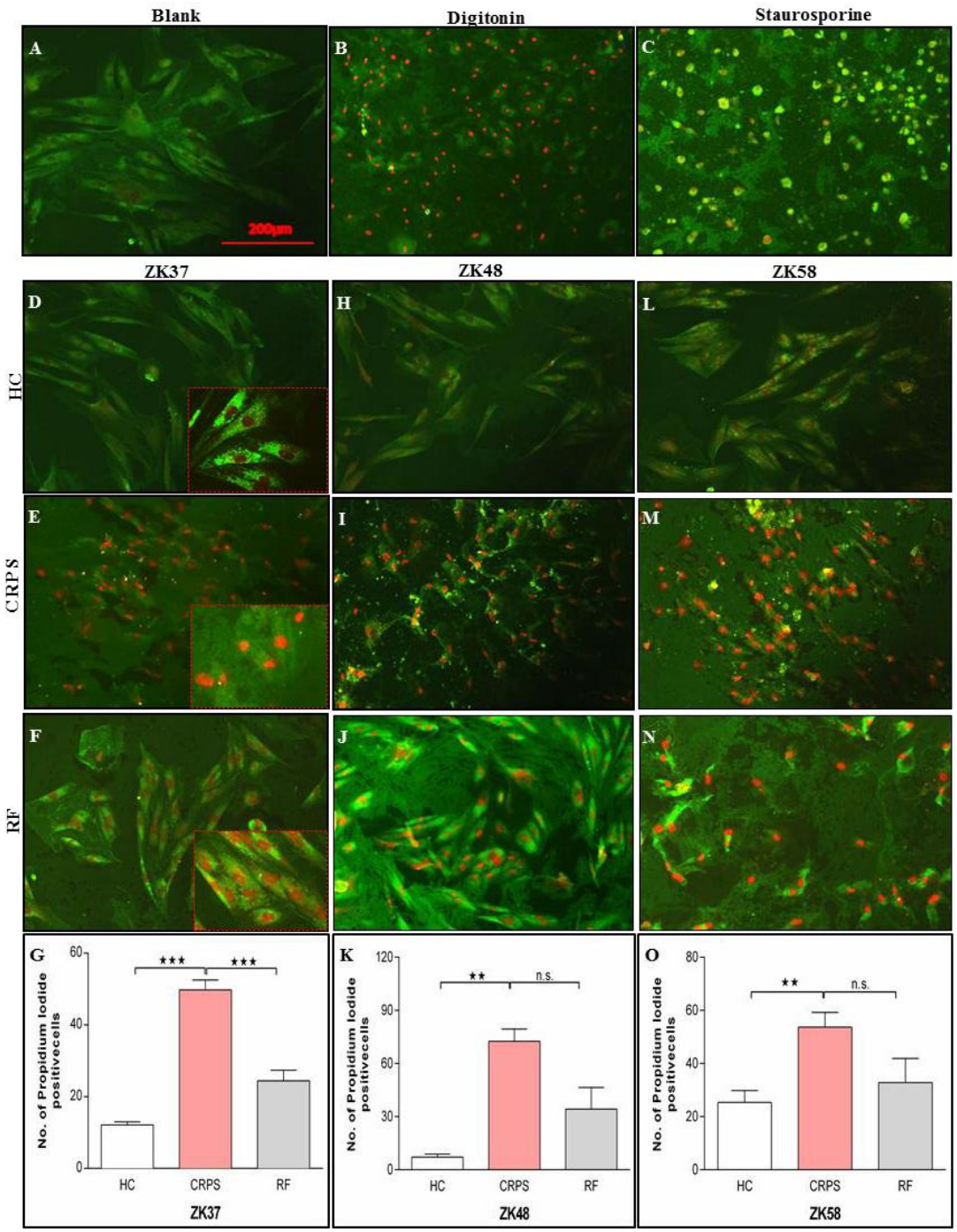

Figure 20: Quantification of apoptotic and necrotic cells by Annexin-V FLUOS immunofluorescence staining. In all the three different osteoblasts, CRPS IgG mediated a significantly increased number of propidium iodide positive necrotic cells. Images were taken at 10x objective with the scale of 200 µm and the inner square is the same representative image taken in 40x objective. (A) Blank, (B) 1 µM Digitonin (necrotic positive control), (C) 1µM Staurosporine (apoptotic positive control), ZK37 (D) HC, (E) CRPS, (F) RF IgG treated cells stained for Annexin – V and propidium iodide, (G) Quantification bar graph of ZK37 to show the number of propidium

iodide positive cells, $p < 0.001$. ZK48 (H) HC, (I) CRPS, (J) RF IgG treated cells stained for Annexin – V and propidium iodide, (K) Quantification bar graph of ZK48 to show the number of propidium iodide positive cells, $p < 0.01$. ZK58 (L) HC, (M) CRPS, (N) RF IgG treated cells stained for Annexin – V and propidium iodide, (O) Quantification bar graph of ZK58 to show the number of propidium iodide positive cells, $p < 0.01$. (HC n= 10; CRPS n= 10; RF n= 7).

4.2.4. Gene expression study of osteoblast markers

Decreased expression of osteoblast specific markers in primary osteoblasts might explain the reason for patients with CRPS developing bone-related disturbances.

Primary osteoblasts differentiated from MSCs were treated with HC, CRPS and RF IgG for 24 h and the relative gene expression of osteoblast specific markers such as Collagen, type I, alpha-1 or COL1A1 (Col-1), dickkopf-1 (DKK-1), osteocalcin or bone gamma-carboxyglutamic acid-containing protein (BGLAP) and alkaline phosphatase (ALP) were analyzed. An irregularity in the expression of these genes alters the proper bone building and regulating processes. Primary osteoblasts (ZK37, ZK48 and ZK58) were incubated with HC, CRPS and RF IgG for 24 h and the expression of the above-mentioned genes were studied. ZK37 showed statistically significant decrease in all osteoblast specific markers in CRPS IgG treated group, ALP ($p < 0.01$, fig 21A), Col-1 ($p < 0.05$, fig 21B), osteocalcin ($p < 0.05$, fig 21C) and DKK-1 ($p < 0.05$, fig 21D).

