Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

Name: Dr Dean Huggard

Title: Characterisation of innate immune responses in children with Down syndrome

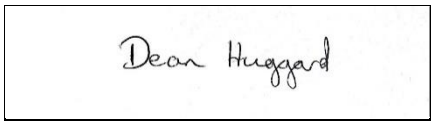
Student Number: 17342339

Degree Programme: PhD in Paediatrics 2020

PhD Supervisor: Professor Eleanor Molloy

Declaration and Permission

This thesis has not been submitted as an exercise for a degree at this or any other University. It is entirely my own work. I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement. I consent to the examiner retaining a copy of the thesis beyond the examining period, should they so wish.



This work is dedicated to my wife Clodagh for her unwavering support, and to our children Beth, Jake, and Abbie, who always manage to put a smile on my face.

Contents

List of Tables and Figures.

Acknowledgements

Firstly, I want to sincerely thank my supervisor Professor Eleanor Molloy for giving me this fantastic opportunity, the confidence to succeed, her wisdom, and for always being so understanding and supportive in all matters both professional and personal.

I would like to thank Professor Derek Doherty for always being available, his invaluable help and guidance in the laboratory as well as an insightful reviewer of my scientific writing.

I would also like to pay special thanks to Dr Lynne Kelly who provided indispensable teaching and guidance on lab methodology. Lynne was a constant source of support and knowledge throughout this journey.

There were many other significant collaborators who I would like to acknowledge: All those working in the Down syndrome clinic, Tallaght, Nurse specialist Fiona McGrane, Dr Niamh Lagan, Dr Joanne Balfe, Prof Edna Roche, and all the phlebotomy staff. Chief Biochemists in Tallaght, Dr Michael Kelly and Siobhan Rooney. All working in the Cardiology department Crumlin especially Dr Orla Franklin, and Dr Ronan Leahy (Immunology) from Children's Health Ireland, Crumlin. Colin Kirkham from the rotunda and Ashanty Melo, Ana Moreno for the flow cytometry tutorials in TTTMI.

Thanks to the National Children's Research Centre and the National Children's Hospital Foundation for the financial support, without which I could not have achieved this goal.

Having a few good mates in the same boat makes the odyssey less onerous and provided us with a great cathartic outlet! To Mary, Matt, Emer, Lynne and new kid on the block Tim, thanks for the support, banter and the friendship.

Lastly, to all the children and their parents who helped us with this research, a heartfelt thanks for your significant contribution. Your enthusiasm and willingness to get involved inspired me to complete this PhD and ensure their message is publicised.

Prizes and Grants during Thesis

- Shortlisted Poster: Vaccination and Palivizumab in Down syndrome, what is the evidence?
  Irish Paediatric Association 2018

- Rosette winning poster: Immunodeficiency in children with Down syndrome
  Irish Paediatric Association 2017

- NCRC Clinical fellowship 2018-2020 - €161,020. Supervisors: Prof Eleanor Molloy & Prof Derek Doherty.

- National Children's Hospital Foundation Research project grant 2017 - €39,741. Supervisor: Prof Eleanor Molloy.

International presentations during Thesis

- Altered monocyte function in children with Down syndrome
  European Academy of Paediatrics, Paris (Oral) 2018

- Differences in innate lymphocytes in Down syndrome
  European Academy of Paediatrics, Paris (Oral) 2018

- Immunodeficiency in children with Down syndrome
  World Down Syndrome Congress, Glasgow 2018

- Altered immune function and endotoxin responsiveness in children with Down syndrome
  Paediatric Academic Society, Toronto 2018

- Immunodeficiency in children with Down syndrome
  RCPCH conference, Glasgow 2018

National presentations during Thesis

- Increased pro- and anti- inflammatory cytokines in children with Down syndrome

  Irish Paediatric Association, Westmeath (Oral) 2019

- Clinical associations with immune screening for children with Down syndrome

  Irish Paediatric Association, Westmeath (Oral) 2019

- Increased Toll like receptor 2 expression in children with Down syndrome

  Irish Paediatric Association/Welsh Paediatric Society, Galway (Oral) 2018

- Vaccination and Palivizumab in Down syndrome; what is the evidence?

  Irish Paediatric Association/Welsh Paediatric Society, Galway 2018

- Altered Toll like receptor signalling pathways in Down syndrome

  Irish Paediatric Association/Welsh Paediatric Society, Galway 2018

- Differences in innate lymphocytes in Down syndrome

  OLCHC Research & Audit Conference 2018

- Immunodeficiency in children with Down syndrome

  Irish Paediatric Association, Kilkenny 2018

Publications during Thesis

- **Huggard D,** Kelly L, Ryan E, McGrane F, Lagan N, Roche E, Balfe J, Leahy TR, Franklin O, Doherty DG, Molloy EJ. *Increased systemic inflammation in children with Down syndrome.* Cytokine. 2019 Nov 27;127:154938.
- **Huggard D**, Kaoy WJ, Kelly L, McGrane F, Ryan E, Lagan N, Roche E, Balfe J, Leahy TR, Franklin O, Moreno-Oliveira A, Melo AM, Doherty DG, Molloy EJ. *Altered TLR signalling in children with Down syndrome.* Mediators of Inflammation 2019 Sep 12; 2019:4068734.
- Lagan N, Grane FM, **Huggard D**, et al. P147. *The development of the down syndrome health surveillance clinic.* Archives of Disease in Childhood 2019;104:A216.
- **Huggard D**, McGrane F, Lagan N, Roche E, Balfe J, Leahy TR, et al. *Altered endotoxin responsiveness in healthy children with Down syndrome.* BMC immunology. 2018;19(1):31.
- **Huggard D**, Molloy EJ. *Palivizumab for all children with Down syndrome?* Archives of disease in childhood. 2018.
- **Huggard D**, Molloy EJ. *Do children with Down syndrome benefit from extra vaccinations?* Archives of disease in childhood. 2018;103(11):1085-7.

Papers submitted for publication

- **Huggard D,** Molloy EJ. *Immune dysregulation in children with Down syndrome: a review.* Frontiers in immunology, January 2020.
- **Huggard D,** Lagan N, McGrane F, Roche E, Balfe J, Leahy TR, Molloy EJ. *Clinical Associations of immune screening for children with Down syndrome.* Archives of disease in childhood, January 2020.
- **Huggard D,** Kelly L, Worrall A, Gallagher E, McGrane F, Lagan N, Roche E, Balfe J, Leahy TR, Franklin O, Doherty DG, Molloy EJ. *Melatonin as an immunomodulator in Down syndrome.* Journal of Pineal research. January 2020.

Abstract

Introduction: Down syndrome (DS) is associated with increased incidence of infections in childhood, higher mortality from sepsis, and other inflammatory conditions such as arthropathy, thyroid and coeliac disease. Several immunodeficiencies have already been described in DS, however we evaluated novel aspects of their immune system; neutrophil and monocyte function, genes involved in Toll like receptor (TLR) pathway signalling, a broad panel of pro-and anti-inflammatory cytokines, and characterisation of the NLRP3 inflammasome. Furthermore, the clinical significance of immune screening and the latest evidence around vaccination in DS was evaluated.

Methods: Children with DS and age matched controls were recruited to examine neutrophil and monocyte cell surface markers CD11b, TLR2 and TLR4 by flow cytometry. Enzyme linked immunosorbent assay (ELISA) was utilised to evaluate an extensive panel of inflammatory cytokines, and quantitative polymerase chain reaction (qPCR) to quantify fold change in expression of key genes involved in TLR signalling and the inflammasome. All experiments featured immunomodulation with pro- (LPS, Pam3Csk4) and anti-inflammatory (Melatonin, Sparstolonin B (SsnB)) stimuli.

Results: Children with DS displayed further dysregulation of their immune system and inflammatory response: neutrophil endotoxin hyperresponsiveness, increased TLR2 expression on neutrophils and monocytes with abnormal TLR signalling; and further evidence of excess circulating inflammatory cytokines. Melatonin and SsnB were effective at abrogating the inflammatory response. Both the white cell (WCC) and neutrophil count were associated with poor respiratory outcome. Children with DS are at increased risk from vaccine preventable diseases like, respiratory syncytial virus (RSV), influenza, and pneumococcus.

Conclusion: Children with DS have a complex and profound immune dysregulation which contributes to respiratory morbidity, mortality from sepsis and autoimmunity. Melatonin and SsnB may have potential therapeutic benefit as immunomodulators. The WCC and neutrophils may be used as biomarkers to help predict prognosis and allow timely intervention for high risk individuals. Tailored vaccination programmes should be endorsed and administered for all children with DS.

Chapter 1: Immune dysregulation in children with Down syndrome

1.1 Introduction

Down syndrome (DS) is caused by extra genetic material from chromosome 21 and occurs in all ethnicities and across different species. It occurs in approximately 1 in 700 births in the USA, and 1 in 546 births in Ireland, which is the highest rate in Europe(1, 2). There are many co-morbidities associated with DS including; developmental delay, congenital heart disease, gastrointestinal anomalies, increased risk of haematological malignancy and several autoimmune conditions(3). It is also the most common genetic syndrome associated with immune deficits, with both the innate and adaptive responses being affected(4).

Children with DS are a high-risk group who get more severe infections and have poorer outcomes. In addition, they are more likely to be admitted to hospital, have an increased length of stay due to respiratory tract infection (RTIs) and a greater chance of requiring ventilatory support and intensive care (PICU) (5, 6). Infants with DS are more susceptible to severe respiratory syncytial virus (RSV) bronchiolitis with worse clinical outcomes and are more likely to be admitted to PICU with a higher overall mortality rate(7). Garrison et al(8) reported that children with DS had a 30% increased mortality risk from sepsis than children without DS who also had sepsis. There is also evidence that their response to immunisations is sub-optimal, and that adaptive immunity may wane overtime contributing to their vulnerability to infection(9).

There are some anatomical considerations which may contribute to an increased predisposition to infection. Children with DS have a shorter midface and a relative macroglossia making it more difficult to clear secretions, leading to aspiration and LRTI development. Furthermore, they have a relatively short eustachian tubes which facilitates migration of pathogens into the middle ear(10), resulting in recurrent otitis media with effusion (OME) and sensorineural hearing loss (SNHL).

Autoimmune conditions such as hypothyroidism, coeliac disease and arthropathy are more prevalent in DS. These chronic inflammatory conditions are as a

result of unchecked and persistent inflammation which can have significant long-term health complications(11). For example, in later life, this cohort represent the largest group of people with dementia under the age of 50 years(12). This neurodegenerative disorder is driven by aberrant neuroinflammatory processes which are exacerbated in DS(13), and may be driven by indolent chronic infection such as periodontitis, which is also more common in DS(14).

1.2 T lymphocytes

T lymphocytes are crucial parts of the adaptive immune system and are dichotomised into CD4+ or CD8+ based on their T cell receptor (TCR) and expression of CD4 or CD8. The former binds MHC class 2 molecules while CD8 interacts with MHC class 1(15). During the first year of life there is normally a large expansion in the number of circulating T & B lymphocytes, however in DS there is an absence of these immunological changes(16). The fate of differentiating T and B cells over time is quite different in this population. de Hingh et al(17) demonstrated that T lymphocyte numbers gradually increase towards the normal range over time, but B lymphocyte levels remain markedly reduced. These findings do point towards an inherent dysfunction of adaptive immunity in DS.

The thymus gland is a primary organ of lymphoid origin which is the site of T cell development and also has a crucial role in ensuring immune tolerance. Abnormalities of the thymus gland in DS including the smaller size of the organ have been known for some time (10). There is evidence of reduced T cell receptor excision circle (TREC) counts which represent recent emigrants from thymus gland and are a surrogate for T cell turnover(18). However, an appraisal of thymocyte development as well as Regulatory T cell (Treg) functionality has been less well studied. Tregs are a key subtype of T lymphocytes which maintain self-tolerance and prevent autoimmunity by suppressing the immune system(19). Marcovecchio et al(20) examined histological thymic samples from children with DS (n=9), DiGeorge syndrome (DGS) (n=10), controls (n=26) and demonstrated that thymus in DS is hypocellular, smaller and with a reduced number of mature thymocytes. In the periphery there were reduced

lymphocytes and Tregs which demonstrated decreased suppressive ability in patients with DS. These abnormalities may alter thymic selection of T lymphocytes and the Treg population leading to a greater propensity to develop autoimmune conditions.

In DS there are decreased T lymphocyte numbers, both of CD4+ and CD8+ cells. Although absolute numbers will increase over time, deficient stimulation in response to circulating antigens may render the functionality and phenotype of these cells impaired(21, 22). Infants and children with DS have a reduced lymphocyte proliferative response to stimulation with phytohaemagglutinin (PHA)(23). Further evaluation of the function of T cell subpopulations on children and adults with DS (n=40) and controls (n=51), in response to pathogen specific stimulation with varicella zoster virus (VZV) and cytomegalovirus (CMV) found that the DS cohort could demonstrate an efficient effector T cell response with an equivalent phenotype and function to controls. However, the DS cohort needed greater effector T cell frequencies to eliminate pathogens(24). Noble et al(25) reported a decrease in the number and functionality of helper T cells in children with DS cohort age matched controls. Furthermore, there may be an inherent defect in T helper cell responses to stimulation in DS in view of the normal levels of IL-2.

1.3 B lymphocytes

B lymphocytes are key players in all aspects of the adaptive immune response, they are derived from haematopoietic stem cells and following antigen recognition undergo proliferation, differentiation, and class switching to produce specific antibodies, and also retain memory to rapidly produce a high affinity response on subsequent encounter with the previous stimulating antigen(26). There are 4 main subpopulations of B lymphocytes in peripheral blood; IgM memory B cells, switched memory B cells, mature naïve B cells and transitional B cells which have recently emigrated from the bone marrow(27). The switched memory cells are important as they represent the previous antigen experience of the individual and are imperative for an appropriate antibody response on encountering pathogens or following vaccination(28).

Several deficiencies have been described in B lymphocyte number and function in DS. Verstegen et al(29), found a decrease in peripheral numbers of naïve and memory B cells. Furthermore, this study found that molecular defects of Immunoglobulin A and M were present, reflecting impaired antigen selection in memory B cells. These findings are mirrored in those with common variable immunodeficiency (CVID). This may be a contributory factor to the increased burden of infections in DS, particularly respiratory tract infections. However, children with DS, in contrast to CVID tend to have less severe infections overall and have adequate serum immunoglobulin G and A levels, furthermore, their response to vaccines although occasionally suboptimal and varied is probably largely protective(9). Martinez et al(30) reported an increased number of B cells with CD18 positivity versus controls. This integrin has been shown to be key in the function and translocation of lymphocytes(31), adding weight to the claim that a dysregulation of B lymphocytes is a significant cause of immunodeficiency in DS.

Further evidence of dysfunction of B lymphocytes in children with DS was demonstrated by Carsetti et al(27) who found that DS is in fact a primary immunodeficiency disorder characterised by a fundamental defect in the differentiation of B cells leading to a significant decrease in switched memory B cells. These cells play a crucial role in the response to immunisation and the secondary response to infectious organisms. The levels of immunoglobulins in DS were not profoundly different from controls. However given their increased susceptibility to infection it can be argued therefore that switched memory B cells are important in the fight against infection and developing long term immunity post immunisation, despite apparently normal serum immunoglobulins(32). Therefore, these cells are important in the response to vaccination and maintenance of adequate titres. Carsetti et al demonstrated that transitional and mature naïve B cells are reduced by 50% in children with DS, and that switched memory B cells were decreased by 85-90% versus controls. Although the total numbers of certain classes of B lymphocytes were found to be low, following stimulation with TLR-9 agonists children with DS mounted an exaggerated response and produced increased numbers of antibody generating cells

from IgM and switched memory B lymphocytes. This demonstrated that children with DS can respond to antigenic stimulation.

There is conflicting evidence regarding serum immunoglobulin levels in DS. Valentini et al(33) found that overall serum immunoglobulin levels were in the normal range, except for IgA which was found to be 40% lower compared with controls. They also found that salivary IgA was normal, despite the contrary being reported in other studies(34). Other research has shown adequate immunoglobulin levels in most children with DS(16, 35). Hypergammagloublinaemia of IgG and IgA after 5 years of age has been described in DS as well as decreased IgG2 and IgG4 and elevated IgG1 and IgG3(36). Despite relatively normal immunoglobulins in DS, the important clinical questions surround whether protection from pathogens is conferred and the maintenance of robust long-term immunity.

A possible explanation for the observed differences in T and B lymphocyte number and function may be due to a tendency towards apoptosis in these cells. Apoptosis, also known as programmed cell death, is a cellular mechanism to remove unwanted cells. In the immune system it is important in eradicating poorly responsive B lymphocytes in germinal centres and deleting auto-reactive T lymphocytes in the thymus gland(37). There are reports that a propensity towards apoptosis in lymphocytes in children with DS may be a factor in the lymphopaenias described. Gemen et al(38) examined apoptotic markers (propidium iodide and Annexin V) by flow cytometry on peripheral lymphocytes in controls (n=32) and children with DS (n=72). There were greater levels of apoptosis in the DS cohort, which increased with age, and especially within B cells. This may be a cause for the reduced B lymphocytes seen in DS(29). Elsayed et al(39) evaluated apoptosis by immunophenotyping and annexin V in 17 children with DS (n=17) and controls (n=17) found that there was also increased rates of apoptosis in DS, but contrary to the former publication, T cells were more profoundly affected. The authors concluded that it is the impairment of functionality in these cells that leads to the immune dysregulation and that cellular immunity was more markedly affected than humoral(39).

1.4 Neutrophils

Neutrophils are a crucial effector cell, are the main phagocyte of the innate immune system and play an important role in clearing pathogenic micro-organisms(40). Izumi et al(41) found significantly impaired neutrophil chemotaxis and periodontal disease in adults with DS versus healthy controls (n=14 in both groups) which they suggested may have a role in the poorer oral health of this population. Licastro et al(42) found that phagocytic activity in children with DS (n=27) was significantly decreased compared with controls (n=23) which may point to an inherent defect in neutrophil functionality in DS. At the cell surface, receptors like CD11b (Mac-1) are important in the activation and migration of cells towards the site of infection or injury(43). Novo et al(44) found no significant differences in CD11b expression on neutrophils in children with DS (n=12, age 8-16 years) versus controls(44). Regarding metabolic activity, Khocht et al found increased oxidative burst capacity from neutrophils which correlated with clinical evidence of chronic inflammation and periodontitis(45). This suggests neutrophils may play a key role in the pathogenesis of persistent inflammation.

1.5 Monocytes

The monocyte is another crucial innate immune cell that has several roles. They protect against foreign pathogens, clear dead cells, contribute to tissue repair and stimulate the adaptive immune system(46). Monocytes exist as a malleable, heterogeneous population and it is now accepted that there are 3 distinct subtypes, based on their relative CD14/CD16 surface positivity, which have distinct functions and are context dependent(47). The classical monocyte accounts for approximately 80% of the total monocyte population and expresses high levels of CD14 and is bereft of CD16 on their surface. The remainder have CD16 surface positivity and are separated based on the level of CD14 expression. More commonly the non-classical monocyte has very low CD14 and raised CD16 expression and the intermediate or inflammatory monocyte which has both cell surface markers increased(48).

The classical monocyte displays cell surface markers associated with antigen presentation, and the highest levels of CD163, CD36 which points to these cells having a major role in phagocytosis. The relative numbers are reduced in the setting of acute infection like sepsis or in chronic disease. The intermediate monocyte has multi functionality in phagocytosis and antigen presentation, but also in the production of several cytokines such as Interleukin (IL)-10, increased Toll like receptor (TLR) cell surface expression and increased numbers in acute inflammation. The non- classical monocyte is pro-inflammatory and is the chief producer of pro-inflammatory cytokines IL-1β and Tumour Necrosis Factor (TNF-α). Its numbers increase in both acute and chronic disease(49, 50).

Although paediatric research on monocyte subtypes in sepsis is limited, Skrzczynsk et al reported that infants (n=30) had more CD14+/16+ and 14dim/16+ (intermediate and non-classical types), a reduced ability to phagocytose *E.coli in vitro* and produced less IFN-γ, IL-1 and more IL-10(8). Monocytes in children with DS have similar anomalies. Bloemers et al(51) examined the innate immune system in children with DS in detail and found that total leukocyte, lymphocyte and monocyte counts were decreased in the DS cohort compared to controls. Although total monocyte counts were reduced, there was a significant increase in the absolute number and overall percentage of non -classical or CD14dimCD16+ monocyte sub-population. Non-classical monocyte have been implicated in various disease states such as cancer, sepsis and chronic inflammation(52). Like neutrophils, there is evidence demonstrating deficient monocyte chemotaxis in children with DS, which may be disadvantageous in the setting of acute infection(53).

1.6 Dendritic cells

The dendritic cell is an innate immune cell that has also been described as being decreased in DS compared with controls. These antigen presenting cells are key in propagating a Th1 response from CD4+ T lymphocytes, aid in co-ordinating an effective immune response and are important in combatting pathogenic viruses. In addition, functionally impaired dendritic cells may lead to persistence of viral

infections by impairing host specific T lymphocyte responses(54). Bloemers et al(51)enumerated innate immune cells in DS (n=41) and controls (n=41) reporting that myeloid dendritic cells were decreased in DS, especially in the first 24 months of life. This may contribute to the increased burden of viral infections incurred in children with DS during this time.

1.7 Natural killer cells

Natural killer (NK) cells arise from haematopoietic stem cells, and function in an effector and regulatory capacity. NK cells work by a combination of cytolysis and cytokine production (e.g. IFN-γ) and have anti-neoplastic, anti-viral and anti-bacterial actions(55). Bloemers et al(51) reported a higher NK cell percentage in children from 1-9 y with DS (n=41) versus age-matched controls (n=41) but not to a significant level. However, in the under 2-year olds a higher percentage of NK cells in the DS group was found (10.3 versus 5.7% $p<0.01$)(51) . Maccario et al(56) found a significant increase in the NK cell percentage in patients with DS (n=25: (n=11 <10 y; n= 5 11-20yrs; n=9 21-42 y) compared with controls (n=25 age and sex matched) which did not increase with age.

The functionality of NK cells in DS has been studied; Maccario et al(56), described that NK cells in DS displayed a hypersensitivity to interferon stimulation. Cossariza et al(57) evaluated numbers and function of lymphocytes in children (n=10, average age=9.2 y) and adults (n=7, average age 43.2 y) with DS versus age-matched controls found a significant increase in NK cell percentage in both DS groups. The proportion of NK cells was significantly higher in the adults with DS. There was a significant decrease in the cytotoxic activity in both DS age-groups compared with controls. The samples were also incubated with stimulatory cytokines IL- 2, IFN-γ, IFN-β, after which normal cytotoxicity was recorded, suggesting that in patients with DS, NK cells can respond to stimulation. There is conflicting evidence about the degree of aberrant NK functioning. Nurmi et al(58) found deficient NK cell activity post stimulation with interferon-α in adults with DS compared with controls. Nair et al(59) found that NK activity against target cells (K562) was reduced in DS and that the

response to IL-2 was impaired versus the control group. In contrast, there were no differences in the effects of Interferon alpha (IFN-α) on NK between children with DS and controls(60). Abnormal NK function adds to the evidence of a dysregulated innate immune system in DS.

1.8 Gamma delta (γδ) T cells

The majority of the T lymphocyte population including CD4+ helper and CD8+ cytotoxic T cells express a CD3+ associated α/β T cell receptor. A smaller subset of T lymphocytes utilise heterodimeric T cell receptors composed of γ/δ chains, which recognise non-peptide antigens without the need for major histocompatibility complex (MHC) presentation(61). These γδ T cells have a varied tissue distribution in the body and are mostly enriched in several gastrointestinal and epithelial tissues, as well as in the epidermis. In peripheral blood they account for approximately 0.5-5% of the total lymphocyte count(62).

γδ T cells have a myriad of different functions and they are an important first line of defence from invading pathogens. They release several chemokines which increase neutrophil concentration at the site of infection and can also serve as antigen presenting cells, stimulate other adaptive and innate immune cells, while also retaining immunological memory. γδ T cells are key first responders to inflammation and propagate an early cytokine response(62). Cytokines such as IFN-γ, TNF-α, IL-17 and the anti-inflammatory IL-10 are known to be secreted by γδ T cells in the setting of autoimmunity or infection(63, 64).

There is a paucity of studies in the literature looking at γδ T cells in DS. Bertotto et al(65) examined the proportion of blood lymphocytes bearing the γδ T receptor in this population and showed a significant increase in γδ T cells in adults with DS versus controls. This was mainly attributed to a larger number of cells that express non-covalently bound γδ chains on their cell surface, in contrast to controls, where most of these cells had the disulphide-linked form of the receptor. These cells appear to be different in number and perhaps function and are an important link between the innate and adaptive immune response.

1.9 CD11b

CD11b/CD18 (also known as Mac-1) is a surface receptor involved in propagating monocyte and granulocyte adhesion and diapedesis(66) and is a marker of cell activation. Dysregulation in granulocyte adherence and translocation has been shown to increase the risk of infection in adults and neonates(67). CD11b has potential clinical utility, as well as other biomarkers (CRP, CD64) to predict the likelihood of sepsis (68). Comparing CD11b expression post LPS stimulation, it has been reported that infants with neonatal encephalopathy have a significantly greater response than healthy term controls(69). Autoinflammation is more prevalent in DS, and CD11b, and its genetic variants have now been implicated in the development of autoimmune disease, as it functions as a key regulator of crucial immune processes such as Toll like receptor (TLR) signalling, pro-inflammatory cytokine production and in the maintenance of autoreactive B cell tolerance(70, 71).

1.10 Toll like receptors

A key mechanism linking the innate and adaptive immune response is via Toll Like Receptor (TLR) signalling. TLRs are pattern recognition receptors (PRRs) located on the cell membrane cells including neutrophils, monocytes, macrophages, lymphocytes, dendritic and epithelial cells. They are located at the cell membrane where they can recognise and bind signal molecules. These molecules can be derived from microorganisms such as bacteria, viruses or fungi exhibiting pathogen associated molecular patterns (PAMPs e.g. LPS, peptidoglycan, flagellin) or from dying endogenous cells bearing damage associated molecular patterns (DAMPs e.g. heat shock proteins, oxidative stress)(72).

Activation of TLRs causes downstream signalling pathways which require a variety of five adaptor proteins. The Toll/interleukin-1 receptor (TIR) domain, which is found on the cytosolic face of both the TLRs and the adaptors is the main signalling area. The four remaining adaptor proteins involved in TLR downstream signalling are as follows: myeloid differentiation primary-response gene 88 (Myd88), MyD88-adaptor-like protein (MAL), TIR-domain-containing adaptor protein inducing

interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM). Their activation eventually results in increased production of the interferon regulatory factor (IRF) family and nuclear factor kB (NF- kB)(73), ultimately leading to inflammatory cytokine release.

Two important receptors involved in recognising pathogenic ligands and maintaining host defence are TLR2 and TLR4, which predominantly bind to constituents of gram positive and gram-negative bacteria respectively(74). However, there are several studies showing that a dysregulation in these receptors can cause excess pro-inflammatory cytokines and chemokines, leading to autoimmunity, sepsis and multi-organ dysfunction(75, 76). These clinical sequelae are particularly relevant for children with DS. Infections from gram positive bacteria like *Streptococcus pneumoniae* and *Staphylococcus aureus* causing lower respiratory tract infections (LRTIs), and recurrent otitis media are more prevalent in children with DS and associated with poorer outcomes(5, 10). TLR2 is the major PRR involved in binding gram-positive bacteria and is strongly implicated in chronic inflammation. It is possible that dysregulation of this receptor and its pathways may be abnormal in DS(77). Indeed, anomalous TLR2 signalling has been associated with unregulated pro-inflammatory cytokine production and autoimmunity(78).

TLR4 is of interest as plays a key role in fighting infection, however its aberrant activation can also lead to excess pro-inflammatory cytokine release, persistent inflammation leading to septic shock and autoimmunity(79, 80). In mice with LPS-induced lung injury the benefits of utilising TLR4 monoclonal antibodies to block the receptor have been observed. There was reduced inflammation and pulmonary oedema in those who had TLR4 attenuation(81). In adults with DS and periodontal disease compared to controls without periodontitis, there was no difference in the expression of TLR2 or TLR4 single nucleotide polymorphisms (SNPs)(82).

Dysregulation of TLRs can lead to excess pro-inflammatory cytokine release and damage to tissue, consequently appropriate regulation of TLR signalling is crucial in maintaining homeostasis. There are many regulators described for TLRs, with microRNAs (miRNAs) now being described as key controllers of signals from these receptors(83). O'Neill et al(84), in their review of miRNAs and their influence on fine

tuning TLR responses, described several key miRNAs that attenuate signalling. The following have been implicated in the control and reduction of TLR responses by manipulating transcription: Mal- miR-145, MyD88 – miR-155, and TLR2 – miR-105. TLR signalling is tightly controlled to prevent persistent inflammation with many negative regulators interacting at many levels of the TLR pathways to maintain a balance. (85). These pathways may be aberrant in DS and contribute to a dysregulated immune response.

1.11 Cytokines

One of the key outputs following cell surface receptor and TLR pathway activation is cytokine production. Cytokines are proteins secreted by various cells and result in specific communications and interactions between cells of the immune system(86). A regulated system is required to prevent chronic inflammation and autoimmunity and also to ensure an appropriate response to pathogenic insults. Indeed, if there is dysregulated pro and anti-inflammatory cytokine release in the setting of infection systemic inflammatory response syndrome (SIRS) and or compensatory anti-inflammatory response syndrome (CARS) may occur, which can lead to deleterious consequences for certain patients(87). Both pro and anti-inflammatory mediators were elevated in in a murine model of sepsis with early deaths and high Interleukin- 6, Tumour necrosis factor α (TNF-α), Macrophage inflammatory protein 2 (MIP-2) , Interleukin 1 receptor antagonist (IL-1ra) predicted mortality within 24 hours(88). In adult humans with abdominal sepsis, elevated pro and anti-inflammatory mediators were also associated with increased mortality (TNF-α, Interleukin- 8, Interleukin- 10, IL-1ra)(87). In a paediatric cohort admitted to PICU with influenza (n=52), it was shown that significant early immune suppression (leucopaenia, low TNF-α) was linked to concomitant *S.aureus* infection and death(89).

A large meta-analysis (19 papers, DS n=957, Controls n=541) examining circulating cytokines in children and adults in DS concluded that TNF-α, IL-1β, IFN-γ were significantly raised in DS(90). These mediators have been implicated in the development of chronic inflammation and autoimmune disease which are more

common in our population of interest(91). Early onset Alzheimer's disease is another clinical feature of DS and IL-6 is a key cytokine associated with this neurodegenerative process(92). IL-10 serves to dampen down the inflammatory response by attenuating cytokines like IL-6 and TNF-α. There is evidence that IL-10 is elevated in DS, and it is hypothesised that the pronounced anti-inflammatory signals could be a contributor in the increased prevalence of respiratory tract infections and pneumococcal lung disease(35, 93). Overall, an excess of circulating inflammatory cytokines appears to predominate in DS.

1.12 The Inflammasome

Inflammasomes are multi-protein complexes that generate IL-1 family cytokines. Their activation results in an innate inflammatory cascade involving caspases and the cleavage of pro IL-1β and pro IL-18 to their active forms(94). The NLRP3 inflammasome has been well characterised and is associated with several medical conditions such as metabolic disorders, inflammatory bowel disease, multiple sclerosis and other autoimmune diseases(95). It is found mainly in innate immune cells, such as macrophages, dendritic cells, monocytes and neutrophils following inflammatory stimuli(96). As children with DS are reported to have significantly elevated levels of IL-1β(97), and are more prone to autoimmune conditions the inflammasome and its potential immunomodulation is potentially important target for further research in DS. Currently, there is a lack of research on the inflammasome in DS.

1.13 Complement pathway

The complement system is another critical component of innate immunity. Complement factor H (CFH) is secreted by the liver and after albumin is the most common plasma protein, and functions to inhibit conversion of C3 to C3b on the complement pathway. This results in dampening down and preventing spontaneous activation of the immune system. Deficiencies in CFH are associated with increased risk of persistent inflammation and autoimmunity(98). DS results in increased expression

of certain genes and micro RNAs (miRNAs) on chromosome 21. miRNA-155 has been shown to be significantly increased in DS, and this causes a significant down-regulation of CFH mRNA which may partly explain the increased prevalence of chronic inflammation and autoimmunity in this cohort(99).

Alzheimer's disease (AD) is extremely common in DS, occurring with an earlier age of onset than the general population. The classical complement cascade and activation of the membrane attack complex in neurons in response to amyloid beta plaque deposition has been implicated in the development of AD in DS(100). Another study examining complement and AD in DS reported that C1q, which is the initial factor in the complement pathway, was increased in neurons with activated microglia and Abeta plaque accumulation, highlighting the importance of a dysregulated complement cascade in neurodegeneration(101).

There may indeed be a fundamental problem with the complement system in DS. Sullivan et al(102) examined proteomics of blood samples from 263 people, 165 of whom had DS, and pointed towards an overall deficiency of complement factors or hypocomplementaemia C1QA, C1R, C3 and C6 were deficient. Indeed, hypocomplementaemia is associated with type 1 interferonopathies, (disorders which result from dysregulation of the type 1 interferon response, leading to significant autoinflammation(103)), and are also strongly associated with DS (104), suggesting that this persistent inflammation leads to the consumption of complement factors. Another clinical sequala associated with DS and reduced complement levels is otitis media and bacterial pneumonia(105).

