Downregulation of *SOCS1* gene in the development of Guillain-Barré syndrome

By

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Declaration

It is hereby declared that

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2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Approval

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Ethics Statement

No animal model was used in this study.

Abstract

Guillain-Barre syndrome (GBS), is a post-infectious disorder of the peripheral nervous system mediated by an aberrant immune response where cytokines play an imperative role in disease pathogenesis. Suppressor of cytokine signalling (SOCS1) proteins, a classical inhibitor of the JAK-STAT signalling pathway, which regulates down-regulates cytokine signalling and suppresses autoimmunity. Therefore, we aimed to determine the association of SOCS1 protein gene expression with GBS immunopathogenesis. This prospective study included 33 GBS patients and 33 healthy controls (HC) in Bangladesh. Detailed clinical investigations and blood samples were collected. Expression of *SOCS1* gene was determined by qPCR and measured by using the $2^{-\Delta CT}$ method. Mann-Whitney U-test was performed to measure statistical difference of the $2^{-\Delta CT}$ values among the case and controls. The Pearson correlation was performed to find the association of *SOCS1* mRNA expression with the response of regulatory T cells (Tregs) in GBS and healthy individuals. The median age of patients was 31 years (IQR: 22–40), with male predominance (65%). 69% of patients had antecedent events, mostly diarrhea (60 %), and 33% were severely affected. We found significant downregulation of *SOCS1* mRNA expression in GBS patients compared to HCs (median: 0.7582 vs. 1.198, P = 0.0463). We also found a statistically insignificant trend of decrease in the expression of *SOCS1* mRNA in severe GBS patients compared to mild GBS (median=0.7582 vs. 2.370). However, there were no significant associations between *SOCS1* gene expressions and other clinical manifestations of GBS. In addition to that, no significant correlation was found between *SOCS1* mRNA expression and $CD4^+CD25^+$Tregs, $CD4^+FOXP3^+$Tregs, and $CD4^+CD25^+FOXP3^+$Tregs cell responses in patients with GBS and HCs. Thus, downregulation of the *SOCS1* gene may play a pivotal role in developing this autoimmunity in patients with GBS. A large sample size is required to validate the association of the *SOCS1* gene with GBS pathogenesis and further clinical progression of the disease.

Keywords: Guillain-Barre syndrome; Suppressor of cytokine signalling (SOCS); Down-regulation; *SOCS1*; mRNA expression.

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-Sarah Khurshid

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Dedication

I dedicate this work to my beloved parents.

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Abbreviation

GBS	Guillain-barré syndrome
AIDP	Acute inflammatory demyelinating polyneuropathy
AMAN	Acute motor axonal neuropathy
AMSAN	Acute motor sensory axonal neuropathy
C. jejuni	*Campylobacter jejuni*
CMV	cytomegalovirus
Tregs	T regulatory cell
APCs	Antigen presenting cells
NKs	Natural killer cell
DC	Dendritic cell
EAN	Experimental autoimmune neuritis
FACS	Fluorescent activated cell sorting
HC	Healthy control
CSF	Cerebrospinal fluid
GBS-DS	GBS disability score
IVIG	Intravenous immunoglobulin
MRC	Medical research council
PBMC	Peripheral blood mononuclear cell
NINDS	National institute of Neurological Disorders and Stroke
PBS	Phosphate buffered saline
LOS	Lipo-oligosaccharide
TCR	T cell receptor
BCR	B cell receptor
TLR	Toll-like receptors
SOCS1	Suppressor of cytokine signalling
DMSO	Dimethyl Sulfoxide
RA	Rheumatoid Arthritis
SLE	Systemic lupus erythematosus
MS	Multiple sclerosis

Chapter 1

Introduction

Guillain-Barré syndrome is an immune-mediated, post-infectious, consequential disorder of the peripheral nervous system caused by the infiltration of inflammatory cells in almost every person around the world (Geleijns, 2005; Grozdanova et al., 2011; Heikema et al., 2015; Hughes et al., 2007; Z. Islam et al., 2012a). It is witnessed that around 50–70% of cases enroll in GBS after encountering respiratory or gastrointestinal infections or additional immunostimulants (Greene et al., 2013; Hughes et al., 2016). The nature of the disease is developing inflammation in the peripheral nervous system via carbohydrate mimicry (Hughes & Rees, 1997; Moran et al., 1996), involving the extent of sensory deficits and cranial nerve involvement (Geleijns, 2005). Patients usually feel symmetrical weakness in a limb, which progresses throughout 12 to 28 days (M. B. Islam et al., 2016). The weakness ultimately affects all the voluntary muscles, eventually resulting in paralysis for the patient (Grozdanova et al., 2011; M. B. Islam et al., 2020). To some extent, they are required to have artificial ventilation due to being unable to breathe (Hughes et al., 2016). In addition, it is believed that *Campylobacter jejuni* is the most frequently causing infectious agent for developing GBS (Islam et al., 2012). The autoantibodies are more common in individuals infected with *C. jejuni* strains that have surface lipooligosaccharides (LOS) or lipopolysaccharides (LPS) that imitate host gangliosides (Z. Islam et al., 2012b; Moran & Prendergast, 2001). Also, there are a few more infectious agents that are also connected with GBS, for instance, cytomegalovirus, Epstein-Barr virus, etc. (Burns, 2008). The precise pathogenesis of GBS is still unknown, despite some research suggesting that immune responses such as complement systems, T cell-mediated cytotoxicity, and macrophage activation cause demyelination and axonal damage in the peripheral nervous system (Atkinson et al., 2006; Geleijns, 2005; Meche & Doorn, 1995). It is said that the cytokines generated during an immune response will determine how the response develops in autoimmunity (Li et al., 2023). Several investigations have been done regarding the action mechanism of cytokines and their inflammatory pathways in patients with GBS (Z. Islam et al., 2020; Tsang & Valdivieso-Garcia, 2003). Interestingly, some cytokines may transform the illness process by interacting with infectious risk determinants of GBS, such as *c. jejuni*, zika virus,

and so on, and cytokines and their receptors are increasingly being investigated as potential treatment targets for GBS (Li et al., 2023; Russell et al., 2020). Considering the involvement of inflammatory pathways, a type of cytokine signalling pathway that activates to suppress inflammatory or pro-inflammatory cytokines has been discovered as the JAK-STAT signalling pathway (Naka and Fujimoto, 2010). The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway was learned in the activation of genes by interferons (IFNs) (Yan et al., 2018). Conversely, the JAK-STAT signalling pathway is controlled by the suppressors of cytokine signalling (SOCS), a family of intracellular proteins that limits the impact of cytokines on immune cells (Naka and Fujimoto, 2010). According to Shuai and Liu, SOCS proteins suppress cytokine signalling in various ways (Shuai and Liu, 2003). While SOCS-1 and SOCS-3 have a limited ability to affect or interfere with TLR-mediated STAT-1 activation and the IFN-signaling pathway, they have the most homology with JAKs and can interact with them directly or indirectly to inhibit their catalytic activity (Wang et al., 2011). Through the JAK-STAT and nuclear factor-B (NF-B) pathways, respectively, both IFN- and LPS can stimulate the expression of suppressor of cytokine signalling 1 (SOCS1) (Kinjyo et al., 2002). Overall, IFN and LPS pathways are negatively regulated by *SOCS1* (Naka and Fujimoto, 2010; Wu et al., 2015).

In this study, we will determine whether *SOCS1* gene downregulation for GBS affects regulatory T cell expression and the precursor or subtypes or outcome.

1.1 History:

About a century earlier, in 1916 three French neurologists Guillain, Barré, and Stroh described an acute paralysis disease with spontaneous recovery (van Doorn, Ruts, and Jacobs, 2008). Their surveillance shows a significant level of expanding protein present in CSF with normal cell count (Geleijns, 2005). Apart from this fact, Jean Baptiste Octave Landry de Thezillat already established similar cases in 1859, which he termed as “ascending paralysis” (Paralysis, 1859). Subsequently, several cases with similar manifestations had been testified, and this scientific entity was named after Guillain and Barré. In 1927, Dragonescu and Claudian first used the term Guillain-Barré syndrome at a presentation. It was presented by Barré where Strohl’s name was mislaid from the title of the presentation and the list of authors in the reference to the 1916 paper. Later, different types of the syndrome with distinguishing clinical points were identified. Based on

clinical features, etiology, and electrophysiological traits, today distinguishes are viable.

1.2 Epidemiology:

1.2.1. Worldwide incidence:

The epidemiological study shows that Guillain Barré syndrome has been most intriguing yet challenging even though the treatments intended for this disease have developed over the years. Study shows, around 1–3/100,000 population from Europe, the USA, and Australia are affected with GBS (Hughes and Cornblath, 2005). Whereas around the globe, about 1.3 cases out of 100,000 population (range, 0.4–4.0) with a median incidence happen to be GBS. It is claimed that males are more susceptible to GBS rather than females. The ratio of males commonly affected by GBS compared to females is 1.5 to 1 (Martyn and Hughes, 1997). According to 2008 data about 40,000 to 120,000 cases have been diagnosed with a slightly increased frequency of GBS (1.5) in men than women (Hughes and Cornblath, 2005). However, age also has been a factor in developing GBS. It seems to play a bimodal role between elders and young adults. The frequency of developing GBS is mostly between 15-24 and 65-74 years aged people. Another study demonstrated that about 0.6/100,000/year are reportedly children less than 16 years of age in Europe and North America (Mcgrogan et al., 2009). In spite of this, about 70 % of cases illustrate an association between emerging respiratory or gastrointestinal infections and the upsurge in age (more likely starting from 50 years of age and above) (Mcgrogan et al., 2009). The incidence of GBS increased with age after 50 years from 1.7/100,000/year to 3.3/100,000/year (Mcgrogan et al., 2009). Above all, the recovery rate for GBS is reported to be higher while the mortality percentage is approximately 8 % (Winer et al., 2005).

1.2.2. GBS in Bangladesh:

In Bangladesh, more than half of the GBS patient is prompted by *Campylobacter jejuni* (Zhang et al., 2010). About 59% of GBS patients are enlisted as male (Islam et al., 2011). It has been claimed, the frequency of having GBS is 3.25 cases per 100,000 children less than 15 years of age. In 2006 and 2007, 608 (37%) and 855 (46%) cases of children less than 15 years of age, have fulfilled GBS symptoms. Whereas, 1,619 and 1,844 cases were enrolled as AFP (acute flaccid paralysis) patients. Moreover, it is believed that about 1.5 to 2.5 cases per 100,000 population per year among all divisions of Bangladesh are children less than 15 years of age, who have encountered GBS.

Perhaps, seasonal variants may affect the percentage of Guillain Barré syndrome. In Bangladesh, most cases enrolled as GBS in May (n = 159) and the lowest in February (n = 84) (Islam et al., 2011). The incidence of GBS seems to be highest between January and March, especially in the AMAN subgroup in Bangladesh. Whereas, the majority of the GBS cases are pure motor variants (92%) with predominant axonal degeneration (67%) (Zhang et al., 2010).

1.3 Clinical features of GBS:

Table 1.1. Clinical features of Guillain-Barré syndrome (Hughes and Cornblath, 2005):

Motor dysfunction:
Symmetrical limb weakness: proximal, distal or global
Neck muscle weakness
Respiratory muscle weakness
Cranial nerve palsies: III-VII, IX-XII
Areflexia
Wasting of limb muscles
Sensory dysfunction:
Pain Numbness
Pain Numbness, paraesthesiae
Loss of joint position sense, Vibration,
Ataxia
Autonomic dysfunction:
Sinus tachycardia and bradycardia
Other cardiac arrhythmias (both tachy and brady)
Hypertension and postural hypotension
Wide fluctuations of pulse and blood pressure
Tonic pupils
Hypersalivation
Anhydrosis or excessive sweating
Urinary sphincter disturbances

Constipation Gastric dysmotility Abnormal vasomotor tone causing venous pooling and facial flushing
Other: Papilloedema

1.4 Diagnosis:

The significance of diagnosing GBS was felt after the swine flu incidence of 1976–1977. In 1978, the explanation and criteria of GBS were published for the first time in the Annals of Neurology, as requested by the team of the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS, now NINDS) (Asbury and Cornblath, 1990). Gastrointestinal infections or respiratory infections are said to be noticeable as initial symptoms of developing GBS. The onset of symptoms involved symmetrical limb weakness, areflexia, mild to severe sensory dysfunctions, and multiple autonomic dysfunctions. An elevated level of protein is more likely to be present in CSF with a normal cell count (Joshi et al., 2016). It is also demonstrated that CSF protein content is closer to being normal during the first days of the disease (Hughes and Cornblath, 2005). Perhaps, at the end of the second week, the protein level of CSF increases by more than 90%. Further, an electrophysiological and pathophysiological study is required for the proper diagnosis.