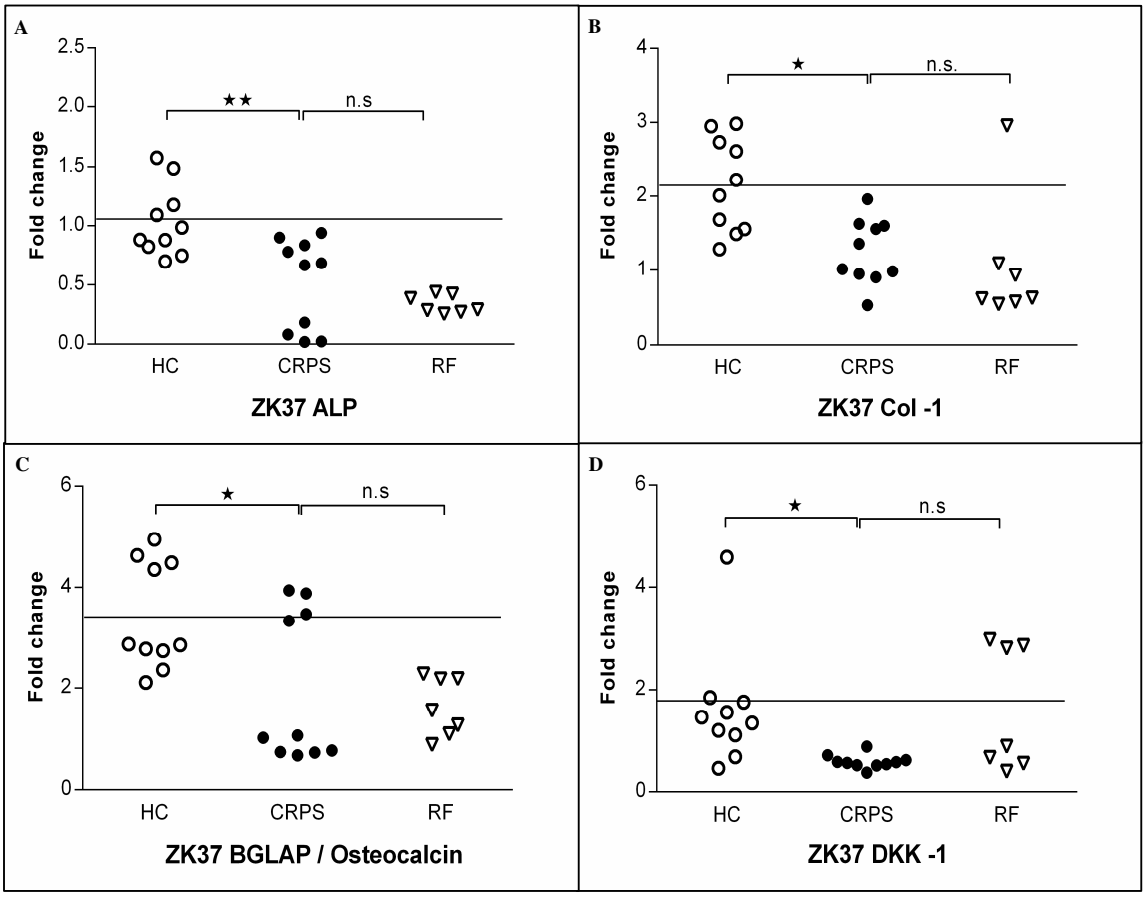

Figure 21: Relative mRNA expression of osteoblast specific markers in ZK37 primary osteoblast. ZK37 showed statistically significant decrease in all osteoblast specific markers in CRPS IgG treated group. Expression of the genes are shown in the figure (A) ALP ($p < 0.01$), (B) Col-1 ($p < 0.05$), (C) osteocalcin ($p < 0.05$) and (D) DKK-1 ($p < 0.05$). (HC n= 10; CRPS n= 10; RF n= 7). Experiments are carried out in triplicates and the average of each sample is plotted in the graph. All data normalized to GAPDH and blank is set to 1.

On the other hand, ZK48 did not show statistically significant difference in ALP ($p > 0.05$, n.s., fig 22A) and DKK-1 ($p > 0.05$, n.s., fig 22D) between the different groups but with a significant decrease in Col-1 and osteocalcin ($p < 0.05$ each, fig 22B and 22C) in CRPS IgG treated group. Additionally, the RF IgG treated group in ZK48 had higher expression of DKK-1 ($p < 0.05$, fig 22D). Finally, ZK58 when treated with different IgG did not alter the expression of ALP ($p > 0.05$, n.s., fig 23A) but had a statistically significant decrease in the expression of Col-1 ($p < 0.01$, fig 23B), osteocalcin ($p < 0.001$, fig 23C) and DKK-1 ($p < 0.05$, fig 23D) in the CRPS IgG stimulated group leaving no significant difference between the CRPS and RF IgG stimulated groups. (HC n= 10; CRPS n= 10; RF n= 7). Experiments

are carried out in triplicates and the average of each sample is plotted in the graph. All data normalized to housekeeping gene GAPDH and blank is set to 1.

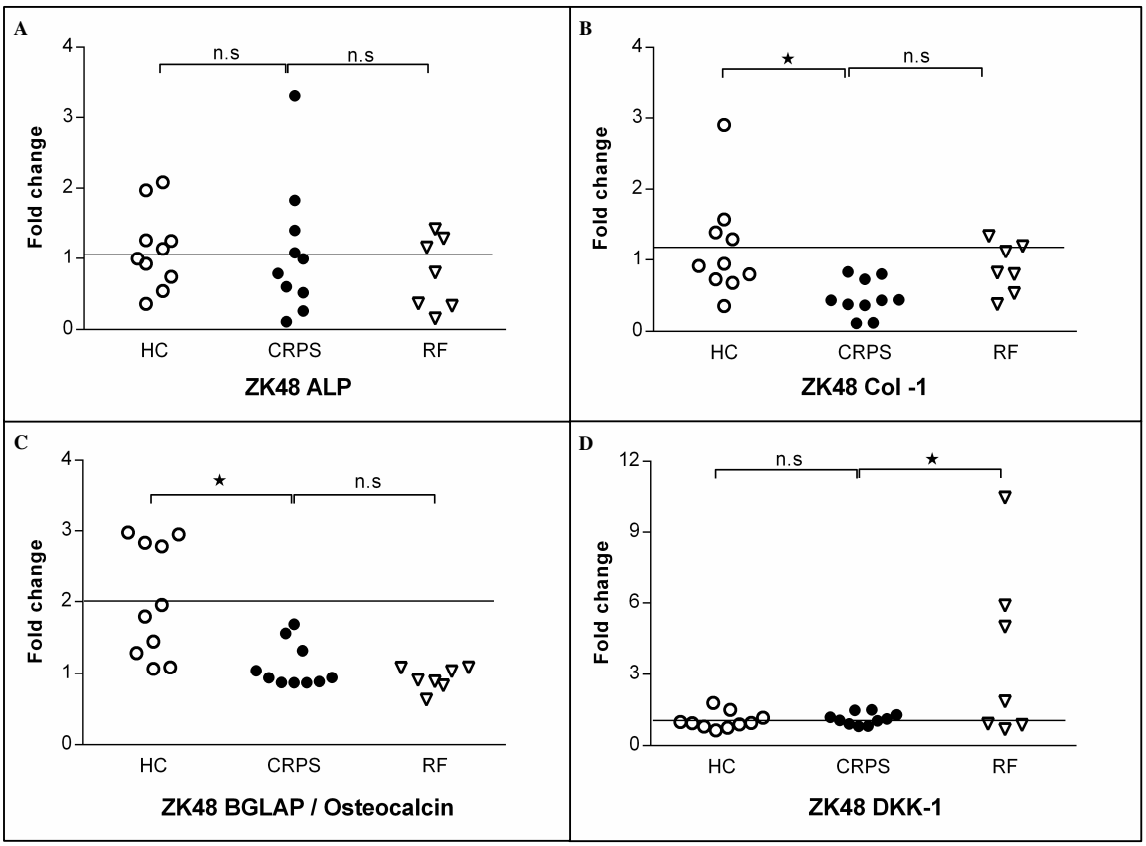

Figure 22: Relative mRNA expression of osteoblast specific markers in ZK48 primary osteoblast. ZK48 did not show statistically significant difference in (A) ALP ($p > 0.05$, n.s.) and (D) DKK-1 ($p > 0.05$, n.s.) between the different groups but with a significant decrease in (B) Col-1 and (C) osteocalcin ($p < 0.05$ each) in CRPS IgG treated group. Additionally, the RF IgG treated group in ZK48 had higher expression of (D) DKK-1 ($p < 0.05$). (HC n= 10; CRPS n= 10; RF n= 7). All data normalized to GAPDH and blank is set to 1. Experiments are carried out in triplicates and the average of each sample is plotted in the graph.