1.14 Circadian Rhythm

Almost all mammals including humans display physiology and time dependant functioning over a 24-hour period, the circadian rhythm. There are several genes expressed which inherently govern this daily pattern, and they are modulated by a core transcription/translation feedback loop (TTFL). This results in a cycle between gene expression of a forward arm and a negative arm that act to negate the actions of the former. Abnormalities of this TTFL have been implicated in several disease

processes(106). The TTFL or core oscillator functionality is dependent on negative feedback circuits, and several key genes. BMAL1 and CLOCK form a heterodimeric link which stimulate transcriptional activation and the expression of the repressor proteins Period (PER) and Cryptochrome (CRY), which in turn feedback and inhibit their own transcription by disrupting the BMAL/CLOCK complex(107). Further proteins like RAR-related orphan receptor (ROR) and REV-ERB also play a role in modulating BMAL1 expression; the former activating and REV-ERBs downregulating its expression(108). The higher centre controlling the circadian rhythm is located in the suprachiasmatic nucleus of the hypothalamus. It ensures peripheral (immune cell) clock homeostasis by regulating important messengers such as glucocorticoids from the hypothalamic pituitary axis (HPA), and circulating catecholamines from the autonomic nervous system(109).

The influence and connection between immunity and the circadian rhythm has been well studied; it is now known that individual cells and components of the immune system have their own sub-cellular clockwork, and that cell counts, and cytokine levels may vary depending on the time or the stage of sleep-wake cycle. The dysregulation of the circadian rhythm has also been linked to a higher incidence of malignancy and autoimmunity(110). Further effects of disruption to circadian clocks and the immune response is discussed by Labrecque et al(111), who report that multiple cellular functions are disrupted; cellular migration, proliferation in response to pathogens, phagocytosis and cytolysis. The importance and association of an appropriate circadian rhythm and improved outcomes in sepsis has been demonstrated by Carlson et al(112). In rodents recovering from sepsis following caecal ligation and puncture, those who had significant disruption to the normal light: dark cycle had poorer outcomes versus those who did not. Given that DS is associated with higher mortality in sepsis, disturbed sleep, and potential circadian rhythm and melatonin anomalies, we wanted to examine this further by appraising genes involved in regulating this important physiological phenomenon(8, 113).

1.15 Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is a multifunctional hormone which is secreted in the pineal gland at the base of the brain(114). The effects of melatonin on regulating circadian rhythm and sleep wake cycles are well described(115, 116), however, research has now demonstrated that there is a myriad of immunomodulatory effects of this molecule, from treating chronic inflammation to adjuvant therapy in managing sepsis(117). As children with DS have poorer outcomes in sepsis and are more likely to incur autoimmunity, exploring the potential clinical utility of this agent in this cohort is worthwhile.

Melatonin has been shown to have robust antioxidant anti-apoptotic effects via prevention of caspase 3 cleavage, and anti-inflammatory actions through inflammasome deactivation(118). Other mechanisms of action that have been postulated include; binding to MT1 and MT2 cell membrane receptors resulting in a decrease of cGMP and cAMP production, and engaging nuclear receptors retinoid orphan receptors (ROR) and retinoid Z receptors (RZR) directly(119, 120). Although several immunomodulatory functions of melatonin have been described, it's potential role in mediating CD11b, TLR4 cell membrane function, as well as TLR signalling, pro and anti-inflammatory cytokine release and inflammasome deactivation has not, to our knowledge, been previously described in DS.

1.16 Sparstolonin B (SsnB)

Another potentially beneficial immunomodulator is Sparstolonin B (SsnB), a natural isocoumarin compound derived from the roots of plant species such as *Sparganium stoloniferum* and *Scirpus yagara* it has been shown to reduce inflammation(121). The molecular mechanisms by which this compound works has been described in the literature. Liang et al examined the effects of SsnB on mice macrophages; TLR2 and TLR4 induced cytokine production (IL-1β, TNF-α, IL-6) was significantly reduced following SsnB incubation, in a dose dependant manner (100uM greater effect v 10uM). The authors also showed this compound reduces general Nf-kB activity and has also been demonstrated to act as selective TLR2 and TLR4 antagonists

by preferentially limiting the association of MyD88 with TLR2 and TLR4 but not TLR9(122). Its effects on macrophages treated with LPS or Pam3csk4, was to reduce the expression of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α. In a simulated mouse model of endotoxin shock there was reduced mortality in mice treated with SsnB, and an inhibition of LPS induced cytokines IL-6 and TNF-α in lung, liver and serum(123). These findings were mirrored in another study looking at the effects on a mouse endotoxin model, where treatment with Scirpus yigara (SsnB being a key component) resulted in better outcomes in LPS sepsis and protected against cytokine production (TNF-α and IL-6) and end organ damage(124). Therefore, SsnB may have a role in modulating the response to infection and in the treatment of sepsis.

1.17 Vaccine response

From a clinical perspective, anomalous memory B lymphocytes may contribute to a varied response to vaccination in children with DS, and there are numerous papers citing suboptimal immune responses in this cohort(9, 125-127). This may have significant clinical consequences for a high-risk cohort more prone to severe RTIs and hospitalisations from vaccine preventable diseases like influenza and pneumococcus(5, 6). There is also evidence that despite initial adequate titres, the immune response may wane over time and that long-term immunity in DS may not be preserved as well as controls(128). Therefore, through public health campaigns it is imperative that immunisation against these pathogens is highlighted and delivered routinely, and that this vulnerable cohort is studied and followed over time to ensure a robust immunity is maintained. Tailored vaccination programmes may need to be considered.

1.18 Conclusion

The extent of immune dysregulation in DS is substantial, spanning the innate and adaptive systems [Figure 1.1], which contribute to a phenotype at risk of increased infections, poorer clinical outcomes and chronic inflammation. Other aspects of innate immunity may also be abnormal and contribute to the increased morbidity and warrant further interrogation such as: neutrophil and monocyte function, the

inflammasome, TLRs and their pathways. Pharmacotherapies such as pavilizumab, pneumococcal and influenza immunisations, as well as potential immunoprophylactic agents such as azithromycin and Brancho-Vaxam may help alleviate the infectious burden. Consequently, these children need to be managed with a heightened sense of awareness and urgency acutely, in the setting of sepsis, and signs of chronic inflammation need regular screening and appropriate follow up.

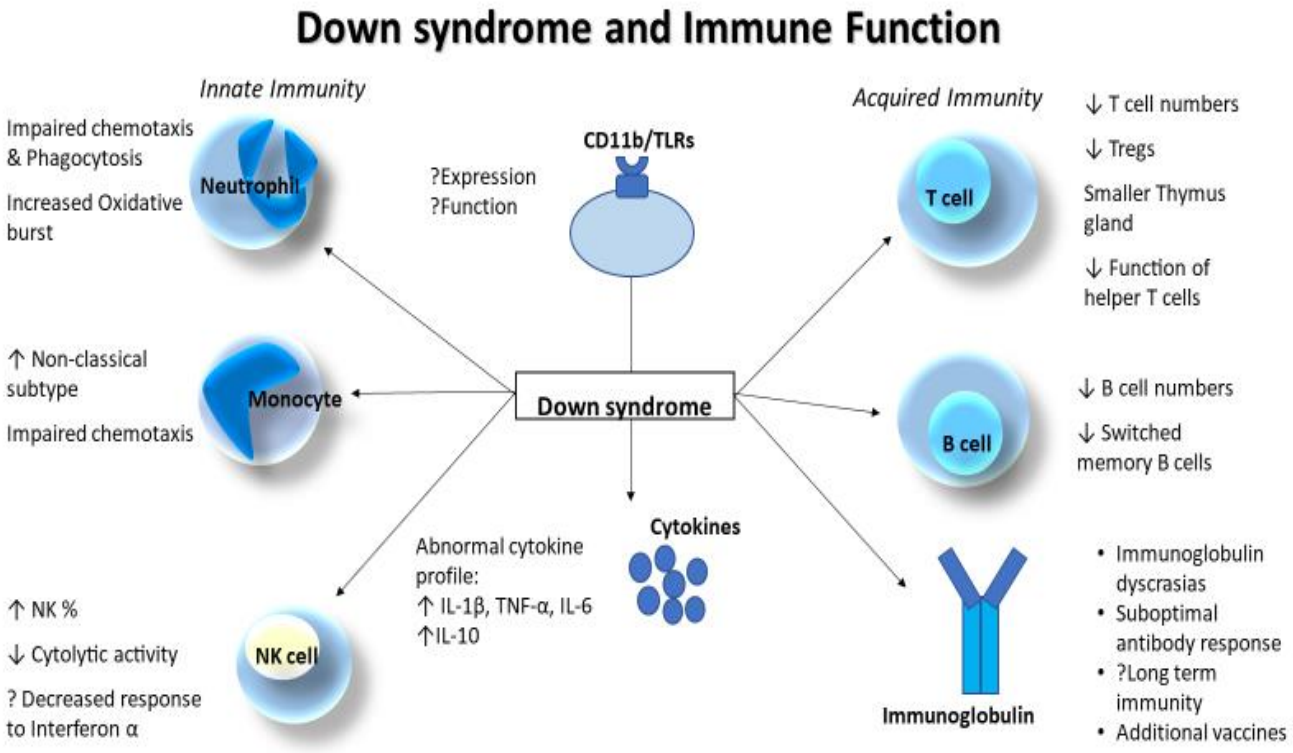


Figure 1.1 Down syndrome and immune function.

Abnormalities of the innate (Neutrophil, Monocyte and Natural killer cell) and acquired (T and B cell, and immunoglobulins) immune system in Down syndrome.

Abbreviations; NK=Natural Killer cell; TLR= Toll like receptor; TNF= Tumour necrosis factor; Tregs= Regulatory T cells.

1.19 Hypothesis

Altered inflammation in children with Down Syndrome may contribute to persistent inflammatory complications and be amenable to immunomodulation.

1.20 Aims

1.20.1 Aim 1: Improving understanding of immunodeficiency in children with Down Syndrome.

Rationale: Although Children with DS have certain immunodeficiencies described, there are aspects of their immunity which have not been examined in detail. In addition, no clinical management guidelines exist guiding clinical immunology assessment and management.

Objectives: The variation of immunodeficiencies will be defined in this population of children with DS. Perform a literature review of immunisation response and vaccination advice in DS. This will also allow guideline development and rationalisation of immunodeficiency screening and optimisation of vaccination in collaboration with national and international groups including the DS Medical Interest Group in the UK (DSMIG) and DS Ireland (DSI).

Deliverables: Clinical Immunology: a) primary immunisations given; b) incidence of respiratory tract infection (otitis media, admission for lower respiratory tract infection (LRTI)LRTI's, prescriptions of oral antibiotics for RTI's); c) other infections; d) serum IgG, IgA, IgM; e) basic lymphocyte subsets; f) vaccine specific antibody responses, namely pneumococcus, tetanus, and serotype specific antibody levels.

1.20.2 Aim 2: To assess Inflammatory responses in children with Down syndrome.

Rationale: Innate immune function and serum cytokines are abnormal in children with Down syndrome. These children are also at increased risk for auto inflammatory disorders, an important focus for research is the implication of the NLRP3 inflammasome in this condition.

Objectives: To evaluate neutrophil and monocyte function, measure pro and anti-inflammatory cytokines, in children with Down syndrome. To characterise the NLRP3 inflammasome in DS.

Deliverables: (i) Neutrophil and monocyte function: response to bacterial stimuli (LPS), through CD11b, Toll like receptor 4 (TLR-4), Toll like receptor 2 (TLR-2) expression by flow cytometry on BD FACS Canto II. (ii) Pro and anti-inflammatory cytokines: Evaluate pro- and anti-inflammatory cytokines post stimulation with bacterial endotoxin (LPS) with multiplex ELISA including the following cytokines: IL-6, IL-8, IL-18, IL-10, Tumour Necrosis Factor (TNF)-α, Interferon (IFN)-γ, Vascular Endothelial Growth Factor (VEGF), Granulocyte-Colony Stimulating Factor (G-CSF), and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) will be evaluated to quantify the systemic inflammatory response. (iii) Evaluation of NLRP3 inflammasome components and associated genes such as NLRP3, ASC, IL-1β and caspase-1 following stimulation with ATP and LPS via TaqMan® Real time PCR.

1.20.3 Aim 3: To assess the potential of immunomodulatory therapies in children with Down syndrome.

Rationale: Although children with DS have immunodeficiencies in childhood they also have a marked proinflammatory response to specific stimuli. Therefore, immunomodulation is a feasible target. Melatonin has been shown to have notable anti-inflammatory effects: reducing the effects of endotoxaemia, decreasing NLRP3 activation and proinflammatory cytokine production(129, 130). Sparstolonin B is a natural isocoumarin compound which acts as a TLR antagonist, abrogating pro-inflammatory cytokine production and reducing inflammation(121, 123)

Objectives: Immunomodulation following dose-response curves using whole blood will be performed with the TLR antagonist SsnB. Immunomodulation with SsnB and melatonin is being performed on experiments involving neutrophil and monocyte function, multiplex cytokine analysis and on the inflammasome. The potential therapeutic benefits of melatonin and SsnB will be examined.

Deliverables: To assess the potential immunomodulatory effects *in-vitro* of melatonin and SsnB on TLR4 and CD11b expression on neutrophils and monocytes, genes

involved in the inflammasome (NLRP3, ASC, IL-1β), TLR signalling (MyD88, IRAK4, TRIF), and on a broad panel of pro and anti-inflammatory cytokines (IL-2, IL-6, IL-8, IL-10, IL-1β, IL-1ra, IFN-γ, GM-CSF, VEGF, Epo).

Chapter 2: Materials and Methods

2.1 Ethical approval

This project was approved by the Ethics Committees of Children's Health Ireland (CHI) at Tallaght (formerly the National Children's Hospital, Tallaght) and CHI at Crumlin (formerly Our Lady's Children's Hospital, Crumlin) Dublin, Ireland from July 2017 to July 2020 (appendix i).

- **CHI at Tallaght:**
  This is a tertiary paediatric hospital in south Dublin. There is a dedicated clinic for children with Down syndrome with 350 patients attending annually. The majority are well patients who undergo routine follow up and review.
- **CHI at Crumlin:**
  This is the largest paediatric hospital in Ireland, with 240 inpatient beds, and 26 paediatric intensive care (PICU) beds. The cardiac team look after a large number of patients with DS, on both an elective and emergency basis, and many of whom will require a stay in PICU for management of CHD at some point.

2.2 Consent and data protection

All families and participants received verbal and documented information on the study and written consent was obtained prior to recruitment. In Tallaght, in advance of attending the dedicated DS clinic, the parents were sent out an information pack which included; an information sheet on the study, vaccination advice for children with DS, and consent forms to bring along to clinic for further discussion (appendix ii). Secondly, a face to face consultation with the patient and families took place in the outpatient department where any queries or concerns could be explored and written informed consent was taken on the day. In CHI Crumlin, a patient list of the scheduled or current inpatients with DS was sought and a face to face consultation took place, whilst giving the information leaflet out. Time was allowed for parents to decide and written consent obtained.

The consent forms were GDPR compliant and all clinical and laboratory data was completely anonymised. Clinical data and consent forms were stored in a locked filing cabinet in a secure office in Tallaght, the clinical data was then transferred to an anonymised study specific database saved on a password protected computer. Biobanked samples were anonymised and stored in a -80C freezer (Isotemp, Fischer Scientific) pending further analysis.

2.3 Clinical data

Each patient had a thorough clinical appraisal to assess the degree of co-morbidity in a systematic fashion. This was achieved by using a clinical questionnaire (appendix iii) evaluating each organ system which was used as part of the DS clinic in Tallaght. The cohorts were subdivided based on age and clinical features e.g. congenital heart disease, recurrent respiratory tract infections, or number of hospitalisations. This data was coded and anonymised and transferred to a password protected computer. Clinical outcome measures included: Gestational age and birth weight; Special care/Neonatal intensive care admission; Transfer to a tertiary surgical centre; Congenital heart disease (CHD) and type of lesion; Corrective surgery for CHD; Thyroid dysfunction; Coeliac disease; Gastrointestinal anomalies; Arthropathy; Recurrent respiratory tract infections (RRTIs); Hospitalisations due to RTI; Obstructive sleep apnoea (OSA); Requiring non-invasive ventilation (NIV); Ophthalmological abnormality; Hearing loss; Seizures; Haematological abnormality (Transient abnormal myelopoiesis, acute leukaemia); Full blood count.

2.4 Study population

2.4.1 Children with DS

Children with DS were recruited from 2 separate sites (CHI – Tallaght, and CHI-Crumlin). Those from Tallaght were clinically stable children from infancy to ≤ 16 years old attending the dedicated DS clinic. The other smaller DS cohort were children from infancy to ≤ 16 years old who were admitted to hospital on the ward or in Paediatric intensive Care (PICU) in CHI at Crumlin, with a significant CHD lesion.

2.4.2 Controls

Age-matched Paediatric healthy Controls with no significant medical history: children from infancy to ≤16 years old attending CHI-Tallaght or CHI-Crumlin for routine phlebotomy or day case elective procedures and whose results were subsequently normal. Children in both groups were excluded if they had recent fever or evidence of infection.

2.4.3 Adult controls for Sparstolonin B (SsnB) dose response

Healthy adults (age range 23-33 years; 3 female) with no recent evidence of infection or underlying inflammatory illness.

2.5 Blood sampling

Blood samples (3ml) were obtained from patients or controls and collected in a sodium citrate anti-coagulated blood bottle. Samples used for assessing neutrophil and monocyte function, cytokine levels and genes involved in toll like receptor (TLR) signalling and the inflammasome were transported to the Trinity Translational Medicine Institute (TTMI) at the St James's Hospital campus and were analysed within 2 hours of phlebotomy. Samples used for immune screening bloods; CD3+, CD4+, CD8+, CD19+, CD56+, (or T and B cell subsets) immunoglobulin M (IgM), IgG, IgA, and antibody titres to pneumococcus and tetanus, were analysed in the main immunology lab in St James's Hospital, Dublin. Full blood counts (FBC) were done in the haematology lab in Tallaght hospital, Dublin. Samples for the dose response analysis of SsnB underwent processing for flow cytometry immediately after phlebotomy.

2.6 Whole blood processing

Whole blood was incubated at 37◦C for 1 hour with various combinations of pro- and anti- inflammatory stimulants depending on the experiment: Lipopolysaccharide (LPS; *E.coli* 0111:B4: SIGMA Life Science, Wicklow, Ireland) 10 ng/mL, Pam3Cys-Ser-(Lys)4. trihydrochloride (Pam3Csk4) (TOCRIS bio-techne, Abingdon, UK) 5ng/ml, Melatonin (SIGMA Life Science, Wicklow, Ireland) at 42 μM and the TLR antagonist

Sparstolonin B (SsnB; SIGMA Life Science, Wicklow, Ireland) 10µM. After 1 hour of incubation 50μl of whole blood was aliquoted from each treatment sample into different Eppendorf tubes for further processing and analysis of neutrophil and monocyte function by flow cytometry. Next, 300µl of blood was placed into Eppendorf tubes containing 1ml of Trizol® (ThermoFisher) and stored at -80^{o}C (Isotemp, Fischer Scientific). These samples were subsequently processed to isolate RNA, cDNA and finally analysed to evaluate genes involved in TLR signalling and the inflammasome by reverse transcriptase polymerase chain reaction (RT-PCR). The remaining whole blood volume from each Eppendorf tube was then spun down in the centrifuge (Thermo Scientific, Hereaus Fresco 17) at1,500rpm for 10mins at room temperature, the serum isolated was then stored at -80◦C for subsequent analysis of pro and anti-inflammatory cytokines by enzyme linked immunosorbent assay (ELISA). Figure 2.1. Please see flow diagram of whole blood processing, Figure 2.2.

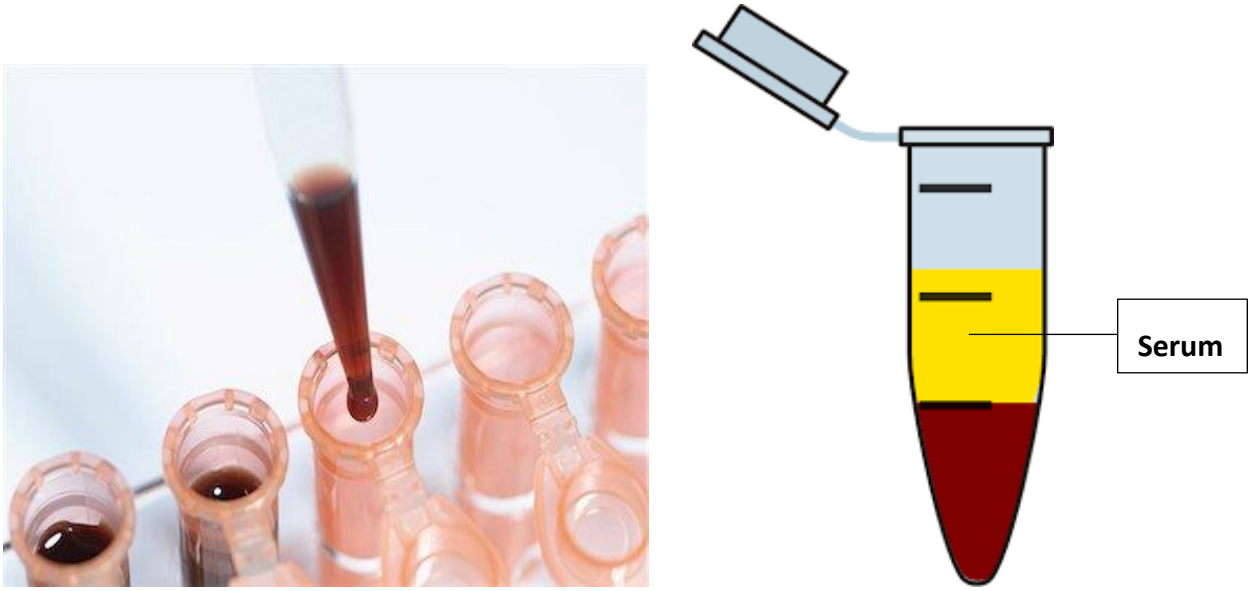


Figure 2.1 Pipetting of whole blood & isolated serum layer.

Figure 2.2 Flow diagram of whole blood processing.

2.6.1 Dose optimisation of Sparstolonin B (SsnB)

For the dose response optimisation of SsnB (SIGMA Life Science, Wicklow, Ireland), the initial concentration was 5mg, 286.6 g/mol. Three increasing concentrations (10uM, 100uM, 1000uM) of SsnB were made up using dimethyl sulfoxide (DMSO) (SIGMA Life Science, Wicklow, Ireland) as per manufacturer's instructions. 50µl of whole blood was placed into 8 Eppendorf tubes and to each one the following immunomodulators were added: Pam3Csk4 5ng/ml; SsnB @10µM; SsnB @10µM + Pam3Csk4, SsnB @ 100µM; SsnB @100µM + Pam3Csk4; SsnB @1000µM; SsnB @@1000µM + Pam3Csk4. The whole blood was then incubated for 1 hour at 37◦C and processing for flow cytometry continued as outlined below.

2.7 Flow cytometry

Flow cytometry allows the measurement of several chemical and physical characteristics of a cell by measuring variances in light scatter as cells travel in suspension through laser sensors. Cell size (forward scatter; FSC), cytoplasmic granularity (side scatter; SSC), and the intensity of fluorescent antibodies bound to the cells permit quantification of individual cells, and discrimination between subpopulations based on these factors(131). In our lab the expression of CD11b, TLR2 and TLR4 on the surface of neutrophils and monocytes, the dose response of SsnB to Pam3Csk4 stimulation, and the enumeration of gamma delta 1 t cells (γδ1), gamma delta 2 t cells (γδ2), natural killer (NK), CD19+, CD4+, CD8+ cells in DS was quantified by flow cytometry. After incubation with pro- and anti-inflammatory stimuli at 37◦C for 1 hour, blood samples were incubated with a dead cell stain (100 µL; Fixable Viability Dye eFlour 506, Invitrogen, California USA), diluted to working concentration (1/1000) in phosphate buffered saline (PBS). The following fluorochrome-labelled monoclonal antibodies (mAb) were added to each sample. Firstly, for neutrophil and monocyte CD11b, TLR2, and TLR4 expression (2.5 µL per tube): CD14-PerCP, CD15-PECy7, CD16-FITC, CD66b-Pacific Blue and TLR2-APC or TLR4-APC (BioLegend®, California, USA) and PE labelled CD11b (BD Biosciences, Oxford, UK; 10 µL per tube. Secondly for enumeration of lymphocytes: γδ1-FITC, γδ2-PE, NK-APC-Cy7, CD19-APC, CD4-PE-Cy7,

CD8-PerCp. For both experiments the following steps were carried out. PBA buffer (PBS containing 1% bovine serum albumin and 0.02% sodium azide) was used to make up the antibody cocktail. Samples were incubated with antibodies in the dark for 15 minutes. Next 1mL of FACS lysing solution (BD Biosciences, Oxford, UK) was added to each tube to lyse red blood cells, the samples were then incubated for 15 minutes in the dark. Cells were pelleted by centrifugation at 450g for 7 minutes at room temperature, washed twice with PBA buffer and fixed in 300 µL of 1% paraformaldehyde. The final cell pellet was resuspended in 100 µL PBA buffer and analysed on a BD FACS Canto II flow cytometer Figure 2.3.

Figure 2.3 BD FACS Canto II Flow Cytometer.

2.7.1 Quantification of cell surface antigen expression

Neutrophils were delineated based on SSC-A and CD66b+ positivity as previously described(30), monocytes were defined based on SSC-A, CD66b- and subsets based on relative CD14+ CD16+ populations: classical (CD14+/CD16-), intermediate (CD14+/CD16+), non-classical (CD14dim/CD16+; Figure 2.2). Lymphocytes were initially selected out based on FSC-A versus SSC-A and subpopulations were defined (figure 2.3

and 2.4) as: γδ1 (CD3+/γδ1+), γδ2 (CD3+/γδ2+), NK (CD3-/CD56+), CD19 (CD3-/CD19+), CD4 (CD3+/CD4+), CD8 (CD3+/CD8+). A minimum of 10,000 events were collated and relative expression of CD11b TLR2 and TLR4 was expressed as mean channel fluorescence (MFI), which is the mean intensity of fluorescence emitted by all cells selected and is comparable to the relative number of receptors on the surface of each cell(132). For the enumeration of innate lymphocytes cell populations were expressed as a percentage of total lymphocytes. The data was analysed using FloJo software (Oregon, USA). Every sample was processed and analysed by the same researcher (DH) thereby reducing variability in results.

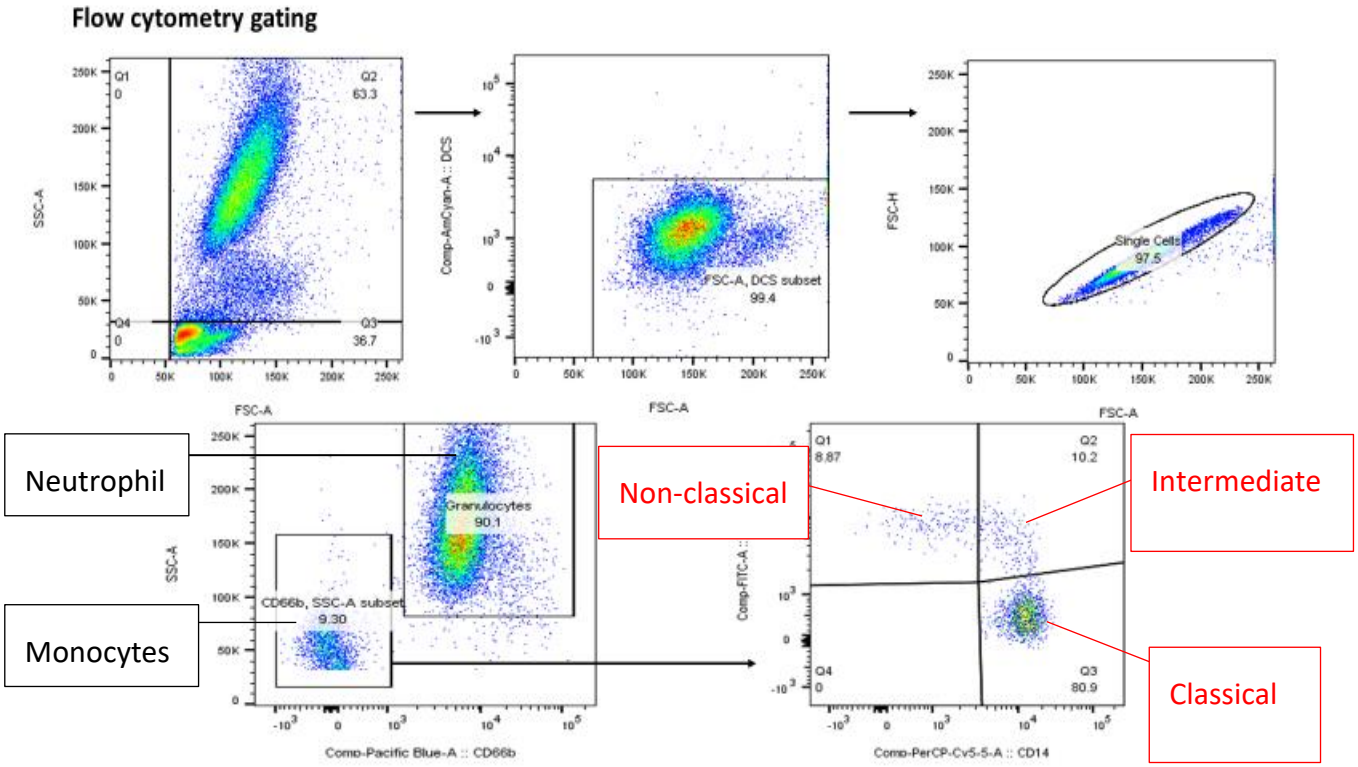


Figure 2.4 Gating strategy for isolation of granulocytes and monocyte sub-populations.

Neutrophils were delineated based on SSC-A and CD66b+ positivity. Monocytes were defined based on SSC-A, CD66b- and subsets based on relative CD14+ CD16+ populations; classical (CD14+/CD16-), intermediate (CD14+/CD16+), non-classical (CD14dim/CD16+).

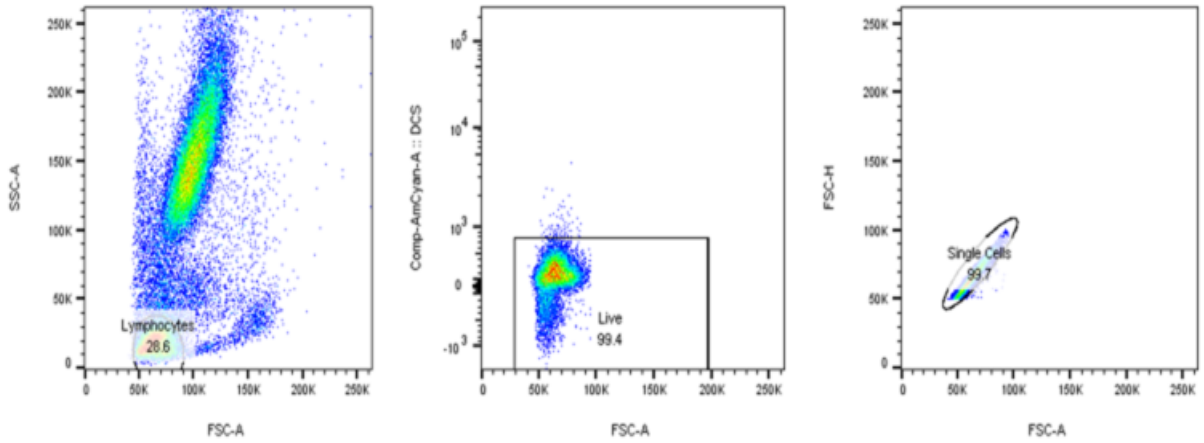


Figure 2.5 Gating strategy for delineating lymphocytes, dead cells and doublets.

Lymphocytes (FSC-A v SSC-A), Dead cells (FSC-A v DCS), Doublets (FSC-A v FSC-H).