- **Features required for diagnosis:** - Progressive motor weakness of more than one limb - Low or absent reflexes - No other identifiable cause
- **Features strongly support the diagnosis:**
- **Clinical:** - Progression of symptoms over days to 4 weeks - Symmetry of weakness - Mild sensory signs - Cranial nerve involvement - The onset of recovery 2-4 weeks after progression stops

- Autonomic dysfunction - pain
- **Cerebrospinal fluid (CSF):** - high concentration of CSF protein after the first week - Less than 50 mononuclear leukocytes per µl CSF
- **Electrodiagnosis:** - Conduction slowing or block conduction slowing or block
- **Features casting doubt on the diagnosis:** - Bladder or bowel dysfunction at the onset - Sharp sensory level - Persistent asymmetry of weakness - Persistent bladder or bowel dysfunction

1.5 Disease course and prognosis:

The study shows GBS is influenced by age, gender, season, or vaccination. However, the geographical location plays a major part. The prevalence of GBS subtypes varies in western countries compared to Asian countries. In Asian countries, the pure motor forms, the Miller Fisher variant, and axonal subtypes of GBS are higher, perhaps due to the type of preceding infection and host-dependent factors (McKhann et al., 1993; Asbury, 2000; Islam et al., 2010). Despite the distinctions around the world, the severities of the disease are almost the same, which has been evaluated by the GBS disability score (Hughes et al., 1978) and the Medical Research Council (MRC) sum-score (van Koningsveld et al., 2007).

The major progress starts after 1-3 weeks of the disease. The maximum level of progression is observed within four weeks. As clinical results illustrate variability, precise prospects are required for the diagnosis of GBS.

1.6 Preceding events:

1.6.1. Antecedent infections:

GBS is a syndrome that involves a variety of motor and sensory deficits. Additionally, preceding

events show that it is considered a post-infectious disease because of the commentary on the enrolment of two-thirds of patients showing GBS illness (Jacobs, 1997). These infections may affect the immune reaction against peripheral nerve antigens in the human body, as suggested by the intermission of 1 to 4 weeks between the precursor of the infection and the onset of weakness (Jacobs, 1997). However, a sustainable study shows that the root of GBS is involved with a range of infectious agents. In patients with GBS, *Campylobacter jejuni* is the most common infectious pathogen. However, Cytomegalovirus, Epstein-Barr virus, Mycoplasma pneumonia, and Haemophilus influenza are also responsible for causing GBS (van Doorn, Ruts, and Jacobs, 2008). In addition, it is reported that cytomegalovirus is the second most common infectious pathogen causing GBS (Seneviratne, no date). Also, it has also been described in association with Human Immunodeficiency Virus (HIV) infection (Burns, 2008). Hepatitis A, B, C, and D, Typhoid, and Falciparum malaria are restrained to anecdotal case reports (Seneviratne, no date). Furthermore, a study in Japan shows that most of the antecedent events in GBS and related disorders are fever (52%), cough (48%), sore throat (39%), nasal discharge (30%), and diarrhoea (27%) (van Doorn, Ruts, and Jacobs, 2008)

1.6.2. Non-infectious events:

Many reports show that surgery, vaccination, and parturition have also been related to GBS (Burns, 2008). However, it is also said that vaccines for simple rabies, oral polio, influenza, measles, mumps, and rubella (MMR), tetanus toxoid, and hepatitis B may have a possible association with emerging GBS, even though the association is being compared after two events. However, consideration mainly ascended after the surveillance of a slight upsurge in the occurrence of GBS after swine influenza vaccines in the USA in 1976 (van Doorn, Ruts, and Jacobs, 2008). In 1992–1994, vaccine campaigns placed in the USA recognized that vaccines were related to a very minor, but major, increased risk of emerging GBS of about one GBS case per million vaccines above the background incidence. An alternative study of patients who had GBS did not show significant development of GBS again after a vaccination. However, special caution might be obligatory when recapping a tetanus vaccination: we have encountered a relapse of GBS two times after tetanus vaccinations (van Doorn, Ruts, and Jacobs, 2008). However, this does not demonstrate that tetanus is a GBS-associated agent. However, precautions should be maintained prior to any exposure.

1.7 Pathophysiology:

1.7.1. Subtypes:

a) Acute Inflammatory Demyelinating Polyneuropathy (AIDP):

The most common type of GBS is AIDP. However, it displays establishment in the western countries rather than Asia. Immunologically, it is triggered by an auto-immune response engaged against Schwann cell membranes. It not only reduces conduction velocity but also conduction block or abnormal progressive spreading. However, pathological changes take place by the humoral and cellular response (Seneviratne, 2000). Studies prove that T cell infiltration in endoneurium and infiltration in myelin sheath by macrophages causes de-myelination (Hughes, Cornblath and Willison, 2016). Additionally, Axonal damage may also arise as a secondary event, in severe cases.

b) Acute Motor Axonal Neuropathy (AMAN) and acute Motor Sensory Axonal Neuropathy (AMSAN):

The major difference between AMAN and AMSAN is the Absent or reduced compound muscle in AMAN but Absent or reduced SNAP and CMAP amplitude in AMSAN (Seneviratne,2000). However, in Bangladesh, about 67% of GBS patients had an axonal variant, where 56% were AMAN and 11% were AMSAN. In addition, AMAN seemed to be found more frequently from January to March (Zhang et al., 2010). However, AMAN is differentiated by speedily progressive weakness, often with respirational failure but usually has a good recovery, whereas AMSAN typically causes slow recovery (Seneviratne,2000). Moreover, in both cases, peri-axonal macrophages are found in peri-axonal space (Burns, 2008). However, acute motor axonal neuropathy often follows infection with *C. jejuni*, which comprises a molecular mimicry between lipo-oligosaccharides LOSs and GM1gangliosides (monosialotetrahexosylganglioside, a prototype of gangliosides) of nerve.

c) Miller-Fisher syndrome (MFS):

Even though MFS shares similar pathophysiological events with AMAN & AMSAN, it gets the initiation by anti-GQ1b and anti-GT1a antibodies that aim towards oculomotor and bulbar nerves in where GQ1b and GT1a ganglioside concentrations are supposed to have moderately high (Burns, 2008). However, it is a rare disorder. In this syndrome, the resolution occurs after 2-3 months.

Table 1.3. The range of GBS subtypes and serum anti-ganglioside antibodies (van Doorn, Ruts, and Jacobs, 2008)

Subtypes	Antibodies
Acute Inflammatory Demyelinating Polyradiculoneuropathy (AIDP)	Unknown
Acute Motor (and sensory) Axonal Neuropathy (AMAN or AMSAN)	GM1, GM1b, GD1a, GalNAc-GD1a
MFS and GBS overlapping syndrome	GD3, GT1a, GQ1b

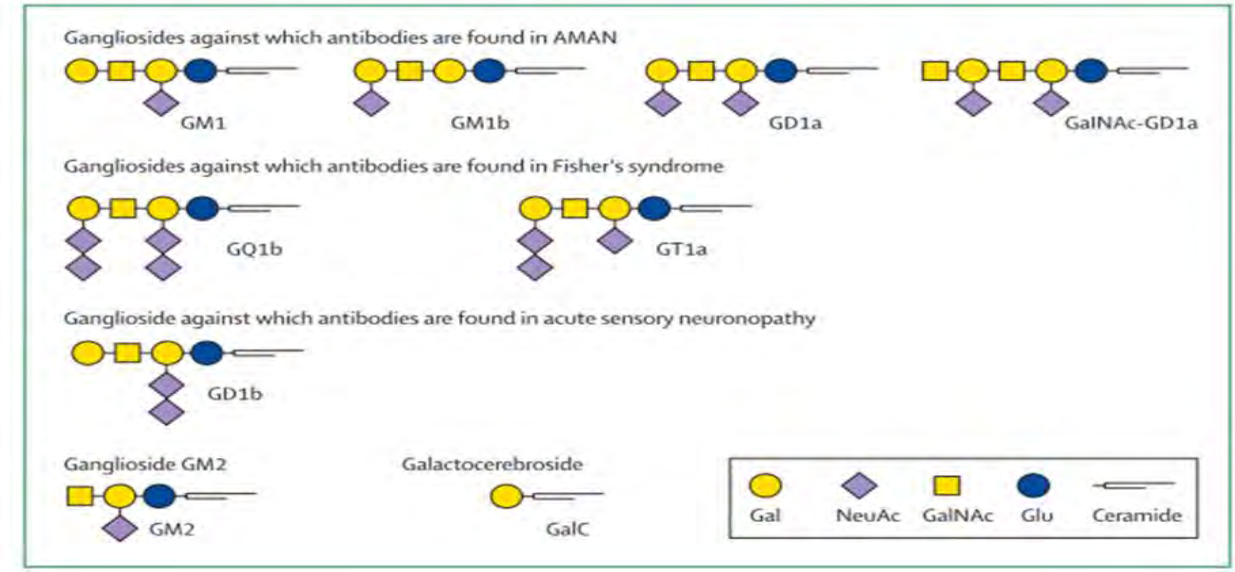

Figure 1.1. Structures of gangliosides and galactocerebroside associated with different subtypes of GBS (R. A. C. Hughes and Cornblath, 2005)

1.8 Treatment of GBS:

1.8.1. Steroid: Six qualified preliminaries have tended to the estimation of steroids in treating intense GBS. These included 195 patients. Mean inability at about a month, the extent of patients who were enhanced by one review at about a month, and the enhancement in review at a year all stayed unaltered by steroids, which have all the earmarks of being protected yet insufficient. This

appears differently in relation to the treatment of patients with more incessant demyelinating neuropathies, who react well to steroids. This absence of reaction to steroids isn't effectively clarified; it might be that any advantage that steroids have in decreasing irritation is exceeded by some other impending impact on the fix forms. A Cochrane investigation uncovered that a solitary pilot considers tending to consolidated treatment with methylprednisolone and intravenous immunoglobulin was not randomized. Notwithstanding, it recommended a conceivable favorable position. A randomized report that has as of late been displayed but not yet distributed just neglects to locate a critical favorable position of the blend. Post-hoc control of the information for known hazard factors suggests leverage blend treatment; nonetheless, such examinations are known to be or maybe untrustworthy and can be misdirecting (Pithadia and Kakadia, 2010). GBS is an immune system ailment influencing the peripheral nervous framework that is generally activated by an acute infectious process. It is also known as an inflammatory disorder (Pithadia & Kakadia, 2010). The steroid was found to not be an appropriate drug for treating GBS (Pithadia and Kakadia, 2010).

1.8.2. Plasma exchange: Plasma exchange is useful for extremely influenced patients in the main week or two of the ailments (Rac, 2009). Plasmapheresis is planned to evacuate the antibodies assaulting the nerves from your blood (Pithadia and Kakadia, 2010). Plasma exchange includes being associated with a machine that can isolate the platelets from the liquid or plasma. About 250ml of blood is evacuated at once, the plasma is disposed of and the platelets have come back to the patient with clean plasma. The method is rehashed a few times for around five days until the point when adequate plasma has been traded (Rac, 2009). The dangers of the method are to a great degree little and present-day disinfection has for functional purposes killed the danger of transmitting disagreeable diseases in the perfect plasma.

1.8.3. Intravenous immunoglobulin (IVIg): Another treatment for GBS is an intravenous organization of immunoglobulin (IVIg). The antibodies utilized have appeared to balance the humoral reaction in their capacity to repress autoantibodies and smother autoantibody creation. By hindering autoantibodies, the supplement interceded harm can be constructed (Pithadia and Kakadia, 2010). It blocks the required of Fc (gamma) receptors, avoiding phagocytic damage by macrophages. In addition, the management of IVIG is more modest than plasma exchange and may be the favored treatment in hospitals that have neither the plasma exchange equipment nor the expertise.

1.9 Immune mechanisms of GBS:

1.9.1. Molecular mimicry & cross-reactivity:

Patients who enrolled as GBS are found to face molecular mimicry and cross-reactivity in their bodies. More than half of the patients with GBS are attacked by *C. jejuni* (Zhang et al., 2010), which shows that lipo-oligosaccharides (LOS) of *C. jejuni* mimics the carbohydrates of gangliosides (Pithadia and Kakadia, 2010). The sort of ganglioside mimicry in *C. jejuni* appears to decide the specificity of the antiganglioside antibodies and the related variation of GBS. *C. jejuni* diseased patients with pure motor or axonal GBS much of the time express a GM1-like and GD1a-like LOS, though those disengaged from patients with ophthalmoplegia or MFS normally express a GD3-like, GT1a-like, or GD1c-like LOS. Antibodies in these patients are typically cross-reactive and perceive LOS and also gangliosides or ganglioside buildings (van Doorn, Ruts, and Jacobs, 2008). However, it also shows similar activities against mice models, which proves that it truly has molecular mimicry activity against gangliosides (van Doorn, Ruts, and Jacobs, 2008).

1.9.1.1. Anti-ganglioside antibody:

There are various types of gangliosides, which have specific distribution in tissues of the peripheral nervous system and play a major part in maintaining cell membrane structure. On the other hand, these various types of gangliosides including LM1, GM1, GM1b, GM2, GD1a, GalNAc-GD1a, GD1b, GD2, GD3, GT1a, and GQ1b are found in half of the GBS patients (van Doorn, Ruts, and Jacobs, 2008). However, antibodies to GM1, GM1b, GD1a, and GalNAc-GD1a are found in pure motor axonal variants of GBS (Pithadia and Kakadia, 2010). However, Hostile to ganglioside antibodies that respond to self-gangliosides are found in immune system neuropathies (Pithadia and Kakadia, 2010). These antibodies were first found to respond with cerebellar cells. These antibodies demonstrate the most grounded relationship with specific types of GBS. Auto-antigenic gangliosides that are right now known are GD3, GM1, GQ3, and GT1 (Pithadia and Kakadia, 2010).