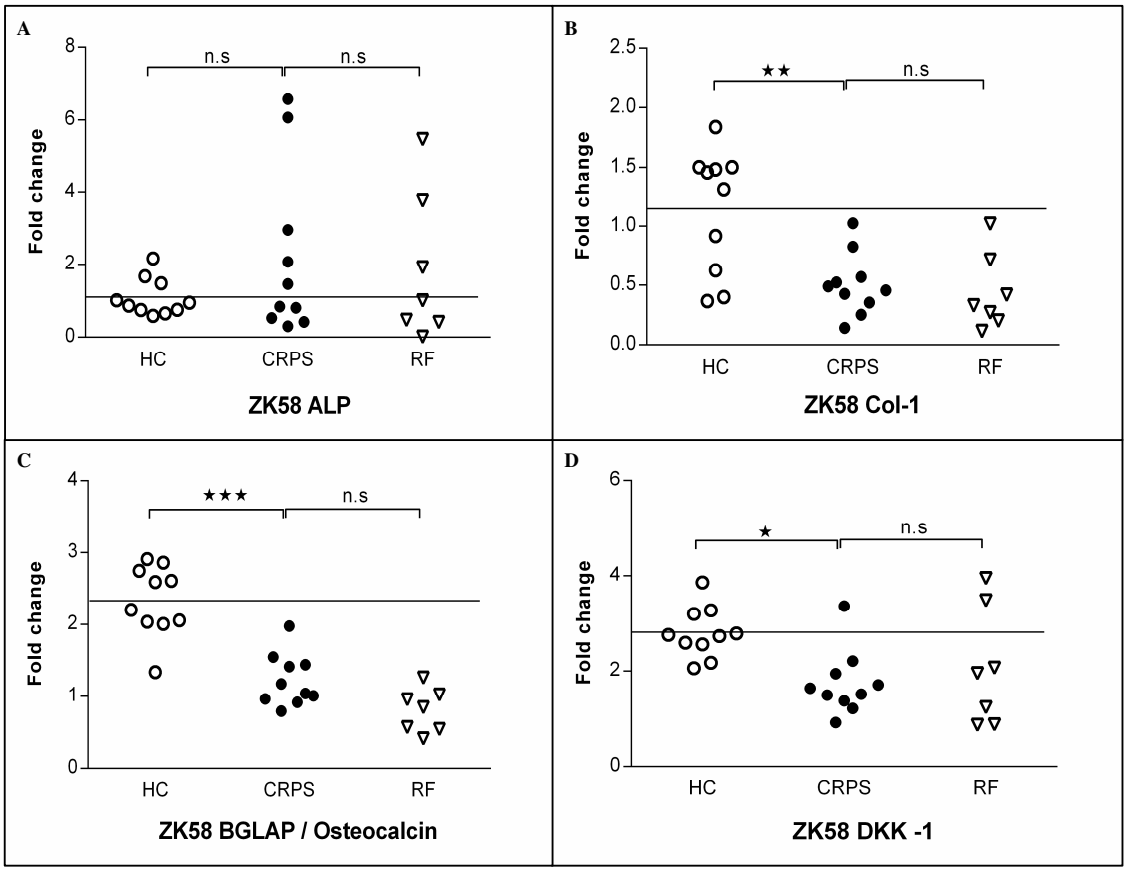

Figure 23: Relative mRNA expression of osteoblast specific markers in ZK58 primary osteoblast. ZK58 treated with different IgG did not alter the expression of (A) ALP ($p > 0.05$, n.s.) but had a statistically significant decrease in the expression of (B) Col-1 ($p < 0.01$), (C) osteocalcin ($p < 0.001$) and (D) DKK-1 ($p < 0.05$) in the CRPS IgG stimulated group leaving no significant difference between the CRPS and RF IgG stimulated groups. (HC n= 10; CRPS n= 10; RF n= 7). All data normalized to GAPDH and blank is set to 1. Experiments are carried out in triplicates and the average of each sample is plotted in the graph.

4.2.5. Immunoblotting of Collagen-1 in primary osteoblasts

ZK37 (primary osteoblasts) were treated with HC, CRPS and RF IgG for 24hrs and collagen protein expression was analyzed by immunoblotting (Fig. 24A). Four randomly selected IgGs from each group were incubated with ZK37 (primary osteoblasts) and the cell lysate was analyzed in immunoblotting for collagen-1 (col-1) protein. We found that CRPS IgG treated ZK37 showed significantly reduced levels of col-1 ($p < 0.001$, Fig. 24B) comparing to the HC and RF IgG treated groups. Reduced levels of col-1 in CRPS IgG treated primary osteoblasts clearly explains that CRPS IgG has a pathological role in the col-1 expression and synthesis.

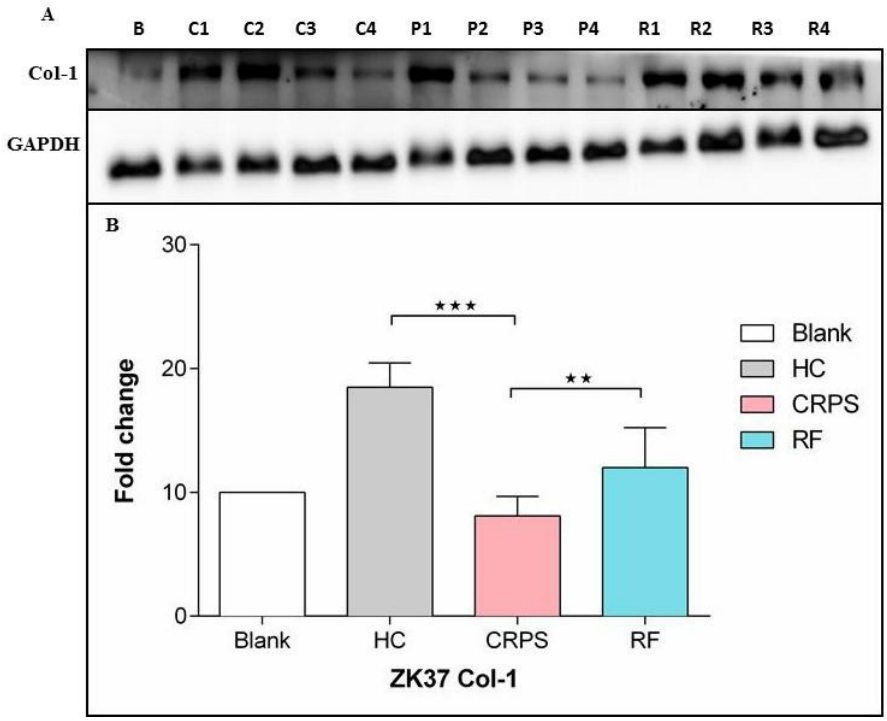

Figure 24: **Immunoblotting of Collagen-1 in ZK37. A)** CRPS IgG has the ability to reduce the collagen-1 protein level in primary osteoblasts. **B)** Quantification graph showing the ZK37 treated with CRPS IgG had significantly reduced levels of Col-1 ($p < 0.001$) comparing to the HC and RF IgG treated groups.

4.3. *Expt. set 3:* Intravenous Immunoglobulin as therapeutic approach for CRPS

Recently, IvIg is used to treat many autoimmune and inflammatory diseases including CRPS. In this queue, studies are carried out in the view to identify the mechanism of IvIg in treating the autoimmune diseases but their complexity in behavior and the signaling pathway underlying is not yet clearly understood. Our 13 CRPS patients IgG reported that when endothelial cells incubated with CRPS IgG, higher cytotoxicity is observed compared to the healthy control. Captivatingly, when the cells were incubated with CRPS IgG + IvIg fractions at 100mg/ml substantially reduced the cytotoxicity levels in HCMEC, HEK 293 and A10 cells. However, the proliferation rate of the cells that were incubated with CRPS + IvIg fractions does not show any statistically significant difference between the groups. Thus, we were interested to study the effect of IvIg along with CRPS patients IgG in various other cell types, which are believed to contribute vascular, bone and connective tissue metabolism and thereby lead to local trophic changes.

4.3.1. IvIg mediated cytotoxicity

The cytotoxic effect of HC, CRPS IgG, IvIg alone and CRPS IgG + IvIg on HCMEC and HEK293 cells were analyzed. After incubating HCMEC with IgG, CRPS IgG incubated cells showed higher cytotoxic effects compared with both, HC ($p < 0.001$) and CRPS IgG + IvIg ($p < 0.001$, Fig. 25A). Incubation of HEK293 with the HC and CRPS IgG-fractions showed no difference between HC and CRPS (n.s) but a significant decrease in cytotoxicity was seen in cells treated with CRPS IgG + IvIg ($p < 0.01$, Fig. 25B). A10 cells also showed reduced cytotoxic effects when treated with IvIg along with the CRPS IgG ($p < 0.001$, fig 25C). 2% Triton X-100 was used as positive control and all the experiments were repeated in triplicates.