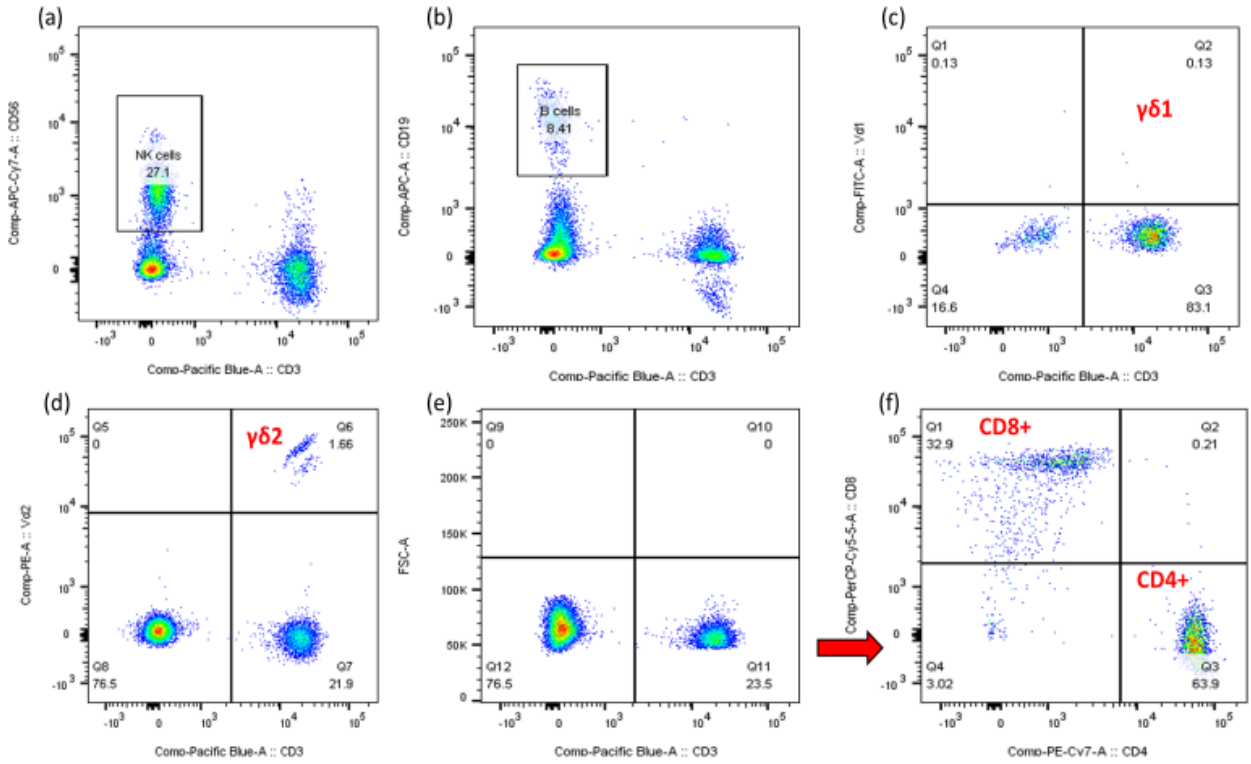


Figure 2.6 Gating strategy for delineating innate lymphocyte populations.

(a) NK cells (CD3-/CD56+), (b) B cells (CD3-/CD19+), (c) γδ1 (CD3+/γδ1+), (d) γδ2 (CD3+/γδ2+), (e) CD3 (CD3+/FSC-A), (f) CD4 and CD8 cells (CD4/CD8+).

2.8 RNA extraction, cDNA synthesis, RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) is a laboratory method involving reverse transcribing RNA into DNA (complimentary DNA or cDNA), and then amplifying the specific genetic targets using polymerase chain reaction(133). There are many significant applications of this technique, notably, in clinical microbiology RT-PCR is used in the detection and monitoring of pathogenic organisms(134). Our whole blood in Trizol samples were allowed to thaw at room temperature following removal from the -80◦C freezer. RNA extraction steps as follows were as per manufacturer's instructions (Invitrogen, USA). Firstly, 200µl of Chloroform per 1ml of Trizol was added, the sample was vortexed for 15 seconds and was incubated at room temperature for 2 – 3 minutes. This was then centrifuged at 12,000rpm at 4°C for 15 minutes to separate the RNA (aqueous phase) from DNA and protein (organic phase). The aqueous phase was then transferred to a fresh sterile RNase-free tube and 500µL of Isopropyl alcohol/1ml Trizol added. Following a third room temperature incubation step for 10 minutes, sample was then centrifuged at 4°C at 12,000 rpm for 10 minutes to isolate the RNA. The RNA pellet was washed with 75% Ethanol (minimum of 1ml/ml of Trizol used) and vortexed to dissolve, after which the sample was micro-centrifuged at 7,500rpm at 4°C for 5 minutes. Ethanol was aspirated without disturbing the RNA pellet which was then allowed to air-dry for 5 minutes to remove the last traces of Ethanol. The pellet was finally re-dissolved in 30 µl RNase-free DEPC water (3% Diethyl-pyrocarbonate) and heated at 65°C for 10 minutes. Following this, the sample was assessed for RNA purity using the NanoDrop as described below.

RNA purity and concentration were determined by using the NanoDrop ND-100 Spectrophotometer and analysed using ND-1000 Ver.3.1.2 software. This produces an absorbance curve for each sample, and RNA purity can be calculated using the ratio of absorbance at 260 and 280 nm. A ratio of ≥ 1.6 for RNA suspended in water was considered the acceptable purity level. Samples with a ratio of < 1.6 were suboptimal and indicated possible contamination with phenol or protein, these were discarded. Final RNA samples were stored in the -80◦C freezer.

2.8.1 Synthesis of cDNA from template RNA

To enable conversion of total RNA into single stranded cDNA the High Capacity Archive kit (Applied Biosystems, CA, USA) was utilised. Accordingly, 1µg of RNA is transcribed into a final volume of 40µL. A No template control (NTC) was also included ((The amount of RNA required to give 1µg was calculated and added to RNAase free water giving a total volume of 20µl.)). The volumes required to prepare the 2X RT mastermix for one sample was as per table 2.1:

Component	**µl**
10X buffer	4
25X dNTPs	1.6
10X RT Random Primers	4
Multiscribe Reverse transcriptase	2.0
Rnase-free water	8.4
Total per reaction(µl)	20

Table 2.1. Volumes of each component required for RT mastermix for each sample.

20µl of 2X master mix was combined with each sample, centrifuged briefly before placing in the thermal cycler. The programme settings were as follows; 10 minutes at 25◦C, 120 minutes at 37◦C, 5 minutes at 85◦C, then hold at 4◦C. On completion of this step the samples were stored at -80◦C (The final concentration of each sample was $1\mu g/20\mu l$ = 50ng/µl).

2.8.2 Taqman Real Time PCR®

The evaluation of gene expression was performed by Taqman® RT-PCR. Commercially available TaqMan® primer and probe combinations were used to detect expression of the following TLR signalling genes, MyD88 (NM 001172567.1), TRIF (NM_182919.3) and IRAK4 (NM_001114182.2), the following inflammasome genes, NLRP3 (NM_001079821.2), IL-1β (NM_000576.2), ASC (NM_ 013258.4), and the following circadian rhythm genes, BMAL (NM_001030272.2), Clock (NM_001267843.1), CRY (NM_004075.4), REV-ERB-α (NM_021724.4). The endogenous control selected was GAPDH (NM_002046.3). All samples were assayed in triplicate on a 384-well plate. Thermal cycling conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, and for 40 cycles, 24 seconds at 95°C and 1 minute at 60°C, using the 7900HT Fast Real-Time PCR System. Relative quantification (RQ) values were calculated using the 2-ΔΔCt method(135), (where ΔΔCt represents the threshold cycle [Ct] of the target minus that of the internal reference [ΔCt]). Therefore, the relative amount of a gene expressed in the control is equal to 1, and the expression quantities of the stimulated counterpart are expressed relative to this (e.g. 2-fold).

Real time PCR is utilised to measure gene expression and amplified DNA is quantified during the exponential phase of the PCR, allowing the amount of a particular target DNA or RNA relative to a standard to be quantified. The larger the initial concentration of target sequences in the reaction mixture the fewer the number of cycles needed to attain a particular yield of amplified product. The PCR reaction exploits the 5´nuclease activity of AmpliTaq Gold® DNA Polymerase to cleave a TaqMan® probe during PCR. The TaqMan® probe exhibits a reporter dye at the 5´ end and a quencher dye at the 3´end of the probe. During the reaction, cleavage of the probe splits the reporter dye and the quencher dye, which results in greater fluorescence of the reporter. Accumulation of PCR products is detected directly by quantifying the increase in fluorescence of the reporter dye.

The preparation of samples for RT-PCR was performed on ice in the laminar flow cabinet. Every cDNA sample was diluted with RNAase free water to give a final concentration of 5ng/µl per well. For each gene to be quantified 6 µl of sample at

5ng/µl was needed. The amount of mastermix for the quantity of samples to be analysed was prepared as per table 2.2:

Component	1x (µl)	Manufacturer
TaqMan® Universal PCR buffer mix	5.0	Applied Biosystems
TaqMan® gene expression assay	0.5	Applied Biosystems
RNase free water	2.5	Qiagen
Total	8.0	

Table 2.2. Volumes of each component needed for TaqMan assay in one well of 384-well plate.

2.9 Multiplex Enzyme Linked Immunosorbent Assay (ELISA)

To quantify a panel of pro and anti-inflammatory cytokines we employed a Multiplex ELISA technique. ELISA involves a double sandwich antibody; with one attached to the well providing immune specificity and antigen capture, while another antibody is bound to a detector enzyme and an amplification factor, permitting accurate quantification of the cytokine in question. We utilised chemiluminescence multiplex ELISA technology with our assay, which allows detection of multiple cytokines at one time by having several specific capture antibodies coated on corresponding points on a microplate. A proprietary tag is linked to the detection antibody which emits a signal once excited(136).

The following cytokines; tumour necrosis factor alpha (TNFα), interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), interferon gamma (IFN-γ), interleukin 18 (IL-18), vascular endothelial growth factor (VEGF), erythropoietin (Epo), interleukin 1

receptor antagonist (IL-1ra), and interleukin 10 (IL-10) were analysed using a custom-made MSD®MULTI-SPOT assay plate from Mesoscale (MSD Diagnostics, USA). Extracted peripheral blood serum, was transferred to a 96 well MSD plate and these cytokines were assessed as per manufacturer's instructions. The plate was then analysed on the SECTOR Imager and validated (Meso Scale Discovery, Rockville, MD, USA; www.meso-scale.com). The limits of detection for the individual assays were within expected ranges.

Firstly, the individual U plex-Coupled antibody solutions were prepared. The U plex plate was prepared by creating the U-plex Coupled Antibody Solutions. 200µl of each individual biotinylated antibody was coupled to 300 µl of a unique linker. The appropriate linker was used for each antibody. This mixture was vortexed and incubated at room temperature for 30 minutes. 200 µl of Stop Solution was then added, vortexed and incubated at room temperature for 30 minutes. The multi plex coating solution was prepared by adding 600µl of each U-Plex coupled antibody solution into a single tube and vortexed. Up to 10 antibodies were pooled. The antibody solution with the same liner were not combined. 50µl of multiplex coating solution was added to each well. The plate was sealed with an adhesive plate seal and incubated with shaking at room temperature for an hour. The plates were washed 3 times with 150µl /well of 1xMSD wash buffer solution. The plate was then coated and ready to use.

To prepare the assay, 25µl of Diluent 43 was added to each well. The plate was tapped gently on all sides. 25µl of the prepared Calibrator solution was added to the calibration wells and 25µl of serum samples were added to each serum well. The plate was sealed by an adhesive seal and incubated at room temperature with shaking for 1 hour. The plate was washed 3 times with MSD Wash Buffer. 50µl of detection antibody solution was added to each well. The plate was sealed by an adhesive seal and incubated at room temperature with shaking for 1 hour. Read Buffer was added to each well. The plate was analysed on an MSD instrument. The results were displayed in picograms/ml and analysed on GraphPad prism software (www.graphpad.com).

2.10 Statistical analysis

2.10.1 Sample size and power calculation

The sample size estimation and statistical analysis plan for the project were designed with the statistical support of CSTAR. In this observational study, we anticipated recruiting around 100 children with DS and 60 paediatric controls. Using the rule of thumb of 15 patients/predictor this will allow us to evaluate 10 Predictors. Many of the cytokines and markers of neutrophil function have not been studied in this population so we cannot calculate a sample size for all parameters. We have statistical support from University College Dublin, and Colin Kirkham research officer from the Rotunda Hospital. In total we recruited 165 children with DS and 60 controls.

2.10.2 Statistical analysis.

Data was analysed with SPSS version 25 (IBM USA), GraphPad Prism, and Microsoft Excel. A Chi-square test was employed to compare categorical variables and assess equality of proportions. Continuous/ nominal results were first evaluated for normality of distribution by generating and inspecting histograms. The Kolmogorov-Smirnov and Shapiro-Wilk tests of normality were also employed to confirm whether the data was normally distributed. For comparison of normally distributed data, the Student's t-test was used to appraise mean values between two independent groups. Non-normal data was interrogated with a non-parametric test: the Mann-Whitney U test. These tests were performed in assessing CD11b, TLR2, TLR4, cytokine levels, genes involved in TLR signalling and the inflammasome between children with DS and controls.

Logistic regression was used to evaluate the possible relationship between a binary categorical variable or outcome (children with DS who were hospitalised or did not require hospitalisation due to a RTI) and independent variable(s) which can be categorical or continuous (WCC – low or normal, or exact WCC value). This gives the probability of requiring hospitalisation due to a RTI (at some point) for any given WCC. The exponentiated B is the odds ratio per unit increase or decrease in the explanatory variables. For significant variables the 95% confidence interval does not cross 1. Multiple logistic regression was performed to appraise several explanatory variables

and their relationship with a binary outcome of interest. This also controls for the independent effect of each of those variables controlling for the others. In all cases, a p value threshold of 0.05 was considered statistically significant.

Chapter 3: Altered endotoxin responsiveness in children with Down syndrome

3.1 Introduction

Down syndrome (DS) is caused by an extra copy of genetic material from chromosome 21, and is the most prevalent chromosomal abnormality, affecting approximately 1 in 550 births in Ireland(137), and 1 in 700 births in the USA(1). Co-morbidities associated with DS include developmental disability, congenital heart disease (CHD), gastrointestinal tract anomalies, and an increased risk of haematological malignancy(3). In addition, it is the most common genetic syndrome associated with abnormal immune function and immune defects(4). There is significant evidence of immune dysregulation in Down syndrome including T-cell and B-cell lymphopenia due to impaired expansion of these cell lines in infancy(17), a smaller thymus gland with reduced naïve T cell and regulatory T-cell numbers(138), suboptimal antibody responses to vaccination(33, 125, 127, 128), and abnormal levels of serum cytokines(35, 90, 139).

Children with Down syndrome are, therefore, at increased risk of infection, especially in early childhood, particularly respiratory tract infections (10). Hilton *et al.*(5) reported a higher risk of admission to hospital and intensive care with respiratory tract infections (RTIs) in children with DS. Mortality from sepsis is 30% greater in patients with DS in comparison to children without DS who also had sepsis(8).

It is challenging to attribute causation to a specific deficit of the immune system with the increased incidence of infections and sepsis seen in this cohort. A normal innate immune system is crucial in providing first line defence against infection. Neutrophils and monocytes are crucial cellular components of the innate immune system. Defective phagocytic activity and neutrophil chemotaxis have previously been reported in DS (42, 44). Monocyte function in DS is poorly described. Increased numbers of the non-classical (CD14dim/CD16+) monocyte pro-inflammatory sub-type have been described in DS in comparison to controls(51). This monocyte population has previously been implicated in sepsis and chronic disease(49).

CD11b is a cell surface marker involved in mediating neutrophil and monocyte adhesion and diapedesis(66) and is an indicator of activation. Dysfunction in neutrophil adherence and migration has been shown to increase the risk of infection in adults and neonates(67). Toll-like receptor 4 (TLR-4) is the key receptor involved in lipopolysaccharide (LPS) endotoxin recognition and activation of the innate immune system(140), and has also been implicated in the pathogenesis of autoimmune conditions such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (141).

Immunomodulators can alter responses to infection, alleviate autoimmunity and ultimately improve patient care. Melatonin is an endogenous hormone which mediates its anti-inflammatory effects by modulating pro-inflammatory cytokines and inflammasome de-activation, thereby ameliorating results in endotoxaemia (129, 142). Melatonin has a very good safety profile and is used in paediatrics in sleep management (116). Clinical trials in adults and neonates with sepsis have demonstrated improved clinical outcomes(143, 144).

3.2 Hypothesis

We hypothesized that children with DS have altered neutrophil and monocyte function which contributes to their increased susceptibility to infection and increased mortality from sepsis.

3.3 Aims

We aimed to evaluate the *in vitro* effect of LPS endotoxin, and the anti-inflammatory melatonin on CD11b and TLR4 expression on neutrophils and monocytes in children with DS.

3.4 Results

3.4.1 Patient characteristics & Experimental method

There were 23 healthy children with Down syndrome (DS) with a mean $\pm$SD age of 8.67 $\pm$ 4 years(y) of which 13 were female (57%), and 21 healthy controls with a mean age of 7.4 $\pm$ 4.60 y, of which 10 were female (48%). In the DS cohort, children with a history of significant congenital heart disease requiring surgery in infancy (n=7) were all clinically stable with no further cardiology intervention. All control participants had no significant medical history. Both groups were well at the time of blood sampling with no recent history of infection.

Whole blood samples were treated with LPS (10 ng/ml), Melatonin ($42\mu M$), and LPS plus melatonin for 1 hour. Samples were processed for flow cytometry as described in chapter 2 and CD11b and TLR4 expression on neutrophils and monocytes was quantified. Values were expressed as mean channel fluorescence (MFI).

3.4.2 Effects of LPS endotoxin on CD11b expression

Neutrophil baseline CD11b expression in children with DS was significantly lower compared with controls (p=0.045). Following incubation with LPS, CD11b significantly increased in both groups (Figure 3.1(a): DS p<0.0001; Control p=0.0001). When comparing the fold increase in CD11b expression from baseline, children with DS had a significantly higher rise after LPS stimulation [DS: Controls: 116% versus 62.4%; p=0.03; Figure 3.2(a)].

CD11b expression on total monocytes showed no difference at baseline or after LPS stimulation between both groups [Figure 3.1(b) p=0.48]. The percentage rise of CD11b expression after LPS was similar in both groups also [DS versus Control: 53 v 55%; p=0.92 Figure 3.2(b)]. Monocyte subset CD11b expression analysis revealed no significant differences at baseline or after LPS stimulation in classical (CD14+/CD16-) (p=0.74), and non-classical (CD14dim/CD16+) (p=0.21) sub-populations in children with DS versus controls [Figures 3.1(c)(e) & 3.2(c)(p=0.55)] (e)(p=0.56)]. Intermediate monocytes (CD14+/CD16+) demonstrated no difference in CD11b expression at baseline in children with DS and controls (p=0.87). After LPS stimulation there was a significant increase in CD11b in children with DS (p=0.004) but not in controls [p=0.78;

Figure 3.1(d)]. The mean percentage rise in CD11b expression in children with DS was not significantly increased compared to controls [DS vs controls: 31.8 v 5.8%; p=0.088; Figure 3.2(d)].

Classical monocytes (CD14+/CD16-) exhibited the highest CD11b expression at baseline compared with the other sub-populations in both cohorts (DS vs Control - classical vs intermediate: p=0.009 v 0.01). This sub-population also demonstrated the largest mean percentage rise in CD11b after LPS treatment in both children with DS and controls. (DS – classical % rise vs intermediate vs nonclassical = 52.1 vs 31.8 vs 15.3%; Controls - 44 vs 5.8 vs 24%). Non-classical monocytes (CD14dim/CD16+) demonstrated the lowest mean CD11b expression at baseline of any sub-population, in both children with DS and controls. This was significantly lower compared to both intermediate and classical monocyte CD11b in both cohorts.

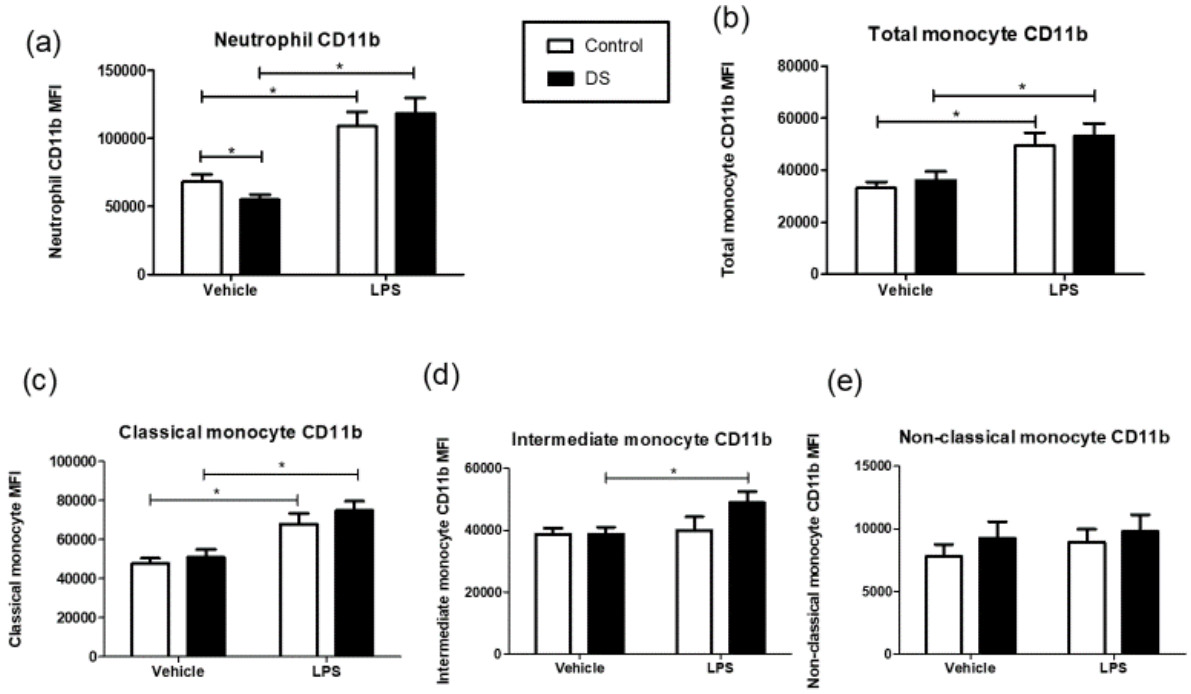


Figure 3.1: Reduced neutrophil CD11b and LPS hyperresponsiveness on intermediate monocytes in whole blood of children with DS compared to controls.

Whole blood was incubated with LPS (10 ng/ml) for 1 hour. Samples processed for flow cytometry and CD11b expression on neutrophils and monocytes quantified. Values expressed as mean channel fluorescence (MFI). Unpaired t-test (mean +/- SEM). *p < 0.05. (a) Neutrophil CD11b (DS n=23; Controls n=16) (b) Total monocyte CD11b (DS n=19; Controls n=21) (c) Classical monocyte CD11b (DS n=19; Controls n=21; (d) Intermediate monocyte CD11b (DS n=18; Controls n=20); (e) Non-classical monocyte (DS n=19; Controls n=21).

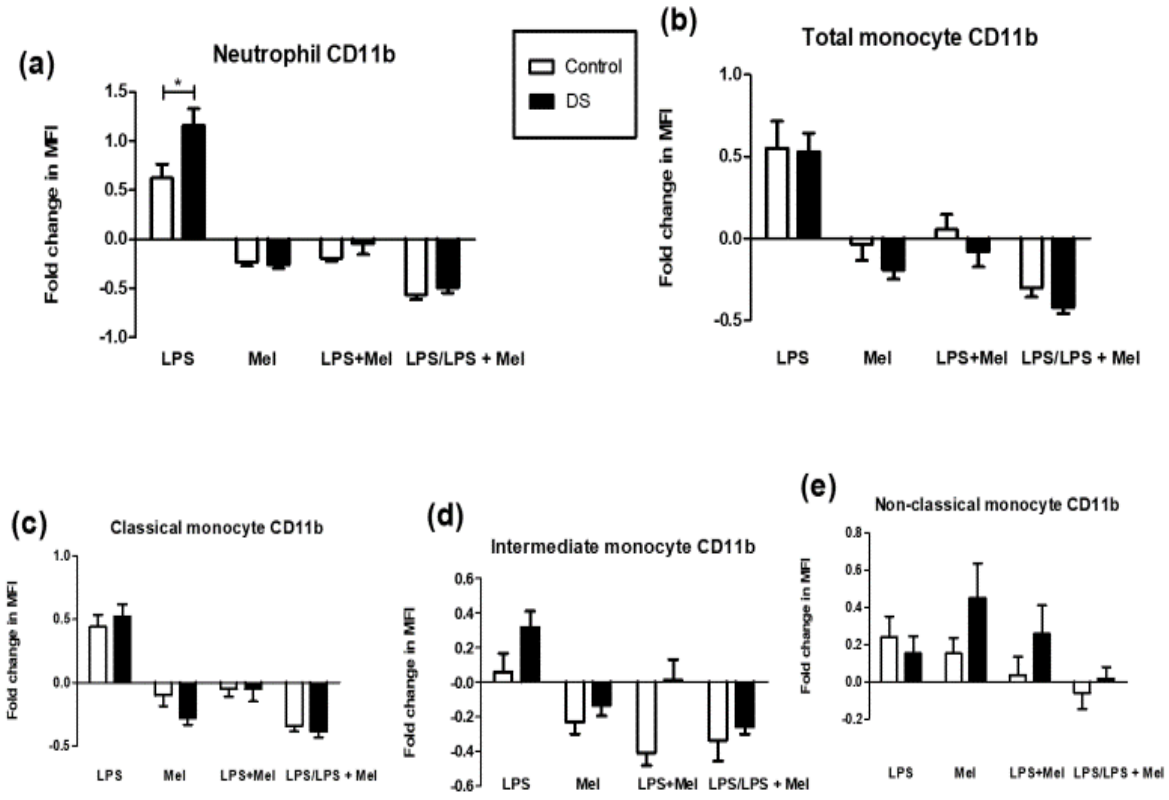


Figure 3.2: Melatonin reduces neutrophil and monocyte CD11b in children with DS and controls.

Whole blood samples were treated with Lipopolysaccharide (LPS) (10 ng/ml), Melatonin (Mel) ($42\mu M$), Lipopolysaccharide and melatonin (LPS+Mel) for 1 hour. Samples processed for flow cytometry and CD11b expression on neutrophils and monocytes quantified. Values expressed as fold changes in mean channel fluorescence (MFI). Unpaired t-test (mean +/- SEM). p* <0.05. (LPS/ LPS + Mel) = Lipopolysaccharide versus Lipopolysaccharide and melatonin. (a) Neutrophil CD11b (DS n=23; Controls n=16); (b) Total monocyte CD11b (DS n=19; Controls n=21); (c) Classical monocyte CD11b (DS n=19; Controls n=21); (d) Intermediate CD11b (DS n=18; Controls n=20); (e) Non-classical monocyte CD11b (DS n=19; Controls n=21).

3.4.3 Effects of LPS endotoxin on TLR4 expression

Neutrophil TLR-4 expression at baseline was not significantly different between children with DS compared to controls (p=0.57). After LPS incubation there was no significant response in TLR4 expression in either cohort [DS p=0.15 v Control p=0.057; Figure 3.3(a)]. On comparing the mean percentage rise in TLR-4 expression after LPS, there was a 9.4% rise in children with DS versus 28.7% in the control group [Figure 3.4(a)(p=0.23)].

TLR-4 expression on total monocytes did not show any difference at baseline between children with DS and controls (p=0.24). TLR-4 expression post LPS treatment increased significantly in controls (p=0.016) but did not reach significance in the children with DS [p=0.07; Figure 3.3(b)]. The mean percentage rise after LPS stimulation was 8.4% in children with DS versus 17.2% in controls [(p=0.2)) Figure 3.4(b)]. Monocyte subset TLR-4 expression analysis revealed no significant differences at baseline or after LPS treatment in classical (CD14+/CD16-) or intermediate (CD14+/CD16+) subpopulations between children with DS and controls [Figures 3.3(c)(p=0.51) (d)(p=0.4) & 5(c)(p=0.75) (d)(p=0.84)]. Non-classical monocyte (CD14dim/CD16+) TLR-4 expression was found to be significantly higher at baseline in children with DS compared to controls [p=0.02; Figure 3.3(e)]. There were no significant differences in TLR-4 expression after LPS stimulation in either cases or controls [Figure 3.4(e) (p=0.96)].

The classical monocytes in both cohorts exhibited the largest mean percentage rise in TLR-4 expression after LPS treatment (DS – classical vs intermediate vs non-classical = 15.2 vs 3.6 vs 9.9%; Control = 17.9 vs 1.2 vs 10.7%). Intermediate monocytes had the largest mean TLR-4 MFI at baseline of any monocyte subpopulation in both children with DS and the control group (DS v control: intermediate vs classical p=0.003 versus 0.005). Non-classical monocytes displayed the lowest mean TLR-4 at baseline of the three monocyte subsets and was significantly lower than intermediate monocyte TLR-4 in both cohorts.

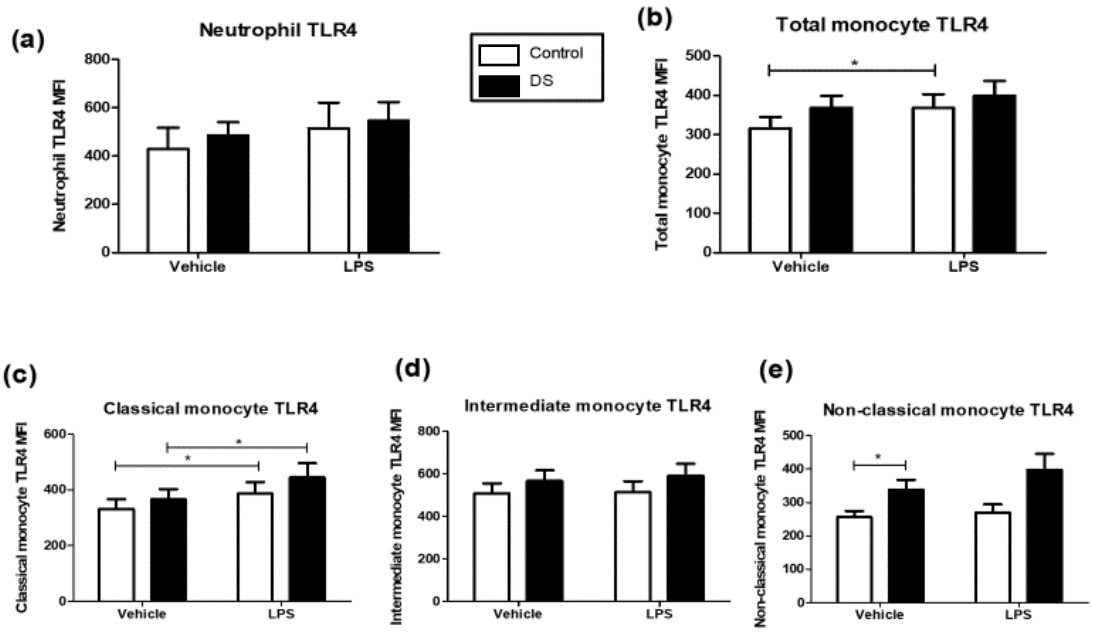


Figure 3.3: Increased non-classical monocyte TLR4 expression in children with DS compared to controls.

Whole blood was incubated with LPS (10 ng/ml) for 1 hour. Samples processed for flow cytometry and TLR4 expression on neutrophils and monocytes quantified. Values expressed as mean channel fluorescence (MFI). Unpaired t-test (mean +/- SEM). *$p < 0.05$. (a) Neutrophil TLR4 (DS n=19; Controls n=10); (b) Total monocyte TLR-4 (DS n=22; Controls n=15); (c) Classical monocyte TLR4 (DS n=16; Controls n=15); (d) Intermediate monocyte TLR4 (DS n=15; Controls n=14); (e) Non-classical monocyte TLR-4 (DS n=16; Controls n=20)

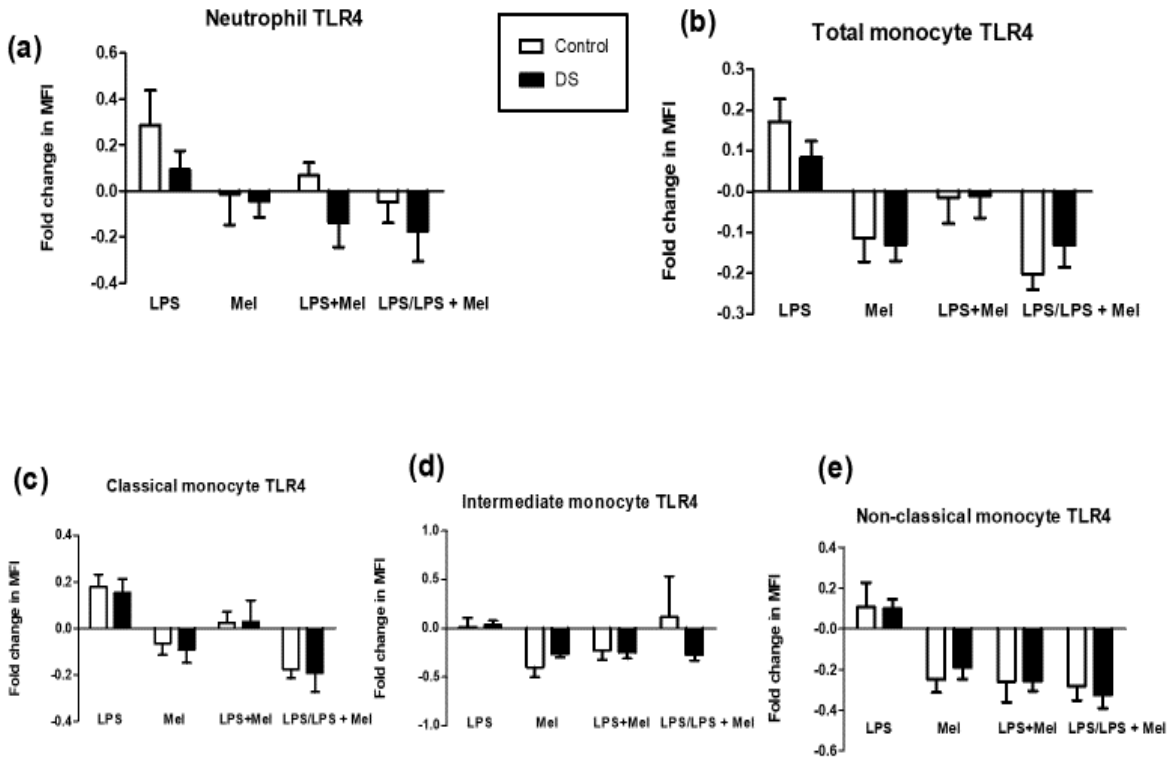


Figure 3.4: Melatonin reduces TLR4 expression on monocytes in children with DS and controls.