1.9.2. Complement activation:

It has been found that supplement actuation happens at the site of nerve harm after death, such as the axolemma in patients with AMAN and the Schwann cell film in patients with AIDP(Pithadia & Kakadia, 2010). However, it has been observed in a mouse model of GBS which demonstrated that some antiganglioside antibodies are extremely dangerous for peripheral nerves(Sekiguchi et

al., 2012). An α-latrotoxin inserted in a mouse model can result in the arrival of acetylcholine and also transporting exhaustion of this synapse at the nerve terminals, and blockade of nerve transmission and loss of motion of the nerve-muscle promptness, which destroys the nerve terminal and peri-synaptic Schwann cell(Susuki et al., 2007). Antibodies to GM1 affect the sodium channels at the hubs of Ranvier of rabbit peripheral nerves(Islam et al., 2012). All these effects appear to be reliant on supplement actuation and, what's more, the arrangement of the film assault complex. The neurotoxic effects of these antibodies were repressed by immunoglobulin and the supplement inhibitor eculizumab.

1.9.3. Cell-mediated inflammation:

1.9.3.1. Macrophages Activation:

Macrophages perform a variety of activities, including antigen presentation, elimination of microorganisms and tumor cells, and tissue remodeling (Tsang & Valdivieso-Garcia, 2003). Macrophages, which have a diverse set of pathogen recognition receptors, play a role in both innate and adaptive immune responses through phagocytosis and identification of pathogen-associated molecular patterns(Winer, 2001). The macrophages responsible for antigen presentation express and upregulate the major histocompatibility complex (MHC) I and II antigens during the immunological response(Chu et al., 2018). Furthermore, macrophages are involved in the activation of T helper (Th) cells as well as the generation of pro-inflammatory cytokines and chemokines(Ebrahim Soltani et al., 2019). They also express adhesion molecules, which are crucial in lymphocyte recruitment into inflammatory lesions(Park et al., 2014). Furthermore, macrophages protect the brain by secreting neurotrophic substances and suppressing the inflammatory response. They exhibit a wide range of roles and phenotypes based on the various cytokines and pathogens they encounter in the body's microenvironment.

GBS is characterized by macrophage-mediated nerve damage. Two putative immunological pathways for macrophage-mediated nerve invasion have been hypothesized in AIDP. According to the first hypothesis, macrophages enter the peripheral nerve basement membrane and target antigens on the surface of the SC or myelin sheath to induce SC damage via activated CD4+ T cells and inflammatory mediators. Furthermore, macrophages produce and release inflammatory mediators like as MMPs or toxic nitric and oxide radicals, which promote SC damage and myelin sheath invasion. An alternate idea argues that antibodies may stimulate macrophages to myelin or

axonal antigen binding sites and improve macrophage phagocytosis via Fc/complement receptors in antibody-dependent macrophage cytotoxicity or by activating complement-dependent macrophage phagocytosis. AIDP GBS has been discovered to have complement deposition on the outer surface of SC as well as increased complement levels in serum and CSF. It induces early vesicular alterations in myelin, macrophage build-up, and demyelination. AMAN's pathogenic traits differ from those of AIDP. AMAN is distinguished by axon malfunction with minimal demyelination caused by anti-GM1 antibodies and complement-mediated attack on the axolemma of the Ranvier nodes.

1.9.3.2. T-Lymphocytes activations:

A. Th1 and Th2 paradigm: The imbalance between Th1 and Th2 cells is directly linked to Guillain-Barré syndrome. During the acute phase, the Th1 response predominates, whereas, during the recovery phase, the Th2 response predominates. Th0 cells may develop into Th1 or Th2 cells depending on whether their environment contains exogenous or endogenous cytokines. During the acute phase, Interferon-gamma (IFN y) generated mostly by natural killer (NK) cells (Martín-Fontecha et al., 2004) and IL-12 produced by dendritic cells (DCs) following Toll-like receptor (TLR) activation govern Th1 differentiation. Low amounts of IFN-g and high levels of IL-4 are required for the differentiation of Th2 cells (Mowen & Glimcher, 2004; Trinchieri, 2003). IFN-y and IL-4 may function as autocrine growth factors for themselves and autocrine inhibitory factors for the opposing cell group. Th1 cells stimulate immunoglobulin (Ig) G2a synthesis in B cells through IFN-g, while Th2 cells induce B cell to produce IgE and IgG1 by IL-4 (Liew, 2002, p. 2). Included among Th1 cytokines are IL-12, IFN-y, TNF-a, and IL-1b. These cytokines may activate macrophages to create reactive oxygen intermediates and NO, promote their phagocytic activities, and increase their antigen-presenting ability by upregulating the expression of MHC II (H. Zhang et al., 2011). Th2 cytokines, such as IL-4, IL-5, IL-10, and IL-13, promote B cell activation, Ig class switching to IgE and IgG1, and suppress proinflammatory macrophage activity (H. Zhang et al., 2011).

B. Th17: RORα and RORyt are transcription factors that promote the differentiation of Th0 cells into Th17 cells. Th17 cell cytokines may cause local inflammation in the target organ while simultaneously helping B cells in antibody production. Th17 produces IL-17, a cytokine that assists in the body's defence against germs and fungi. Activated Th17 cells additionally produce IL-6, IL-17F, IL-21, IL-22, TNF-, and granulocyte-macrophage colony-stimulating factor (GM-

CSF), all of which enhance inflammatory cell recruitment and induce massive tissue abnormalities (Elkarim et al., 1998; Hartung et al., 1990). During the acute phase of GBS, a recent research revealed a significantly greater number of Th17 cells in the peripheral blood and IL-17A in the plasma (1–14 days following the onset of sickness). Furthermore, a link has been found between IL-17A levels and GBS severity scale scores.

C. Regulatory T cells (Tregs): Tregs are CD4+ T cells that exhibit high amounts of CD25 as well as the transcription factor forkhead box P3 (Foxp3). Tregs play an important role in immune homeostasis and the prevention of autoimmunity by inhibiting self-reactive T cells (Sakaguchi et al., 2006). Tregs also reduce Th1 and Th2 cytokine release and antigen-specific T cell proliferation. Regulatory T cell (Treg) cytokines include TGF- and IL-10 (an immunosuppressive cytokine), while FOXP3 transcription factor expression is needed for Treg suppressive activity on other cells (Salomon et al., 2018). When compared to patients with other neuropathies, GBS patients had a significant decline in the percentage of CD4+CD25+cells (T regulatory cells).

D. Cytotoxic T cell ($CD8^+$ T cell): Though the significance and biological features of the $CD8^+$ subpopulations are less evident; one intriguing hypothesis of a study is that activated $CD8^+$ cells are cytotoxic/effector cells may play a role in GBS pathogenesis. More research into the presence and function of activated $CD8^+$ cells in GBS is needed. Some studies discovered a decline in $CD8^+$, whereas others discovered an increase in CD8 in 3/14 and a decrease in 2/14 patient samples, or no change overall. Those disparities could be related to the phase of the infection at the time samples are taken, or to triggering factors such as infection or vaccination that occur prior to the onset of GBS (Wanschitz, 2003).

1.9.3.3. B-Lymphocytes activations:

In GBS patients there is an increase of memory B cells in peripheral blood mononuclear cells which is also positively correlated with the clinical severity of this disease. When exposed to infectious pathogens for the first time, B cells often develop into IgM-secreting plasma cells. The host immune system will then maintain the memory of earlier infections by creating memory B cells in the germinal center (Pérez et al., 2014). Following the second exposure, memory B cells proliferate rapidly and switch isotype class to produce complement-fixing IgG1 and IG3-predominant antibodies (Y. J. Liu et al., 1996). Liu et al. In GBS, these antiganglioside antibodies are pathogenic (58, 59). Gangliosides are found on the outside leaflets of plasma membranes in

many tissues, but they are especially plentiful in neuronal cells. Gangliosides form a protective screen against autologous immunity and pathogen adhesion due to their sialic acids and negative charge. Antiganglioside antibodies break this protective coating, allowing complements to easily adhere to neuronal cells and induce significant nerve damage (Wu et al., 2021). Some recent experiments with mice deficient with CD4, CD8, and CD28 by Zhu and colleagues also provided evidence that T-cells possess a vital role in the development of EAN (Sekiguchi et al., 2012). Previous studies in patients with GBS have found IgG-type antibodies predominantly, indicating an association of T helper cell (Sekiguchi et al., 2012).

1.9.3.4. Cytokine production:

Cytokines have a significant role in the initiation, propagation, and modulation of damages specifically associated with tissues, as well as acting as signal molecules between immune system cells. In GBS, IFN- γ has a twofold effect: it can cause inflammation in EAN and GBS, and evidence suggests that it can transform into CD4+CD25+ suppressor T cells from outer CD4+CD25 T cells (Chi et al., 2007). Similarly, TNF-α, likewise, has a double role in GBS, functioning as both causing inflammation and as a protective agent. Since IL-17 and IL-22 are released by Th17 cells, and remains crucial in inflammatory disorders, previous studies have found high level of IL-17A and IL-22 in plasma throughout the severe stage of GBS but the concentration of IL-17A was reduced subsequently next to IVIg therapy (Huang et al., 2009). Furthermore, several other cytokines are involved in some other autoimmune disorders, which could be explored as GBS biomarkers (Huang et al., 2009). To discover more about their potential involvement in GBS, more research is required.

1.10 Signalling pathway involved in GBS:

The primary triggers and pathways involved in the development of autoimmune illness are based on a separate mechanistic basis, however, more than one pathway is likely to contribute to a given disease; like rheumatoid arthritis, or GBS. In laboratory models, these pathways have been well established, although evidence of participation in human illnesses is frequently speculative. Nonetheless, the available data underscore the tremendous progress made thus far in defining the pathophysiology of these highly complex and varied illnesses, and it is obvious that additional diagnostic and therapeutic approaches will be emerging.

Undoubtedly, novel diagnostic and treatment techniques will emerge. The use of new technology

may even allow for the particular removal of auto-reactive cells without suppressing the entire immune system, as is now the case. Studies showing depletion of auto antigen-specific B cells using cytotoxic T cells expressing chimeric antigen receptors143 and population expansion of auto antigen-specific Treg cells (for passive transfer) using nanoparticles displaying disease-relevant self-peptide-MHC complexes144 provide evidence of the feasibility of antigen-specific therapy in autoimmunity. Furthermore, because genetic predisposition is a precondition for most autoimmune disorders, breakthroughs in understanding the significance of genetic variants in these syndromes are likely to alter approaches to diagnosis and therapy. Among them. The JAK/STAT signalling pathway may show a promising function in patients with GBS.

1.10.1. JAK/STAT signaling pathway & inhibitors:

The Janus kinase/signal transduction and transcription activation (JAK/STAT) pathways were formerly assumed to be intracellular signaling pathways in mammals that mediate cytokine signals. Existing research indicates that the JAK/STAT system modulates the downstream signaling of many membrane proteins, including G-protein-associated receptors, integrins, and others. The JAK/STAT pathways have a crucial role in human disease pathophysiology and pharmacological mechanisms, according to mounting data. The JAK/STAT pathways are linked to all areas of immune system function, including fighting infection, maintaining immunological tolerance, improving barrier function, and cancer prevention, all of which are critical components in the immune response. Furthermore, the JAK/STAT signaling pathways are involved in extracellular mechanistic signaling and may be a significant modulator of mechanistic signals that influence disease progression and the immunological environment. As a result, understanding the mechanism of the JAK/STAT pathways is critical for developing new medications that target disorders based on the JAK/STAT route. The importance of the JAK/STAT pathway in molecular signaling, disease progression, immunological environment, and therapeutic targets is discussed in this study. Some JAK/STAT pathway signaling negative regulators have been found, including suppressor of cytokine signalling (SOCS), protein inhibitors of activated STAT (PIAS), and protein tyrosine phosphatase (PTP).

1.10.1.1. Suppressor of cytokine signal (SOCS):

SOCS proteins have been linked to the regulation of over 30 cytokines during the last decade, including interleukin (IL)-6, leukemia inhibitory factor (LIF), leptin, granulocyte colony-

stimulating factor (G-CSF), IL-10, growth hormone, interferon (IFN), and IFN. Cell lines and overexpression systems have been utilized extensively to uncover both interaction partners and cytokines suppressed by SOCS proteins. While this overexpression technique has clearly established their potential actions, notably for the therapeutic administration of supraphysiological doses of SOCS proteins, it has been somewhat useless in discovering physiologically relevant cytokine signalling networks.