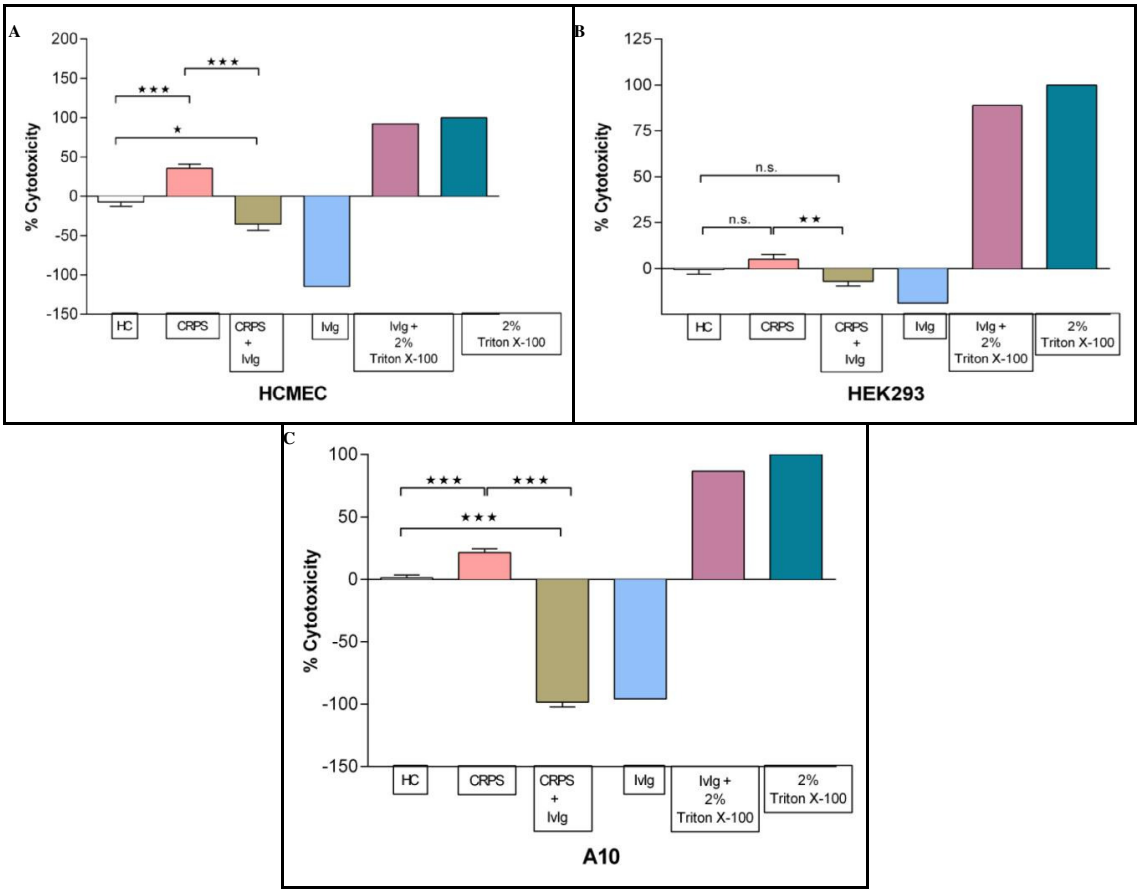

Figure 25: IvIg mediated cytotoxic effects of HC, CRPS and CRPS + IvIg to human microvascular endothelial cells HCMEC, HEK293 and A10 cells. CRPS IgG when treated with endothelial HCMEC cells induced higher

cytotoxicity compared to HC ($p < 0.001$) and CRPS + IvIg ($p < 0.001$). CRPS - IgG did not induce any significant cytotoxicity compared to HC ($p > 0.05$) in the HEK293 cells, but a significantly reduced cytotoxicity is observed in the CRPS + IvIg group ($p < 0.01$). A10 cells when treated with IvIg along with CRPS IgG showed less cytotoxic effects compared to CRPS IgG alone group ($p < 0.001$).

4.3.2. IvIg mediated proliferation

After four hours treatment with WST-1 proliferation reagent, HCMEC incubated with CRPS IgG showed less proliferation when compared to the healthy control ($p < 0.001$), Fig. 26A), without difference between other two pairs (CRPS vs CRPS + IvIg and HC vs CRPS + IvIg, $p > 0.05$). No statistically significant difference in proliferation rates was observed in HEK293 cells between the HC and CRPS groups but a statistically significant decrease of proliferation is observed in the CRPS + IvIg samples when compared to other two groups ($p < 0.001$), Fig 26B). In A10 cells, CRPS + IvIg treated group showed a statistically significant decrease in the proliferation rate when compared to HC and CRPS IgG treated groups ($p < 0.001$, Fig 26C).

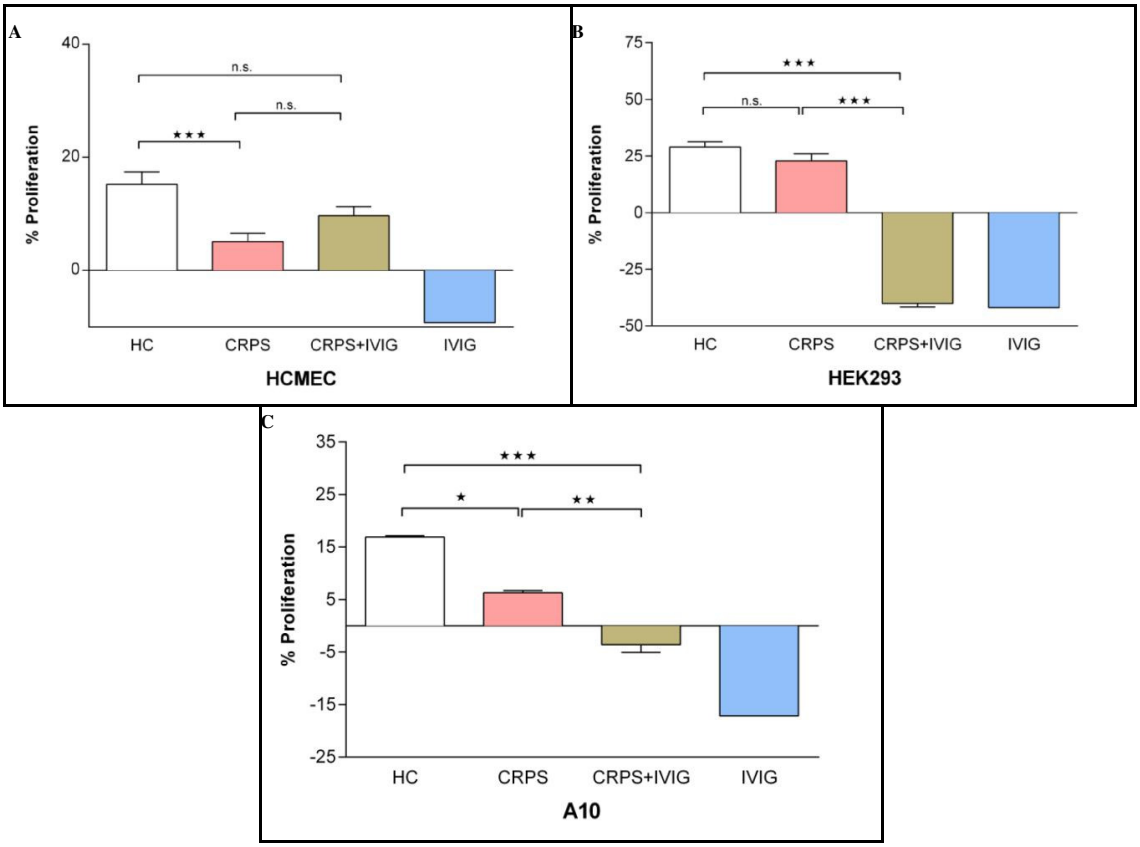

Figure 26: IvIg mediated proliferation rate of HCMEC, HEK293 and A10 cells after incubation with HC, CRPS and CRPS + IvIg is measured after 4 h treatment with WST-1 proliferation reagent. (A) HCMEC showed less proliferation when incubated with CRPS IgG compared to HC, ($p < 0.01$), whereas no difference is seen between CRPS and CRPS+IvIg ($p>0.05$). (B) HEK293 showed no significant effects between the HC and CRPS IgG however a statistically higher difference is seen between the CRPS and CRPS+IvIg groups ($p < 0.001$). (C) A10 cells showed a statistically significant decrease in proliferation in the CRPS + IvIg treated group, ($p < 0.001$).

5. DISCUSSION

CRPS is a multifactorial disease, including different pathogenic mechanisms. Neurogenic inflammation following trauma is the main peripheral mechanism in CRPS. However, central sensitization and genetic factors are also involved in the pathogenesis of the disease. In the recent years, an involvement of the immune system has been suspected to be an important factor in CRPS development. In this thesis, I have studied the involvement of autoantibodies in this process and their effects on various cell types affected in CRPS. This study is an approach to gain the deeper insight in the immunological part of pathophysiology of CRPS.

5.1. Effects of CRPS IgG on endothelial cells

CRPS is a painful, debilitating, and often-chronic condition characterized by various sensory, motor and vascular disturbances. Despite many years of study, current treatments are limited due to lack of understanding of the underlying mechanisms. Limited studies are available that attempted to explain the molecular level changes in gene expression supporting the nociceptive sensitization commonly observed in CRPS limbs, or how those changes might evolve over time (Gallagher et al., 2013). Vascular abnormalities were one of the commonly observed autonomic disturbances in CRPS patients. Vascular breakdown or leakage could be responsible for various symptoms in CRPS patients, such as edema and temperature regulation disturbances. Breakdown of immunologic self-tolerance, development of autoantibodies to β2AR and M2R receptors were also shown to be considerable elements in adult CRPS cases (Kohr et al. 2011) and could be involved in the development of autonomic symptoms in CRPS. These autoantibodies progress to initiate the autoimmune-mediated neuro-inflammatory response.