Whole blood samples were treated with Lipopolysaccharide (LPS) (10 ng/ml), Melatonin (Mel) ($42\mu M$), Lipopolysaccharide and melatonin (LPS+Mel) for 1 hour. Samples processed for flow cytometry and TLR4 expression on neutrophils and monocytes quantified. Values expressed as fold changes in mean channel fluorescence (MFI). Unpaired t-test (mean +/- SEM). (LPS/ LPS + Mel) = Lipopolysaccharide versus Lipopolysaccharide and melatonin. (a) Neutrophil TLR-4 (DS n=19; Controls n=10); (b) Total monocyte TLR-4 (DS n=22; Controls n=15); (c) Classical monocyte TLR-4 (DS n=16; Controls n=15); (d) Intermediate monocyte TLR-4 (DS n=15; Controls n=14); (e) Non-classical monocyte TLR-4 (DS n=16; Controls n=20).

3.4.4 Effects of melatonin on CD11b expression

Neutrophil CD11b expression decreased significantly after melatonin treatment in both cohorts (DS p=<0.0001; Controls p=<0.0001), compared with baseline the mean percentage fall in CD11b expression was 25.8% in children with DS versus 23.1% in controls (p=0.63)). There were no differences in mean percentage fall in CD11b expression when comparing LPS treated samples and those treated with LPS and melatonin in both cohorts [Figure 3.2(a)(p=0.64)].

Total monocyte CD11b expression reduced significantly after melatonin incubation in children with DS (n=12; p=0.02), but not in the control group (n=17; p=0.12). The mean percentage fall in CD11b MFI was 19% in children with DS versus 3.4% in controls [Figure 3.2(b)(p=0.24)]. In classical and intermediate monocytes there were significant decreases in CD11b expression from baseline after melatonin in both cohorts) DS (p=0.001); Control (p=0.05); (d) DS (p=0.02); Control (p=0.03)]. Non-classical monocytes (CD14dim/CD16-) showed a significant increase in CD11b expression after melatonin in children with DS (p=0.03), and in controls but not to a significant level in the latter (p=0.1). The mean percentage rise in CD11b expression after melatonin was 45% in children with DS versus 15.3% in controls [Figure 3.2(e) (p=0.12)].

3.4.5 Effects of melatonin on TLR-4 expression

Neutrophil TLR-4 expression showed no significant change after melatonin treatment in either group. The mean percentage fall in TLR-4 expression was 4.4% in children with DS and 1.3% in controls (p=0.82). Comparing LPS and LPS + melatonin treated samples there was a 17.5% mean reduction in TLR-4 expression on neutrophils of children with DS compared to a fall of 4.8% in controls (p=0.48), Figure 3.4(a).

Total monocyte TLR-4 was significantly reduced after melatonin incubation in both groups (DS p=0.03; Controls p=0.05). The average percentage fall in TLR-4 expression after melatonin treatment was 13% in children with DS versus 11.4% in controls [Figure 3.4(b) (p=0.81)]. Monocyte subset analysis of melatonin on TLR-4 expression showed no significant reduction in either group [Figure 3.4(c) (p=0.74) (d) (p=0.23) (e) (p=0.52)].

3.5 Discussion

Neutrophil CD11b expression at baseline was significantly lower in children with DS compared with controls. Following LPS treatment children with DS upregulated CD11b, and this was significantly greater than controls. Novo *et al.*(44) reported that, at baseline, CD11b expression on neutrophils was not significantly different between children with DS (n=12) and controls, although the smaller numbers and older population in this study may contribute to these findings. Our research suggests that although the level of CD11b may be lower under normal conditions, after contact with endotoxin there is an increased ability to activate and mobilise neutrophils in response to this stimulus. Neutrophils in children with DS may be hyper-responsive to endotoxin, which may have detrimental effects in the setting of sepsis. Adults with sepsis and renal injury in the absence of hypotension, have been shown to have increased activation of neutrophils with upregulation of CD11b(145), worsening prognosis. Furthermore, neutrophil mediated lung injury in sepsis, and multi-organ dysfunction (MODS) have been associated with increased CD11b expression on these cells(146, 147). A blockade of this receptor could have potential benefits in these clinical contexts(43). In paediatric studies LPS hyper-responsiveness has been demonstrated through increased CD11b expression on neutrophils and monocytes of neonates with encephalopathy (148, 149), these infants having developed significant immune dysregulation. Zhou et al(150) examined TLR4 signalling and the CD11b response on polymorphonuclear cells in mice. The authors concluded that TLR4 mediates CD11b upregulation and is key for PMN activation in response to LPS. Further correlation between CD11b and TLR4 has been described by Guang et al(151) who reported that CD11b mediates TLR4 signalling and trafficking in a cell specific manner in dendritic cells and macrophages, having a crucial role in balancing the innate and adaptive response to LPS. It appears that the two receptors are inter-linked and have important regulatory roles on one another in initiating the innate immune response.

Zhang *et al.* (152) demonstrated that mice deficient in CD11b exposed to *Mycobacterium tuberculosis* developed more severe granulomas, higher leucocyte recruitment and elevated pro-inflammatory cytokines. This demonstrates the immunomodulatory effect neutrophil CD11b expression exerts on the host response to

infection. A persistent inflammatory response can be seen in autoimmunity and there is a higher prevalence in DS, recent studies suggest that reduced CD11b is associated with chronic inflammation in SLE and lupus nephritis(70, 153). Neutrophil CD11b is also decreased in septic shock and correlated with poorer outcomes (154, 155). In this context, the increased incidence of both autoimmunity and sepsis in DS is particularly noteworthy(8, 156). We demonstrated that melatonin caused a predominant decrease in CD11b expression in both cohorts; Figure 4. We also showed that children with DS exhibited a hyper-responsive CD11b response to LPS in neutrophils Figure 4(a). In the acute setting of sepsis/SIRS an upregulation of CD11b may be associated with deleterious effects(157), furthermore, a positive correlation between CD11b expression and the degree of systemic inflammation has been described(68), making melatonin a potential adjunct in acute sepsis/SIRS.

The classical monocyte (CD14+/CD16-) accounts for the largest proportion of monocytes (80-85%) and its main functions include antigen presentation and phagocytosis(47). We found classical monocytes exhibited significantly higher CD11b expression at baseline, and greater fold increases in CD11b after LPS than other monocyte sub-populations in both groups. This sub-group also displayed the largest rise in TLR-4 after LPS compared with other monocyte subpopulations in both cohorts. This suggests that classical monocytes are significantly pro-inflammatory with the largest CD11b and TLR4 response to LPS than any other sub-population. Regarding differential CD11b expression on monocyte subsets Tak *et al*(158) reported no significant differences, whereas another study examining differential *in vivo* activation of monocyte subsets reported the most significant rise in CD11b on the intermediate monocyte(159). However, these studies(158, 159) characterised CD11b expression after lower doses of LPS with longer incubations in an adult *in vivo* setting as compared to our study which was undertaken in a paediatric cohort. Monocyte CD11b was highest on classical and intermediate monocytes(46).

Intermediate monocytes (CD14+/CD16+) are elevated in the setting of acute illness such as sepsis in children(160). In our study, there was a significant rise in CD11b expression after LPS stimulation in children with DS but not in controls on intermediate monocytes. This adds to the evidence that there are hyper-responsive

elements to the innate immune system in children with DS. Indeed, intermediate monocytes produce significant quantities of TNF-α once activated(46). Previous studies have demonstrated elevated levels of TNF-α in patients with DS compared with healthy controls(90) at baseline. Intermediate monocytes demonstrated the greatest TLR-4 at baseline compared with other monocytes in both groups which has also been demonstrated in adults (49).

Non-classical monocytes (CD14dim/16+) have been implicated in both acute and chronic disease and have a pro-inflammatory phenotype with increased production of IL-1β and TNF-α(47). This monocyte sub-group had significantly lower CD11b and TLR-4 expression in both groups at baseline. Furthermore, non-classical monocytes demonstrated a relative hypo-responsiveness to LPS versus the other sub-populations. Boyette *et a*l(46) assessed the phenotype, function, and differentiation monocyte subsets, and reported that non-classical monocytes had the lowest CD11b MFI and that there was the smallest response in this subset following TLR-4 stimulation. We found baseline TLR-4 expression was significantly raised in children with DS versus controls. The TLR-4 response plays a significant role in fighting infection but may also be responsible for the dysregulated inflammation seen in septic shock(80). Williams *et al.* noted an increased mortality in mice with polymicrobial sepsis who exhibited early up-regulation of TLR-4, and improved survival in those with suppressed TLR gene expression(76). Suppression of TLR-4 activation, pro-inflammatory cytokine release, and developing endotoxin tolerance is important in limiting the adverse effects of sepsis. Furthermore, a failure of this protective negative feedback process may contribute to increased mortality in sepsis(161).

We demonstrated that melatonin has an anti-inflammatory influence on innate immune function by reducing CD11b expression on neutrophils and total monocytes in children with DS and controls, thereby inhibiting neutrophil and monocyte activation and migration. Although there is a paucity of literature on the effect of melatonin on CD11b, Alvarez-Sanchez et al reported a reduction in CD11b in melatonin-treated mice(162). A significant reduction in TLR-4 expression only occurred in total monocyte populations. Melatonin may act as a TLR-4 antagonist and may be modulated via TLR-4 mediated inflammatory genes through molecule myeloid differentiation factor 88

(MyD88)-dependent and TRIF-dependent signalling pathways(163), thereby attenuating inflammation.

Melatonin has beneficial immunomodulatory effects in the setting of sepsis by inhibiting mitochondrial dysfunction and inflammation, reducing nitrosative and oxidative stress(144). Melatonin has a robust antioxidant or free radical scavenging activity of (164, 165) and melatonin administration also impairs NF-κB transcriptional activity, reducing pro-inflammatory cytokine (IL-1β, TNF-α, IFN-γ) release and inhibiting activation of the NLRP3 inflammasome(166). Melatonin improved survival and clinical outcomes in neonates versus controls in sepsis (143, 167, 168). We have demonstrated that the immunomodulatory effects of melatonin in sepsis can also be broadened to include reducing neutrophil and monocyte activation.

Melatonin increased CD11b expression on non-classical monocytes and to a significant level in the children with DS. However, it has been shown that melatonin can have pro-inflammatory actions in response to endotoxaemia. Effenberger et al(169) reported that melatonin enhanced the general immune response following LPS treatment. Melatonin may have differing actions on distinct cell lines, with the pro-inflammatory non-classical monocyte being preferentially activated. Further evaluation of the immunomodulatory properties of melatonin in children with DS will allow assessment of its potential as a therapeutic agent.

3.6 Conclusion

This research highlights important differences in the innate immunity of children with DS versus age-matched controls. To our knowledge this has not been studied previously in this population. Children with DS have an increased response to LPS in neutrophils and intermediate monocytes, while also having elevated TLR-4 expression on non-classical monocytes compared to controls. These variations may be a contributory factor in a heightened/dysregulated innate immune response, which may have deleterious effects, leading to the worse outcomes seen in sepsis in these children. Lastly, melatonin could represent a useful clinical adjunct in the treatment of sepsis as an immunomodulator and our study suggests its anti-inflammatory effects also influence neutrophil and monocyte function.

Chapter 4: Altered Toll-Like Receptor Signalling in children with Down syndrome

4.1 Introduction

Down syndrome (DS) is caused by extra genetic material from chromosome 21 and is the most common of the chromosomal abnormalities affecting 1 in 700 births in the USA (1). It is associated with a myriad of health complications including, developmental delay, congenital heart disease, gastrointestinal atresias, acute leukaemia and obstructive sleep apnoea (137). Immune dysregulation is another important feature of DS such as reduced T and B lymphocyte counts (10, 30), altered serum cytokines (35, 90), and suboptimal antibody responses to immunisation (125, 126, 128). However, there are key aspects of the immune system that, to our knowledge, have not been examined in this cohort previously. In the last chapter we examined cell surface receptors CD11b and TLR4, here we evaluate another important Toll like receptor (TLR2) and assess some of the associated inflammatory signalling pathways.

Toll like receptors and their activation are crucial in initiating the innate immune response, while also linking the adaptive response to infection (73). Activation of these TLRs initiates downstream signalling pathways and recruitment of a constellation of adaptor proteins; myeloid differentiation primary-response gene 88 (MyD88), MyD88-adaptor-like protein (MAL), IL-1R associated kinase (IRAK) family and TIR-domain-containing adaptor protein inducing interferon-β (TRIF), which in turn stimulate downstream kinases (JNK, ERK, MAPKs), leading to nuclear translocation of an increase in the transcription factors, including nuclear factor kappa B (NF-kB) and interferon regulatory factor-3 (IRF-3), ultimately leading to production of pro-inflammatory cytokines(170). Strict regulation of these TLR pathways is crucial in achieving protection from infection but also avoiding damage from excess cytokine production which can lead to worse outcomes, acutely in sepsis, or in chronic inflammation resulting in autoimmunity, both of these sequelae occurring more frequently in DS (8, 171).

TLR2 is involved in detecting bacterial infection and in chronic inflammation and is located at the cell membrane, where it recognises and binds signal molecules. These molecules derived from microorganisms such as bacteria, viruses or fungi exhibiting pathogen associated molecular patterns (PAMPs e.g. peptidoglycan, lipoproteins, lipoarabinomannan, lipoteichoic acid, zymosan, and glycolipids) or from dying endogenous cells bearing damage associated molecular patterns (DAMPs e.g. endogenous DAMPs, heat shock protein (HSP)70, Snapin, and Hyaluronic acid)(72, 172). Although lipopeptides from gram positive bacteria and commercial TLR2 agonists such as Pam3Cys-Ser-(Lys)4-3HCL (Pam3Csk4)(173) are classically associated with activation of this receptor, there is evidence that lipopolysaccharide (LPS) endotoxin can also produce robust increases in TLR2 expression (174, 175). Both *in vivo* and *in vitro* experiments have demonstrated significant increases in monocyte TLR2 after LPS treatment (174).

The significance of TLR2 and its affinity for constituents of gram-positive bacteria is of particular relevance in DS as they are more prone to infection from this group of pathogens. Children with DS are at increased risk of infections, have greater risk of admission to hospital with respiratory tract infections (RTIs), where they are more likely to have a protracted stay and require intensive care support (5). Gram positive bacteria such as *Streptococcus pneumoniae* and *Staphylococcus aureus* are the causative organisms in many of these cases, with the former also a significant contributor to recurrent otitis media with effusion (OME), which often leads to conductive hearing loss in these children(10). Aggressive longstanding periodontitis resulting from persistent infection and chronic inflammation is seen regularly in this population (176). Elevated TLR2 expression is found in oral mucosa of patients with chronic periodontitis compared to controls (177, 178).

Dysregulated TLR2 signalling causes autoinflammation due to unchecked pro-inflammatory cytokine release. This can lead to several autoimmune and inflammatory conditions such as rheumatoid arthritis, systemic lupus erythematosus, atherosclerosis, and sepsis (78). Children with DS are more susceptible to similar conditions like arthropathy, thyroid and coeliac disease (179), and have increased mortality from sepsis (8). CD11b is a cell surface receptor which indicates activation

and mediates neutrophil and monocyte diapedesis and adhesion (66). Abnormal neutrophil migration and adherence has been linked to an increased risk of infection in neonates and adults (67). Therefore, developing and utilising TLR antagonists or inhibitors of these pathways may prove to be of clinical benefit. Sparstolonin B (SsnB), is a natural isocoumarin compound which is derived from the roots of plant species such as *Sparganium stoloniferum* and *Scirpus yagara* and has been shown to reduce inflammation (121). This compound acts as a selective TLR2 and TLR4 antagonist by preferentially limiting the association of MyD88 with TLR2 and TLR4 and reduces general NF-kB activity (122). SsnB has the potential to abrogate pro-inflammatory cytokine release and macrophages treated with LPS or Pam3Csk4 have reduced expression of pro-inflammatory cytokines such as Interleukin 1β (IL-1β), Interleukin 6 (IL-6), Tumour necrosis factor alpha (TNF-α) (123).

4.2 Hypothesis

We hypothesized that TLR signalling may be abnormal in children with DS and that altered TLR2 contributes to a clinical phenotype at increased risk of gram-positive infection, chronic inflammation and autoimmunity.

4.3 Aims

We aimed to evaluate TLR pathways in 3 ways; by determining the expression of TLR2 on the surface of neutrophils, monocytes, and their subsets; examine gene expression of key regulatory proteins involved in TLR signal propagation, MyD88, IRAK4, and TRIF; and lastly to determine cytokine production at baseline and following immunomodulation with pro-inflammatory stimuli (LPS, Pam3Csk4) and the anti-inflammatory agent SsnB.

4.4 Results

4.4.1 Patient characteristics & Experimental method

There were 20 children with Down syndrome (DS) with a mean ±SD age of 8.79 ± 5.27 years (y) of which 11 were female, and 15 controls with a mean age of 6.22 ± 4.22 y, of which 5 were female. In the DS cohort, children with a history of significant congenital heart disease requiring surgery in infancy (n=8) were all clinically stable with no further cardiology intervention. Both groups were afebrile at the time of blood sampling with no recent history of infection.

Whole blood was incubated with LPS (10 ng/ml), Pam3Csk4 (5ng/ml), LPS + Pam3Csk4, SsnB (10μM), LPS + SsnB, Pam3Csk4 + SsnB, and LPS + Pam3Csk4 + SsnB for 1 hour. Samples were processed for flow cytometry as described in chapter 2, and CD11b and TLR2 expression on neutrophils and monocytes quantified. Values expressed as mean channel fluorescence (MFI).

4.4.2 Monocyte subset analysis

Regarding enumeration of monocyte subsets; although children with DS had a higher percentage of intermediate (DS v control; 5.2%: v 4.3%; p=0.16) and non-classical (DS v control; 18.3% v 11.5%; p=0.06) subpopulations than controls, there were no statistically significant differences between them. The percentage of classical monocytes was lower in children with DS compared to controls (DS v control; 76.4% v 84.2%; p=0.048).

4.4.3 TLR2 expression

Neutrophil TLR2 expression was higher in children with DS compared with controls at baseline (p=0.02). After LPS incubation there was no change in TLR2 expression in either cohort [DS p=0.25 v Control p=0.75; Figure 4.1(a)]. TLR2 expression on total monocytes was also raised at baseline in children with DS versus controls (p=0.04). TLR2 expression post LPS treatment increased in the DS cohort but not in controls [DS p=0.0002; Control p=0.12; Figure 4.1(b)].

Monocyte subset analysis of TLR2 revealed greater expression on intermediate (p=0.01) and non-classical monocytes (p= 0.048) in children with DS when compared to

controls at baseline. Classical monocyte TLR2 was higher in children with DS but did not quite reach significance (p=0.06). [Figure 4.1 (c)-(e)]. There were no rises in TLR2 expression in either cohort post LPS treatment. Intermediate monocytes had the largest mean TLR2 MFI at baseline of any monocyte subpopulation in both children with DS and the control group (DS v DS: control v control: intermediate v classical p=0.001: p=0.005). Non-classical monocytes displayed the lowest mean TLR2 at baseline of the three monocyte subsets and was lower than intermediate monocyte TLR2 in both cohorts [Figure 4.1].

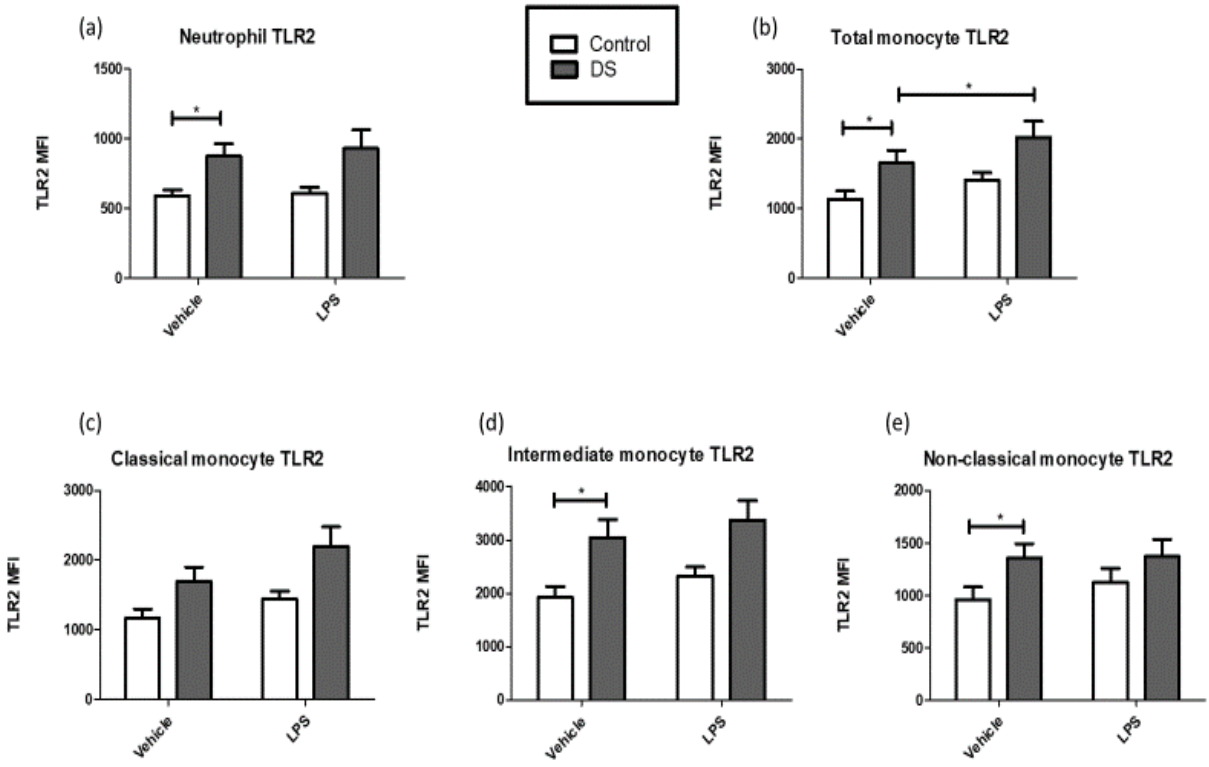


Figure 4.1: Increased neutrophil and monocyte TLR2 in children with DS compared to controls.

Whole blood was incubated with LPS (10 ng/ml) for 1 hour. Samples processed for flow cytometry and TLR2 expression on neutrophils and monocytes quantified. Values expressed as mean channel fluorescence (MFI). Unpaired t-test (mean +/- SEM). *$p<0.05$. Children with DS (n=20), controls (n=15) (a) Neutrophil TLR2; (b) Total monocyte TLR2 (c) Classical monocyte TLR2; (d) Intermediate monocyte TLR2; (e) Non-classical monocyte TLR2.

4.4.4 Effect of Pam3Csk4 and LPS on TLR2 expression

After incubation with Pam3Csk4 and Pam3Csk4 and LPS in combination, there were no rises in TLR2 expression on neutrophils in either cohort [DS p=0.64 v Control p=0.73; Figure 4.2(a)]. TLR2 expression on total monocytes was raised after treatment with LPS and LPS plus Pam3Csk4 in both groups [DS: LPS p=0.002; LPS + Pam3Csk4 p=0.0009; Control: LPS p=0.003; LPS + Pam3Csk4 p=0.0001; Figure 4.2(b)]. Treatment with Pam3Csk4 in isolation did not result in any increase in TLR2 in either cohort. The effect of incubating with both Pam3Csk4 and LPS resulted in higher TLR2 expression than Pam3Csk4 or LPS alone on total monocytes in controls but not in the DS group [v Pam3Csk4: DS p=0.57; Control p=0.0004; v LPS: DS p=0.14; Control p=0.002; Figure 4.2(b)].

Monocyte subset analysis of TLR2 expression revealed significant rises on classical monocytes (CD14+/CD16-) after Pam3Csk4 incubation in the DS group but not controls, [DS p=0.002; Control p=0.22; Figure 4.2(c)] and following Pam3Csk4 plus LPS treatments in both groups [DS p=0.0006; Control p=0.02; Figure 4.2(c)]. Again, the combination of pro-inflammatory stimulants resulted in significant increases in TLR2 expression compared with Pam3Csk4 treatment alone in both cohorts [DS p=0.006; Control p=0.0006; Figure 4.2(c)], however, in comparison to LPS treatment alone, only the control group exhibited significantly greater TLR2 expression (p=0.01).

Intermediate monocyte (CD14+/CD16+) analysis showed no significant TLR2 response following Pam3Csk4 alone. Treating with Pam3Csk4 plus LPS caused significant rises in both cohorts [DS p=0.007; Control p=<0.0001; Figure 4.2(d)]. After comparing TLR2 expression following treatment with both TLR agonists to incubation with Pam3Csk4 alone there was only a significant increase in the control group [DS p=0.32; Control p=0.002]. Comparing dual treatment to single LPS incubation there were rises in TLR2 but not to significance in either group [DS p=0.08; Control p=0.08; Figure 4.2(d]).

Non-classical monocyte (CD14dim/CD16+) TLR2 rose significantly in response to Pam3Csk4, and both treatments in controls [p=0.004; p=0.005 respectively; Figure 4.2(e)], but only in response to Pam3Csk4 plus LPS in children with DS [p=0.59;

p=0.03]. When comparing the TLR2 rise following both treatments to Pam3Csk4 alone there were no significant differences. In comparison with LPS treatment alone, dual incubation resulted in significantly greater rises in TLR2 in both cohorts [DS p=0.02; Control p=0.01; Figure4.2(e)].

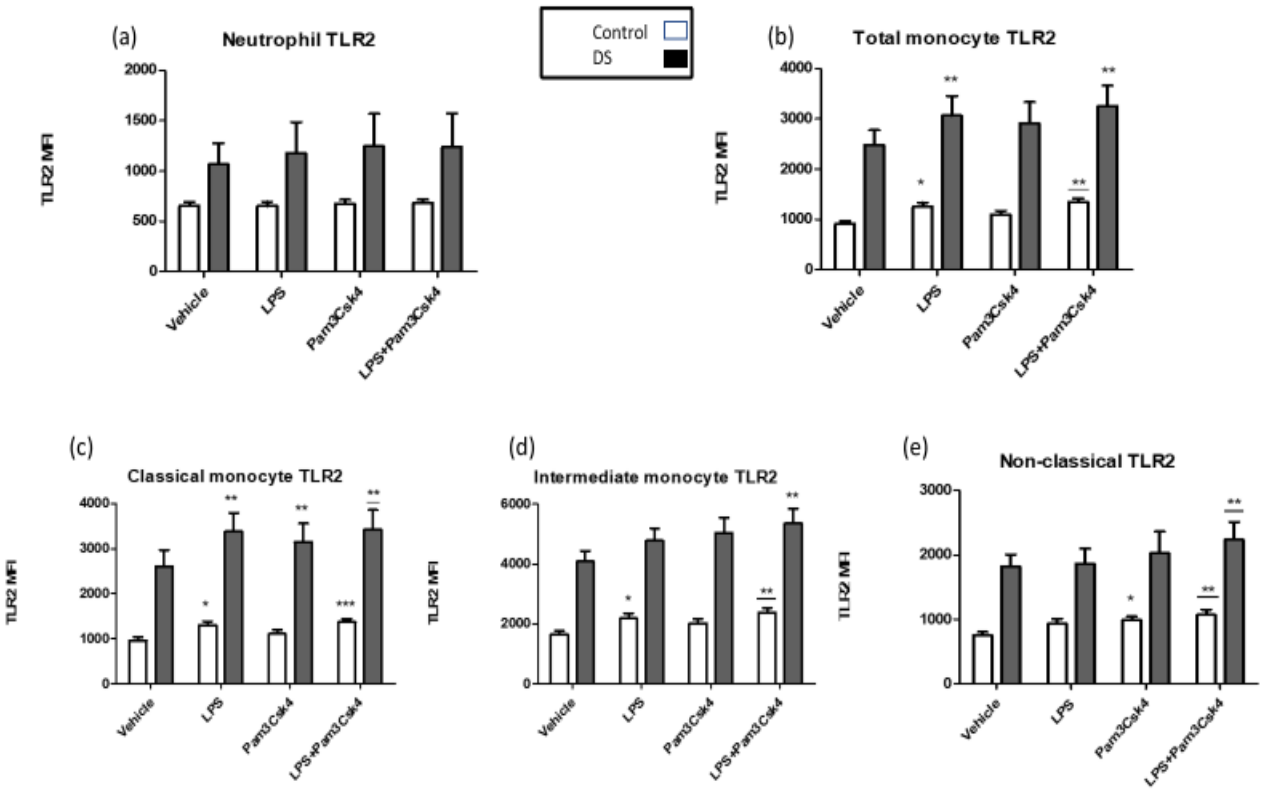


Figure 4.2. LPS and Pam3Csk4 together elicit greater TLR2 expression on monocytes in children with DS and controls.

Whole blood was incubated with LPS (10 ng/ml) and Pam3Csk4 (5ng/ml) for 1 hour. Samples processed for flow cytometry and TLR2 expression on neutrophils and monocytes quantified. Values expressed as mean channel fluorescence (MFI). Unpaired t-test (mean +/- SEM). Children with DS (n=7), controls (n=11). (a) Neutrophil TLR2; (b) Total monocyte TLR2 *p < 0.05 vs vehicle control; **p <0.05 vs vehicle in their respective cohort; ** p <0.05 vs vehicle and Pam3Csk4 in respective cohort; (c) Classical monocyte TLR2 *p < 0.05 vs vehicle control; **p <0.05 vs vehicle in their respective cohort; ** p <0.05 vs vehicle and Pam3Csk4 in respective cohort; ***p <0.05 vs vehicle, LPS and Pam3Csk4 in respective cohort; (d) Intermediate monocyte TLR2 *p < 0.05 vs vehicle control; **p <0.05 vs vehicle in their respective cohort; ; ** p <0.05 vs vehicle and Pam3Csk4 in respective cohort; (e) Non-classical monocyte TLR2 *p <0.05 vs vehicle control; ** p <0.05 vs vehicle and LPS in respective cohort.

4.4.5 Effect of Pam3Csk4 and LPS on CD11b

There were rises in CD11b after Pam3Csk4, LPS, and LPS plus Pam3Csk4 incubation on neutrophils in both cohorts [Pam3Csk4; DS p=0.04; Control p=0.03; LPS; DS p=0.004; Control p≤0.0001; LPS+Pam3Csk4; DS p=0.0004; Control p≤0.0001; Figure 4.3(a)]. Treatment with both stimulants resulted in higher CD11b than with Pam3Csk4 alone in both groups [DS p=0.002; Control p=0.0009].

Total monocyte CD11b increased after all treatments in both cohorts [Pam3Csk4; DS p=0.03; Control p=0.008; LPS; DS p≤0.0001; Control p≤0.0001; LPS+Pam3Csk4; DS p=0.0003; Control p≤0.0001; Figure 4.3(b)], and treatment with both LPS and Pam3Csk4 resulted in higher CD11b expression than with either Pam3Csk4 [DS p≤0.0001; Control p=0.0002] or LPS alone [DS p=0.03; Control p=0.03] also in both groups.

Monocyte subset analysis revealed that classical monocytes demonstrated similar significant increases in CD11b to all treatments in both groups [Pam3Csk4; DS p=0.02; Control p=0.02; LPS+Pam3Csk4; DS p=0.0006; Control p=<0.0001; Figure 4.3(c)], and incubation with both LPS and Pam3Csk4 caused significantly higher expression than with Pam3Csk4 alone in both cohorts [DS p=0.007; Control p=0.0007]. There were no significant changes in CD11b in response to LPS or Pam3Csk4 on intermediate monocytes in both cohorts [Figure 4.3(d)]. Treatment with Pam3Csk4 resulted in significantly raised CD11b on non-classical monocytes in both groups [DS p=0.01; Control p=0.01; Figure 4.3(e)], and both LPS and Pam3Csk4 incubation yielded significantly higher CD11b expression than vehicle and LPS treated samples in controls only (p=0.008; p=0.03; Figure 4.3(e)].