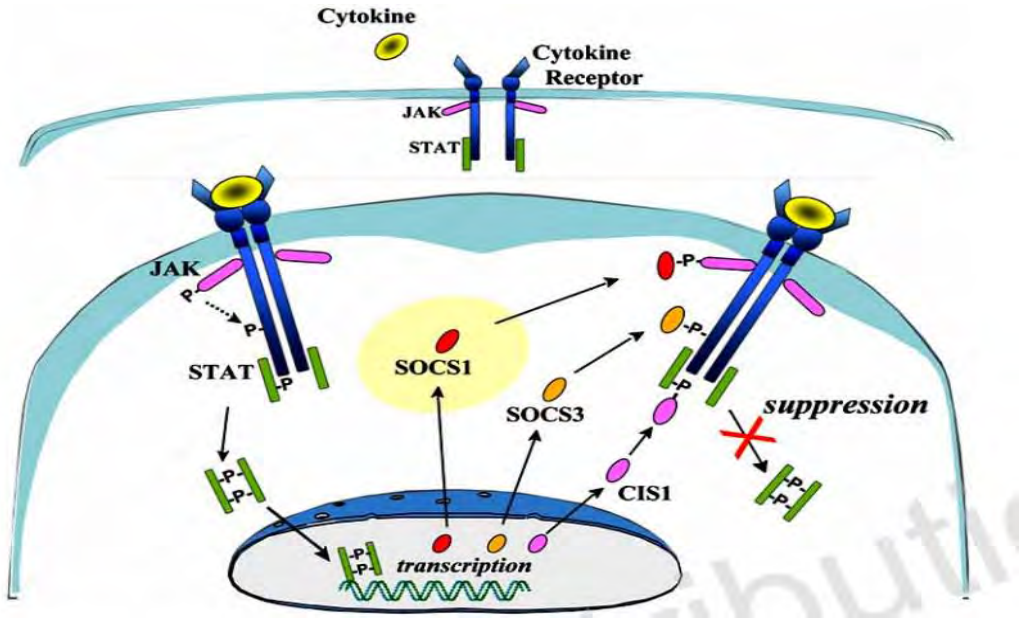

Fig: 1.2. Inhibition of the JAK/STAT signalling pathway by SOCS proteins.(Kato et al., 2008)

1.10.1.1. a. Mechanisms of action of SOCS:

It is also known as STAT-induced STAT inhibitory proteins (SSI), and it is activated by the JAK/STAT signalling pathway (H. Liu et al., 2015). The SOCS family consists of eight members: cytokine-inducible SH2-containing protein (CIS), SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, and SOCS7 (Whyte et al., 2011). According to genetic and biochemical research, they play critical roles in immune control. For example, whereas ectopic SOCS3 expression can decrease IFN signalling, SOCS3-deficient animal models clearly demonstrate that, while SOCS3 is required for G-CSF, IL-6, LIF, and leptin signalling, it is not required for IFN regulation. *SOCS1*

knockout mice exhibit a complicated multi-organ inflammatory infiltration and show crucial roles for *SOCS1* in the regulation of Toll-Like Receptor (TLR) signalling, type I and II interferon signalling, and c-cytokine-dependent T cell homeostasis. Although anecdotal evidence suggests that CIS-deficient animals have no cytokine-related abnormalities, transgenic models imply a role in selective suppression of the JAK-Stat5 signalling pathways. CIS-transgenic animals have growth and lactation abnormalities similar to Stat5-deficient mice due to decreased growth hormone and prolactin signalling. CIS-transgenic mice also have increased TCR signalling and reduced IL-2 responses. SOCS2-deficient animals grow to enormous dimensions and exhibit lower neuronal density and abnormal neuronal differentiation as a result of growth hormone signalling disruptions. There have been no reports of SOCS4-deficient mice. SOCS4-deficient mice have not been documented, and SOCS5 and SOCS6-deficient animals have no obvious phenotype. SOCS7 gene deletion reveals a key function in insulin signalling control. Functional redundancy may explain not only the obvious lack of effect in CIS, SOCS5, and SOCS6-deficient mice but also the apparent lack of involvement for SOCS proteins in the regulation of JAK/STAT dependent cytokines including erythropoietin (EPO) and thrombopoietin (TPO). Other SOCS family members may be able to compensate for the loss of individual SOCS proteins, a hypothesis that has to be properly validated by creating animals with compound SOCS deficits. The remainder of this examination will go into the mechanics of SOCS action in depth and address some of the issues that come with it.

Each SOCS protein has three unique domains: a varied N-terminal binding domain with a low consensus, a conserved core SH2 domain that is responsible for a specific target protein, and a highly conserved C-terminal SOCS box domain that interacts with proteasome components (Morris et al., 2018; Seif et al., 2017). Furthermore, the kinase inhibitory domains of SOCS1 and SOCS3 allow them to inhibit JAK1, JAK2, and TYK2 kinase activity but not JAK3 (Xin et al., 2020). The length of the N-terminal domain varies among SOCS family members. SOCS1-3 and CISH have shorter N-terminal domains than SOCS4-7(Yoshimura et al., 2021). The SH2 domains, which allow them to remain on the cytokine receptor, inhibit JAK kinase activity by hastening proteasome-mediated degradation of the whole signal transduction complex (Morris et al., 2018). Furthermore, SH2 domains, which are important in the recognition and binding of homologous phosphor tyrosine patterns, define the targets of each SOCS/CIS protein in order for it to perform its regulatory function (Russell et al., 2020). The length and structure of the N-terminals, on the

other hand, vary and include extended SH2 subdomains (ESSs), which promote contact with their substrates (Russell et al., 2020). The SOCS box is a ubiquitin-related domain that is linked to elongin C and B complexes, cullin-5, the RING-box, and ligase E2 (Yoshimura et al., 2021). SOCS proteins can be employed as ubiquitin E3 ligands, degrading proteins by binding to their N-termini (Yoshimura et al., 2021). In biochemical investigations, the kinase inhibitory region (KIR) of SOCS3 blocks JAK2's sub layer-binding groove and prevents it from interacting with its substrate (Naka & Fujimoto, 2010). SOCS creates a negative feedback loop in the JAK/STAT signalling pathway: active STATs enhance SOCS gene transcription; on the other hand, SOCS proteins bind to phosphorylated JAK and JAK receptor, shutting down the pathways (Xin et al., 2020). SOCS also has a negative regulatory role in three ways: binding with a phosphate agent at the receptor (SOCS physically prevents the signal transducer from being recruited to the receptor); directly interacting with JAKs; or specifically suppressing the activity of JAK kinase receptor (Morris et al., 2018). In summary, SOCS proteins preferentially regulate the termination of the JAK/STAT signalling transduction process (Xin et al., 2020). Additionally, deficiency or reduction of SOCS expression may result in changes in cytokine response (Y. Liu et al., 2014; Xin et al., 2020). SOCS proteins do not exhibit high levels of expression at unstimulating states, nevertheless, cytokine stimulation and JAK/STAT activation induce their rapid transcriptions (Yoshimura et al., 2021).

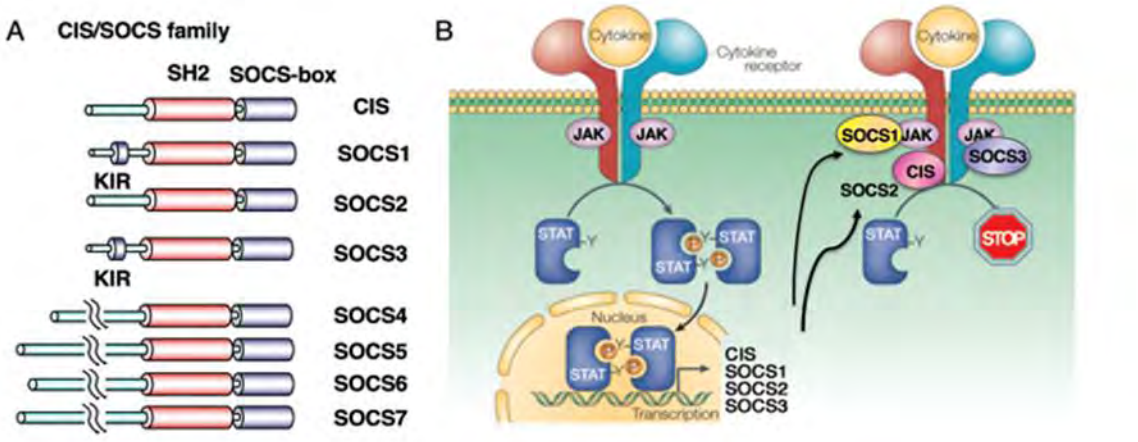

Figure 1.3. The CIS/SOCS family and their mode of action. (A) Basic structures of the CIS/SOCS family proteins. (B) CIS, SOCS1, SOCS2 and SOCS3 are induced by STATs. CIS inhibits STAT5 activation by binding to the receptor, SOCS1 directly binds to JAKs and SOCS3 binds to both the gp130-related receptors and JAKs. SOCS2 also binds to the receptor-like CIS but is relatively specific to the GH receptor(Pfitzner E et al., 2004).

A range of autoimmune disorders are caused by dysregulation of immunological tolerance to self.

Tolerance is maintained in the thymus via "negative selection," or the deletion of self-reactive T cells. The regulatory cells, which include regulatory T lymphocytes (Tregs), maintain peripheral tolerance(regulation of cell plasticity)(Chi et al., 2007). *SOCS1*, a negative regulator of cytokine signalling, has been shown to play a significant role in Treg cell integrity and function by shielding the cells from excessive inflammatory cytokines(Hagen et al., 2018). SOCS1 (Suppressor of cytokine signalling 1) suppresses the JAK-STAT pathway, and a lack of SOCS1 results in uncontrolled IFN-g activity(Morris et al., 2018). SOCS1 is a direct target of the ubiquitin-conjugating enzyme Ubc13, which is involved in TCR-stimulated activation of the IKK-NF-kB pathway, and this targeting of SOCS1 stops them from converting into effector-like T cells (Article et al., 2017).

1.11 Objectives:

1.11.1.General objective:

- In this study, we aimed to determine the expression of the *SOCS1* gene in peripheral mononuclear cells and their association with the pathogenesis of GBS.

1.11.2.Specific objectives:

- Isolate PBMCs from the patients with GBS and healthy controls.
- Determine the expression of *SOCS1* mRNA in isolated PBMCs by using real-time quantitive-PCR in patients with GBS and healthy individuals.
- Investigating the clinical associations of *SOCS1* mRNA expression in patients with GBS.
- Find the correlation between *SOCS1* mRNA expression and responses of $CD4^{+}CD25+$, $CD4^{+}FOXP3^{+}$, $CD4^{+}CD25+FOXP3^{+}$ regulatory T cells in GBS.

Chapter 2

Methods and Materials:

2.1. Study Subject:

Blood samples were collected from 33 patients with GBS from Dhaka Medical College and Hospital (DMCH), and National Institute of Neurosciences & Hospital (NINS), Dhaka and 33 healthy controls from Bangladesh. Based on the National Institute of Neurological Disorders and Stroke (NINDS) criteria stated by Asbury and Cornblath with clinical and electrophysiological data, patients were enrolled. All participants were given the written informed consent before data collection, clinical examination, and specimen collection. The healthy individuals in the control group were ethnically matched and had no history of neurological disease, recent infection, or chronic medical illnesses. The study was reviewed and approved by the Institutional Review Board and the ethical committee of the icddr, b, Dhaka, Bangladesh. This study was carried out in the Laboratory of Gut-Brain Axis, Infectious Diseases Division (IDD), icddr, b, Bangladesh.

2.2. Clinical evaluation of patients with GBS:

A comprehensive data was collected from patients including; medical and demographic data, including sex, age, antecedent events, electrophysiological characteristics, symptoms, and prior infections (diarrhea, any respiratory tract infection, any sort of high temperature, or any other diseases such as rash, dysuria in the past). All the patients of GBS reported antecedent illness within 1-3 weeks prior to the onset of weakness. The disease severity and outcome were evaluated to observe the functional status of individuals by GBS disability score (GBS-DS). The GBS-DS is defined as the capacity to move independently or mildly affected when the GBS-DS score is 0, 1, or 2, whereas a terrible prognosis is defined as the inability to stand and walk independently or death (GBS-DS of 3, 4, 5 or 6). The severely affected patients with GBS are assessed with a GBS-DS score >3. The Medical Research Council (MRC) sum score at baseline and weekly follow-ups, is used to assess the degree of disease severity by observing the strength of muscles (the maximum level of weakness). The Medical Research Council (MRC) sum scale, which ranges from 0 to 60,

was used to measure the disease's severity at the nadir, or the maximum degree of weakness experienced by GBS sufferers. Patients with GBS at nadir who had an MRC sum score of less than 40 were classified as badly affected, while those who had an MRC sum score of 40 or more were classified as mildly affected. When patients comply with the criteria for respiratory failure, mechanical ventilation must be performed; a number of clinical indicators, such as rapidly worsening motor impairments and an ineffective cough, may aid in indicating the impending respiratory failure.