Substantial evidence has been shown that CRPS is a neurogenic inflammatory disorder with a probable autoimmune component in many individuals (Blaes et al., 2007; Goebel, 2011; Goebel et al., 2011). Kohr *et al.* described in their study of adult CRPS patients, that 90% of the cohort had autoantibodies to either the β2AR (Beta-2 adrenergic receptor) or to the M2R (muscarinic acetylcholine receptor) (Kohr et al., 2011). Similarly, our results also show that 83% of total patients taken in the study had autoantibodies against surface epitopes of β2AR and/or M2R. Only one out of 7 patients with radial fracture and none of the controls showed

positive binding to the CHO-cells overexpressing the ADRB2 receptors and none to the CHRM2 receptor, indicating that the autoantibodies are disease-specific. Besides, blocking the receptors with their specific antagonists also blocked binding of autoantibodies to these receptors to about 80%.

In our study, the endothelial cell line HCMEC expresses target receptors, β2AR and M2R. We could demonstrate autoantibody binding of CRPS sera to HCMEC and this binding affects the cell proliferation and led to an increased cytotoxicity.

The expression of pro-inflammatory cytokines was elevated in the CRPS-IgG treated HCMEC endothelial cells. These cells showed elevated levels of adhesion molecules such as ICAM-1, VCAM-1 and the chemokine MCP-1 (CCL2), when incubated with CRPS-IgG. Cytokines play a major role in recruiting monocytes, neutrophils, and lymphocytes, as well as in inducing chemotaxis through the activation of G-protein-coupled receptors that also involves adhesion molecules and glycosaminoglycans. Monocyte chemoattractant protein-1 (MCP-1/CCL2) is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages (Flugel et al., 2001). Both the protein CCL2 and its receptor CCR2 were demonstrated to be induced and involved in pathology of various diseases such as AIDS, autoimmune diseases like multiple sclerosis, psoriasis, RA, pulmonary and vascular disorders. Immunological surveillance of tissues, as well as the generation of pathological inflammatory response requires migration of monocytes from the blood stream across the vascular endothelium. CCL2 secreted in or injected into skin arrives in the draining lymph nodes where it can be presented on the surface of high endothelial venules for recruitment of lymphocytes. MCP-1 expression is usually in peak at day 3 post-operation but their up-regulated levels could be observed for up to several weeks after operation. According to a study by Flügel and collegues (Flugel et al., 2001), neuronal MCP-1 and its corresponding receptor CCR2 is up-regulated in response to remote nerve injury but they could not spot any differential levels of RANTES and IP10. In many peripheral nerve injury models, neither IFN-J nor STAT-1 tyrosine was phosphorylated. The increased expression of various pro-inflammatory mediators in HCMEC endothelial cells after treating with CRPS IgG drives us to speculate that it could be a possible reason for the endothelial dysfunction and vascular disturbances in CRPS patients.

Additionally, we found that these autoantibodies are able to potently induce phosphorylation of the inflammatory cascades such as ERK 1/2, STAT-1 and P38 MAPK pathways by activating β2AR in HCMEC cells. To analyze this effect in detail, we performed immunoblotting by stimulating the IgGs from CRPS patients and HC in HCMEC cells. ERK stimulation was significantly up-regulated in the endothelial HCMEC cells when treated with CRPS IgG when compared to healthy control IgG. To confirm that the ERK stimulation leads to inflammation we also analyzed the major inflammation mediating signaling cascades such as P38 MAPK and STAT-1 and found that P38 and STAT-1 were also up-regulated in the CRPS IgG treated HCMEC cells. P38 phosphorylation is responsible for the production and signal transduction of chemokines and cytokines. All these data suggests that CRPS IgG binding to target receptors (ADRB2 and CHRM2) play important roles in activating the inflammatory mediating cascades and in sustaining the vicious cycle of inflammatory response. On the other hand AKT-1 phosphorylation is significantly reduced in HCMEC cells stimulated with CRPS IgG comparing to the healthy controls. The PI3K/Akt pathway is necessary for several key endothelial cell (EC) functions, including cell growth, migration, survival, and vascular tone. Decreased levels of AKT in CRPS IgG stimulated endothelial cells could disturb all the above-mentioned functions, which may explain the vascular dysfunction in CRPS patients.

Antiendothelial autoantibodies have been described in a variety of diseases, for example Susac syndrome (SS) (Magro et al., 2011), atherosclerosis (Varela et al., 2011), systemic vasculitis (Miura et al., 2012) and undifferentiated connective tissue diseases (Lage et al., 2012). Interestingly, most studies could not identify the underlying target antigens. However, functional effects of these autoantibodies have been found. A specific study highlights that AECA (Anti-endothelial cell antibodies) positive sera from Behcet's disease patients induced elevated levels of ICAM-1 expression and increased phosphorylation of ERK 1/2 in human dermal microvascular endothelial cells (Lee et al., 2002). Another study showed that P38 MAPK activation contributes to autoimmune renal injury in mice (Iwata et al., 2003). AECA plays an important role in mediating connective disease associated with pulmonary arterial hypertension. It has been shown that IgG purified from the sera of AECA positive could increase the expression of ICAM-1 and other chemokines in cultured endothelial cells (Li et al., 2010; Liu et al., 2014).

Apart from this many other cascades might be involved that mediate the local inflammation

in the affected limb. Reports suggest that vascular inflammation can be detected by enhanced circulating IL-6 produced by the activated inflammatory cells within the vessel wall (Brasier, 2010; Steyers and Miller, 2014). IL-6 production, in turn induces the expression of various inflammatory mediators such as C-reactive protein (CRP) and angiotensinogen (Ridker et al., 2002). In our study, we found that IL-6 expression is not significantly increased in CRPS-IgG treated cells. A variety of pro-inflammatory agonists activate inflammation in the smooth muscle cells, fibroblasts and endothelial cells, all of which could be involved in the local inflammation in CRPS. The activation pathway is via the NF-κB transcription factor including TNF-α, local and systemic inflammatory mediators such as CD40 ligands. More downstream this could induce the expression of monocyte adhesion proteins including VCAM-1 and ICAM-1 (Brasier, 2010). Our detailed study about the expression of these adhesion molecules in the vascular endothelial cells (HCMEC) after treatment with CRPS IgG explains that these antibodies could act as possible activators that drive the chemotaxis and activation of vascular inflammation in CRPS.

We included the radial fracture (RF) group as a comparative disease control group to understand whether the autoantibody against the surface receptors residing in the trauma patients in the early stage or they develop later in the disease condition. Unfortunately, we couldn't get consistent results in the binding and functional assays, which left us unable to derive a clear explanation. One possible reason could be, in RF, the IgG itself could have undergone some trauma-induced modifications such as glycosylation that produced varying results in the binding and cell based assays in immortalized cells. Recent evidence from several independent model systems suggests that IgG glycosylation is critical for the immunomodulatory activity of IgG (Bohm et al., 2014). In a pregnancy cohort study, it is shown that N-linked glycosylation of the Fc fragment of IgG changes during the pathological and physiological events and strongly influence antibody mediated inflammatory properties (Bondt et al., 2014).

Activation of one of the target receptors (β2AR) leads to the down regulation of density of receptor and suppresses the immune reactions such as down regulation of cytokine production, suppression of IL-2 receptor expression and inhibit lymphocyte proliferation (Feldman et al., 1987). One major effect of stimulation of β2AR in the downstream of the signaling cascade is the elevation of cyclic AMP level. There are various sources of studies

that explain the immunosuppressive effect of beta-2 agonists may be due to the elevation of cyclic AMP level. Considering beta-adrenergic (β2AR) receptors and muscarinic M2 receptors as target for the autoantibodies from CRPS patients we did block these receptors by their specific blockers (βAR and M2R antagonists, 1 µM (±)-Propranolol HCl and 1 µM Atropine respectively) in the vascular endothelial cells. We found that blocking the receptors significantly reduced the surface binding of CRPS IgG to the HCMEC cells and also increased the proliferation effect in these cells. Although there was reduced cytotoxic effect after blocking the receptors it was not statistically significant in the CRPS IgG treated HCMEC cells. This may be due to a different regulation of proliferation and cytotoxicity by the autoantibodies and needs further investigation.

5.2. Autoantibody effects on osteoblasts

Beta-adrenergic receptors (β2AR) are expressed in many cell types, including osteoblasts, cardiomyocytes, lymphocytes, skeletal muscle, smooth muscle and endothelial cells. The receptor can activate various signaling pathways in these cells. All the main effect of sympathetic neurons on bone cells such as norepinephrine release during bone remodeling is mainly mediated by β2AR expressed by osteoblasts. Recent studies have shown that the involvement of muscarinic acetylcholine receptors (mAChRs) plays an important role in cell proliferation and ALP activity in osteoblasts (Liu et al., 2011; Sato et al., 2010). The expression of our target receptors on the osteoblast cells helps us to study the effects our CRPS IgG on primary osteoblasts derived from different patients. Bone metabolism in CRPS is substantially impaired, leading to severe demineralization in the affected limb in about 40% of the patients. We focused in our study on the effects of the binding of autoantibodies from CRPS patients to osteoblasts expressing the receptor and the probable activating or blocking effect of the autoantibodies.