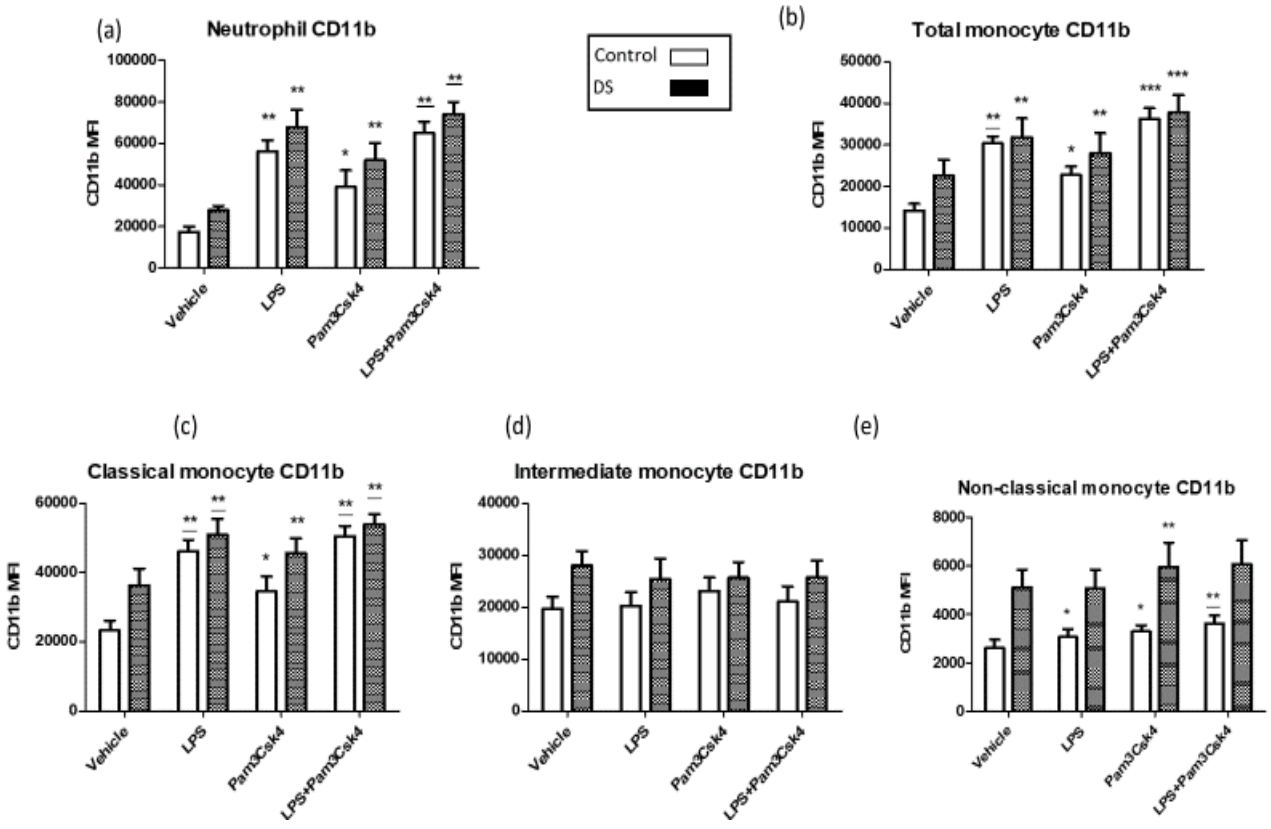


Figure 4.3. Neutrophil and monocyte CD11b expression increased in response to LPS and Pam3Csk4 in children with Down syndrome and controls.

Whole blood was incubated with LPS (10 ng/ml) and Pam3Csk4 (5ng/ml) for 1 hour. Samples processed for flow cytometry and CD11b expression on neutrophils and monocytes quantified. Values expressed as mean channel fluorescence (MFI). Unpaired t-test (mean +/- SEM). Children with DS (n=7), controls (n=11). (a) Neutrophil CD11b *p < 0.05 vs vehicle control; **p <0.05 vs vehicle in their respective cohort; ** p <0.05 vs vehicle and Pam3Csk4 in respective cohort;; (b) Total monocyte CD11b *p < 0.05 vs vehicle control; **p <0.05 vs vehicle in their respective cohort; ** p <0.05 vs vehicle and Pam3Csk4 in respective cohort; ***p <0.05 vs vehicle, LPS and Pam3Csk4 in respective cohort; (c) Classical monocyte CD11b *p < 0.05 vs vehicle control; **p <0.05 vs vehicle in their respective cohort; ** p <0.05 vs vehicle and Pam3Csk4 in respective cohort; (d) Intermediate monocyte CD11b; (e) Non-classical monocyte CD11b *p <0.05 vs vehicle control; **p <0.05 vs vehicle in their respective cohort; ** p <0.05 vs vehicle and LPS in respective cohort.

4.4.6 Effect of SsnB on TLR2 expression

The TLR antagonist SsnB was used to assess its potential as an immunomodulator after stimulation with LPS, Pam3Csk4, and both TLR agonists together. Regarding TLR2 expression on neutrophils, there was no reduction of TLR2 in samples treated with SsnB plus Pam3Csk4 and SsnB plus LPS in children with DS and controls [Pam3Csk4: DS p=0.076; Control p=0.78; LPS: DS p=0.12; Control p=0.73 Figure 4.4(a)]. In total monocytes SsnB reduced TLR2 following incubation with LPS plus Pam3Csk4 in controls but not in children with DS [DS p=0.23; Controls p=0.001; Figure 4.4(b)]. There was also a rise in TLR2 on total monocytes in response to LPS plus Pam3Csk4 in controls. There were no reductions in TLR2 expression following SsnB plus LPS and SsnB plus Pam3Csk4 in both groups. [LPS+SsnB: DS p=0.15; Control p=0.08; Pam3Csk4+SsnB: DS p=0.25; Control p=0.18 Figure 4.4(b)].

Monocyte subset analysis revealed that SsnB significantly decreased TLR2 expression on classical monocytes (CD14+/CD16-), after stimulation with LPS (p=0.04) and LPS plus Pam3Csk4 (p=0.02) in controls but not in children with DS, [Figure 4.4(c)]. There was a significant increase in TLR2 following incubation with LPS plus Pam3Csk4 in controls. Again, there was a decrease in TLR2 expression in children with DS after treatment with SsnB but not to a significant level [LPS+SsnB p=0.1; Pam3Csk4+SsnB p=0.15; LPS+PamCsk4+SsnB p=0.14; Figure 4.4(c)]. Intermediate monocyte (CD14+/16+) TLR2 was decreased following SsnB treatment with Pam3Csk4, and Pam3Csk4 plus LPS in both cohorts but not quite reaching statistical significance [Pam3Csk4+SsnB: DS p=0.12; Control p=0.06; LPS+Pam3Csk+SsnB: DS p=0.07; Control p=0.08; Figure 4.4(d)]. The effect of SsnB on the non-classical monocyte (CD14dim/CD16+) was to significantly reduce TLR2 expression after incubation with LPS in controls but not in the DS group [DS p=0.34: Controls p=0.03; Figure 4.4(e)]. There were reductions in TLR2 in both cohorts, (more marked in the control group) after SsnB and Pam3Csk4, and Pam3Csk4 plus LPS, but not quite to significance in the control group [Pam3Csk4+SsnB; DS p=0.53; Control p=0.16; LPS+Pam3Csk+SsnB: DS p=0.37; Control p=0.07; Figure 4.4(e)].

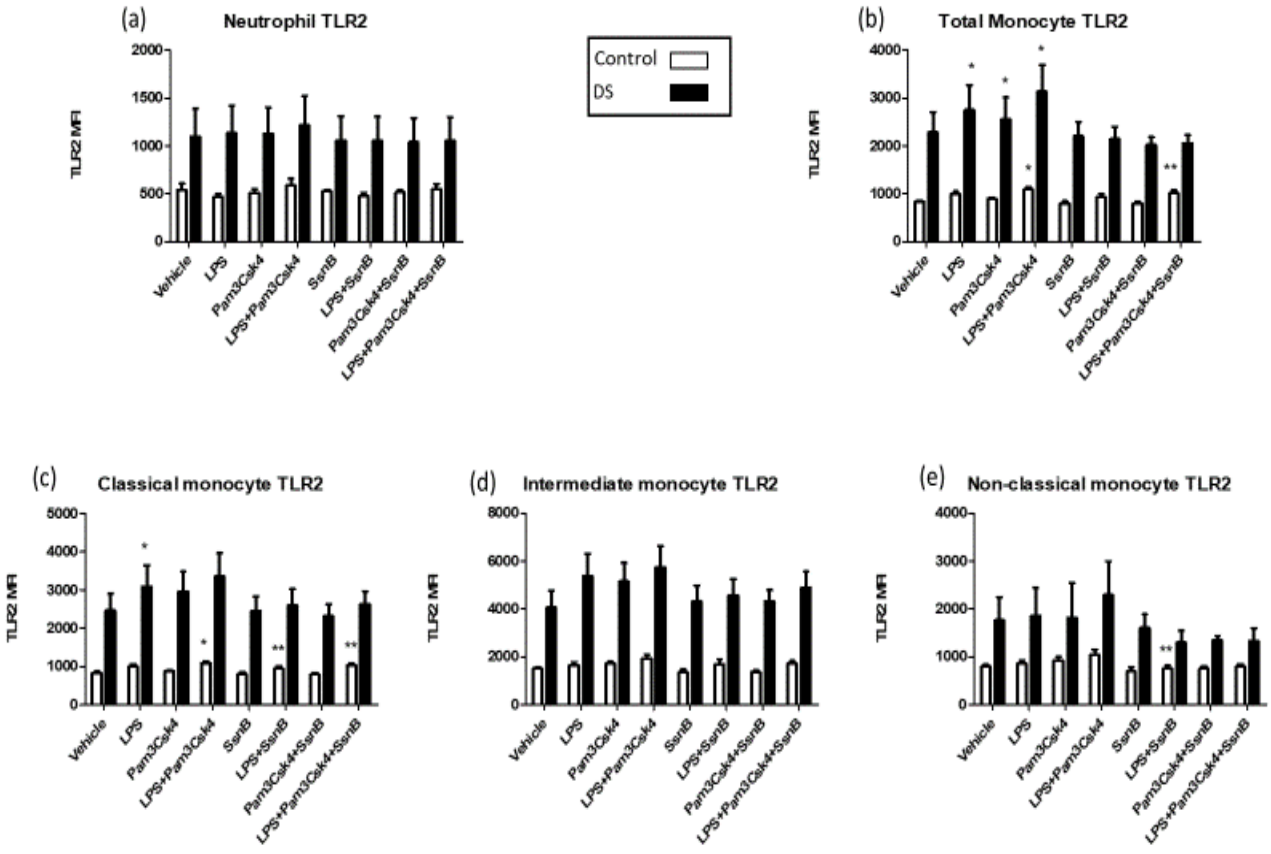


Figure 4.4. SsnB reduces TLR2 expression in response to LPS and LPS plus Pam3Csk4 on monocytes in controls.

Whole blood was incubated with LPS (10 ng/ml), Pam3Csk4 (5ng/ml), LPS + Pam3Csk4, SsnB (10µM), LPS + SsnB, Pam3Csk4 + SsnB, and LPS + Pam3Csk4 + SsnB for 1 hour. Samples processed for flow cytometry and TLR2 expression on neutrophils and monocytes quantified. Values expressed as mean channel fluorescence (MFI). Unpaired t-test (mean +/- SEM). Children with DS (n=5), Controls (n=5). (a) Neutrophil TLR2; (b) Total monocyte TLR2 *p < 0.05 vs vehicle in respective cohort; **p <0.05 vs LPS+Pam3Csk4 in respective cohort; (c) Classical monocyte TLR2 *p < 0.05 vs vehicle in respective cohort; **p <0.05 vs LPS and LPS+Pam3Csk4 in respective cohort; (d) Intermediate monocyte TLR2; (e) Non-classical monocyte TLR2 **p <0.05 vs LPS in their respective cohort.

4.4.7 Effect of SsnB on CD11b expression

CD11b expression was reduced on neutrophils following treatment with SsnB plus LPS (p=0.02) and after incubation of LPS with Pam3Csk4 (p=0.004) in the control group, there were also rises in CD11b following treatment with LPS (p=0.05) and LPS and Pam3Csk4 (p=0.02) in this group. In children with DS there was no change in CD11b on neutrophils following SsnB treatment, [LPS+SsnB p=0.07; LPS+Pam3Csk4 p=0.1; Figure 4.5(a)]. On Total monocytes there was a decrease in CD11b after LPS and SsnB in controls (p=0.01), there was also a rise in CD11b post LPS incubation (p=0.009). In children with DS there was a fall in CD11b after SsnB and Pam3Csk4 treatment (p=0.04), in both cohorts there was no decrease after SsnB and both pro-inflammatory stimulants. [Figure 4.5(b)].

Monocyte subset analysis revealed a trend downwards for CD11b on classical monocytes (CD14+/CD16+) in both cohorts following treatment with SsnB and the pro-inflammatory modulators, although not significantly in either group [DS LPS+SsnB p=0.06; Control p=0.17; DS Pam3Csk4 + SsnB p=0.14; Control p=0.17; DS LPS+Pam3Csk4+SsnB p=0.09; Control p=0.17; Figure 4.5(c)]. Intermediate monocytes (CD14+/16+) exhibited the lowest change in CD11b expression following SsnB in both groups, with no significant changes noted [Figure 4.5(d)]. In non-classical monocytes (CD14dim/CD16+), there was a significant reduction in CD11b expression following SsnB, LPS and Pam3Csk4 incubation in both groups [DS p=0.05; Control p=0.03; Figure 4.5(e)].

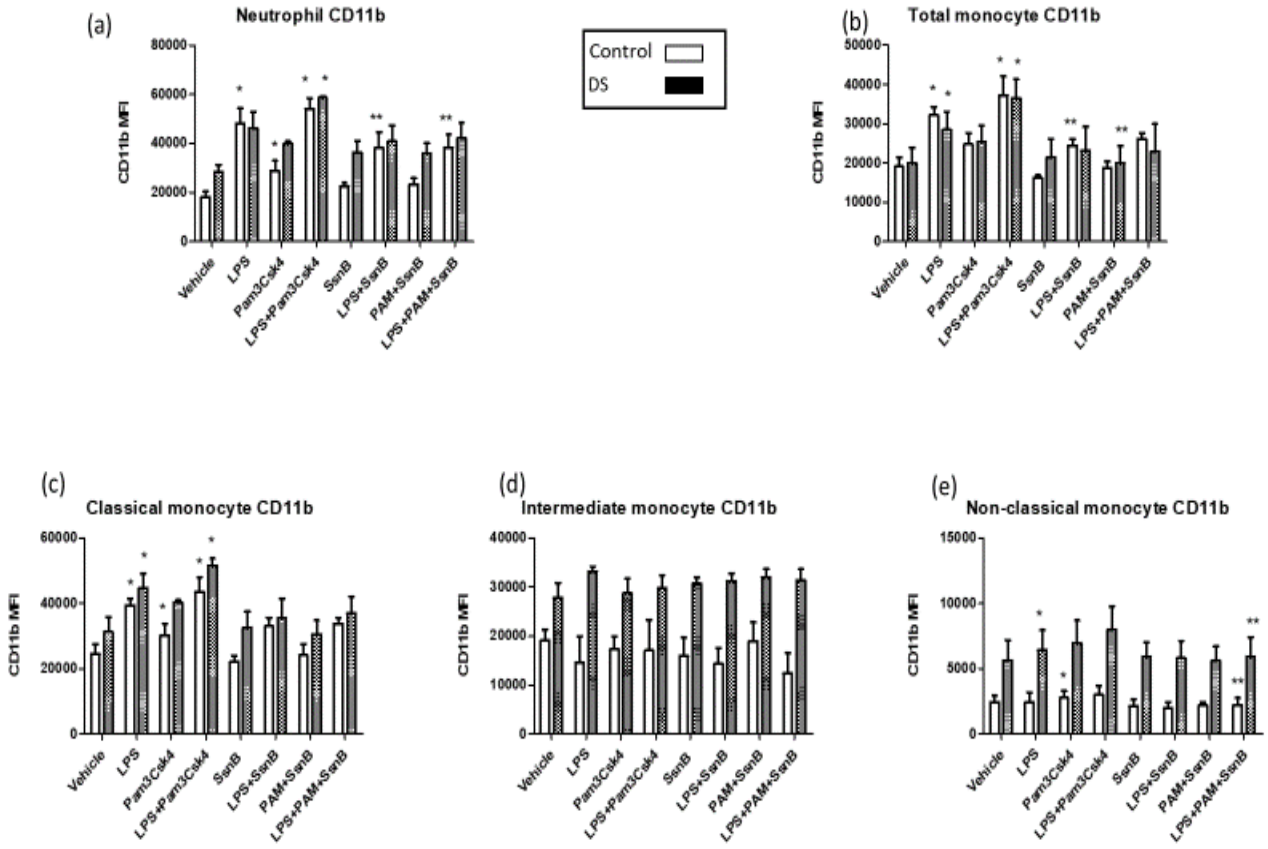


Figure 4.5. SsnB reduces neutrophil and monocyte CD11b in response to LPS and Pam3Csk4 in children with DS and controls.

Whole blood was incubated with LPS (10 ng/ml), Pam3Csk4 (5ng/ml), LPS + Pam3Csk4, SsnB (10µM), LPS + SsnB, Pam3Csk4 + SsnB, and LPS + Pam3Csk4 + SsnB for 1 hour. Samples processed for flow cytometry and CD11b expression on neutrophils and monocytes quantified. Values expressed as mean channel fluorescence (MFI). Unpaired t-test (mean +/- SEM). Children with DS (n=5), Controls (n=5). (a) Neutrophil CD11b *p < 0.05 vs vehicle in respective cohort; **p <0.05 vs LPS and LPS+Pam3Csk4 in their respective cohort; (b) Total monocyte CD11b *p < 0.05 vs vehicle in respective cohort; **p <0.05 vs LPS and Pam3Csk4 in their respective cohort; (c) Classical monocyte CD11b *p < 0.05 vs vehicle in respective cohort; (d) Intermediate monocyte TLR2; (e) Non-classical monocyte TLR2 *p < 0.05 vs vehicle in respective cohort; **p <0.05 vs LPS+Pam3Csk4 in respective cohort.

4.4.8 Gene expression in Toll like receptor signalling pathways

Whole blood was incubated with LPS (10 ng/ml) for 1 hour. Samples were then processed for quantitative RT-PCR as described in chapter 2. The endogenous control was GAPDH (NM_002046.3). All samples were assayed in triplicate and relative quantification (RQ) values were calculated using the 2-ΔΔCt method.

Peripheral blood cell expression of MyD88 was lower in children with DS ($p=0.001$), following LPS treatment there was a rise in MyD88 in controls ($p=0.03$) but this was not seen in children with DS [Figure 4.6(a)]. At baseline, TRIF expression was raised in children with DS compared with controls ($p=<0.0001$), there was no increase in expression following LPS treatment in either group [Figure 4.6(c)]. There were no differences in the expression of IRAK4 between the two groups, and an absence of LPS responsiveness in both cohorts [Figure 4.6(b)].

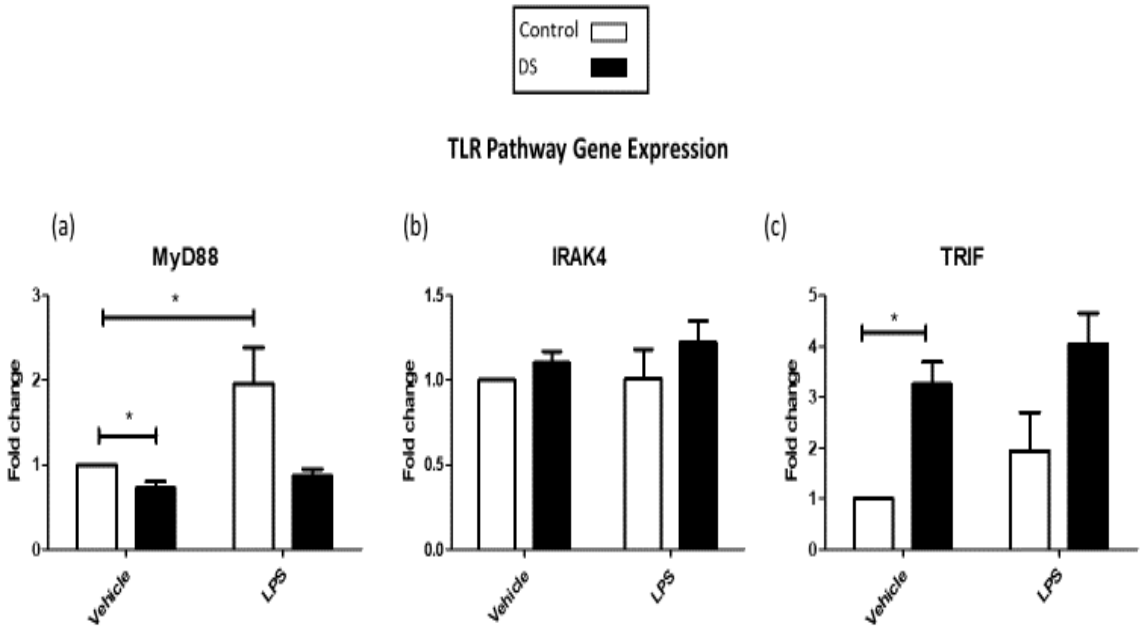


Figure 4.6. Reduced Myd88 and increased TRIF expression in children with DS compared to controls.

Whole blood was incubated with LPS (10 ng/ml) for 1 hour. Samples were then processed for quantitative RT-PCR. The endogenous control was GAPDH (NM_002046.3). All samples were assayed in triplicate and relative quantification values were calculated using the 2-ΔΔCt method. Unpaired t-test (mean +/- SEM). MyD88 (a), IRAK4 (b), TRIF (c), in children with DS (n=10) compared to control samples (n=10) at baseline and following treatment with LPS.

4.4.9 Immunomodulation and Cytokines; effects of SsnB on IL-1β, TNF-α, IL-6, IL-18, VEGF, Epo, IL-8, IFN-γ, IL-10 and IL-1ra

Whole blood was incubated with LPS (10 ng/ml), Pam3Csk4 (5ng/ml), LPS + Pam3Csk4, SsnB (10μM), LPS + SsnB, Pam3Csk4 + SsnB, and LPS + Pam3Csk4 + SsnB for 1 hour. Extracted peripheral blood serum was transferred to a 96 well MSD plate and these cytokines were assessed as per manufacturer's instructions using multiplex ELISA technology (MSD®MULTI-SPOT assay plate from Mesoscale).

Following stimulation of whole blood with LPS there were rises in both cohorts of IL-1β, TNF-α, IL-6, IL-18, IL-8, VEGF. Significant LPS responsiveness was only seen in control samples for Epo and IFN-γ (p value <0.05) [Figures 4.7-4.9]. The addition of LPS and Pam3Csk4 together also resulted in increases in IL-1β, TNF-α, IL-6, IL-8 and VEGF in both groups but not in IL-18 or EPO (p value <0.05), and only significantly in the DS cohort for IFN-γ. [Figures 4.7-4.9]. There were increases in both cohorts for IL-8, and for control TNF-α and VEGF after Pam3Csk4 stimulation alone (p value <0.05), indicating this immunomodulator generates an inferior cytokine response compared with LPS.

When the TLR2 antagonist SsnB was added to LPS, production of TNF-α, IL-18, and Epo was reduced in both cohorts (p value <0.05) [Figures 4.7 & 4.8]. IL-1β and IL-6 levels fell following SsnB in the DS cohorts only, while VEGF levels were reduced in the control group only. The effects of LPS and Pam3Csk4 together were abrogated by SsnB for IL-1β, IL-6, and VEGF in the control group only (p value <0.05). SsnB had the most marked effect on TNF-α production, resulting in diminution after its addition to LPS, Pam3Csk, and LPS plus Pam3Csk4 in both cohorts (p value <0.05). SsnB had no effect on IL-8 and IFN-γ production [Figures 4.7-4.9].

IL-10 release increased following LPS and was reduced by SsnB in controls. Pro-inflammatory simulants LPS and Pam3Csk4 resulted in rises in IL-1ra in both cohorts, and this was diminished after addition of SsnB to LPS and LPS plus Pam3Csk4 in both groups (p value <0.05), [Figure 4.8].

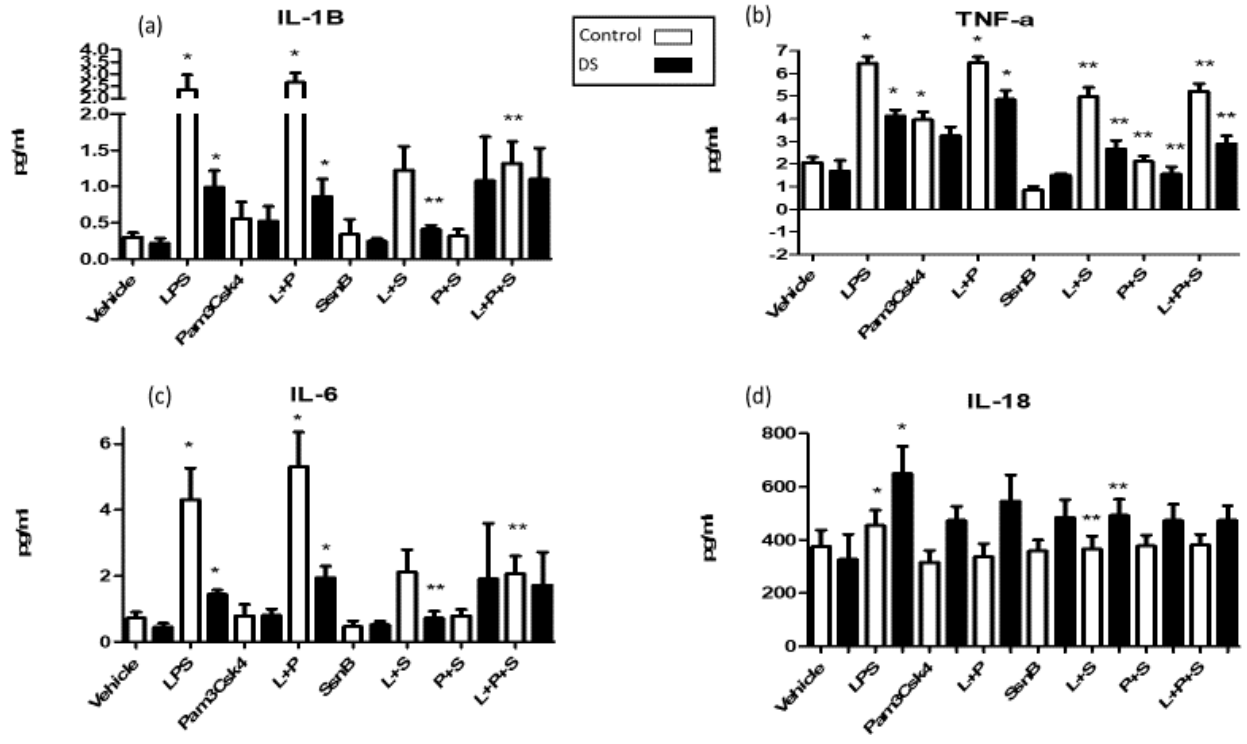


Figure 4.7. SsnB reduces pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and IL-18 in response to LPS and Pam3Csk4 children with DS and controls.

Whole blood was incubated with LPS (10 ng/ml), Pam3Csk4 (5ng/ml), LPS + Pam3Csk4, SsnB (10µM), LPS + SsnB, Pam3Csk4 + SsnB, and LPS + Pam3Csk4 + SsnB for 1 hour. Cytokines assessed using multiplex ELISA. Values expressed as pg/ml or in logarithmic scale equivalent. Unpaired t-test (mean +/- SEM). *p < 0.05 vs vehicle in respective cohort; ** p <0.05 vs respective treatment. Children with DS (n=7), controls (n=7).

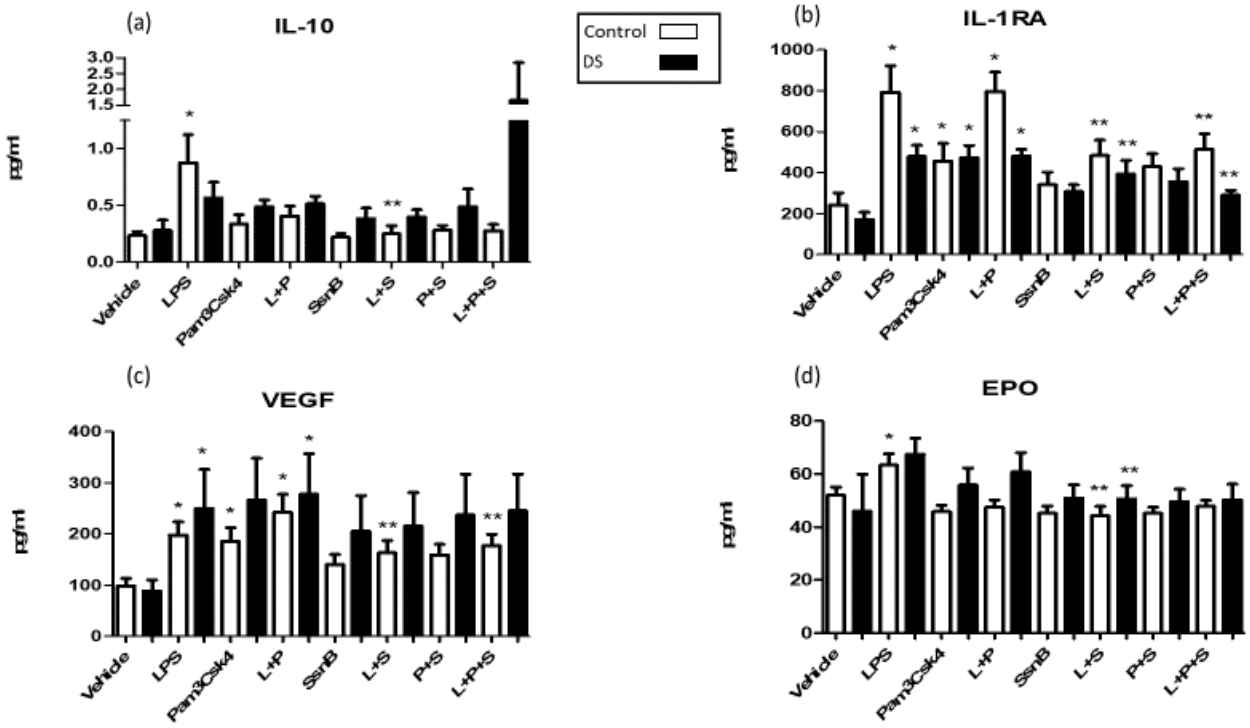


Figure 4.8. SsnB reduces anti-inflammatory (IL-10, IL-1RA), and cardiovascular (VEGF, EPO) cytokines in response to LPS and Pam3Csk4 in children with DS and controls.

Whole blood was incubated with LPS (10 ng/ml), Pam3Csk4 (5ng/ml), LPS + Pam3Csk4, SsnB (10µM), LPS + SsnB, Pam3Csk4 + SsnB, and LPS + Pam3Csk4 + SsnB for 1 hour. Cytokines assessed using multiplex ELISA. Values expressed as pg/ml or in logarithmic scale equivalent. Unpaired t-test (mean +/- SEM). *p < 0.05 vs vehicle in respective cohort; ** p <0.05 vs respective treatment. Children with DS (n=7), controls (n=7).

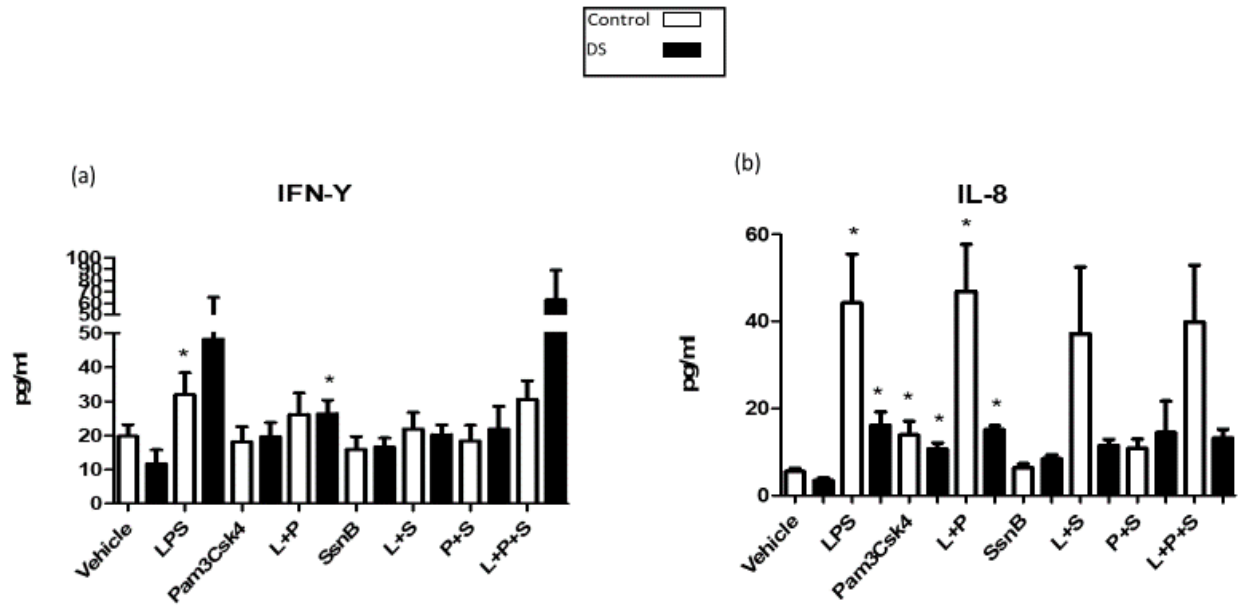


Figure 4.9. SsnB did not alter IFN-γ or IL-8 levels in response to LPS and Pam3Csk4 in children with DS and controls.