2.3. Study workflow:

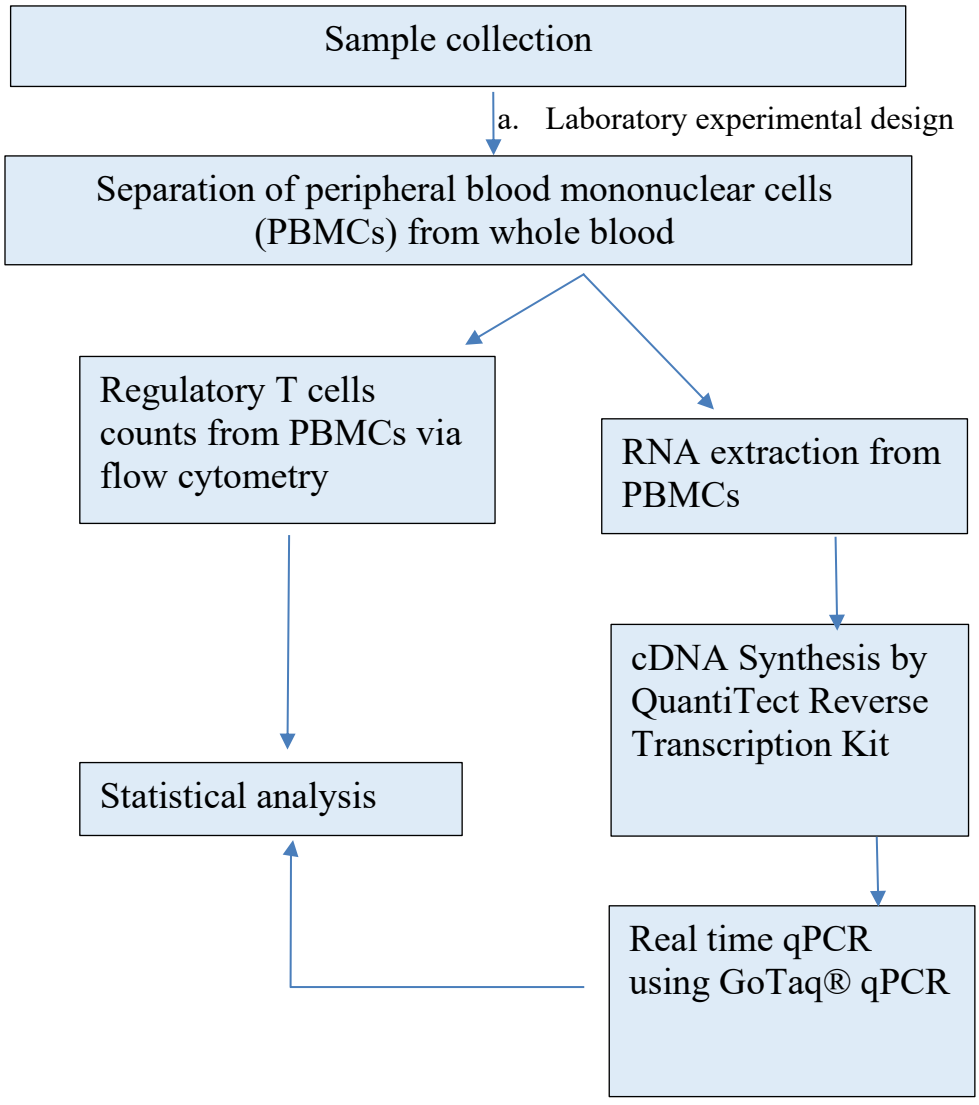

Figure 2.1. The flowchart highlights the workflow of the study design.

2.3.1. Sample collection:

Around 8-13 ml of blood was obtained on average from various EDTA commercial tubes from both GBS and healthy individuals for separation of peripheral blood mononuclear cells. The blood was warmed until it reached room temperature.

a. <u>Laboratory experimental designs:</u>

In this case-control study, the collection of blood from every individual has been taken to isolate PBMCs. After that, the counting of regulatory T cells (Tregs) from PBMCs by Flow cytometry assay, and RNA extraction from PBMCs is performed. Finally, the First-Strand cDNA Synthesis is executed from extracted RNA and real-time quantitative PCR is done.

2.3.2. Separation of peripheral blood mononuclear cells (PBMCs) from blood:

- Using a serological pipette, collected blood was first gently mixed inside the tube and its volume was measured.
- The blood was then collected in a falcon tube and underlaid with a 2:1 ratio of blood to ficoll on a ficoll plaque.
- The blood was then transferred to a density gradient centrifuge machine for a break-free centrifugation.
- After the falcon was withdrawn from the centrifuge, it was placed in a biosafety cabinet, and distinct blood component bands developed, as depicted in the image.
- The top plasma layer was carefully divided.
- Then, using a micro-pipette, the layer of PBMC bands was collected, moved to a falcon tube, and washed with washing buffer.
- The previous washing was then carried out a second time.
- The supernatant was once more discarded, and cells were then once more suspended in a washing buffer.
- Cell suspension was taken for cell counting in a hemocytometer under a microscope using the trypan blue method.
- The cell suspension was centrifuged once again and the PBMC pellet was re-suspended in cold PBS at a desired concentration.
- After that 5 x 10^6 cells were transferred to 15 ml of falcon tube for RNA extraction.

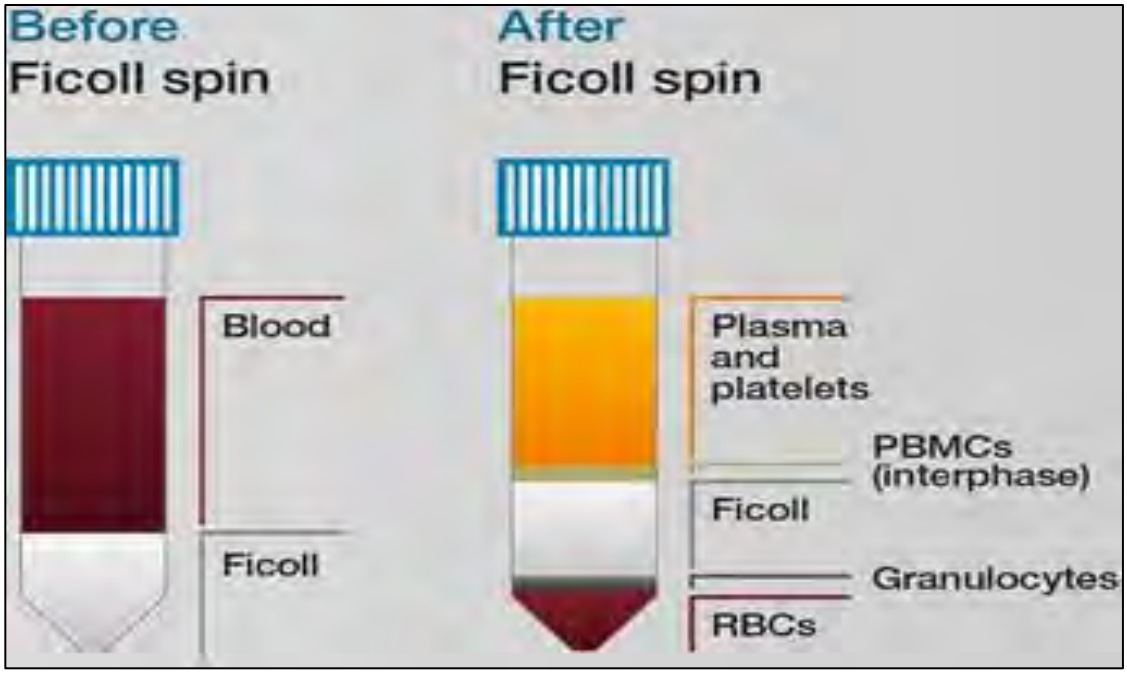

Figure 2.2: Isolation of PBMCs from whole blood.

2.3.3. RNA extraction from PBMCs by QIAamp® RNA Blood Mini kit:

- Determine the number of cells. Pellet the appropriate number of cells by centrifuging for 5 min at 300 x g in a centrifuge tube. Carefully remove all supernatant by aspiration, and proceed to step 2.

  Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the QIAamp membrane. Both effects may reduce RNA yield.

- Before the lyse of cells directly, Aspirate the medium, and wash the cells with PBS (2X). Aspirate the PBS, and transfer the cell into RNase-free falcon tube, and centrifuge at 300 x g for 5 min again. Completely aspirate the supernatant, and proceed to step 2.

  Note: Incomplete removal of PBS will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the QIAamp membrane. Both effects may reduce RNA yield.

- Disrupt cells by adding Buffer RLT.

Note: For cell lines rich in RNases, ensure β-ME (or DTT) is added to Buffer RLT before use.

- For pelleted cells, loosen the cell pellet by flicking the tube and adding Buffer RLT. Vortex or pipet to mix. No cell clumps should be visible before proceeding to the next step.
- Pipet lysate directly into a QIAshredder spin column in a 2 ml collection tube (provided) and centrifuge for 2 min at maximum speed to homogenize. Discard QIAshredder spin column and save homogenized lysate.
- To avoid aerosol formation, adjust pipet to ≥750 µl to ensure that the lysate can be added to the QIAshredder spin column in a single step. If too many cells have been used, after homogenization the lysate will be too viscous to pipet.
- Add 1 volume (usually 350 µl or 600 µl) of 70% ethanol to the homogenized lysate and mix well by pipetting. Do not centrifuge. If some lysate was lost during homogenization, adjust the volume of ethanol accordingly. A precipitate may form after the addition of ethanol but this will not affect the QIAamp procedure.
- Carefully pipet the sample, including any precipitate which may have formed, into a new QIAamp spin column in a 2 ml collection tube (provided) without moistening the rim. Centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). The maximum loading volume is 700 µl. If the volume of the sample exceeds 700 µl, successively load aliquots onto the QIAamp spin column and centrifuge as above.

** Discard flow-through* and collection tube.

- Transfer the QIAamp spin column into a new 2 ml collection tube (provided). Pipet 700 µl Buffer RW1 into the QIAamp spin column and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash. Discard flow-through and collection tube.

  ** Flow-through contains Buffer RW1 and is therefore incompatible with bleach. See page 5 for safety information.
- Transfer the QIAamp spin column into a new 2 ml collection tube (provided). Pipet 500 µl Buffer RPE into the spin column and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash. Discard flow-through.

  Note: Ensure ethanol is added to Buffer RPE before use.

- Carefully open the QIAamp spin column and add 500 µl Buffer RPE. Close the cap and centrifuge at full speed (20,000 x g, 14,000 rpm) for 3 min.

  Note: Some centrifuge rotors may distort slightly upon deceleration, resulting in flow-through, containing Buffer RPE, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column.

  Recommended: Place the QIAamp spin column in a new 2 ml collection tube (not provided)

- Discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. This step helps to eliminate the chance of possible Buffer RPE carryover.
- Transfer the QIAamp spin column into a 1.5 ml collection tube (provided) and pipet 30–50 µl of RNase-free water (provided) directly onto the QIAamp membrane. Centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute. Repeat if the expected RNA yield is >30 µg.
- If a second elution step is performed, elute into the same collection tube using another 30–50 µl RNase-free water.
- RNA purity (260/280 and 260/230 ratio) and concentration were measured by using Nanodrop.

2.3.4. First-Strand cDNA Synthesis by QuantiTect Reverse Transcription Kit:

The following procedure was designed to convert total RNA into first-strand cDNA from 5x 10^6 PBMC.

1. Thaw template RNA on ice. Thaw gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water at room temperature (15–25°C). Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes, and then store on ice.
2. Prepare the genomic DNA elimination reaction on ice according to Table 1. Mix and then store on ice.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than that required for the total number of reactions to be performed. Then distribute the appropriate volume of master mix into individual tubes followed by each RNA sample.

Keep the tubes on ice.

Note: The protocol is for use with 10 pg. to 1 µg RNA. If using >1 µg RNA, scale up the reaction linearly. For example, if using 2 µg RNA, double the volumes of all reaction components for a final 28 µl reaction volume.

Table 2.1. Genomic DNA elimination reaction components:

Component	Volume/reaction	Final concentration
gDNA Wipeout Buffer	7x 2 µl	1x
Template RNA	Variable	(up to 1 µg*)
RNase-free water	Variable	
Total volume	14 µl	–

**This amount corresponds to the entire amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present, regardless of the primers used or cDNA analyzed.

3. Incubate for 2 min at 42°C. Then place it immediately on ice.

   Note: Do not incubate at 42°C for longer than 10 min.

4. Prepare the reverse-transcription master mix on ice according to Table 2. Mix and then store on ice. The reverse-transcription master mix contains all components required for first-strand cDNA synthesis except template RNA.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than that required for the total number of reactions to be performed.

   Note: The protocol is for use with 10 pg. to 1 µg RNA. If using >1 µg RNA, scale up the reaction linearly. For example, if using 2 µg RNA, double the volumes of all reaction components for a final 40 µl reaction volume.

Table 2.2. Reverse-transcription reaction components:

Component	Volume/reaction	Final concentration
Reverse-transcription master mix		
Quantiscript Reverse Transcriptase*	1 µl	
Quantiscript RT Buffer, 5x†‡	4 µl	1x
RT Primer Mix‡	1 µl	
Template RNA		
Entire genomic DNA (Template RNA)	14 µl (add at step 5)	
elimination reaction (step 3)		
Total volume	20 µl	

5. Add template RNA from step 3 (14 µl) to each tube containing the reverse-transcription master mix. Mix and then store on ice.
6. Incubate for 15 min at 42°C. In some rare cases (e.g., if the RT-PCR product is longer than 200 bp or if analyzing RNAs with a very high degree of secondary structure), increasing the incubation time up to 30 min may increase cDNA yields.
7. Incubate for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase.
8. Add an aliquot of each finished reverse-transcription reaction to the real-time PCR mix. Store reverse-transcription reactions on ice and proceed directly with real-time PCR, or for long-term storage, store reverse-transcription reactions at –20°C.