We were interested to study the bone-related disturbances in CRPS patients in two ways: 1) to study the pathophysiological role of various CRPS IgG in osteoblasts and 2) to track the functional differences that might alter the disease complexity in individuals by using osteoblasts from various trauma patients (ZK22, ZK34, ZK38, ZK41, ZK37, ZK48 and ZK58). We used reaming debris collected from different trauma patients and differentiated to primary osteoblasts. When treated with HC or CRPS IgG, amongst seven different trauma

patients derived osteoblasts three of them did not show statistically significant binding between the groups but the other four showed higher binding of the CRPS IgG to osteoblasts. From this we have taken three trauma patients (ZK37, ZK48 and ZK58) derived osteoblasts that showed higher binding in flow cytometry for further examination. In cell based functional assays such as cytotoxicity CRPS IgG treated osteoblasts (ZK37 and ZK58) had significantly increased cytotoxicity compared to the HC group. Although in ZK48, CRPS IgG treated group showed higher binding of functionally active antibodies to the surface receptors they did not show any statistical difference in the cytotoxicity assay between the groups. Additionally, CRPS IgG treated ZK48 had less proliferation property. CRPS IgG by binding to the surface receptors expressed by the bone cells mediates severe necrosis that is almost similar to the effect of 10 µM digitonin, which is a potential necrosis mediator. All three different trauma patient derived osteoblasts showed increased necrotic effect in the CRPS IgG treated group. The main reason of doing immunofluorescence staining with Annexin-V in these primary osteoblasts was to differentiate whether the cytotoxicity caused by CRPS IgG were due to necrosis or apoptosis. It was clear from our results that all the three different osteoblasts on treatment with CRPS IgG had severe necrosis and not apoptosis. This could be another possible explanation for CRPS patients with bone related disturbances such as osteoporosis.

In addition to all the above-mentioned observations in primary osteoblasts, we also investigated the expression of various osteoblast specific markers at mRNA and protein level. Osteoblast specific markers such as collagen - 1, alkaline phosphatase, osteocalcin, dickkopf-1 were studied in all the three different trauma patient derived osteoblasts after treating them with different IgG for 24 h. All the three different osteoblasts showed individually different expression pattern of these markers at baseline. *COL1A1* gene is responsible in encoding the alpha-1 type I collagen protein. This protein strengthens and supports many tissues in the body, including bone and skin. Mutation or abnormal polymorphism of this gene can result in various disorders such as osteogenesis imperfecta type I to type IV and is also involved in the development of osteoporosis. In our study, all the three different patient derived osteoblasts, when treated with CRPS IgG, had statistically significant decrease in the expression of collagen-1 both in mRNA level and protein level.

Alkaline phosphatase (ALP) is another important enzyme important for normal calcification of bone. It is considered as one of the main bone turnover marker that mediates the formation

of bone. In our study containing different trauma patient derived osteoblasts, we couldn't observe any statistically significant difference in their expression between the three IgG groups (CRPS and control groups) in ZK48 and ZK58 whereas a significant decrease in the ALP-1 expression is seen in CRPS IgG treated ZK37 osteoblasts. This variable expression by individuals is yet to be studied in detail. Apart from this other important bone formation markers such as BGLAP or osteocalcin (OC) and dickkopf-1 (DKK-1), a WNT signaling mediator showed also a reduced expression in the osteoblasts with the CRPS IgG. Reduced expression of these bone formation markers could be a possible explanation for CRPS patients who have been reported for reduced BMD (bone mineral density) and patchy osteoporosis (Kumar et al., 2001; Moriwaki et al., 1997). Chronic constriction injury (CCI) is a model for CRPS (Type 2), in which the sciatic nerve in rats will be ligated. Several studies showed, that injured limbs of animals with CCI in the sciatic nerve had reduced BMD and bone mineral content as compared to the contralateral side of the animal (Suyama et al., 2002). Other studies also reported the evidence of osteoporosis in CRPS patients and an improvement of pain using bisphosphonates, a osteoporosis drug, has been described in four randomized controlled studies in CRPS patients (Abe et al., 2011).

A clear thread that bridges the vascular and bone related disturbances in CRPS with an inflammatory effect of autoantibodies against the target receptors (β2AR and M2R) was not yet explained elsewhere. However, few studies explain the beta-adrenergic receptor (β2AR) signaling in osteoblasts might contribute to the catabolic effect of glucocorticoids in bone and leads to osteoclastogenesis-mediated bone loss (Ma et al., 2011). During normal bone remodeling, norepinephrine released in bones by sympathetic neurons suppresses bone formation and promotes bone resorption (Elefteriou et al., 2005; Takeda et al., 2002). Another study showed that stimulating the β2AR by its agonists such as isoproterenol or norepinephrine stimulates osteoclastogenesis and inhibits osteoblast proliferation. This process is mediated in bone cells by stimulating the beta-adrenergic receptors (β2AR) expressed by osteoblasts (Fu et al., 2005; Katayama et al., 2006). This observation would support our hypothesis, that an agonistic β2AR antibody could contribute to the osteoporosis seen in CRPS. Few in vivo studies have demonstrated pharmacological blockers of E-blocker or specific deletion of ADRB2 gene increased the bone mass. On the other hand, some studies showed that β2AR agonists increased the bone resorption instead of increasing the bone mass (Bonnet et al., 2007).

Various studies have shown that β2AR signaling is regulated by exposure to catecholamines and its expression at the cell membrane to enable cells to maintain stringent control over hormonal or neuropeptide responsiveness (Mak et al., 1995). Endogenous hormones like glucocorticoids and thyroid hormones also regulate β2AR signaling and the response of cells to catecholamines. Glucocorticoids can counteract the down-regulation of *Adrβ2* expression induced by β2AR agonists *in vivo* (Hadcock et al., 1989) and can increase *Adrβ2* expression in multiple cell types (Cheng et al., 1980; Collins et al., 1988; Nakada et al., 1989; Norris et al., 1987).

To have a more standardized model we used the SAOS-2 osteosarcoma cells and the A10 smooth muscle cells and determined the surface binding of autoantibodies and their functional effects in the cells after treating them with CRPS IgG and controls. The IgG from CRPS patients showed statistically higher binding to the cell surface of these cells. Moreover, SAOS-2 osteosarcoma cells had higher cytotoxicity and very less proliferation activity when exposed to CRPS IgG. Obviously, cells that express our target receptors (either β2AR or M2R or both) had higher binding of IgG from CRPS patients to the cell surface. The antibody binding is activating the signaling mechanism that is followed through these receptors. Interestingly, one study shows specific antibodies against human cultured chondrosarcoma (HCS2/8) and osteosarcoma (SAOS-2) cells in the serum of patients with osteoarthritis (Kuboki et al., 1999). However, the underlying autoantigen in these cases has never been determined. The β2AR pathway was extensively studied in various cell types regarding its expression, activation and its response elements (Cornett et al., 1998; Feve et al., 1990; Rodan and Rodan, 1986; Takahashi and Iizuka, 1991; Zajac et al., 1986). In ROS17/2.8 osteosarcoma cells, glucocorticoids increased PTH and isoproterenol-mediated activation of adenylate cyclase activity (Rodan and Rodan, 1986). Together these observations suggest that antibody-mediated increased signaling activity of β2AR in HCMEC endothelial, A10 smooth muscle, SAOS-2 and also in primary osteoblasts (ZK37, ZK48 and ZK58) can extend the responsiveness of these cells to the local trophic disturbances, bone catabolic and antianabolic effect of sympathetic neurons.