Whole blood was incubated with LPS (10 ng/ml), Pam3Csk4 (5ng/ml), LPS + Pam3Csk4, SsnB (10µM), LPS + SsnB, Pam3Csk4 + SsnB, and LPS + Pam3Csk4 + SsnB for 1 hour. Cytokines assessed using multiplex ELISA. Values expressed as pg/ml or in logarithmic scale equivalent. Unpaired t-test (mean +/- SEM). *$p < 0.05$ vs vehicle in respective cohort; ** $p < 0.05$ vs respective treatment. Children with DS (n=7), controls (n=7).

4.5 Discussion

We have demonstrated increased TLR2 expression on neutrophils, monocytes, and monocyte subsets in children with DS compared to controls. This could be clinically important as excess TLR2 expression is associated with morbidities seen more commonly in DS, such as gram positive infections (180), autoimmunity(181), and poorer outcomes in sepsis (8). This is the first time to our knowledge, that a dysregulation in TLR2 and its pathways have been described in DS.

In chapter 3 we have previously described an increase in TLR4 expression on non-classical monocytes at baseline, and a hyper-responsiveness in neutrophil CD11b following LPS in children with DS(182). TLR2 and TLR4 expression is altered in other diseases. In term neonates with late onset sepsis, TLR4 expression was increased in infants with positive blood cultures and TLR2 expression was raised in those with clinical sepsis (74). In adult patients with pneumonia, Tang et al (183) reported an increase in TLR2 and TLR4 expression, as well as raised IL-1, IL-6, and TNF-α. Future studies are required to determine the significance of our finding of increased TLR2 expression in DS. Children with DS are at increased risk of RTIs and admission to hospital with this presentation (5, 184). Gram positive bacteria such as *S. pneumoniae* and *S. aureus* are the causative organisms in many of these cases, and TLR2 plays a pivotal role in defence against these pathogens. Studies examining the effects of *S. aureus* and *S. pneumoniae* infection on mice deficient in TLR2 showed a decrease in proinflammatory cytokines and increased mortality (185, 186), highlighting the importance of TLR2 in this clinical context.

Children with DS are also prone to develop recurrent otitis media with effusion (OME) often resulting in conductive hearing loss (187). *S. pneumoniae* is a significant contributor to this condition and can stimulate persistent low-grade inflammation via TLR2 (41, 42). Chronic inflammation resulting from persistent infection by many Gram-positive and Gram-negative bacteria is also seen with increased prevalence in the oral cavity of people with DS (14). There are studies demonstrating an increase in TLR2 expression in oral mucosal tissue in patients with chronic periodontitis compared with controls (177, 178). This chronic inflammation and infection secondary to periodontitis may lead to increased numbers of pro-inflammatory non-classical monocytes locally

and in peripheral circulation(188), furthermore a study by Bloemers et al suggests an increased number of this monocyte subtype in circulating in DS versus controls(189). We found no significant difference in the cell numbers between the monocyte subpopulations, but the percentage of classical monocytes was significantly less in children with DS compared to controls, as has been described by Bloemers et al(51). The differences seen in TLR2 expression between both cohorts could therefore also be influenced by the change in monocyte subpopulations following more frequent bacterial infections in DS. Furthermore, it is difficult to say whether excess gram-positive infection caused greater TLR2 expression or if this receptor is inherently upregulated and or defective from an early age, leading to an increased infectious burden in DS. Overall, persistent inflammation seen in DS could be in part as a result of a dysregulation of TLR2.

In DS, autoimmunity such as arthropathy is also well described (190, 191), with the prevalence being 6 times higher than juvenile idiopathic arthritis in the general population. Increased TLR2 expression on monocyte subsets in patients with active rheumatoid arthritis (181), in the mucosa of duodenal samples of patients with coeliac disease (CD) (192, 193), and in PBMCs of those with autoimmune thyroid disease have also been reported (194). Our findings that TLR2 and TLR4 expression and induction are altered in children with DS suggests a possible role for these pathogen receptors in the development of autoimmune diseases in this group.

Taken together the burden of evidence points to TLR2 as a possible mediator in the pathogenesis of multiple clinical conditions seen more frequently in DS. We have demonstrated that the TLR2/TLR4 antagonist Sparstolonin B is effective in reducing TLR2 and CD11b expression in neutrophils and monocytes in children. It has been previously shown that SsnB mediates its anti-inflammatory properties by reducing NF-kB production and attenuating pro-inflammatory cytokines such as, IL-1β, IL-6, TNF-α(121). It appears that SsnB can also abrogate anti-inflammatory cytokine release as it reduces IL-10 and IL-1ra levels in these children. CD11b is a key surface marker upregulated during activation and migration of innate immune cells(152), it also serves to modulate TLR signalling via both MyD88 dependent and MyD88 independent pathways(151). We have shown some significant decreases in CD11b expression with

SsnB, this to our knowledge is the first experiment outlining the effects of SsnB on CD11b.

The TLR receptors propagate signals through two main pathways dependent on the utilisation (MyD88 dependent) or not (MyD88 independent) of MyD88. The former is employed by all TLRs except TLR3. The MyD88 dependant pathway results in NF-kB and TNF production. The MyD88 independent pathway signals via TRIF inducing IRF3 and finally leading to IFN-β being produced (195). MyD88 deficiency has been shown to have various clinical consequences; from decreased response to IL-1 and IL-18, increased susceptibility to infectious pathogens and resistance to LPS endotoxin (73, 196, 197). MyD88 has been shown to be key in mounting an appropriate immune response to bacterial pneumoniae and streptococci (198). The deficiency of MyD88 in DS described in our experiment could represent a possible reason for the increased prevalence of infections and poorer clinical outcomes.

While MyD88 gene expression is decreased in children with DS, we found that TRIF expression is increased. Signalling via the TRIF dependent pathway leads to a number of important immune responses to infection, enhancing type 1 interferon production, mediating apoptosis, caspase activation and necroptosis, ultimately leading to a reduction in viral replication and increased bacterial clearance (199). Children with DS are more susceptible to RTIs, most of viral aetiology, and a paper appraising viral sepsis in children has reported increased expression of TRIF in those affected by this clinical condition (200). Indeed, TRIF increases the production of important antiviral mediators such as IRF3 and type 1 interferons (201). However, research in murine models of viral respiratory infection found that excess TRIF signalling had deleterious effects on the host due to excess inflammation, neutrophil recruitment, lung inflammation and pulmonary oedema (199).

During our experiments we observed that for some cell populations stimulation with pro-inflammatory agents did not result in a significant response in both cohorts; for example, non-classical monocyte TLR2, and intermediate monocyte CD11b. We previously reported similar hyporesponsiveness to LPS in TLR4 expression on non-classical and intermediate monocytes, and CD11b on non-classical monocytes in children with DS and controls(182). The reasons for this are unclear as other

populations respond significantly, it could be that receptors on these subsets are inherently hyporesponsive or a degree of endotoxin tolerance may be present; resistance to LPS stimulation causing a blunting of the cellular pro-inflammatory response(202). There is some evidence that LPS may act to decrease TLR4 expression on monocyte subsets at 2h instead of upregulating this receptor. However, there may be a temporal component, as this study points out that TLR2 expression at 20h on classical monocytes was increased 20-fold, whereas this did not occur for CD16+ subpopulations(161).

4.6 Conclusion

In conclusion, we describe a dysregulation of TLR pathways in DS. There is greater expression of TLR2 on the surface of neutrophils and monocytes. Downstream signalling is altered with reduced MyD88 and increased expression of TRIF, which may represent compensatory upregulation of MyD88 independent pathways. This altered innate immunity may contribute to an increased burden of infections and chronic inflammation in DS. SsnB attenuates pro-inflammatory mediators and could be of potential therapeutic clinical benefit.

Chapter 5: Increased systemic inflammation in children with Down syndrome

5.1 Introduction

Down syndrome (DS) is the most common chromosomal anomaly and results from extra genetic chromosome 21 material, affecting approximately 1 in 700 births in the USA (1). DS is associated with several co-morbidities including, congenital heart disease, developmental delay, obstructive sleep apnoea, gastrointestinal atresias, and abnormal myelopoiesis (137). Dysregulation of the immune system is another common feature of DS, including lower T and B cell numbers (10, 30), a smaller thymus gland, reduced regulatory T lymphocytes and suboptimal antibody responses to immunisation (125, 126, 128). In chapter 4 we looked at TLR signalling, now we concentrate on the end point of many of these pathways by evaluating cytokine production in this cohort. Although altered serum cytokines in DS(35, 90) have been described, these mainly evaluate pro-inflammatory mediators in older cohorts.

Children with DS develop more infections, are more likely to be admitted to hospital with respiratory tract infections (RTI)(5), and have a 30% increased mortality from sepsis(8). In addition, children with DS have an increased incidence of chronic inflammatory conditions and autoimmunity(156). A recent meta-analysis evaluating the cytokine profile in DS showed increased pro-inflammatory mediators IL-1β, TNF-α and IFN-γ (90). In sepsis there is significant cytokine release which can lead to an acute systemic inflammatory response (SIRS), which may be protective but if prolonged or potentiated may be deleterious. Crucial to a good outcome is a controlled dampening of the immune response. However, a prolonged compensatory anti-inflammatory response (CARS) may lead to an immunodeficient state and poorer outcomes(203). Therefore, understanding the inflammatory response to LPS is important in children with DS. IL-10 and IL-1ra are two anti-inflammatory cytokines that are implicated in fine tuning the balance between SIRS and CARS. Children with DS have increase mortality in the context of sepsis, and perhaps it is an imbalance of these signals which leads to poorer clinical outcomes.

Approximately 50% of all children with DS have cardiovascular disease(204). Cytokines important in responding to hypoxia or promoting new blood vessel growth like EPO and VEGF could have significant impacts in the pathogenesis of this disease and potentially on outcomes. Indeed, children with DS have better post cardiac surgery outcomes compared with patients without DS(205). Pulmonary hypertension is also commonly seen in children with DS(206); and both Epo and VEGF have been associated with this disorder(207, 208). GM-CSF is another vascular cytokine which mediates its effects by promoting white blood cell production. It has been linked to the development of transient abnormal myelopoiesis (TAM) in DS, which affects around 10% of infants in this cohort, and can ultimately lead to myelodysplasia and acute leukaemia(209).

5.2 Hypothesis

We hypothesised that children with Down syndrome have excess cytokine levels, altered inflammatory responses, and this may have an impact on clinical outcomes.

5.3 Aims

To examine anti-inflammatory signals (IL-10 and IL-1ra), a comprehensive array of inflammatory cytokines (IL-2, IL-6, IL-8, IL-18, IL-1β, TNF-α, IFN-γ), cytokines involved in inflammation in response to hypoxia (EPO) propagating angiogenesis (VEGF) and myelopoiesis (GM-CSF), as well as discussing the potential clinical impact of significant congenital heart disease in DS, and LPS endotoxin on these mediators.

5.4 Results

5.4.1 Patient characteristics

There were 114 children with Down syndrome (DS) enrolled with an age (mean ±SD) of 5.7 ± 4.7 years(y) of which 55 were female (48.2%), and the controls (n=60) with a mean age of 6.9 ± 4.8 y, of which 28 were female (46.7%). In the DS cohort,

74% (n=84) had a diagnosis of congenital heart disease with 39% (n=33) of these requiring surgery in infancy. The following was the distribution of cardiac lesions in this group (n=33): Atrioventricular septal defect (AVSD) (n=17); Ventricular septal defect (VSD) repair (n=4); Tetralogy of Fallot (n=4); Atrial septal defect (ASD) and VSD repair (n=3); ASD repair (n=2); Patent ductus arteriosus (PDA) coil (n=1). The time from surgery to sampling was 51.1+/-22.5 months. For comparison in the cohort of children with DS we compared those with congenital heart disease (CHD) requiring surgery (n=33) versus those with CHD not requiring surgery (n=51) and those without CHD (n=30).

5.4.2 Cytokines at baseline in Children with DS and controls

Whole blood was incubated with LPS (10 ng/ml), Pam3Csk4 (5ng/ml), LPS + Pam3Csk4, SsnB (10μM), LPS + SsnB, Pam3Csk4 + SsnB, and LPS + Pam3Csk4 + SsnB for 1 hour. Extracted peripheral blood serum was transferred to a 96 well MSD plate and these cytokines were assessed as per manufacturer's instructions using multiplex ELISA technology (MSD®MULTI-SPOT assay plate from Mesoscale). Values expressed as pg/ml. *p ≤0.05, **p<0.01, ***p<0.001.

There were significant increases in children with DS versus controls at baseline in the following: Epo, VEGF, GM-CSF, IL-10, IL-1ra, IL-6, IL-2 (p ≤0.05) (Table 5.1 & Figure 5.1-3 (a-c), Figure 5.4 (a-b)). There were no significant differences seen in the levels of IL-1β, TNF-α, INF-γ, IL-8 or IL-18 (p>0.05; Table 5.1).

Table 5.1. The mean, standard error of the mean (SEM) and p-values of cytokine levels (pg/ml) in plasma of children with Down syndrome and the control group.

Cytokine	Controls	DS	p- value
IL-2	0.36 ± 0.07	0.68 ± 0.11	**0.05**
IL-6	0.67 ± 0.06	1.88 ± 0.28	**0.02**
IL-8	5.34 ± 0.50	8.16 ± 1.40	0.12
IL-10	0.41 ± 0.06	0.70 ± 0.57	**0.002**
IL-18	529.6 ± 37.58	508.8 ± 17.87	0.57
IL-1β	0.35 ± 0.06	0.57 ± 0.08	0.07
TNF-α	4.72 ± 0.71	8.97 ± 3.12	0.33
IFN-γ	24.21 ± 5.06	22.95 ± 2.85	0.81
IL-1ra	331.2 ± 38.48	497.1 ± 44.25	**0.01**
GM-CSF	0.14 ± 0.04	0.27 ± 0.03	**0.02**
Epo	67.0 ± 4.87	116.2 ± 6.72	**<0.0001**
VEGF	88.88 ± 5.13	181.0 ± 14.48	**<0.0001**

Table 5.1: Interleukin 2 (IL-2); Interleukin 6 (IL-6); Interleukin 8 (IL-8); Interleukin 10 (IL-10); Interleukin 18 (IL-18); Interleukin 1β (IL-1β); Tumour necrosis factor α (TNF-α); Interferon γ (IFN-γ); Interleukin 1 receptor antagonist (IL-1ra); Granulocyte macrophage – colony stimulating factor (GM-CSF); Erythropoietin (Epo); Vascular endothelial growth factor (VEGF). p values in bold denotes significance; p≤0.05.

5.4.3 Effects of LPS on Cytokine responses

Following LPS stimulation of plasma there were significant elevation in VEGF, IL-10, IL-1ra, IL-6, IL-2, TNF-α, IFN-γ, IL-8 in both cohorts ($p \leq 0.05$; Figure 5.1-3 (a-c), Figure 5.4 (a-b)). In children with DS, EPO and GM-CSF were significantly increased after incubation with LPS, which was not observed for controls (Figure 5.1 (b) & (c)). There was no response in IL-18 following LPS in either cohort. (Figure 5.2 (b)).

5.4.4 CHD and cytokine responses

To further examine the differences in the cytokine profile in children with DS and CHD the cohort was divided as follows: CHD requiring surgical intervention and CHD not requiring surgery and children without CHD. There were no significant differences in cytokine levels between children with no CHD and those with CHD not requiring surgery.

Comparing the subpopulations with DS and the control group showed that for Epo, VEGF, IL-6, IL-10, IL-1ra, both DS cohorts had significantly higher levels than controls [Figure 5.1,5.2 (d-f) & 5.4 (c-d)]. Children with DS and CHD had significantly higher IL-8 compared with controls, even though the total levels in children with DS were not significantly different for this cytokine [Figure 5.3 (d)]. GM-CSF was significantly raised in children requiring surgery compared to children who did not require intervention or had no underlying CHD [Figure 5.1 (f)].

Further analysis demonstrated that for every cytokine the children with DS and CHD had the highest mean value. For children with DS and CHD requiring surgery; Epo, VEGF, IL-6, IL-1ra, IL-1β, and IL-8 were significantly greater than for children with DS and CHD not requiring surgery and children without CHD. IL-18, TNF-α, and IFN-γ were not different in the DS subpopulations irrespective of CHD. There were no differences in cytokine levels between children with DS with CHD not requiring surgery or no CHD and those with DS and CHD who had surgery more than 1 month ago (Figure 5.1-3 (d-f), Figure 5.4 (c-d)) once these infants with recent surgery (<1 month; n=7) were excluded.

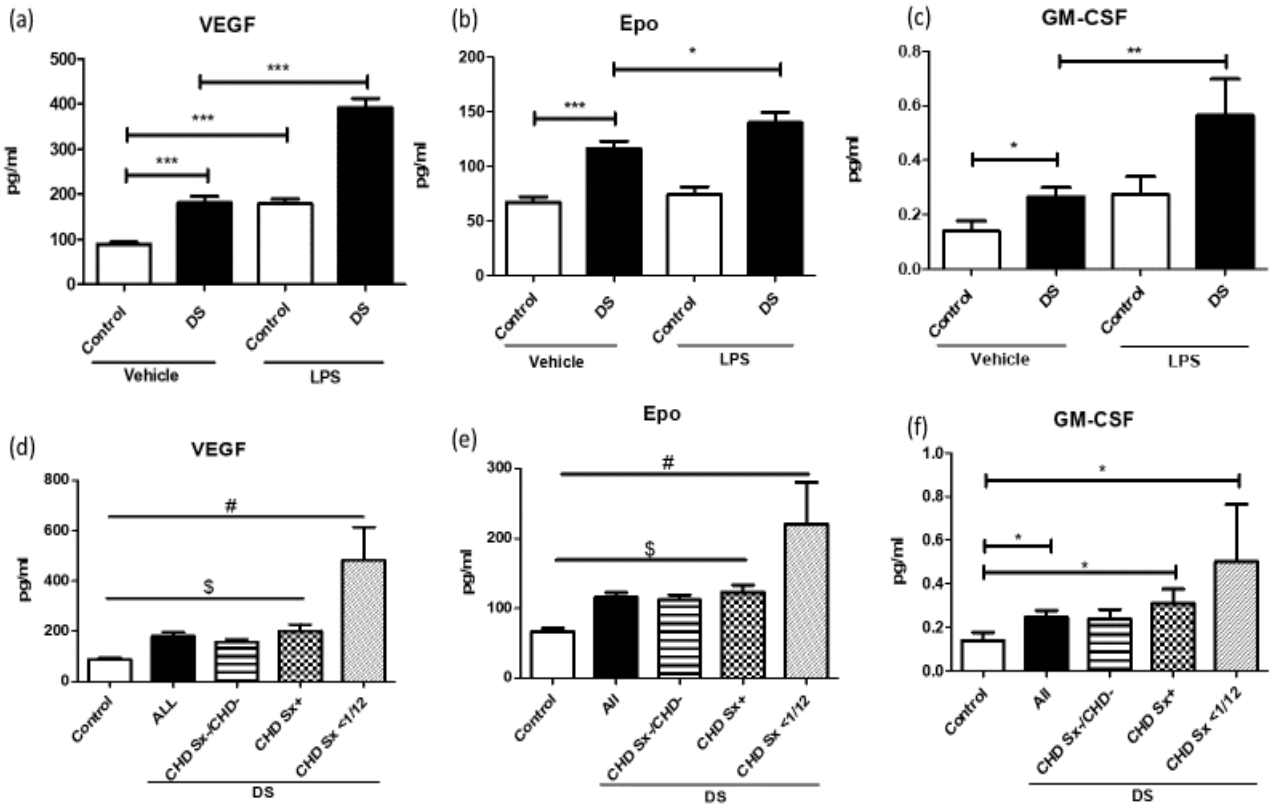


Figure 5.1. (a-c). Raised cytokine levels in plasma of VEGF (a), Epo (b), GM-CSF (c) at baseline in children with DS compared to controls. (d-f). No significant difference in cytokine levels in plasma of VEGF (d), Epo (e), GM-CSF (f) comparing DS subpopulations.

Whole blood was incubated with LPS (10 ng/ml) for 1 hour. Cytokines assessed using multiplex ELISA. Values expressed as pg/ml. Unpaired t-test (mean +/- SEM). *p ≤0.05, **p<0.01, ***p<0.001. Regarding DS subpopulations: All = all children with DS; CHD Sx-/chd- = children with DS and congenital heart disease (CHD) not requiring surgery, and children with DS without CHD; CHD Sx+ = children with DS and CHD requiring surgery; CHD Sx < 1/12 = children with DS and CHD who underwent surgery in the past month. *p ≤0.05. $= p<0.05 all DS subpopulations versus control. #= p<0.05 Children with CHD who underwent surgery in the past month versus the other DS subpopulations and controls.

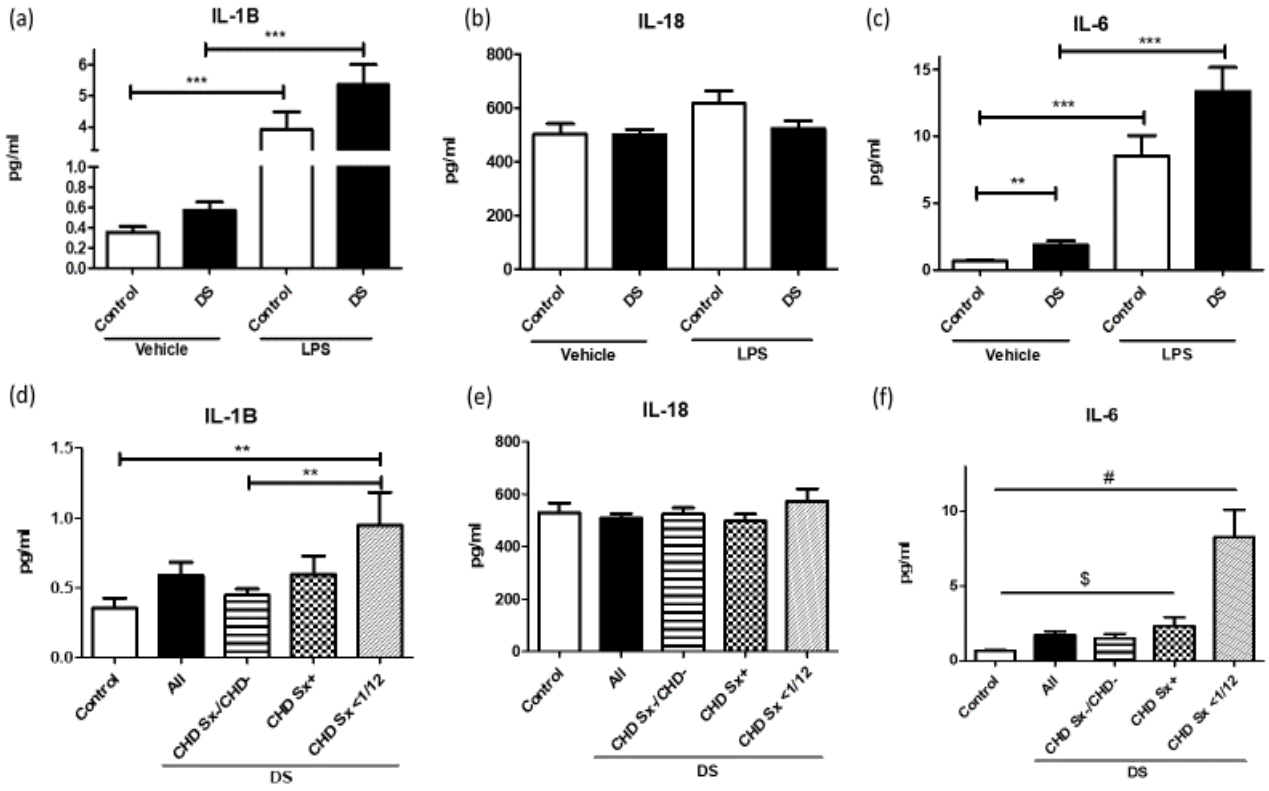


Figure 5.2. (a-c). Raised IL-6 in children with Down syndrome compared to controls. (d-f). No significant difference in Cytokine levels in plasma of (d) IL-1β, (e) IL-18, (f) IL-6 comparing DS subpopulations.

Whole blood was incubated with LPS (10 ng/ml) for 1 hour. Cytokines assessed using multiplex ELISA. Values expressed as pg/ml. Unpaired t-test (mean +/- SEM). *p ≤0.05, **p<0.01, ***p<0.001. Regarding DS subpopulations: All = all children with DS; CHD Sx-/chd- = children with DS and congenital heart disease (CHD) not requiring surgery, and children with DS without CHD; CHD Sx+ = children with DS and CHD requiring surgery; CHD Sx < 1/12 = children with DS and CHD who underwent surgery in the past month. *p ≤0.05. $= p<0.05 all DS subpopulations versus control. #= p<0.05 Children with CHD who underwent surgery in the past month versus the other DS subpopulations and controls.

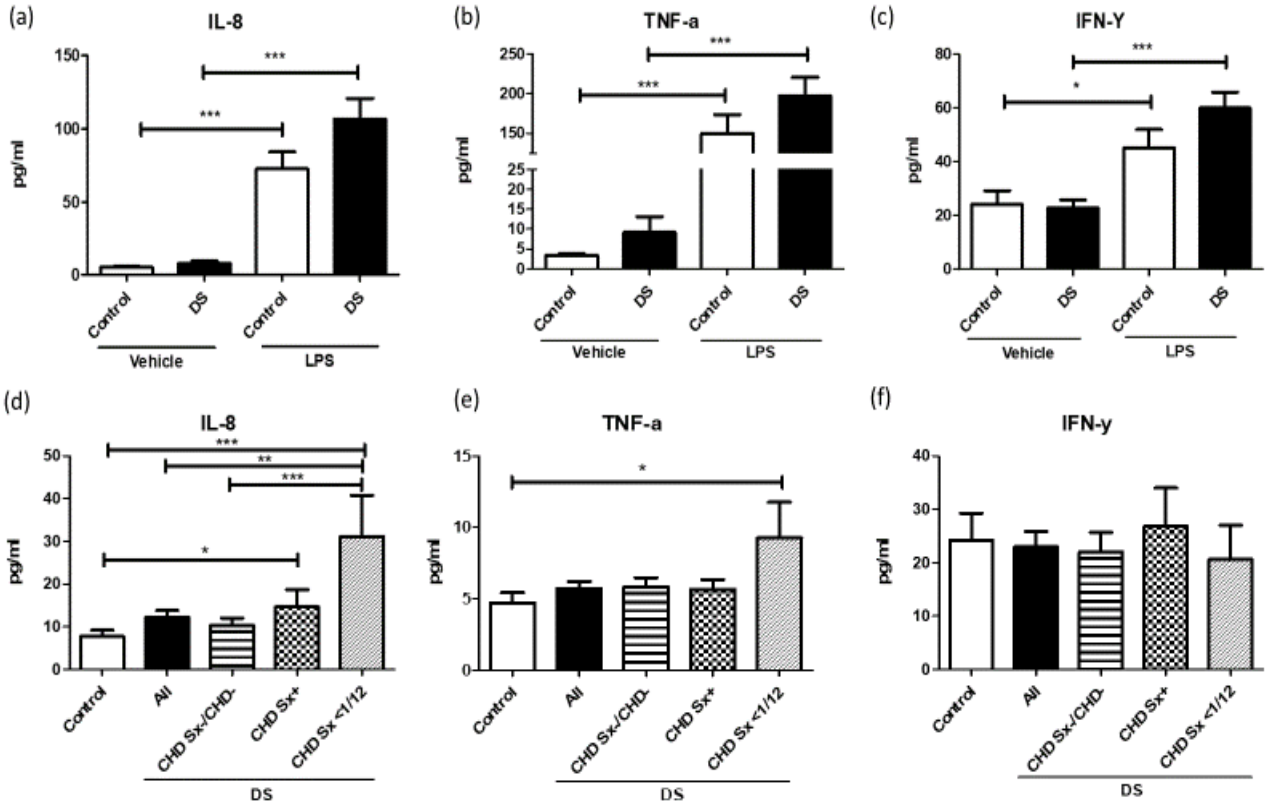


Figure 5.3. (a-c). Equivalent LPS response of cytokines in plasma of (a) IL-8, (b) TNF-α, (c) in children with Down syndrome compared to controls. (d-f). Cytokine levels in plasma of (d) IL-8, (e) TNF-α, (f) INF-γ comparing controls and Down syndrome subpopulations.

Whole blood was incubated with LPS (10 ng/ml) for 1 hour. Cytokines assessed using multiplex ELISA. Values expressed as pg/ml. Unpaired t-test (mean +/- SEM). *p ≤0.05, **p<0.01, ***p<0.001. Regarding DS subpopulations: All = all children with DS; CHD Sx-/chd- = children with DS and congenital heart disease (CHD) not requiring surgery, and children with DS without CHD; CHD Sx+ = children with DS and CHD requiring surgery; CHD Sx < 1/12 = children with DS and CHD who underwent surgery in the past month. *p ≤0.05. $= p<0.05 all DS subpopulations versus control. #= p<0.05 Children with CHD who underwent surgery in the past month versus the other DS subpopulations and controls.

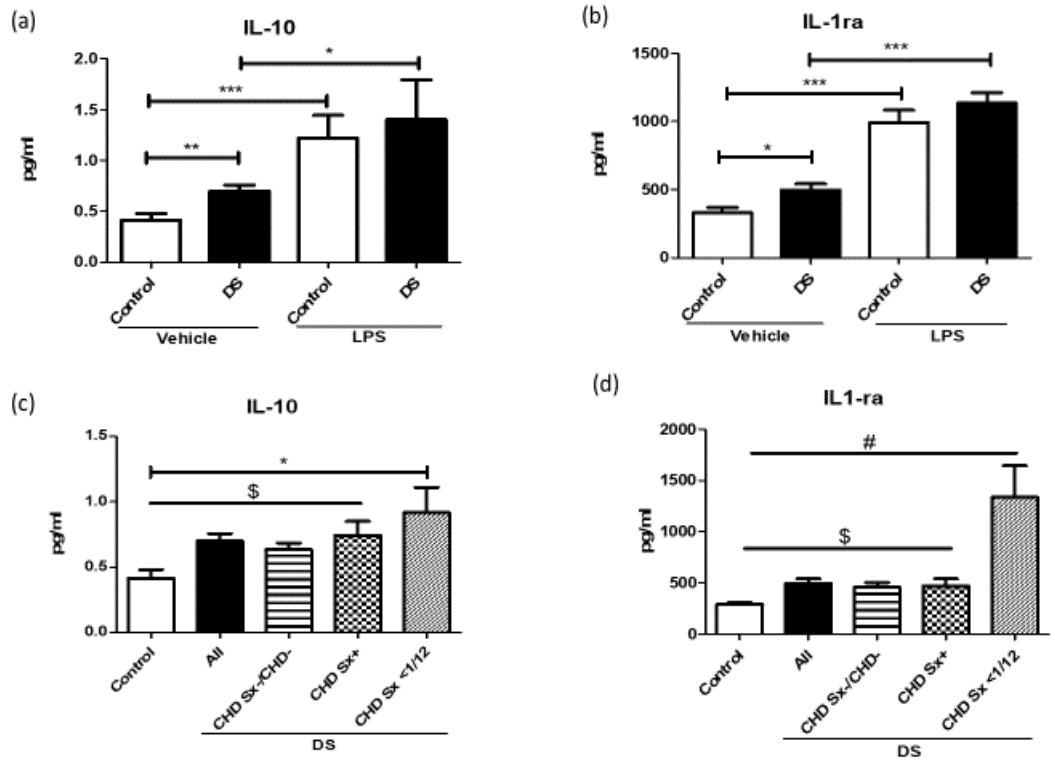


Figure 5.4 (a-b). Raised cytokine levels in plasma of (a) IL-10 and (b) IL-1ra at baseline and in children with DS and controls. (d-e). No difference in cytokine levels in plasma of (d) IL-10 and (e) IL-1ra comparing DS subpopulations.

Whole blood was incubated with LPS (10 ng/ml) for 1 hour. Cytokines assessed using multiplex ELISA. Values expressed as pg/ml. Unpaired t-test (mean +/- SEM). *p ≤0.05, **p<0.01, ***p<0.001. Regarding DS subpopulations: All = all children with DS; CHD Sx-/chd- = children with DS and congenital heart disease (CHD) not requiring surgery, and children with DS without CHD; CHD Sx+ = children with DS and CHD requiring surgery; CHD Sx < 1/12 = children with DS and CHD who underwent surgery in the past month. *p ≤0.05. $= p<0.05 all DS subpopulations versus control. #= p<0.05 Children with CHD who underwent surgery in the past month versus the other DS subpopulations and controls.

5.5 Discussion

We have demonstrated that children with DS have increased pro and anti-inflammatory cytokine levels compared with age-matched controls; IL-2, IL-6, IL-10, IL-1ra, Epo, VEGF and GM-CSF. Although there is definite immune dysregulation in children with DS(10, 16) they had robust LPS responses similar to controls. CHD requiring surgery did not appear to have a significant influence on cytokines in DS except in the acute post-operative period. However, IL-8 was the exception and was significantly raised in children with DS who required surgical intervention versus controls. Children without significant CHD also still exhibit a significantly elevated cytokine profile compared to controls.