2.3.5. qPCR using GoTaq® qPCR Master Mix:

The GoTaq® qPCR Master Mix uses hot-start chemistry, allowing reaction setup to be performed at room temperature. The final reaction volume in this protocol was 20µl.

At first, the GoTaq® Master Mix and Nuclease-Free Water were thawed at room temperature. The GoTaq® Master Mix was vortexed at low speed for 3–5 seconds to mix. The number of reactions was determined to be set up, including positive and negative control reactions. Then according to the total number of reactions, the reaction mix (minus DNA template) was prepared by combining the GoTaq® qPCR Master Mix, PCR primers (forward-reverse primer), and Nuclease-Free Water to a final volume of 16 µl. Then 4 µl of reverse transcriptase inactivated cDNA template was added to the reaction mix for a final volume of 20 µl to each reaction tube. The tubes were sealed properly and centrifuge briefly to collect the contents of the wells at the bottom. During this preparation, all the samples protected from extended light exposure or elevated temperatures. Then, the samples were proceeded for thermal cycling.

Table 2.4 List of primers:

Gene	Primer Name	Primer Sequence	Primer length
SOCS-1	**SOCS-1 Forward**	**5-CCCCTTCCAGATTTGACCG-3**	**19**
	SOCS-1 Reverse	**5-TGAAGAGGTAGGAGGTGCGAGT-3**	**22**
GAPDH2	**GAPDH2 Forward**	**5-TCCCTGAGCTGAACGGGAAG-3**	**20**
	GAPDH2 Reverse	**5-GGAGGAGTGGGTGTCGCTGT-3**	**20**

Table 2.5. Thermal cycling program for amplification of SOCS1 cDNA:

Standard Cycling Conditions Step	Steps	Temperature	Time
GoTaq® Hot Start Polymerase activation	1	95°C	2 minutes
Denaturation Annealing and extension	40	95°C 60°C	15 seconds 1 minute

2.3.6. Flow cytometry assay to measure Tregs:

a) PBMCs staining with both surface and intracellular markers:

1. 1 X 10^6 cells were taken in a FACS tube and 1 ml of FACS staining buffer was added. It was taken for centrifugation at 500 g at room temperature for 5 minutes.
2. The cells were re-suspended in 100 µl FACS staining buffer after the supernatant was removed.
3. A recommended amount (2µL) of anti-CD4 antibody was added to cells for the analysis of CD4+ T cells. A pulse vortex was done to gently mix.
4. Then it was then kept at 2-8°C for at least 30 minutes, covered from light.
5. After incubation, the samples were washed with 100 µl FACS staining buffer, left for centrifugation for a duration of 5 minutes at room temperature at 400-600 x g, and then the supernatant was removed.
6. The cell samples were then re-suspended in FACS staining buffer in a volume of 300 µl.
7. The recommended amount (3µL) of anti-CD25 and anti-FOXP3 antibody were added to cells for the analysis of regulatory T cells. A pulse vortex was done to gently mix. Steps 4-6 were carried out in a similar way for the samples coated with regulatory T cell antibodies.
8. Acquisition of data is done by BD Accuri C6 Software.

b) Gating strategy for Tregs analysis:

The BD Accuri C6 Plus flow cytometer is the platform's most recent generation. Flow cytometry is now much more accessible to novice and veteran flow cytometry researchers with improved sensitivity, reliability, and capabilities. It can detect up to 4 fluorochromes at a time. Before running the experiment, we did compensation by single staining to subtract the unwanted spillover on the filter. For everyday experiments, we also performed Fluorescence Minus One (FMO) for each of the fluorochrome to fix the gate. In the experiment, we first locate the lymphocyte by considering the side scatter, and forward scatter optical detector. Then we isolate the singlet cells by exclusion the doublet cells considering forward scatter (FSC-A) and forward scatter (FSC-H). CD4+populations were determined from the singlet cell gate. Accordingly, for CD4+ populations, we first located the lymphocyte by considering the side scatter (SSC-A) and forward scatter (FSCA) optical detector. Then we isolate the singlet cells by exclusion the doublet cells considering the side scatter (SSC-A) and forward scatter (FSC-A). Lastly, CD4+populations were determined from the singlet cells. Cells on CD4-FITC are simultaneously displayed on both the CD25-APC dot plot and FOXP3-PE dot plot. CD4+CD25+FOXP3+ T cells combined appear in FOXP3-PE-CD25-APC gate for regulatory T cells detection, and subsets of CD4 + CD25 − and CD4 + FOXP3- were gated on dot plot to visualize FMO, respectively. FlowJo Software is used for Data analysis and statistical analysis is done by Graph Pad Prism software.

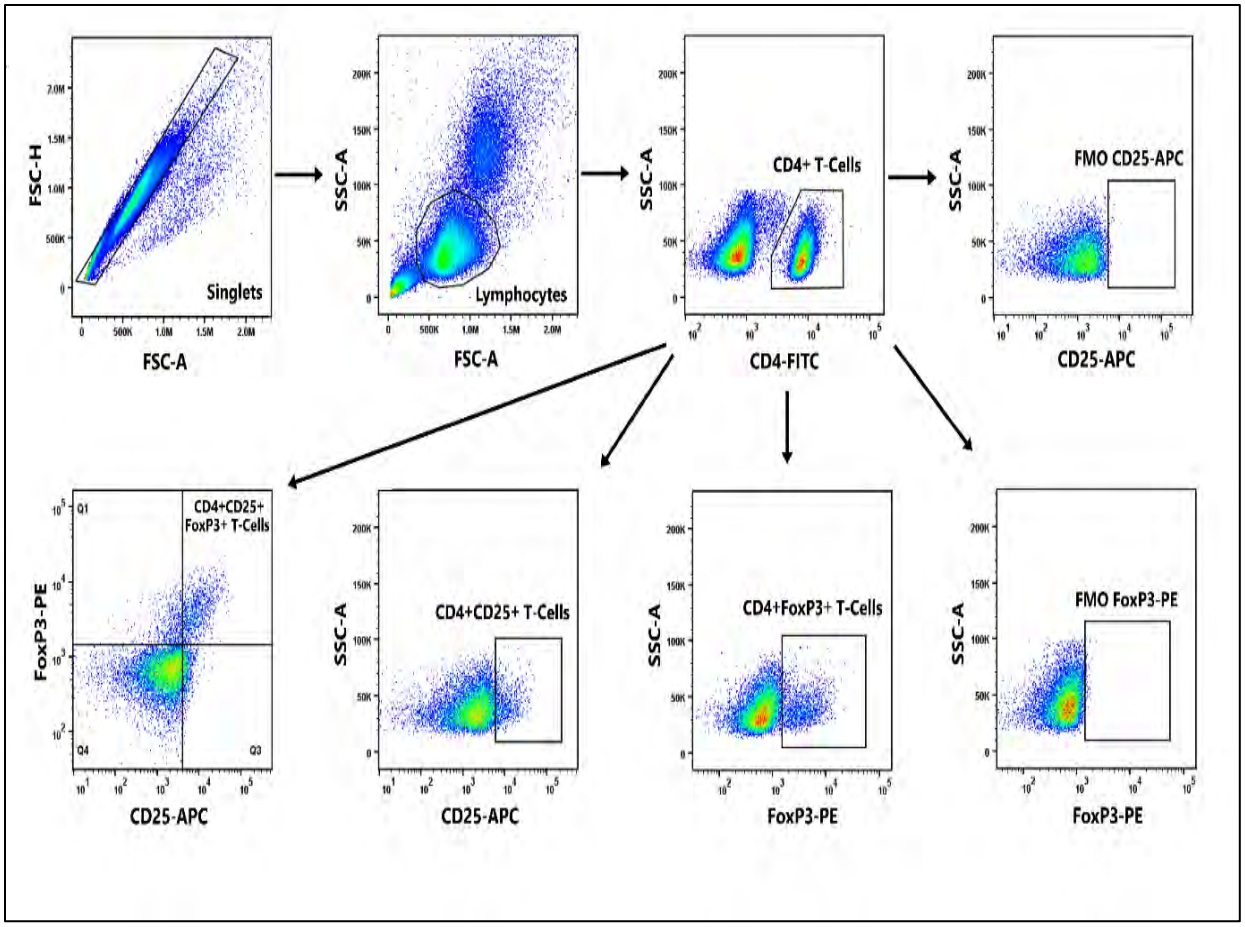

Fig 2.2. Gating Strategy for Tregs analysis in BD Accuri C6 Plus. After removal of the doublets and cellular debris, on both the FSC-H/ FSC-A and FSC/SSC graphs, a lymphocyte population were defined. This population was measured for CD 4 cell surface expression as CD4+T cells were gated. Later, CD 4+ lymphocytes with CD 25 were gated and with FOXP3+ were gated, respectively. Treg cells were gated as lymphocyte with a CD4+ CD25+FOXP3+T-Cells. The Tregs cells were compared with the FMO CD4+CD25-APC and FMO CD4+FOXP3-APC gating.

2.3.6. Statistical analysis:

The cycle threshold (Ct) values were normalized before doing statistical analysis on them because these values couldn't be evaluated as raw data. We used primer against GAPDH2 small nuclear RNA for normalizing the data. We followed a universal method named as delta-delta Ct method (2 –ΔΔCt method) to finally calculate the fold change or expression of *SOCS1*. ΔCt is calculated as the difference between the Ct values of the *SOCS1* gene and the corresponding endogenous control (GAPDH2 gene). ΔΔCt is calculated by subtracting the average ΔCt value of healthy controls from the ΔCt value of the control and experimental sample both. The relative expression

level or the fold changes of *SOCS1* was calculated by using (2-ΔΔCt) this universal formula for both patient and healthy subjects. Comparison between the relative SOCS1 expressions was estimated using non-parametric t-tests in Graph pad prism software version 9. Correlation of the relative *SOCS1* mRNA expressions among regulatory T cells was performed using the Pearson Correlation test in graph pad prism software version 9.

Chapter 3

Results:

3.1 Clinicodemographic characteristics of patients with GBS:

The median age (interquartile range) of 33 patients with GBS was 31 years (IQR: 22-40) with male predominance (65%). Among them, 11 patients were axonal subtypes and 14 cases were demyelinating subtypes of GBS. 69% of patients had antecedent events, mostly diarrhea (60%), and 33.3% were severely affected. At the time of enrolment, the majority of GBS patients (54%) exhibited moderate muscle weakness, as measured by the MRC sum score, which was between 21 and 40. The remaining 42% of GBS patients exhibited mild muscle weakness. In addition, 23 patients (69%) had severe GBS, which was assessed with a GBS-DS score >3. All of the GBS patients were experiencing pain. Fifteen patients with GBS (41%) required mechanical ventilation and had 5 GBS-DS score. Furthermore, only three patients (8.3%) had reported sensory deficiency.

Table 3.1. Clinicodemographic characteristics of GBS patients:

Variables	Patients with GBS (n=33)
Age	31 (22-40)
Male	23 (65%)
Clinical_manifestation	
Antecedent event	25 (69 %)
Autonomic dysfunction	6 (30%)
Mechanical ventilation	15 (41 %)
MRC Sum score **0-20** **21-40**	 14 (42 %) 18 (54 %)
Severely affected (GBS-DS ≥ 3)	23 (69 %)
Mildly affected (GBS-DS<3)	10 (30 %)
Sensory deficient	3 (8 %)

3.2. *SOCS1* mRNA expression in PBMCs in patients with GBS and healthy controls:

The expression level of *SOCS1* mRNA in PBMC was measured and compared between GBS patients and healthy controls. The relative *SOCS1* mRNA expression level was significantly lower among GBS patients (median; 0.7582 vs.1.198, p= 0.0463) than healthy controls (Fig: 3.1)

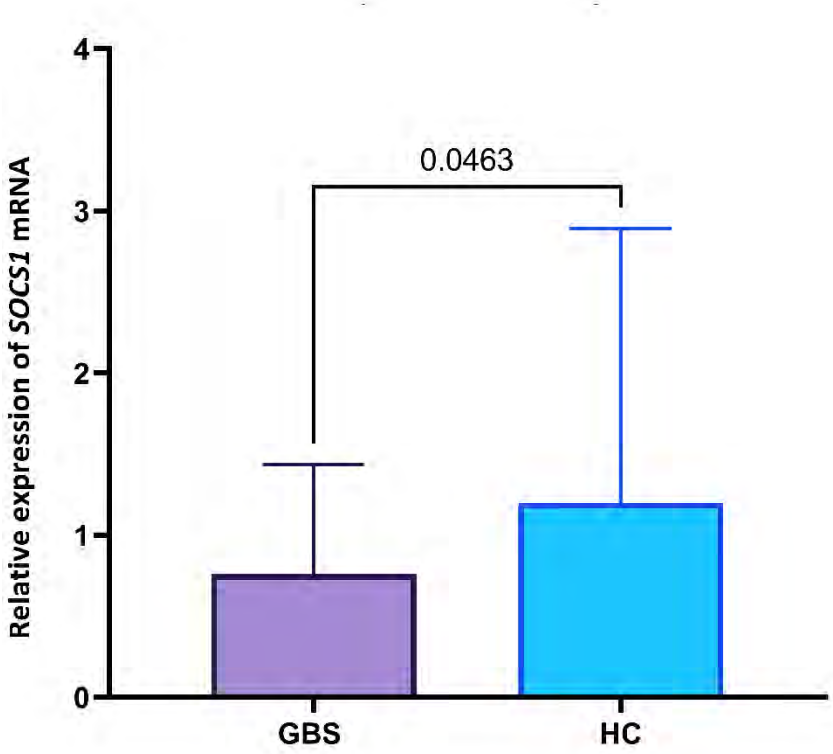

Figure 3.1: Comparison of relative expression of *SOCS1* mRNA in 33 patients with GBS and 33 healthy individuals. The plot represented the *SOCS1* ratio in median with 95% confidence interval (CI) where it signified the association with $p< 0.05$.