5.3. Intravenous immunoglobulin (IvIg) - a potential treatment for CRPS

In the recent years, high-dose intravenous immunoglobulin (IvIg) is used for the treatment of autoimmune and inflammatory diseases. Currently few autoimmune diseases were effectively

treated by IvIg therapy, for example, Guillian-Barre syndrome, Kawasaki disease and chronic inflammatory demyelinating polyneuropathy (CIDP) (Durandy et al., 2005; Nimmerjahn and Ravetch, 2008). Despite numerous studies demonstrating efficacy, the precise mode of action is unclear. Paradoxically, IvIg can exert both pro-inflammatory and anti-inflammatory activities based upon its concentration. On one hand, low-dose IvIg requires complement activation or binding of the Fc fragment of IgG to IgG-specific receptors (FcJR) and elicits a pro-inflammatory activity on innate immune effector cells. On the other hand, when administered in high concentrations, IvIg has anti-inflammatory activities (Durandy et al., 2009). The exact mechanism of action of IvIg as an anti-inflammatory agent remains to be elucidated fully. Some studies have proposed that the anti-inflammatory activity of IvIg can be attributed to a minor species of immunoglobulins (IgGs) that is modified with terminal sialic acids on their Fc-linked glycans. It has also been proposed that these Fc-sialylated IgGs engage a unique receptor on macrophages that, in turn, leads to the up-regulation of an inhibitory FcJ receptor, thereby protecting against autoantibody-mediated pathology (Nimmerjahn and Ravetch, 2007).

There are very few studies that have shown the efficacy of IvIg treatment for CRPS. Medlin *et al.* (Medlin et al., 2013) treated a young patient with high-dose IvIg who developed CRPS secondary to a sciatic compression and found a favorable outcome in this particular patient. Another study attempted to explain the mechanism and mode of action of IvIg and its immunosuppressive activity in Experimental Autoimmune Myasthenia Gravis (EAMG) by isolating IvIg a disease-specific suppressive fraction. This IvIg were passed through columns of IgG from rats with experimental autoimmune MG (EAMG) or from MG patients and found that these fractions resulted in depletion of the suppressive activity of IVIG on rat EAMG whereas the minute amounts of IgG fractions eluted from the EAMG- or MG-specific columns retained the immunosuppressive activity of IVIG (Fuchs et al., 2008; Zhu et al., 2006). Apart from this, few other studies after having used IvIg in their RCTs proposed that high-dose IvIg could be a treatment option for CRPS (Goebel et al., 2010; Goebel and Blaes, 2013; Goebel et al., 2013). Additionally, our group has also shown that IvIg inhibits the BAFF (B-cell activating factor) production in chronic inflammatory demyelinating polyneuropathy (CIDP) and explained BAFF as a new target for IvIg in CIDP treatment (Bick et al., 2013). Considering all these previous studies and the anti-inflammatory properties of IvIg, we also studied the role of IvIg (Intravenous immunoglobulin) in IgG mediated damage of endothelial, fibroblast and smooth muscle cells in CRPS. We could

observe CRPS IgG was able to induce higher cytotoxic effects in these cells and the effect was reversed with the administration of IvIg along with the CRPS IgG. A similar effect have been described in literature that shows that administration of IvIg has reversed the cytotoxic effects of endothelial cells and prevents them from inflammation (Nimmerjahn and Ravetch, 2007, 2008). Although, the proliferation property did not gain any improvement, IvIg administration did recover the cells from being damaged. There are hopes as well as doubts in using IvIg in treating CRPS, because it is very expensive and relatively less percentage of patients were benefited by the treatment as compared to the randomized trials who received ketamine, magnesium or tadalafil (Birklein and Sommer, 2010; Collins et al., 2009; Groeneweg et al., 2008; Schwartzman et al., 2009). The exact mechanism of action of IvIg has to be studied in detail in order to explain how they suppress the inflammation in CRPS patients.

Understanding the complex symptoms in the clinical progression of CRPS also requires understanding about the modeling of the immunologic and integrative physiology. Achieving a cellular and molecular understanding of the clinical progression of CRPS is a complex mechanism. Distressed neurons and glial cells release numerous factors into the parenchyma of the central nervous system from the bloodstream, which stimulate the extravasation of leukocytes and autoantibodies (Watkins et al., 2007). When β2AR and M2R autoantibodies exudate from blood vessels along with leukocytes and complement proteins, serious neuroinflammatory consequences would be expected to arise (Cooper and Clark, 2013). Several studies witnessed that remote immune activation play a potential role in evoking trans-synaptic microglial activation in numerous neurological disorders (Banati, 2003). Considering these previous literatures as evidence for the remote neuroimmune activation, possibilities are there that the functionally active autoantibodies against β2AR and M2R could also recruit similar activation. These results strongly support the autoimmune hypothesis in CRPS. Binding of these functionally active autoantibodies from CRPS IgG to the target receptors (β2AR and/or M2R) might trigger various transcription factors responsible for inflammation, cell proliferation, differentiation, survival or programmed to cell death.

In conclusion, our study highlights the role of autoantibodies against the β2AR and M2R in the CRPS pathophysiology. It also reveals how the participating cells expressing β2AR or M2R were influenced not only by the local neurogenic inflammation, but also by

autoantibody-mediated effects. However, the complete role of these autoantibodies against needs to be elucidated in detail. A simplified pictorial representation of my finding is shown in **Fig. 27**.

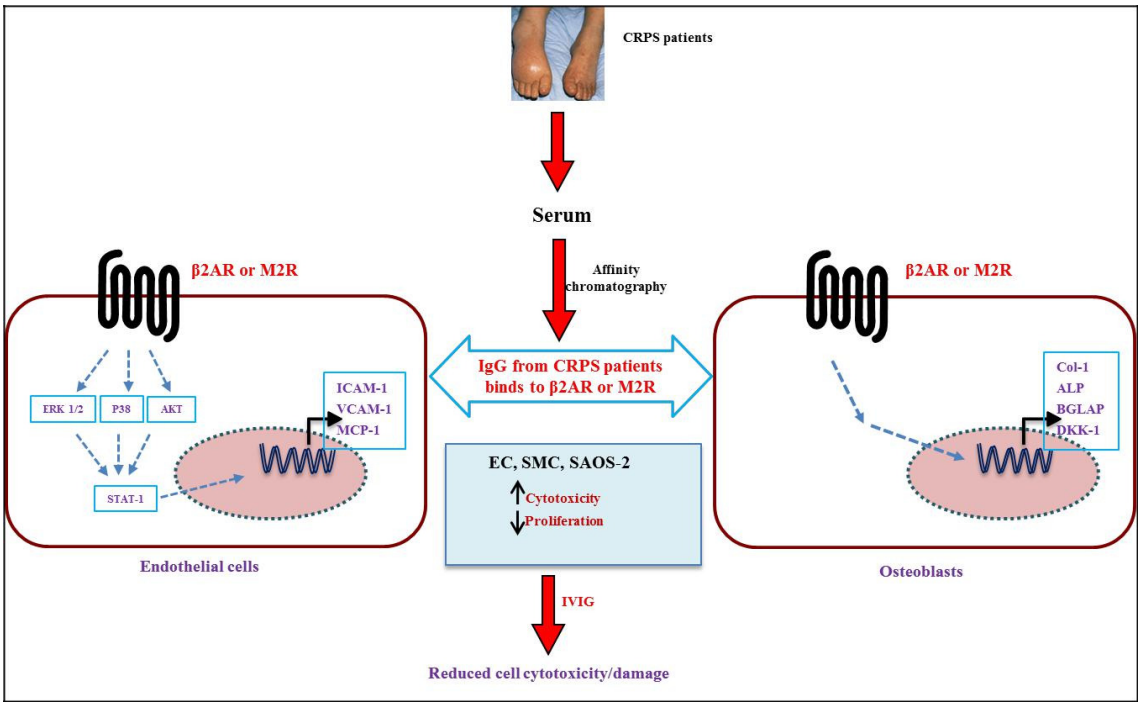

Figure 27: Pictorial representation of findings originated from this thesis. IgG from CRPS patients binds effectively to cells expressing β2AR or M2R receptors and has pathogenic effects in CRPS patients.

One crucial question is the time and the reason of this autoantibody response. The antibodies could possibly be present before the trauma occurs and could become pathogenic by a dramatically changed receptor expression induced by the trauma. Surprisingly, our radial fracture control group also had varying results that we failed to explain in detail. Although, a general mechanism that explains the complexity of complex regional pain syndrome is missing, this study is first step to approach the disease with a deeper molecular insight. An elaborative study of beta-adrenergic receptors and the muscarinic receptors and their signaling mechanism in this disease condition may extend our knowledge on the subtle interaction between the receptor autoantibodies and the immune system.