A recent meta-analysis examining circulating serum cytokine levels in patients with DS found significant differences compared to controls. The analysis reviewed 19 studies involving 957 patients with DS and 541 controls, and concluded that significantly higher levels of Il-1β, TNF-α, IFN-γ, and neopterin were present in the population with DS(90). The authors found that circulating levels of IL-4, IL-6, IL-8 and IL-10 were not statistically different between the DS and control groups. However, it was suggested that one study(35) significantly altered the result for IL-6, and when this outlier was omitted from analysis, IL-6 was significantly raised compared with controls. It is worth noting that although results of some cytokines differ from our findings, for example, IL-1β, a key pro-inflammatory cytokine implicated in chronic disease(210) almost reached significance in our study (p value=0.07). The population with DS in the meta-analysis was predominantly adults as 6 studies were included involving adults (>16 mean age), and of the paediatric studies only one study(35) had a mean age lower than in our study. Furthermore, we had the largest cohort described and although there have been other studies evaluating cytokines in DS(35, 90, 93, 97, 139) this paper examines a comprehensive array of cytokines including anti-inflammatory and cardiovascular mediators.

Increased levels of VEGF and Epo were significantly greater in children with DS than controls DS, irrespective of CHD. Erythropoietin (EPO) has a crucial role in red blood cell production, however, it is now being recognised as having a myriad of effects such as altering the response to inflammation or injury, inhibiting apoptosis in

the setting of hypoxia and thus improving tissue survival (211). In newborn infants with DS, excess Epo levels in plasma and consequent elevated circulating haemoglobin are reported(212). Infants (n=11) with persistent pulmonary hypertension of the newborn (PPHN) or cyanotic congenial heart disease (CCHD) had increased hypoxia-inducible factor (HIF)-1α, VEGF and Epo mRNA expression compared to healthy newborns(213). PPHN and CCHD are seen with increased frequency in DS(214).

We describe increased VEGF levels in our DS cohort. VEGF is an important in regulating angiogenesis, lymphangenesis and is associated with chronic inflammation and tumour growth (215, 216). VEGF-A is a significant mitogen involved in the development of atrioventricular valvular cushions and abnormal expression is associated with CHD(217). Sanchez et al(218) found that DS results in abnormal angiogenesis *in utero*, increased expression of VEGF-A, which is also linked to congenital heart disease lesions. Increased expression of HIF-2α has been associated with excess VEGF production(219), and HO-1 has been shown to be key in cardiac embryonic development(220). VEGF is also associated with pulmonary hypertension and this complication as mentioned is seen more frequently in DS(208).

We report significantly elevated GM-CSF levels in children with DS. GM-CSF is implicated in the pathogenesis of transient abnormal myelopoiesis (TAM) in DS. This is a form of leukaemia occurring in infancy, can resolve spontaneously, and may arise from the foetal liver. GM-CSF from stromal tissue of the foetal liver promotes foetal blast proliferation by creating a suitable haematopoietic microenvironment (209). None of the patients in this study had TAM but may have not been detected as no universal neonatal screening. However, their matched full blood counts and differentials did not have any evidence of TAM at the time of cytokine analysis. There has been associations made with raised GM-CSF and poorer neurological outcomes in neonatal encephalopathy(69) and this may implicate this cytokine in adverse neuroinflammation, although the results of a 5 year follow up of ex preterm small for gestational age (SGA) (n=216) children who were randomised to either receive GM-CSF or not, found no neurodevelopmental or adverse outcome differences between groups(221). GM-CSF is also important to facilitating cross-talk between myeloid cells and T lymphocytes which if dysregulated can lead to chronic inflammation(222).

Although a pro-inflammatory phenotype in children with DS has been described (223), there is a less information on anti-inflammatory cytokines such as IL-10 and IL-1ra. We demonstrated that both IL-10 and IL-1ra are significantly raised in children with DS. IL- 10 is an anti-inflammatory cytokine which reduces inflammation by inhibiting the propagation of pro-inflammatory cytokines such as TNF-α and IL-6(35). There are conflicting results regarding IL-10 in cohorts with DS as Nateghi et al found a lower serum level of IL-10 when compared with controls (DS n=24, Controls n=24), and a reciprocal increased level of the pro-inflammatory cytokine TNF-α and IFN-γ(139). However, Cetiner et al, reported an increased level of IL-10, and IL-4 in serum and a reduction of TNF-α and IL-6(35), hypothesising that this anti-inflammatory picture may contribute to the increased level of RTIs seen in childhood. Indeed, Broers et al(93) described a significant increase in IL-10 after stimulation of whole blood with *Streptococcus pneumoniae* in children with DS versus their healthy siblings, suggesting a heightened anti-inflammatory response which may be contributory to more severe pneumococcal pneumonia in this cohort. Further research by Guazzarotti et al (DS n=24, Control n=42), and Iulita et al (DS n=31, Controls n=31) has reported an increase in serum IL-10 in DS versus controls(224, 225).

Interleukin 1 receptor antagonist (IL-1ra) is an anti-inflammatory cytokine that abrogates IL-1 signalling, has TNF-α inhibitory effects, and suppresses T cell responses(226). Our findings suggest that although pro-inflammatory signals are elevated, so too are the counter-regulatory cytokines IL-10 and IL-1ra. It is a key regulator in maintaining a balance between pro and anti-inflammatory stimuli. Excess IL-1ra has been associated with worse outcomes in the setting of gram-positive arthritis and sepsis. Ali et al(227) reported more aggressive disease and increased mortality in a murine model of *Staphylococcus aureus* arthropathy and sepsis. There is a higher incidence of inflammatory arthritis in children with DS(179), and elevated levels of IL-1ra have been shown in adults with rheumatoid arthritis(228).

Interleukin 6 (IL-6) is a key pro-inflammatory mediator released by leucocytes, fibroblasts and endothelial cells. It stimulates the production of IL-1, TNF-α and other pro-inflammatory cytokines to potentiate an inflammatory response(229). We found greater IL-6 levels in DS, this provides evidence of a hyper-inflammatory response.

Clinically, IL-6 is crucial in mounting an appropriate defence to pathogenic organisms, while if aberrantly produced can lead to chronic inflammation and autoimmunity(91). This cytokine plays a role in Alzheimer's disease, early onset being ubiquitous in DS, and involves degenerative processes in the central nervous system(230). Liscastro et al found elevated IL-6, soluble IL-6 receptors and soluble intracellular adhesion molecule-1 (sICAM-3), soluble vascular adhesion molecule-1 (sVCAM-1) and CRP were produced in children with DS compared to controls(92). The increase of IL-6 and CRP from DS children was similar to that found in elderly patients with clinical AD. IL-6 has also been described as significantly raised in response to influenza A virus. An *ex vivo* cytokine response following stimulation with live Influenza A virus reported the following to be increased in children with DS compared with controls; IL-6, IL-1β, IL-8, and TNF-α(97).

We have demonstrated increased IL-2 in children with DS, although studies to date involving IL-2 and DS show no significant difference in this cytokine versus controls(35, 231). Interleukin 2 (IL-2) is causes proliferation of T lymphocytes, memory and effector cells, and has an essential role in the propagation and functionality of regulatory T cells (Treg cells). The latter important in controlling immunity, inflammation and promoting tolerance. Therefore IL-2 is key in promoting other T cells to provide an immune response, whilst also enabling Tregs to limit the degree of inflammation(232).

A possible explanation for the elevated cytokine profile demonstrated in children with DS, regardless of CHD, is a dysregulation of Toll like receptor (TLR) signalling. We have recently reported increased TLR2 expression on neutrophils and monocytes and altered gene expression of key regulatory proteins involved in signal propagation(77). Excess cytokine levels in DS could reflect anomlaous signalling of TLR pathways as the end point of these pathways results in the release of inflammatory mediators. Furthermore, children with DS may display a hyperresponsive immune reaction as we have previously reported increased neutrophil activation post LPS, and greater TLR4 on non-classical monocytes(182). Indeed, a pro-inflammatory phenotype may predominate in this population leading to excess circualting cytokines.

Although this study represents the largest most comprehensive apprasial of cytokines in a paediatric population, there were several limitations. The exact cellular source for the differing cytokine levels between children with DS and controls was not established as whole blood was used. We did not measure oxygen saturations of our cohorts while awake or while asleep (increased risk of obstructive sleep apnoea in children with DS) and this may be significant given that hypoxia is a key mediator in VEGF and Epo production(213, 233). It would be interesting to appraise oxygen saturations and VEGF/Epo levels in children with DS. Serial cytokine quantification following differing endotoxin incubations would be useful to understan the evolution of the inflammatory response in children with DS especially following cardiac surgery.

5.6 Conclusion

Overall this study demonstrates that children with Down syndrome exhibit significantly greater levels of pro and anti-inflammatory cytokines as well as increased Epo, VEGF and GM-CSF at baseline. From a clinical perspective CHD does not seem to have an impact on circulating cytokines beyond the acute surgical phase. The response to LPS stimulation is largely similar between cases and controls. The differences in circulating cytokines may contribute to varied clinical outcomes, acutely like in sepsis, as the imbalance between SIRS and CARS becomes detrimental, and over time in chronic inflammation. Epo & VEGF have been associated with vascular remodelling and may be associated with pulmonary hypertension. Given the high incidence of this disorder in our cohort and the excess Epo and VEGF demonstrated, further research is needed to evaluate the role of these mediators in the development of pulmonary hypertension.

Chapter 6: Melatonin as an immunomodulator in Down syndrome

6.1 Introduction

Down syndrome (DS) is the most prevalent chromosomal abnormality worldwide, affects 1 in 700 births in the United States, and is due to extra genetic material from chromosome 21(1). It is associated with several of co-morbidities including, low IQ, cognitive delay, congenital heart disease, gastrointestinal anomalies, obstructive sleep apnoea, and acute leukaemia(137). The immune system is also dysregulated in DS with many abnormalities described; decreased T and B lymphocyte counts (10, 30), smaller thymus gland, suboptimal antibody responses to vaccination (125, 126, 128), altered serum cytokines (35, 90) and abnormal neutrophil chemotaxis((41). In the previous three chapters we have reported further immune dyscrasias in DS; anomalous CD11b, TLR2, TLR4 expression, TLR signalling and additional evidence of cytokine dysregulation.

From a clinical perspective these immune deficits contribute to a vulnerable population as children with DS are more prone to infections, admission to hospital with respiratory tract infections (RTIs), and more often require longer hospital stays(5, 6). Furthermore, in the setting of sepsis a 30% increased mortality has been reported in children with DS(8). This may partly be due to the high circulating levels of pro-inflammatory cytokines in this cohort(90, 97), leading to uncontrolled inflammatory cascades, systemic inflammatory response syndrome (SIRS), sepsis and ultimately poorer outcomes.

Another consequence of an altered immune system is that chronic inflammation and autoimmunity; arthropathy, coeliac and thyroid disease, are found more commonly(11, 179, 193). Periodontal disease is due to persistent inflammation in the oral cavity and is also more prevalent in DS, this and other chronic peripheral infections have been implicated in the possible pathogenesis of neurodegenerative changes or Alzheimer's disease, which occurs with regularity and at an earlier onset in DS(14, 176). Trisomy 21 also causes overexpression of the enzyme superoxide dismutase, consequently an excess of free radicals and increased oxidative stress is hypothesised to occur in this cohort, resulting in early onset AD and cataracts(234).

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous multifunctional hormone which is secreted in the pineal gland and other organs by animals, including humans. This versatile molecule has also been preserved in more primitive species such as bacteria, and phylogenetics has tracked its origins to be over 3 billion years old(114). The effects of melatonin on regulating circadian rhythm are well described(115, 116). However it is now known that there are several important immunomodulatory effects of this molecule, especially in relation to benefits as an adjuvant in treating sepsis, chronic inflammation, and as an abrogator of neurodegeneration(117, 235, 236), all of which occur more frequently in DS. Melatonin has been shown to have strong antioxidant effects, antiapoptotic activity through inhibition of caspase 3 cleavage, and anti-inflammatory actions via inflammasome deactivation(118).

Melatonin has a myriad of immune functions and we aimed to evaluate if its effects extend further by examining neutrophil and monocyte function, genes involved in TLR signalling, the inflammasome, and pro and anti-inflammatory cytokines following immunomodulation with lipopolysaccharide (LPS; endotoxin) and melatonin. We hypothesised that melatonin could potentially mediate aspects of the innate immune system in children with DS. This is especially pertinent as DS is characterised by marked immune dysfunction, increased mortality from sepsis, chronic inflammation, prone to oxidative stress, sleep disturbance and possibly abnormal endogenous melatonin levels(113, 237).

6.2 Hypothesis

Melatonin may reduce pro-inflammatory signalling in children with DS and be of potential therapeutic benefit.

6.3 Aims

We aimed to investigate if melatonin could decrease CD11b and TLR4 on neutrophils and monocytes, abrogate genes involved in TLR signalling (MyD88, IRAK4, TRIF), the

inflammasome (IL-1β, ASC, NLPR3), and attenuate pro- and anti- inflammatory cytokines following immunomodulation with LPS endotoxin.

6.4 Results

6.4.1 Patient characteristics

The participants with DS (n=47) had a mean age (mean ±SD) of 5.1 ± 4.3 years (y), of which 42.6% were female, and the controls (n=23) were 7.7 ± 4.2y old of which 52.2% were female(182). In the DS cohort, 74.4% (n=35) had a diagnosis of CHD, with 25.5% (n=12) requiring corrective surgery. The controls were healthy children without DS and no major co-morbidities.

6.4.2 Effects of melatonin on cytokine responses in DS versus controls

Whole blood was incubated with LPS (10 ng/ml), and melatonin ($42\mu M$)for 1 hour. Extracted peripheral blood serum was transferred to a 96 well MSD plate and these cytokines were assessed as per manufacturer's instructions using multiplex ELISA technology (MSD®MULTI-SPOT assay plate from Mesoscale).

Compared with baseline levels, treatment with melatonin resulted in significant decreases in IL-1β and Epo in both cohorts [$p<0.05$; Figures 6.1(b), 6.2(d)]. IL-8, IL-10, and IFN-γ were also reduced compared with baseline after melatonin incubation but only in children with DS [Figures 6.1(d), 6.2(b), 6.3(c)]. Melatonin caused an increase in IL-2 in children with DS, and a decrease in IL-6 in controls versus baseline [Figures 6.3(b), 6.1(c)]. The following cytokines did not change significantly in either group following melatonin incubation: IL-18, TNFα, VEGF, GM-CSF, IL-1ra [Figures 6.1-6.3].

6.4.3 Effects of melatonin on LPS-induced cytokine responses

Comparing cytokine levels after LPS treatment with samples treated with LPS plus melatonin there were significant reductions in TNF-α, IL-8, IL-18, Epo and IL-1ra in both cohorts ($p<0.05$) [Figures 6.1(a), (d), 6.2(d), (b)]. In children with DS there were significant decreases in IL-1β, IL-6 and VEGF comparing LPS treated samples and those treated with LPS and melatonin, this was not replicated for controls [Figures 6.1(b), (c), 6.2(c)]. For IL-2 and GM-CSF, treatment with both LPS and melatonin resulted in

significant increases in these cytokines in both cohorts, and although not quite reaching statistical significance the same trend was observed for IFN-γ and IL-10. [Figures 6.3(b), (a), (c), 6.2(a)].

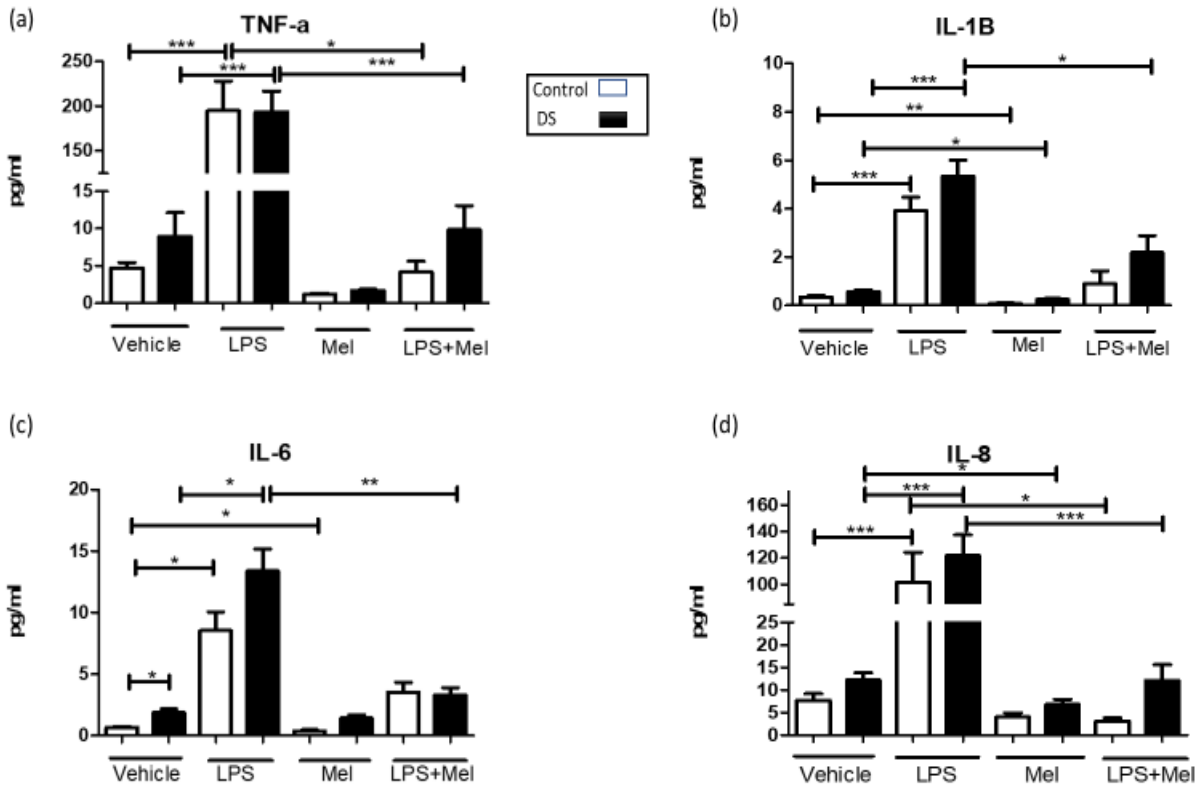


Figure 6.1. Melatonin reduces cytokine levels in plasma of TNF-α (a), IL-1β (b), IL-6 (c), IL-8 (d) at baseline and in response to LPS in children with DS and controls.

Whole blood was incubated with LPS (10 ng/ml), and melatonin (42μM) for 1 hour. Cytokines assessed using multiplex ELISA. Values expressed as pg/ml. Unpaired t-test (mean +/- SEM). Values expressed as pg/ml. *p <0.05, **p<0.01, ***p<0.001. Children with DS (n=47), controls (n=23).

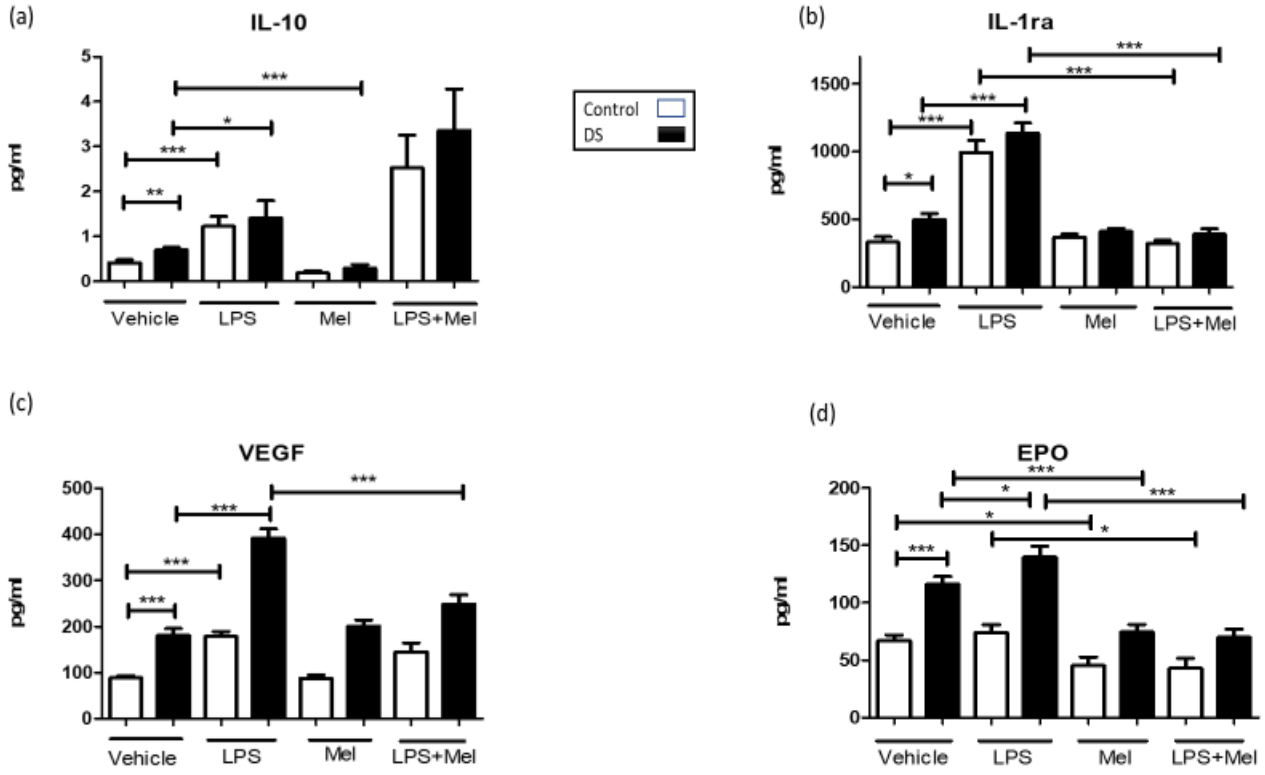


Figure 6.2. Melatonin reduces cytokine levels in plasma of IL-1ra (b), VEGF (c), Epo (d) in response to LPS and in children with DS and controls.

Whole blood was incubated with LPS (10 ng/ml), and melatonin (42μM) for 1 hour. Cytokines assessed using multiplex ELISA. Values expressed as pg/ml. Unpaired t-test (mean +/- SEM). Values expressed as pg/ml. *p <0.05, **p<0.01, ***p<0.001. Children with DS (n=47), controls (n=23).

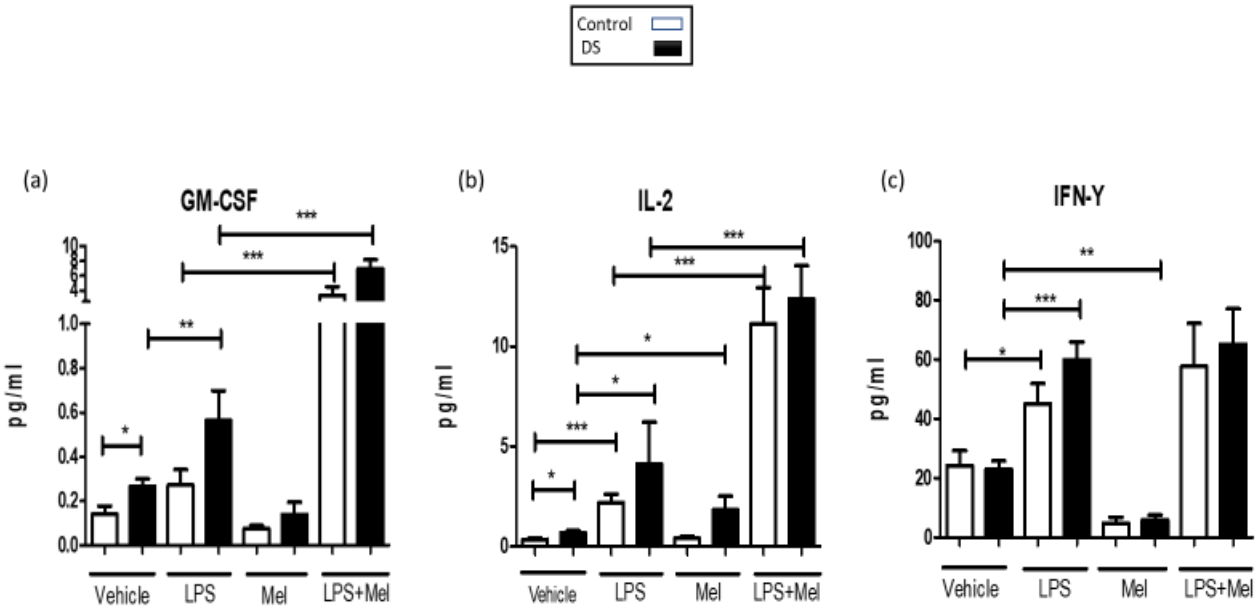


Figure 6.3. Melatonin increases cytokine levels in plasma of GM-CSF (a), IL-2 (b), in response to LPS and melatonin in children with DS and controls.

Whole blood was incubated with LPS (10 ng/ml), and melatonin (42μM) for 1 hour. Cytokines assessed using multiplex ELISA. Values expressed as pg/ml. Unpaired t-test (mean +/- SEM). Values expressed as pg/ml. *p <0.05, **p<0.01, ***p<0.001. Children with DS (n=47), controls (n=23).

6.4.4 Effects of melatonin on CD11b and TLR4

Whole blood samples were treated with LPS (10 ng/ml), Melatonin ($42\mu M$), and LPS plus melatonin for 1 hour. Samples were processed for flow cytometry as described in chapter 2 and CD11b and TLR4 expression on neutrophils and monocytes was quantified. Values were expressed as mean channel fluorescence (MFI).

Compared with baseline expression melatonin caused significant decreases in CD11b on neutrophils and classical monocytes in both cohorts ($p<0.05$) [Figures 6.4(a), (c)]. There was a significant reduction in total monocyte CD11b in children with DS, and a significant reduction in intermediate monocyte CD11b in controls, when compared to baseline levels. [Figures 6.4(b), (d)]. A paradoxical increase in CD11b on non-classical monocytes was observed in the DS cohort [Figure 6.4 (e)].

When comparing samples treated with LPS alone with those incubated with LPS and melatonin, there were significant decreases in CD11b expression in all cell sub-types (Neutrophils, total, classical, intermediate monocytes) in both cohorts, except for the non-classical monocyte. ($p<0.05$) [Figure 6.4 (a-d)]. There was no difference observed in the latter.

Melatonin treatment resulted in significant TLR4 reductions on total, intermediate and non-classical monocytes in both cohorts when compared to baseline ($p<0.05$) [Figures 6.5(b), (d), (e)]. There was no effect of melatonin on neutrophils or classical monocytes [Figure 6.5(a), (c)].

Evaluating TLR4 expression between samples treated with LPS and those treated with LPS plus melatonin revealed significant falls in the receptor on total, classical and non-classical monocytes in both groups ($p<0.05$) [Figures 6.5(b), (c), (e)]. On the intermediate monocytes there was a significant reduction in TLR4 in the DS cohort only. No difference was observed for neutrophils in either group. [Figures 6.5(d), (a)].

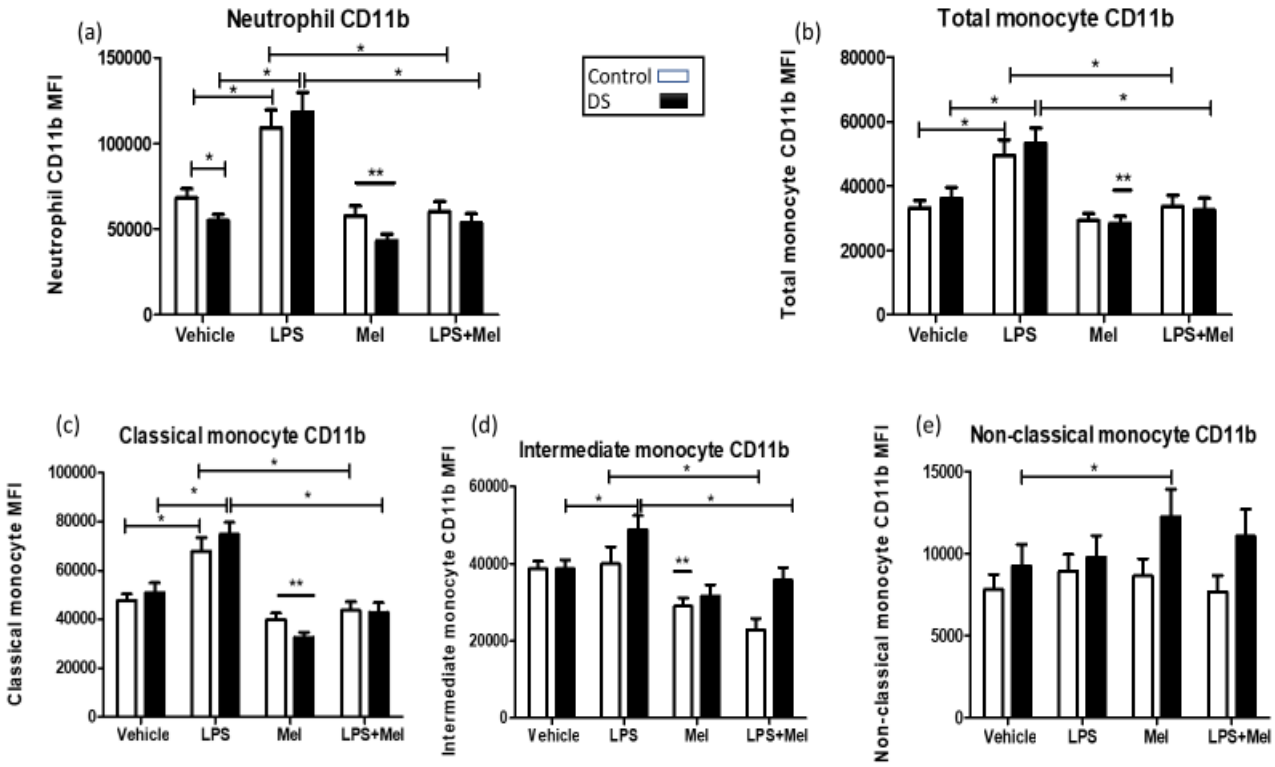


Figure 6.4. Melatonin reduces neutrophil and monocyte CD11b expression in response to lipopolysaccharide (LPS) and in children with Down syndrome (DS) and controls.

Whole blood was incubated with LPS (10 ng/ml), and melatonin (42μM) for 1 hour. Samples processed for flow cytometry and CD11b expression on neutrophils and monocytes quantified. Values expressed as mean channel fluorescence (MFI). Unpaired t-test (mean +/- SEM). *p < 0.05, **p<0.05 versus vehicle in corresponding cohort. (a) Neutrophil CD11b (DS n=23; Controls n=16) (b) Total monocyte CD11b (DS n=19; Controls n=21) (c) Classical monocyte CD11b (DS n=19; Controls n=21; (d) Intermediate monocyte CD11b (DS n=18; Controls n=20); (e) Non-classical monocyte (DS n=19; Controls n=21).

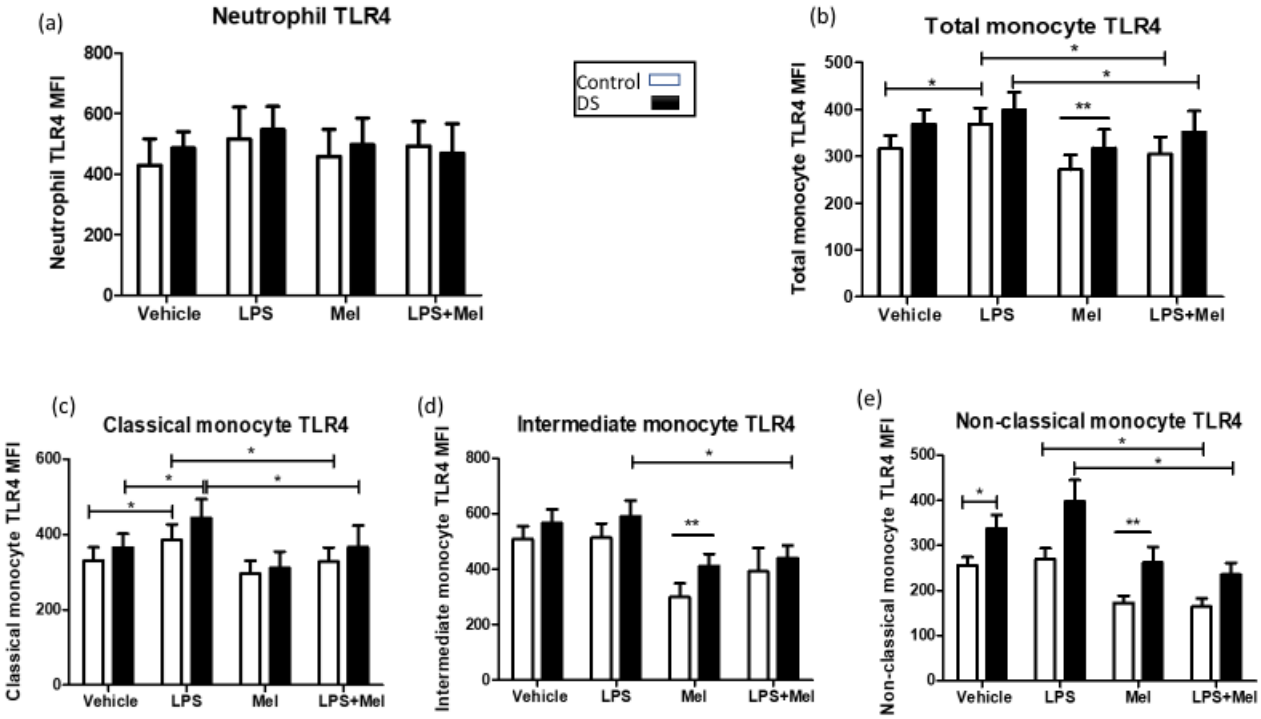


Figure 6.5. Melatonin reduces monocyte TLR4 expression in response to lipopolysaccharide (LPS) in children with Down syndrome (DS) and controls.