3.3. Association of *SOCS1* mRNA expression with GBS disease severity:

The GBS-DS score was determined at the time of patient enrollment which was used to evaluate the status of GBS patients (GBS-DS). According to the GBS-DS, a favorable prognosis is defined as the capacity to move independently when the GBS-DS score is 0, 1, or 2, whereas a terrible prognosis is defined as the inability to stand and walk independently or death (GBS-DS of 3, 4, 5 or 6). The GBS-DS score ≥3 is considered as severely affected (n=33) and GBS-DS < 3 is considered as mildly affected patient. In our findings, there is an inverse correlation between the level of *SOCS1* expression and the severity of the condition but statistically insignificant (p= 0.1423) (Fig: 3.2).

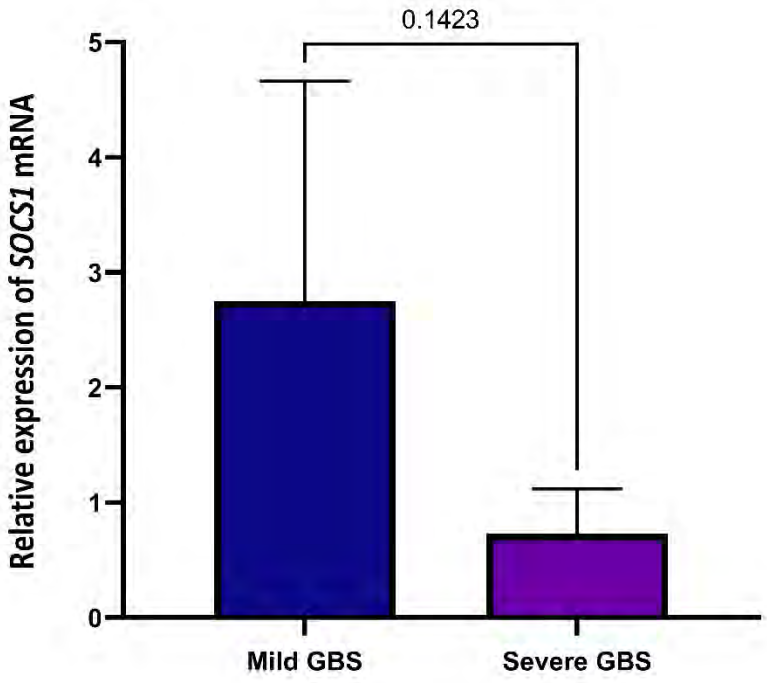

Figure 3.2: Comparative study of *SOCS1* expression between severely affected and mildly affected GBS. Values are presented as a box plot diagram with a median.

3.4. Comparison of *SOCS1* mRNA expression with mechanical ventilation in GBS:

A comparison of the expression fold change between patients who were ventilated and those who were not. Even though the results demonstrated an elevated expression level in individuals who were not ventilated, there was no statistical significance identified (p= 0.5087) (Fig: 3.3).

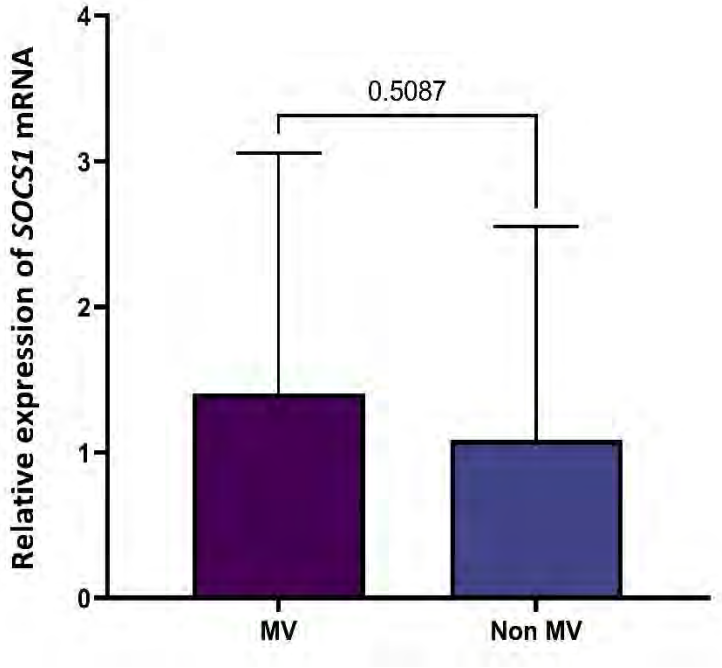

Figure 3.3. Box plots of the median number of *SOCS1* mRNA expression between 15 mechanically ventilated and 18 non-ventilated patients depending on GBS-DS score. The comparative study was observed between mechanically ventilated patients (GBS-DS= 5) with non-ventilated patients (GBS-DS<5).

3.5. Association of *SOCS1* mRNA expression with antecedent events:

A total of 25 individuals who participated in our research, were found to have antecedent infections. The data was evaluated in order to determine whether or not previous infections have an effect on the expression level of *SOCS1*. We conducted a non-parametric t-test between two groups having preceding infections and not having any prior infection reported respectively. It was determined that there was no statistically significant difference between these two groups (p= 0.9836) (Fig: 3.4).

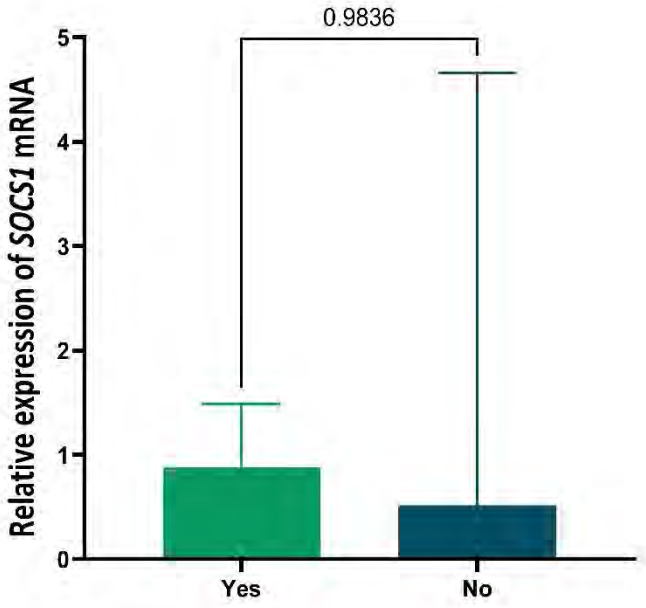

Figure 3.4. A comparative study of *SOCS1* mRNA expression between 33 GBS patients considering the patients having prior infections and without antecedent infection reported.

3.6. Percentage of regulatory T cells between GBS patients and healthy controls:

Between 11 patients with GBS and 15 healthy controls a non-parametric t-tests was performed where the regulatory T cells were less in patients compared to healthy controls. The analysis showed no significance of CD4+CD25+Tregs, CD4+FOXP3+Tregs and CD4+CD25+FOXP3+Tregs cell counts between patients and healthy individual consecutively.

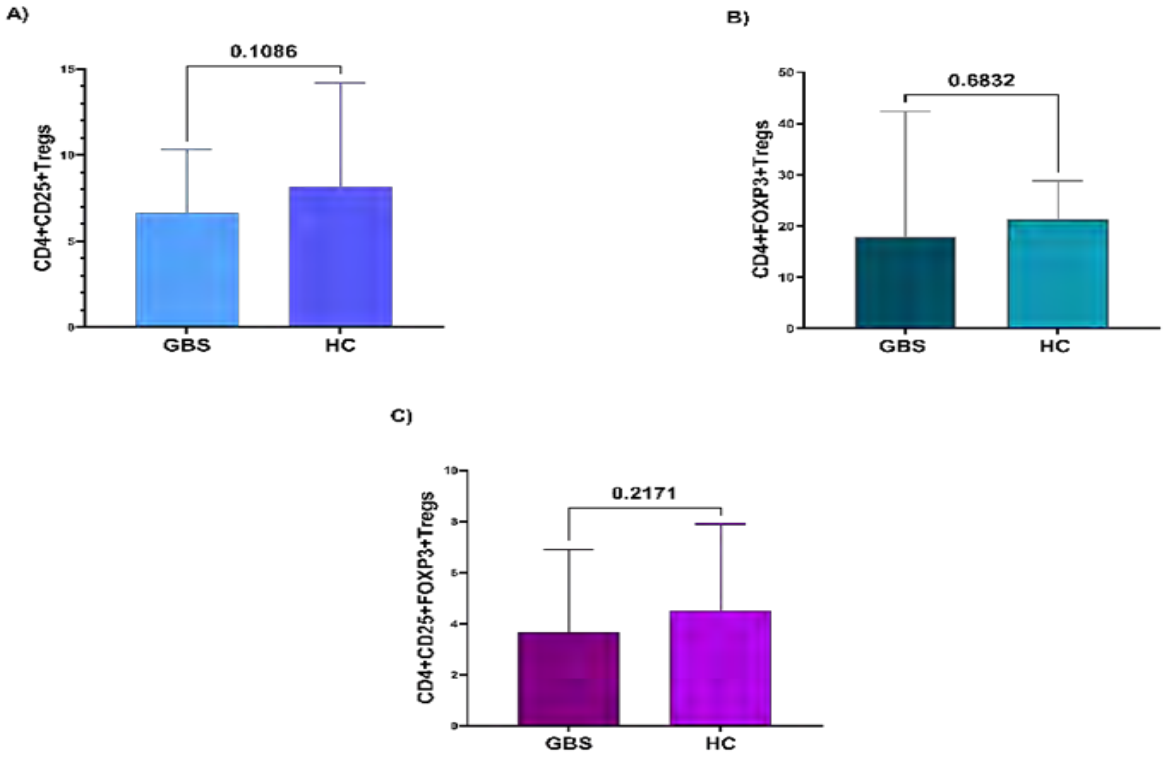

Figure 3.5: A Comparative study between GBS and healthy controls *SOCS1* mRNA expression with three different types of regulatory T cells. The regulatory T cells population includes CD4+CD25^{+}Tregs, CD4+FOXP3+Tregs, CD4+CD25+FOXP3+Tregs.

3.7. Correlation between *SOCS1* expression and regulatory T cell response in GBS and healthy controls:

In total 11 patients with GBS and 15 healthy individuals, regulatory T cells population were compared with their *SOCS1* gene expression (relative Fold change). In this comparison, a Pearson correlation coefficient was performed between the types of regulatory T cells and its *SOCS1* gene expression. The result showed no significant associations between regulatory T cells with *SOCS1* expression. Besides, the correlation between CD4+T cells and *SOCS1* expression had no significance but a weak negative association both in GBS (r= -0.1911, *p*= 0.5734) (Fig3.4:A.) and healthy controls (r= -0.1911, *p*= 0.5734) (Fig3.4:B.). Though, the CD4+FoxP3+ regulatory T cells showed a negative correlation in healthy individuals (r= -0.2074; *p*= 0.4583) (Fig.3.4:B.) associating with the positive correlation in GBS patients (r= 0.1106; *p*= 0.7462) (Fig 3.4:A.) with

SOCS1 gene expression. Conversely, in CD4+CD25+FOXP3+Tregs of GBS and healthy controls, the correlations were weakly associated with *SOCS1* gene mRNA expression although it was not statistically significant. Following the analysis, CD4+CD25+FOXP3+Tregs with patients (r= -0.2267, *p*= 0.5026) and healthy controls (r= -0.2895, *p*= 0.2953) the result showed *SOCS1* expression were reversely proportional with regulatory T cells. The CD4+CD25+ Tregs also showed negative relation with *SOCS1* expression in patients (r= -0.5508, *p*= 0.2023) ((Fig 3.4:A) and control(r= -0.2248, *p*= 0.4206) (Fig 3.4:B.) even though it was statistically insignificant.