6. SUMMARY

CRPS is a neuropathic pain syndrome with severe trophic disturbances, which develops in about 0.2 - 0.5% of all trauma patients following a limb trauma or surgery. In Germany with about 4000-8000 new cases expected per year, about 30-40% of patients show a chronic course, often leading to loss of ability to use the affected limb. The main symptoms include a severe neuropathic pain syndrome, accompanied by severe trophic disturbances such as edema, increased skin temperature, impaired regulation of blood flow and osteoporosis. These autonomic / trophic changes indicate disturbances in the vascular regulation, the connective tissue and bone metabolism. Although neurogenic inflammation in the affected limb is an important factor of the disease, the actual trigger factors, in addition to the trauma, are not yet known. As a new factor we were able to show autoimmune process against autonomic nerve fibers as well as against neuronal cell surface epitopes. This includes functionally active autoantibodies against the ADRB2 (β2 adrenergic receptor) and CHRM2 (muscarinic M2 receptor). An expression of our identified target receptors (ADRB2 and CHRM2) on endothelial cells, smooth muscle, osteosarcoma cells and osteoblasts have been described in previous literature and confirmed by us through the expression studies.

Initially, sera obtained from CRPS patients showed higher binding to the CHO-cells overexpressing the ADRB2 and CHRM2 receptors. Blocking the target receptors with their specific antagonists reversed the binding effects. As our main interest in this study is to address the inflammatory mechanisms in vascular endothelial cells and bone related issues in CRPS, we did further detailed study with HCMEC cells and primary osteoblasts. We were able to find that 86% of patients showed effective binding to the target receptors expressed in endothelial HCMEC cells. Binding of these autoantibodies lead to higher cytotoxic effects in the HCMEC cells and decreased proliferation rate compared to controls. Additionally, expression studies proved that CRPS IgG incubation to vascular endothelial cells stimulated highly elevated levels of pro-inflammatory mediators and adhesion molecules such as ICAM-1, VCAM-1 and MCP-1. We were also able to show that CRPS IgG is a potent stimulator of ERK 1/2, P38 MAPK and STAT-1 pathways. TGF-E1 (transforming growth factor, a key regulator of cell growth, cell proliferation, cell differentiation) and BMP-2 (bone morphogenic protein, key regulator of TGF beta signaling pathway) levels were reduced which could be a possible explanation for less proliferation rate of HCMEC cells. This was

confirmed by the reduced levels of AKT phosphorylation in HCMEC cells that were stimulated with CRPS IgG.

Secondly, our results showed that CRPS IgG binding to primary osteoblasts from different patients varies in their binding, cytotoxic and proliferation properties. Surprisingly, all three different osteoblasts incubated with CRPS IgG showed higher necrosis but not apoptosis, which explains that these IgG has damaging pathogenic effects on the bone cells. Additionally, we were also able to find that CRPS IgG incubated for 24hrs with the primary osteoblasts lead to reduced expression levels of osteoblast-specific markers such as Col-1, DKK-1, ALP and BGLAP/osteocalcin. All these markers are essential for bone mineralization and differentiation process. Reduced expression of these markers explains a possibility for poor bone formation and osteoporotic events in these patients.

Finally, we also studied the mechanism of the immunomodulator IvIg on the CRPS IgG-induced effects on HCMEC, HEK293 and A10 cells and observed CRPS IgG when incubated along with IvIg had reduced cytotoxic effects in these cells. Although there was not much improvement were observed in their proliferation rates. Therefore, IvIg if studied in detail about their mechanism of action, can be used as a potent drug for treating CRPS. One possible mechanism may be the occurrence of idiotypic antibodies in the IvIg preparations as described in the treatment of other autoimmune diseases.

The detection of functionally active autoantibodies in serum samples from CRPS patients against autonomic nervous structures, adrenergic and muscarinic receptor suggests an autoimmune participation as an important pathogenic factor in the pathophysiology of CRPS. Thus, our findings contribute to an understanding of the molecular mechanisms that are involved in inflammation and local trophic changes and possibly drive a way for new treatment strategies focusing on the immune system.

7. ZUSAMMENFASSUNG

Das komplex-regionale Schmerzsyndrom (CRPS) ist ein neuropathisches Schmerzsyndrom, welches sich in etwa 0.2-0.5% aller Traumapatienten nach einem Extremitätentrauma, seltener nach Operationen entwickelt. In Deutschland gibt es etwa 4000-8000 neue Erkrankungen pro Jahr. Etwa 30-40% der Patienten entwickeln ein chronisches Stadium, das oft zur Gebrauchsunfähigkeit der betroffenen Gliedmaße führt. Die wichtigsten Symptome sind ein schwerer neuropathischer Schmerz in der distalen Extremität, sowie trophische und autonome Störungen, wie Ödem, veränderte Hauttemperatur, livide Hautverfärbung und langfristig eine Osteoporose. Diese Symptome weisen auf eine Störung der Gefäßregulation, des Bindegewebes und des Knochenstoffwechsels hin. Eine lokale neurogene Entzündung nach einem Trauma ist ein wichtiger Faktor in der Pathogenese der Erkrankung, der eigentliche Auslöser dieses Prozesses ist jedoch unklar. Wir identifizierten kürzlich Autoantikörper als einen weiteren pathogenen Faktor bei Patienten mit CRPS, welche gegen den muskarinischen M2 Acetylcholinrezeptor (M2R) sowie gegen den adrenergen β2 Rezeptor (β2AR) gerichtet sind. Eine Expression dieser beiden Rezeptoren auf verschiedenen Zelltypen, wie Endothelzellen, glatte Muskelzellen, Osteoblasten und Osteosarkomzellen wurde beschrieben und zu Beginn dieser Studie von uns bestätigt.

Seren von CRPS Patienten zeigten eine höhere Bindung an M2R- oder β2AR-transfizierte CHO Zellen im Vergleich zu Kontroll-Seren. Pharmakologische Blockade der Rezeptoren verhindert die Bindung der Antikörper. Unser Hauptinteresse galt nun der Frage, ob Antikörper gegen diese Rezeptoren an andere (nicht-neuronale) Zelltypen binden und ob dadurch funktionelle Effekte ausgelöst werden. Diese Effekte wurden im Detail bei endothelialen Zellen und Osteoblasten untersucht. 86% der CRPS Patienten zeigten Bindung an die endotheliale Zelllinie HCMEC und diese Antikörper induzierten eine höhere Zytotoxizität und eine reduzierte Proliferationsrate im Vergleich zu verschiedenen Kontrollen. Inkubation mit CRPS-IgG führte dabei zu einer erhöhten Expression von proinflammatorischen Mediatoren und Adhäsionsmolekülen, wie ICAM-1, VCAM-1 und MCP-1. Eine vermehrte Expression oder Sekretion von IL-6 konnten wir nicht beobachten. Im weiteren konnte gezeigt werden, dass CRPS-IgG zu einer Phosphorylierung von ERK-1/2, P38 MAPK und STAT-1 führt. Transforming growth factor (TGF)-β1 (wichtiger Regulator der Zellproliferation und differenzierung) und bone morphogenetic protein (BMP)-2

(wichtiger Regulator des TGF-β1 pathway) waren dabei reduziert, was die Proliferationshemmung der HCMEC Zellen durch CRPS-IgG erklären könnte. Passend dazu war die Phosphorylierung von AKT reduziert.

In einem weiteren Schritt konnte nachgewiesen werden, dass CRPS IgG an primäre Osteoblasten bindet und in diesen Zellen zu einer erhöhten Zytotoxizität führt, wobei hier keine apoptotischen Zellen, sondern eine gesteigerte Nekrose nachgewiesen wurde. Weiterhin konnten wir zeigen, dass CRPS-IgG die Expression von Osteoblastenmarkern (Col-1, DKK-1, ALP und BGLAP/osteocalcin) reduziert. Diese Proteine sind essenziell für eine ausreichende Osteoblastenaktivität und eine entsprechende Mineralisierung des Knochens. Eine Antikörper-medierte Expressionsreduktion könnte die lokale Osteoporose bei CRPS Patienten erklären.

Zuletzt untersuchten wir, ob die pathogenen Effekte der CRPS-IgG Fraktionen durch die immunmodulatorisch wirkende intravenöse Immunglobuline (IVIg, gepooltes Spender-IgG) beeinflusst werden können. Dabei zeigte sich eine Reduktion des zytotoxischen Effekts, weniger jedoch des antiproliferativen Effekts. Ein möglicher Mechanismus dieses Effekts könnte durch das Auftreten antiidiotypischer Antikörper (gegen die entsprechenden Autoantikörper) in den IVIg-Fraktionen erklärt werden.

Die Detektion funktionell aktiver Autoantikörper gegen Rezeptoren des autonomen Nervensystems im Serum von CRPS Patienten weist auf eine pathogenetisch wichtige Rolle dieser Autoimmunphänomene bei CRPS hin. Somit tragen diese Ergebnisse zum Verständnis der Pathophysiologie des CRPS bei und weisen auf eine mögliche Wirksamkeit von Immuntherapien beim CRPS hin.

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