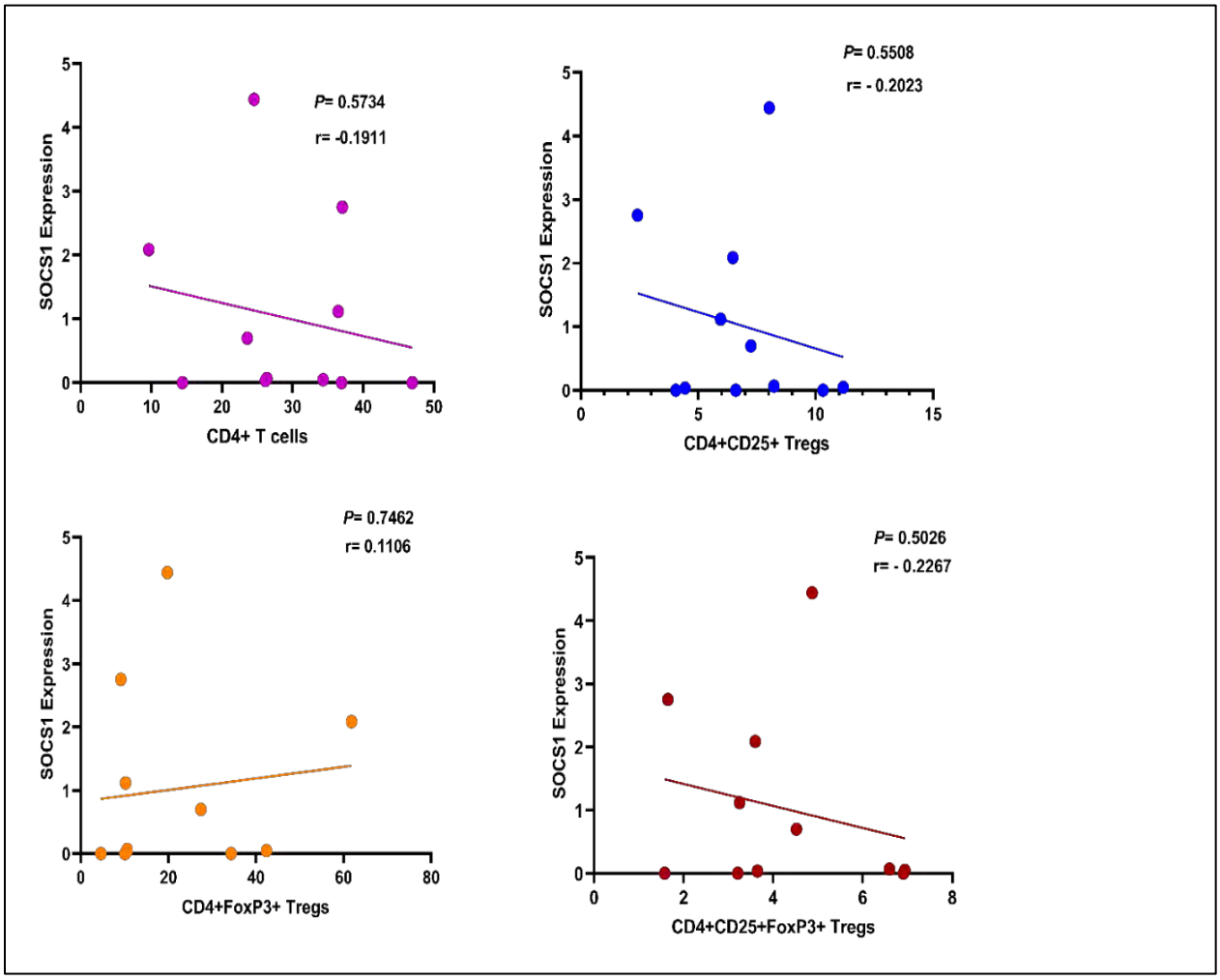

Figure 3.4: A. Relative profiling of patient's CD4+ T cells and CD4+CD25+Tregs, CD4+FOXP3+Tregs, CD4+CD25+FOXP3+Tregs percentages with *SOCS1* expression during enrolment. Values are presented in the XY column graph and the line represents the change pattern between the two variables.

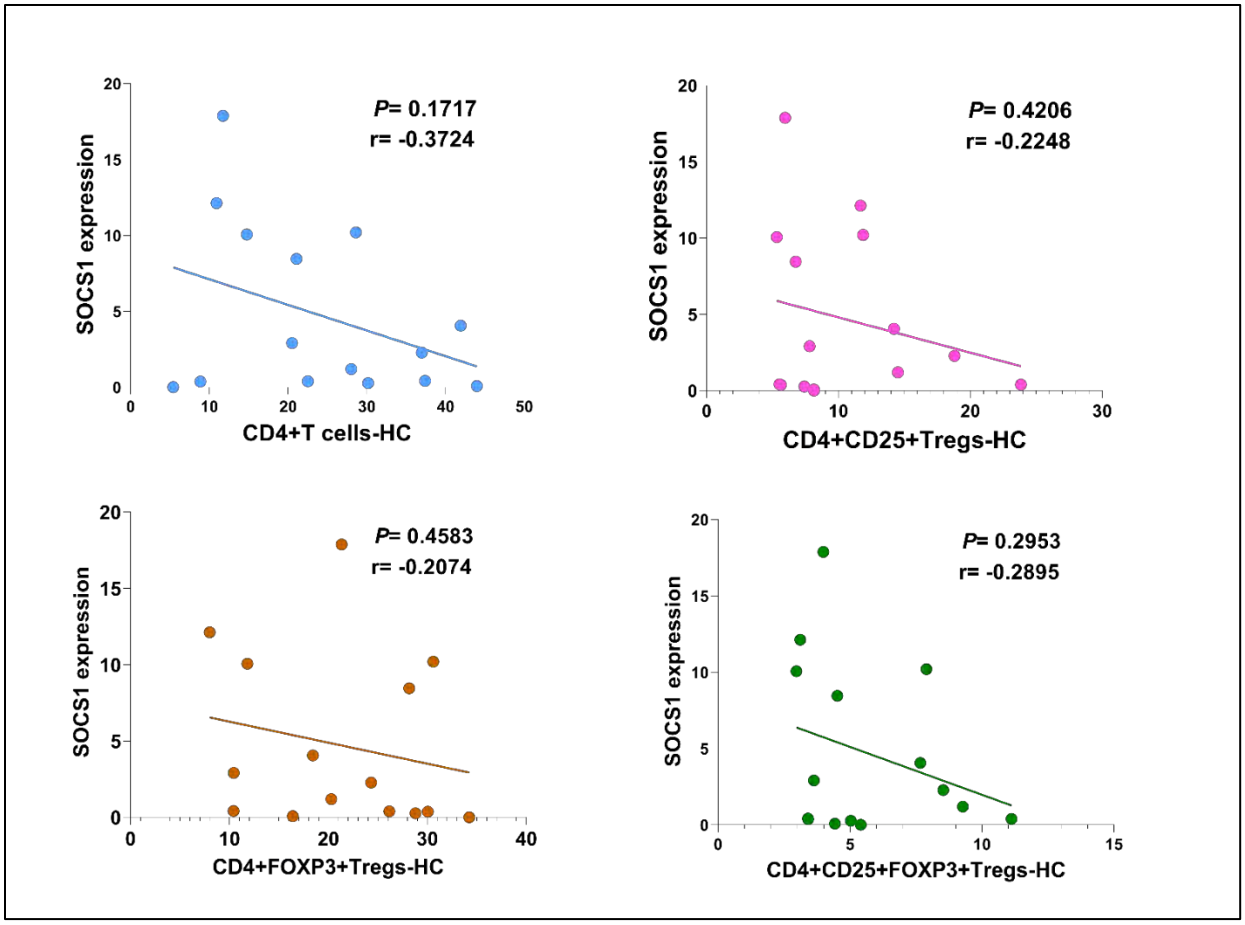

Figure 3.4: B. Relative profiling of healthy individual's CD4+ T cells and CD4+CD25+Tregs, CD4+FOXP3+Tregs, CD4+CD25+FOXP3+Tregs percentages with *SOCS1* expression during enrolment. Values are presented in the XY column graph and the line represents the change pattern between the two variables.

Chapter 4

Discussion:

The pathogenesis of GBS has recently been acknowledged with humoral and cellular immunological dysfunctions. This study demonstrates that the downregulation of the *SOCS1* gene in peripheral blood mononuclear cells is a potential risk factor in the pathogenesis of GBS. There are no significant associations between *SOCS1* gene expression and any clinical manifestations. Besides, downregulation of *SOCS1* gene showed no association with the response of regulatory T cells in patients with GBS and healthy controls.

Previously reported SOCS1 proteins are found to restrict cellular reactivity to the cytokine that triggered its manufacture, thereby averting tissue harm (Whyte et al., 2011). Here, we reported that GBS patients have a discriminating value of *SOCS1* gene expression than healthy controls. The verdicts also correlated with the mechanism of *SOCS1* in distinct autoimmune diseases as well(Liau et al., 2018; Nyati & Prasad, 2014; Yoshimura et al., 2021).

Discrete analysis of relative *SOCS1* gene expression and clinical manifestation of patients with GBS in the population of Bangladesh was perceived consecutively. Earlier studies showed that SOCS proteins are found to inhibit the signaling by direct interaction with intracellular receptors and showcased the decrease of SOCS1 mRNA in peripheral blood mononuclear cells of systemic lupus erythematosus which is negatively associated with the severity of inflammation (Luo et al., 2020). In our study, the downregulation of the *SOCS1* gene is not significantly associated with the severity of GBS. However, the analysis performed in GBS indicated a tendency of depleting *SOCS1* gene expression in severe patients compared to mild patients (Fig.3.2.A). No substantial changes are observed between mechanically ventilated patients or antecedent events of GBS.

The possibility that the function of *SOCS1* gene to prevent abnormal B cells and $CD4^+$ T cell activation highlights the crucial role that *SOCS1* mRNA dysregulation plays in the emergence of autoimmunity like systematic lupus erythematosus (SLE)(Morris et al., 2018; Whyte et al., 2011). In GBS, cellular immune response is associated with an increase in proinflammatory cytokines (for example, TNF-alpha), a decrease in anti-inflammatory cytokines (such as TGF-B1), and an

increase in matrix metalloproteinases (MMP-9-gelatinase B), all of which abnormalities favor immune cell adhesion to and transmigration across(Schmidt et al., 1996). Antibodies against nerve antigens can activate complement, cause antibody-dependent macrophage cytotoxicity, and cause reversible conduction failure(Hu & Ivashkiv, 2009; Matter, 2004). The motive of pursuing the *SOCS1* downstream analysis in this experiment is to underline the impact of the *SOCS1* gene over restraining cytokine signaling via negative regulation, and inhibiting the cross-talks between cytokines that may have divergent effects (Pfitzner E et al., 2004). For instance, the immune-regulatory effect of IL-10-JAK-STAT3 signaling was reduced, which was attributable in part to increased SOCS1 synthesis by IL-6-stimulated CD4+T cells from rheumatoid arthritis patients (Liu et al., 2014).

Besides, a noteworthy correlation of relative *SOCS1* mRNA expression in $CD4^+$ T cells and $CD4^+CD25^+$, $CD4^+FoxP3^+$, and $CD4^+CD25^+FoxP3^+$ regulatory T cells may serve an important insight in terms of finding the role of pathogenesis in GBS disease development and progression. A sub-analysis is implemented between types of regulatory T cells with *SOCS1* gene expression. The experiment included 11 GBS patients with types of regulatory T cells and their *SOCS1* relative fold change of mRNA. Previous studies showed that *SOCS1* gene protects the regulatory T cells from the detrimental effects of inflammatory cytokines which increased the percentage of Foxp3 deletion and conversion into Th1/Th17-like effector cells(Zhang et al., 2013). Another recent research has demonstrated that when Treg cells are transferred into a lymphopenic host or in inflammatory settings, they rapidly lose Foxp3 expression(Whyte et al., 2011). In this investigation, a correlation between $CD4^+FoxP3^+$ regulatory T cells and *SOCS1* gene expression presented a negative relationship in healthy individuals corresponding with a positive correlation in GBS patients. The correlation between $CD4^+FoxP3^+$ regulatory T cells with patients indicates the effect of *SOCS1* gene on certain regulation of regulatory T cells over autoimmunity development. The result showed no significant associations between regulatory T cells with *SOCS1* gene expression. Previously, another investigation regarding $CD4^+CD25^+$ regulatory T cells (Tregs), a kind of $CD4^+$ T cell showed an immunosuppressive properties that are essential for immunological homeostasis(Stephens et al., 2005). This prospective study may play a pivotal role in finding the association of *SOCS1* mRNA in developing GBS. However, the rest of the correlation of regulatory T cell variants with *SOCS1* showed an inversive relation with each other without any statistical significance.

Though GBS is an immune-mediated disease, the findings of cellular response to GBS are still vast and indistinguishable. The contemporary work adds information on the significance of critical innate immune signalling pathways, particularly on negative regulators. As a result, the current study indicated the downregulation of *SOCS1* gene might have an impact on developing GBS as certain types of regulatory T cells have been less expressive.

Chapter 5

Conclusion:

In conclusion, downregulation of the *SOCS1* gene in PBMCs of GBS patients denotes a possibility of developing pathogenesis. Overall, this study specifies that the *SOCS1* gene confers no risk for clinical manifestations of GBS. However, the study performed on a small scale may restrict the findings of other associations in GBS. In future studies with a significant number of patients and healthy controls can confirm the trend observed in our study and explain the underlying role of *SOCS1* gene with regulatory T cell and effector T cell subsets with their association with disease development and progression. Future research in serological subgroups of GBS may detangle the mystery of the *SOCS1* gene mechanism in the development of GBS.

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