Whole blood was incubated with LPS (10 ng/ml), and melatonin (42μM) for 1 hour. Samples processed for flow cytometry and TLR4 expression on neutrophils and monocytes quantified. Values expressed as mean channel fluorescence (MFI). Unpaired t-test (mean +/- SEM). *p < 0.05, **p<0.05 versus vehicle in corresponding cohort. (a) Neutrophil TLR4 (DS n=19; Controls n=10); (b) Total monocyte TLR-4 (DS n=22; Controls n=15); (c) Classical monocyte TLR4 (DS n=16; Controls n=15); (d) Intermediate monocyte TLR4 (DS n=15; Controls n=14); (e) Non-classical monocyte TLR-4 (DS n=16; Controls n=20)

6.4.5 Melatonin and the Inflammasome

Whole blood was incubated with LPS (10 ng/ml) and melatonin ($42\mu M$) for 1 hour. Samples were then processed for quantitative RT-PCR as described in chapter 2. The endogenous control was GAPDH (NM_002046.3). All samples were assayed in triplicate and relative quantification (RQ) values were calculated using the 2-ΔΔCt method.

Compared with baseline expression melatonin caused a significant reduction in IL-1β in both cohorts (DS p<0.0001; Control p<0.0001) [Figure 6.6(a)]. NLRP3 expression was decreased with melatonin in the controls but not in children with DS (p=0.002) [Figure 6.6(b). There were no differences in ASC expression following melatonin treatment in either group.

When samples treated with LPS alone were compared to those incubated with LPS and melatonin there were significant decreases in IL-1β (p<0.0001), and NLRP3 (p=0.0001) in children with DS but not controls [Figures 6.6 (a) & (b)]. There was no change in ASC following the above comparisons.

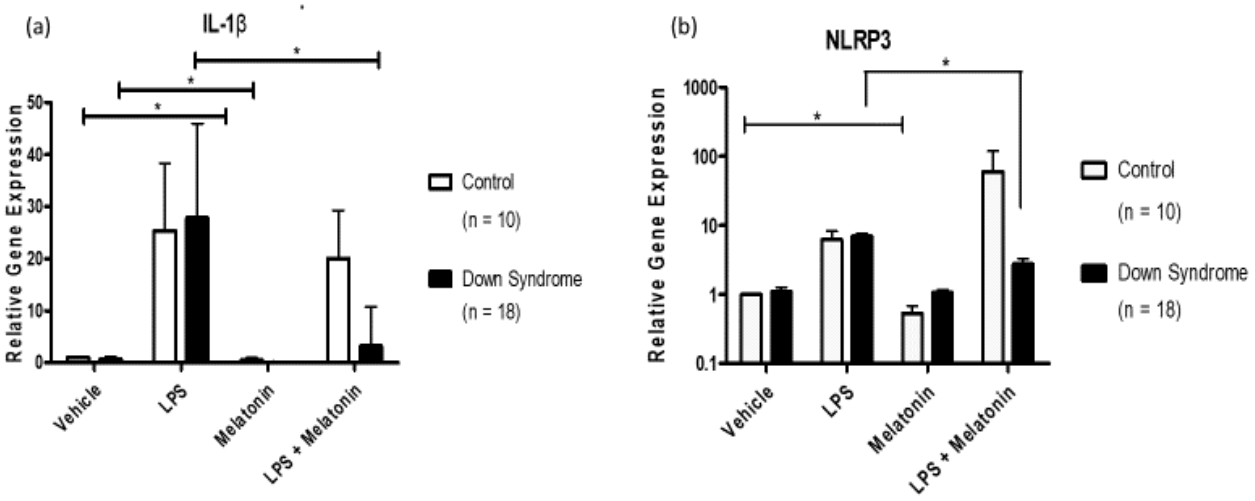


Figure 6.6. Melatonin reduces fold change expression of IL-1β and NLRP3 in response to LPS in children with DS compared to controls.

Whole blood was incubated with LPS (10 ng/ml) and melatonin (42Um) for 1 hour. Samples were then processed for quantitative RT-PCR. The endogenous control was GAPDH (NM_002046.3). All samples were assayed in triplicate and relative quantification values were calculated using the 2-ΔΔCt method. Unpaired t-test (mean +/- SEM). Statistical significance is *p < 0.05. Children with DS (n=18), controls (n=10).

6.4.6 Melatonin and the TLR pathway

Whole blood was incubated with LPS (10 ng/ml) and melatonin ($42\mu M$) for 1 hour. Samples were then processed for quantitative RT-PCR as described in chapter 2. The endogenous control was GAPDH (NM_002046.3). All samples were assayed in triplicate and relative quantification (RQ) values were calculated using the 2-ΔΔCt method.

Melatonin had the effect of reducing MyD88 expression compared to baseline in children with DS but not in controls (p=0.02) [Figure 6.7(a)]. When compared to baseline melatonin did not result in any changes in gene expression for IRAK4 or TRIF in either cohort [Figures 6.7(b), (c)]].

Comparing samples treated with LPS versus those treated with LPS and melatonin, the addition of melatonin resulted in a significant decrease in MyD88 expression in children with DS (p=0.017), but not in controls [Figure 6.7(a)]. There were no changes in IRAK4 or TRIF following the above treatments [Figure 6.7(b), (c)].

There were no significant differences at baseline or comparing LPS to LPS plus melatonin in the expression of BMAL, CLOCK, CRY, REV-ERBα in both cohorts.

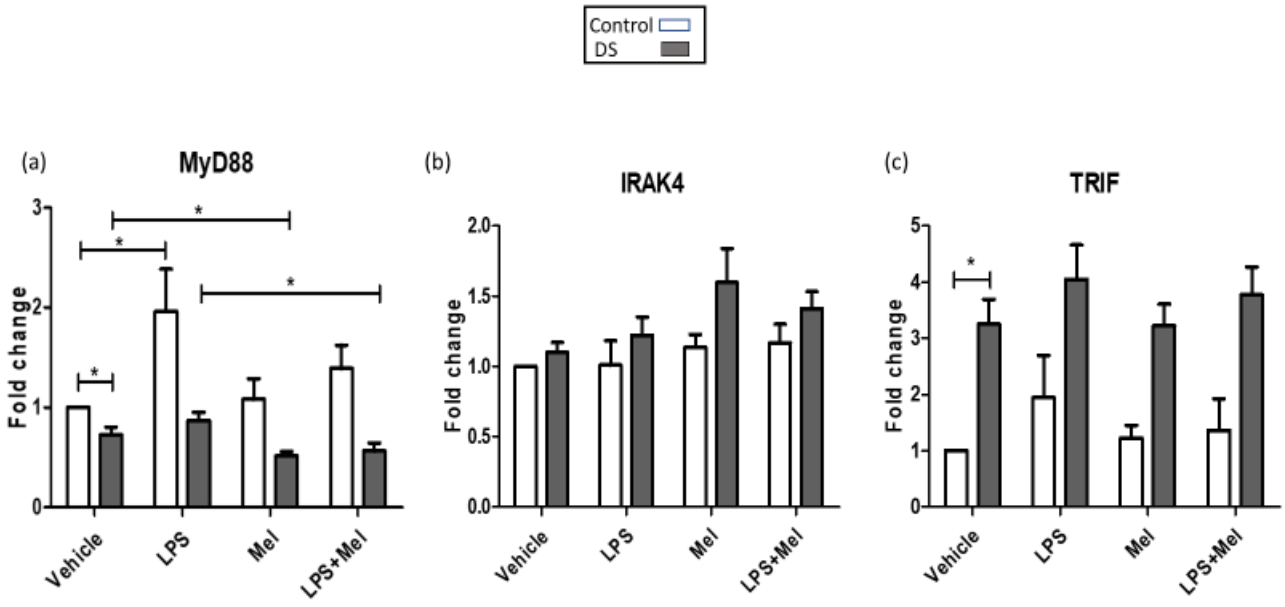


Figure 6.7. Melatonin reduces MyD88 (a) expression at baseline and in response to LPS in children with DS compared to controls.

Whole blood was incubated with LPS (10 ng/ml) and melatonin (42Um) for 1 hour. Samples were then processed for quantitative RT-PCR. The endogenous control was GAPDH (NM_002046.3). All samples were assayed in triplicate and relative quantification values were calculated using the 2-ΔΔCt method. Unpaired t-test (mean +/- SEM). Statistical significance is *p < 0.05. Children with DS (n=10), controls (n=10).

6.5 Discussion

We have demonstrated that melatonin has several significant effects on innate immunity, innate immune cells, receptors, and TLR pathways, and has the potential to reduce endotoxin induced inflammatory responses. Immunomodulatory effects of melatonin were found in both paediatric cohorts; however, it appears there may be more marked effects in the children with DS in relation to cytokine release, TLR signalling and genes involved in the inflammasome. To our knowledge this has not been carried out previously in this cohort or in healthy paediatric controls.

Melatonin is produced in the pineal gland at the base of the brain and exerts its effects through several mechanisms. Melatonin can mediate its actions via direct contact and interaction with molecules; and through antioxidant activity through chelation of reactive nitrogen and reactive species(238). As a hormone melatonin also functions through binding cell membrane receptors; MT1 and MT2 (located on almost all peripheral and CNS tissues), which once activated stimulate phospholipase A and C, ultimately reducing cGMP and cAMP(119). Furthermore, melatonin appears to have activity on receptors located in the nucleus also; retinoid Z receptors (RZR) and retinoid orphan receptors (ROR)(120). However, this special hormone's varied array of effects leads us to further consider other cells, receptors and pathways that may also be affected.

In our study, melatonin lowered CD11b on neutrophils and monocytes and TLR4 on monocytes compared to baseline and following LPS plus melatonin treatment in both cohorts. CD11b is a key receptor involved in the activation and diapedesis of immune cells promoting the inflammatory response(239), TLR4 is crucial in recognising LPS endotoxin, stimulating the innate response, and implicated in septic shock(240). This demonstrates the ability of melatonin to reduce cell activation, migration and abrogate TLR4 signal activation at the cell surface, respectively. This could have implications for improving outcomes in acute inflammation and sepsis or in chronic disease as both CD11b and TLR4 are associated with these processes(80, 157). In the intermediate monocyte population TLR4 expression was significantly decreased comparing LPS to LPS and melatonin samples in children with DS only. This subpopulation of monocyte has a pro- inflammatory phenotype, has the highest TLR4

expression, and is increased in sepsis(49, 182) which leads to worse outcomes in DS(8). This could represent a focus for further research examining the potential clinical utility of melatonin.

The analysis of the influence of melatonin on cytokines yielded perhaps the most significant results. In both cohorts there were decreases in IL-1β and Epo with melatonin and comparing LPS with LPS plus melatonin treated samples there were significant reductions in TNF-α, IL-8, IL-18, IL-1ra, Epo, and significant increases in IL-2 and GM-CSF again in both groups. However, a more pronounced melatonin response was observed in the DS cohort for certain mediators. In response to melatonin IL-8, IL-10 and IFN-γ were reduced compared to controls, whereas IL-6 was decreased in controls only. After samples treated with LPS and melatonin were compared to LPS alone, there were significant decreases in IL-1β, IL-6, and VEGF only in the children with DS. It is well described that melatonin can abrogate pro-inflammatory cytokine release. One mechanism of action is by impairing NF-κB transcriptional activity, reducing pro-inflammatory cytokine (IL-1β, TNF-α, IFN-γ) release(163, 241). It may be that this cohort may respond more effectively to melatonin immunomodulation and should be included in further experiments to elucidate this fully.

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection and it is associated with significant morbidity, mortality and sizeable economic costs(242). The metabolic pathways affected by sepsis lead to imbalances in cytokine production, oxidative stress and mitochondrial function(243, 244). Lipopolysaccharide (LPS) endotoxaemia is the commonest cause of sepsis(130), and the biochemical alterations translate to cell death and apoptosis leading to septic shock and multiorgan dysfunction. Rodent research has now shown that these clinical effects of endotoxaemia can be improved by administration of melatonin in conjunction with standard treatments(245, 246). In sepsis the mechanism of action of melatonin has been studied by Hu et al(144), who reported improved mitochondrial function and an inhibition of oxidative and nitrosative stress. Indeed, the robust antioxidant or free radical scavenging activity of melatonin is highlighted in several studies(164, 165). In animal models of LPS induced mastitis, melatonin has been shown to decrease the inflammatory response by reducing oxidative stress and

pro-inflammatory cytokines such as IL-1β, IL-6, GM-CSF(247, 248). Whilst in murine macrophages incubated with LPS, a reduction in inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) was observed(249). In a study on rats with LPS induced liver disease melatonin was found to result in better outcomes mainly through antiapoptotic and ROS scavenging actions(250). Novel research has demonstrated that in a murine model of polymicrobial sepsis, neutrophils and macrophages exhibit specific melatonin receptors and melatonin can augment neutrophil antimicrobial actions by promoting neutrophil extracellular traps (NET), and improve survival(251).

Much of the literature (above) focuses on animal or *ex vivo* experimentation on melatonin to assess its role as an immunomodulator, however there are some paediatric studies published which examined its use in patients with sepsis. Gitto et al(167), reported improved outcomes and survival in neonates with suspected sepsis (n=10) given melatonin versus controls (n=10). Improved mitochondrial function and restoration of ATP production was cited by the authors as one of the key reasons behind the improvement in clinical status in the treatment arm. A further study evaluating melatonin in a neonatal population (Rx n=25, control n=25) with sepsis found that there were better clinical and laboratory outcomes in those who received melatonin(143). Although these studies highlight the potential utility of melatonin as an adjuvant therapy in managing sepsis, these were non-randomised, non-blinded trials and the need for the establishment of large RCTs to examine melatonin further is needed.

In our study MyD88 gene expression was reduced compared to baseline with melatonin and following LPS and melatonin treatment in the DS cohort only. This suggests melatonin can mediate its effects on TLR signalling in DS. TLR receptors generate signals via two main pathways based on whether the adaptor protein MyD88 is used: MyD88 dependent or MyD88 independent pathways. All TLRs apart from TLR3 are MyD88 dependent and ultimately result in NF-kB and TNF production. The latter pathway signals through another adaptor, TRIF, inducing IRF3 causing IFN-β to be released(195). We previously we found a decrease in MyD88 and an increase in TRIF at baseline in children with DS vs controls(77). This potentially contributed to the increased susceptibility to recurrent infection in children with Ds and suggested

anomalous TLR signalling in DS which can have deleterious consequences due to dysregulated inflammatory cytokine release.

The potential effects of melatonin on the TLR signalling pathways has not been elucidated in detail. However, Kang et al(252) evaluated this by examining protein expression of MyD88, NF-kB, TRIF, IRF3 and IFN-β following liver ischaemia and reperfusion injury in mice. The authors report significant reductions in all of the above TLR signalling proteins, and attenuation of IL-6 and TNF-α, suggesting that melatonin plays a key role as an immunomodulator in TLR pathways. Another study appraised melatonin activity on TLR4 pathways on rodent RAW264.7 macrophages stimulated with LPS. The results showed that melatonin decreased both NF-kB and IRF7 expression, resulting in decreased production of TNF-α, IL-1β, IL-6, IL-8 and IL-10, suggesting that it has crucial inhibitory effects on key TLR4 proteins on both the MyD88 dependant and TRIF dependent pathways(163). Further insights to the mechanism by which melatonin may act on TLR4 and its pathway are provided by Fu et al(253), who describe how TLR4 and MyD88 expression were reduced through inhibition of TLR4 and MyD88 binding, and because of restriction of TLR/MD2/CD14 complex formation by melatonin. This is supported by Tamtaji et al(254), who commented on a similar mechanism and importance of melatonin as a TLR inhibitor. As we have demonstrated an excess of TLR4 on non-classical monocytes in children with DS(182), melatonin may prove to be of therapeutic benefit in this cohort, with further research required.

In evaluating melatonin and genes involved in inflammasome activation we discovered that on comparing LPS and LPS plus melatonin there were significant decreases in IL-1β and NLRP3 expression in the children with DS but not in controls. The inflammasome is part of the innate immune system and is a multiprotein complex that ultimately generates pro-inflammatory cytokines IL-1β and IL-18(94). The NLRP3 inflammasome was a source of interest given its association with chronic inflammation and autoimmunity(95), more prevalent in DS, and the reported greater circulating IL-1β levels in DS(90, 97). There appears to be a potential role for melatonin in inhibiting inflammasome activation; Liu et al(255) in a murine model of sepsis reported that following induction by LPS and melatonin, NLRP3 inflammasome activation was

reduced. Similarly, in Rahim et al(256) reported that melatonin counteracts the NF-kB/NLRP3 connection in response to LPS, and postulate that this agent may have a role in attenuating the inflammatory response by this mechanism. Given that a greater response to melatonin was seen in the DS cohort, and that there is an increased susceptibility to autoinflammation in this population also, specific targeting of the inflammasome could prove beneficial.

As discussed, autoimmunity is more prevalent in DS, and the potential for melatonin to do more harm than good in this context is controversial. Several autoimmune conditions have been associated with both exogeneous and endogenous melatonin(257). Arthropathy is common in DS and there is evidence that excess melatonin seen at higher latitudes, comparing Estonian and Italian patients with rheumatoid arthritis (RA) may be linked to this chronic disease(258). Furthermore, a positive association between disease severity and melatonin levels has been postulated in patients with RA versus controls(259). However, in an animal model of arthritis, melatonin reduced joint inflammation as an adjuvant therapy(260). In a study on human patients with RA given 10mg of melatonin nocte, the authors reported increased ESR, lower antioxidants, but no change in cytokine levels, and no significant change in clinical signs(261).

Melatonin appears to have pro and/or anti-inflammatory actions in different contexts. Human monocytes stimulated with melatonin *in vitro* improved the cytotoxic response to LPS, and also promoted IL-6 and IL-12 levels(262). Conversely, melatonin has been shown to attenuate neutrophil infiltration and reduce inflammation in the settings of experimental acute pancreatitis, lung injury and airway hyperreactivity(263, 264). The immunomodulatory response may also be dose dependent: with a low dose (10 nM) amplifying human neutrophil respiratory burst response, whereas an increased dose (2 mM) having the opposite effect(265). Targeted effects of melatonin on different cytokines in response to LPS sepsis in mice demonstrated that mice treated with the immunomodulator had much higher survival rates (90 versus 20%), and that pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-12 were reduced, the anti-inflammatory IL-10 was significantly raised(266). Shang et al(267) and Xu et al(268), also reported melatonin to result in significant increases in IL-10 in this

context. This may lead to a more balanced cytokine response to infection and improve outcomes in sepsis. In our study, there was a trend towards significant rises in IL-10 in both cohorts, and we did observe increases in IL-2 and GM-CSF for both groups. There is evidence that melatonin can augment IL-2 levels in monocytes *ex vivo* and that it may play a role in regulating CD4+ lymphocyte activity(269). Maestroni et al(270), reported that endogenous GM-CSF was increased with melatonin administered as a chemotherapeutic adjunct to counteract myelosuppressive side effects, adding to the list of immune functions that this hormone displays.

Our evidence suggests that there is a preferential response to melatonin in the DS cohort in relation to certain cytokine responses, TLR signalling and genes involved in inflammasome activation. One potential contributor is that the numbers in the DS group were lower than the controls for the cytokine experiments. Another may be that as a population, those with DS may exhibit altered endogenous melatonin levels. Of the few studies examining this there is conflicting evidence; Uberos et al(237) looked at serum melatonin levels in children with DS (n=15) and controls (n=15), and reported lower levels in the DS group, ((pointing out a possible inherent vulnerability to oxidative stress from free radical accumulation)). Contrary to this Reiter et al(234) examined the chief metabolite of melatonin, 6-hydroxymelatonin sulphate, in urine over 24hours (DS n=12). The absolute levels and variations in this melatonin metabolite were the same as controls. As we have outlined, sleep disruption is common in children with DS, which may potentially disturb their circadian rhythm causing anomalous melatonin levels and immune responses. Effects of disruption to circadian clocks and the immune response is discussed by Labrecque et al(111), who report that multiple cellular functions are disrupted: cellular migration, proliferation in response to pathogens, phagocytosis and cytolysis. In a Down syndrome mouse model evaluating sleep and circadian rhythm it was found that although the mice did exhibit sleep interruptions similar to patients with DS, their circadian rhythm remained unaffected(271). Fernandez et al(272) performed a cross-sectional study examining sleep and circadian rhythm periods in young children with DS (n=66, age range 5-67 months) and controls (n=43, age matched normal development). They observed that children with DS had more sleep fragmentation the circadian rhythms were robust,

and the authors conclude that any resultant cognitive delays are not exacerbated by alterations in circadian rhythms. This has been borne out in our work examining genes involved in regulating circadian rhythm by qPCR. We found that there were no significant differences in key genes BMAL, CLOCK, CRY and REV-ERBα, in children with DS (n=10) and controls (n=10) at baseline or after immunomodulation with LPS and melatonin.

Although not directly examined in our study, it is worth highlighting the potential benefits of melatonin in other aspects of medicine. For example, improving neurodegenerative change, especially relevant given the almost universal progression to Alzheimer's disease in DS(273). Melatonin may also have a neuroprotective role in DS. Corrales et al examined trisomic mice and demonstrated that melatonin treatment can increase the density and actions of neuronal synapses and reduce lipid peroxidation in the hippocampus potentially arresting the advance of neuropathology(235). Parisotto et al(236), reported that long term melatonin treatment decreased oxidative damage and cellular senescence in the brains of mice, and postulate that this therapy could attenuate neurodegenerative changes in adults with DS. However, another murine study found that the administration of melatonin pre and postnatally, somewhat reduced neuronal oxidative stress but failed to result arrest neurodegeneration in trisomic mice(274). It appears that melatonin administration in adulthood may have more neuroprotective benefits, but further trials are warranted.

Augmentation of the immune response to vaccination can potentially achieved with adjuvant melatonin therapy, this may be particularly relevant in DS where there is are known deficiencies following immunisation, with suboptimal antibody responses(125, 127). In animal studies melatonin has increased the humoral response and antibody titres in animals given melatonin versus those solely vaccinated(275, 276). This represents another tantalising avenue for experimentation with this multi-functional immunomodulator.

Overall melatonin mediates its immune response through a wide array of mechanisms, its effects appear be dose dependant and children with DS may be more receptive to treatment with it. Furthermore, this immunomodulator may play a key

role in buffering the inflammatory response by regulating pro and anti-inflammatory signalling depending on the context. A beneficial safety profile also makes this agent the subject of many further clinical trials(277), which are much needed to fully elucidate the actions of this pleiotropic hormone.

Chapter 7: Clinical Immunology in children with Down syndrome

7.1.1 Introduction

The rationale for immune screening for suspected primary immunodeficiency (PID) can be made by demonstrating that early recognition and diagnosis leads to better outcomes. This is particularly true in paediatrics as many of these conditions present in early infancy. The general paediatrician has a key role in being the first responder to further investigate the immune status based on presenting symptoms and signs and a high index of suspicion. Children with DS are an immunodeficient cohort with decreased T and B cell counts, suboptimal response to vaccinations, impaired neutrophil chemotaxis and dysregulated cytokine responses(10, 16). Clinically they are at increased risk of infections, particularly recurrent URTIs, otitis media and pneumonia(278), and more commonly require hospitalisation than other children(6). Therefore, given the increased morbidity, immune screening investigations are often performed to check the underlying immune phenotype of children with DS. A typical panel of tests might include; T and B lymphocyte subset analysis (enumerating CD3, CD4, CD8, CD19, CD56 cells), immunoglobulin levels (IgA, IgM, IgG), and antibody titres to immunisation (anti-pneumococcal, anti-tetanus, anti-haemophilus influenzae). Age appropriate reference ranges for immunological tests in childhood are important, especially in infancy where it is normal to have excess immunoglobulins from placental maternal antibodies and also a lymphocytosis(279). Indeed, the latter immunological phenomenon does not occur to the same extent in infants with DS(16), perhaps making them more vulnerable during this time. Although the clinical significance of these results remains obscure, the hope is they may allow us to focus on the high-risk child with DS, ensuring early prophylactic measures and aggressive treatment of suspected LRTI are initiated.

7.1.2 Hypothesis

We hypothesised that given the abnormalities in immune screening tests of children with DS, that there are potential biomarkers which could allow us to predict the clinical outcomes and focus care for higher risk children.

7.1.3 Aims

We aimed to evaluate the immune phenotype of children with DS and to examine the association of these biomarkers with outcomes such as recurrent respiratory tract infections or previous hospitalisations.

7.1.4 Results

7.1.4.1 Patient Demographics

There were 164 children with DS included in this study, with a mean ± SD age of 5.22 ± 4.34 years (y), of which 47.9% were female. The children were divided into four age categories: < 1 year (n=31;18.9%), 1-5 y (n=62; 37.8%), 5-10 y (n=45; 27.4%), > 10years (n=26; 15.9%).

7.1.4.2 Full blood count

There were 125 children with DS who had a full blood count (FBC), with a mean age of 5.80 ± 3.95 y, of which 45.9% were female. All blood test results were compared to the established age matched reference ranges for children(280-282). Overall 74.2% had a normal white cell count (WCC), 24.2% had a low WCC, and 1.6% had a raised WCC [Figure 7.1(a)]. The cohort was stratified by age into four different groups for further analysis: 0-1, 1-5, 5-10 and those over 10 years of age (up to 16.5 years). There were low WCC observed in 27.3% of infants, 16% of 1-5-year olds, 34.1% of 5-10 year olds, and 23.8% of those over the age of 10 [Figure 7.2(a)]. Neutrophils were low in 12.1% of children with DS (Cut off $<1.5x10^9$/l absolute neutrophil count (ANC)) normal in 83.9%, and high in 4%. Stratifying by age there were low neutrophil counts in: 36.4% of 0-1 year olds, 4% 1-5 years, 17.1% 5-10 year olds, and 9.5% of those over 10 [Figure 7.1(a) & 7.2(b)]. The lymphocyte counts were low in 22.6%,

normal in 73.9% and high in 3.5% [Figure 7.1(a)]. Low lymphocyte counts were found in 22.2% of infants, 20.5% 1-5 year olds, 20% of 5-10 year olds, and 33.3% of those over 10 [Figure 7.2(c)].

7.1.4.3 T and B lymphocyte subsets

There were 82 children with DS who had T and B lymphocyte subset analysis performed. CD3+ T lymphocyte counts were low in 45.1%, normal in 53.7%, and high in 1.2% [Figure 7.1(c)]. Stratifying by age 42.9% of infants, 54.1% of 1-5 year olds, 32% of 5-10 year olds, and 50% of over 10s had low CD3+ counts [Figure 7.3(a)]. CD4+ T cells were reduced in 48.8%, normal in 50%, and increased in 1.2%. Further analysis revealed decreased CD4+ counts in 12.5% of infants, 59.5% of 1-5 year olds, 32% of 5-10 year olds, and 41.7% of those over 10 [Figure 7.1(c) & 7.3(b)]. CD8+ cytotoxic T cell counts were decreased in 34.1%, normal in 61%, and high in 4.9%. Low CD8+ cell numbers were seen in 28.6% of infants, 45.9% of 1-5 year olds, 20% of 5-10 year olds, and 33.3% of over 10s [Figure 7.1(c) & 7.3(c)]. Natural killer cell (CD56+) counts were low in 15.9%, normal in 79.3%, and increased in 4.9% [Figure 7.1c]. NK cell numbers were reduced in 14.3% of infants, 32.4% of 1-5 year olds, 0% of 5-10 year olds, and 14.3% of 10+ year olds [Figure 7.3(d)]. The B lymphocytes (CD19+) were low in 76.8% and within the normal range in 23.2% [Figure 7.1(c)]. Stratifying by age there were low B cell counts in 57.1% of infants, 78.4% of 1-5 year olds, 84% of 5-10 year olds, and 66.7% of those over 10 [Figure 7.3(e)].

7.1.4.4 Immunoglobulins

There were 112 children with DS who had immunoglobulin levels checked. The vast majority of our cohort had normal IgM, IgG, and IgA in serum. IgM levels were normal in 96.1% and low in 3.9%. IgG was normal in 91.3%, low in 1.9% and high in 6.8%. Lastly, IgA was reduced in 2.7%, normal in 88.4%, and increased in 8.9% [Figure 7.1(b)].

7.1.4.5 Antibody titres

There were 61 children with DS who had antibody titres to pneumococcus and tetanus. Regarding total pneumococcal Ig, 98.3% had acceptable titres and 1.7%

had low levels. For pneumococcal Ig2, 11.5% had low antibody titres with 88.5% being acceptable. The tetanus titres were normal in 96.7%, and low in 3.3% [Figure 7.1(d)].

7.1.4.6 Clinical Data

The degree of morbidity due to recurrent respiratory tract infections (RRTIs), and hospitalisations due to RTIs in children with DS was assessed. RRTIs were defined as ≥ 3 episodes of lower respiratory tract symptoms; bronchitis, bronchiolitis or pneumonia per year(283). We also quantified how many of these children required hospital admission due to a RTI, by direct consultation with child and parents in the dedicated DS clinic and review of patient medical records.

There was clinical information available for 151 children with DS and 34.7% of families reported their child had recurrent respiratory tract infections. There were 56 (37.1%) children with DS who required admission to hospital due to a RTI at least once [Figure 7.1(e)]. Multiple hospital admissions were required for (n=27) 17.8% of the population and 56 patients who required admission to hospital with a RTI, (n=26) 46.4% were admitted on more than one occasion.

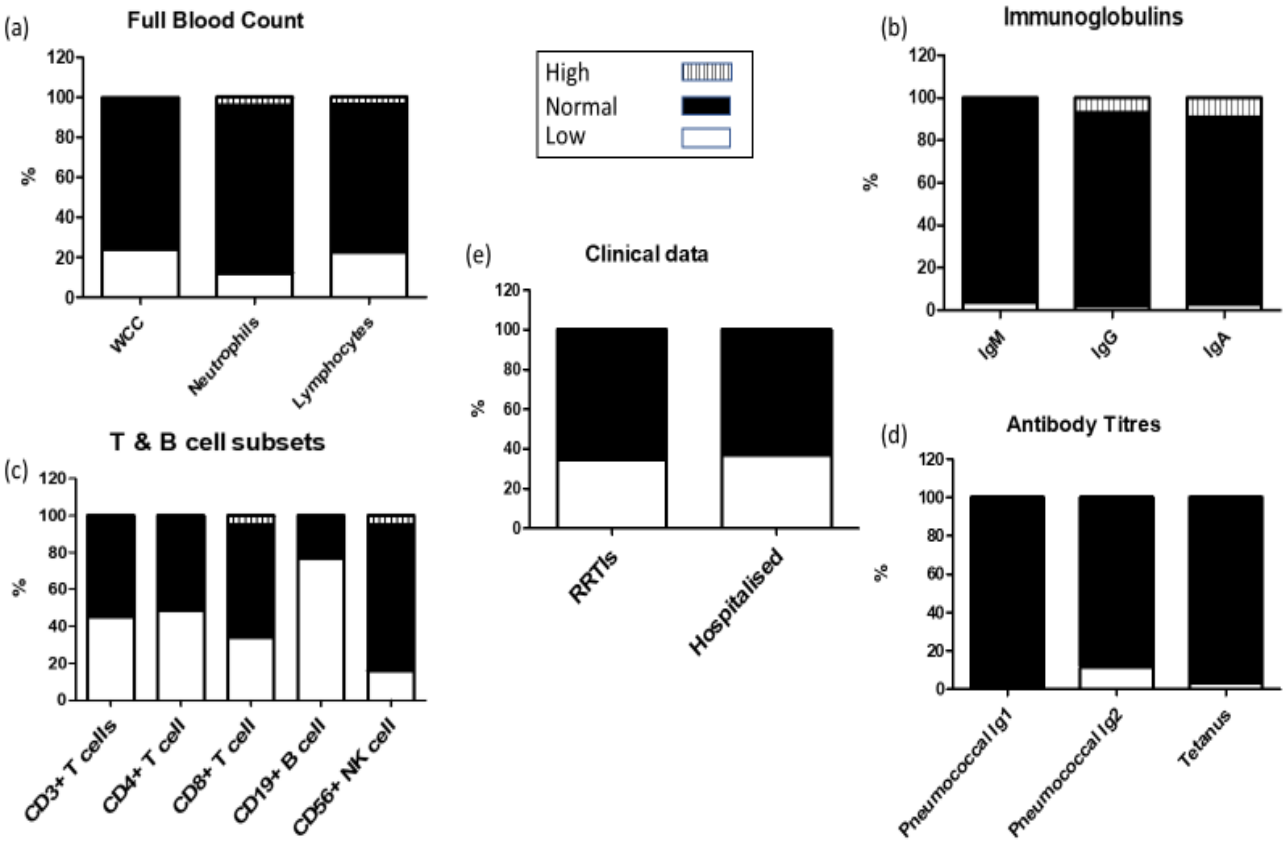


Figure 7.1. Full blood count, Immunoglobulins, T and B cell subsets, Antibody titres, and clinical data for children with DS.

Percentage of children with DS with low, normal or high blood counts for: (a) Full blood count (n=125), (b) Immunoglobulins (n=112) (c) T and B cell subsets (n=82), (d) Antibody titres to pneumococcus and tetanus (n=61), and (e) number of children with DS(n=164) who had a history of recurrent respiratory tract infections and hospitalisation due to respiratory tract infection.

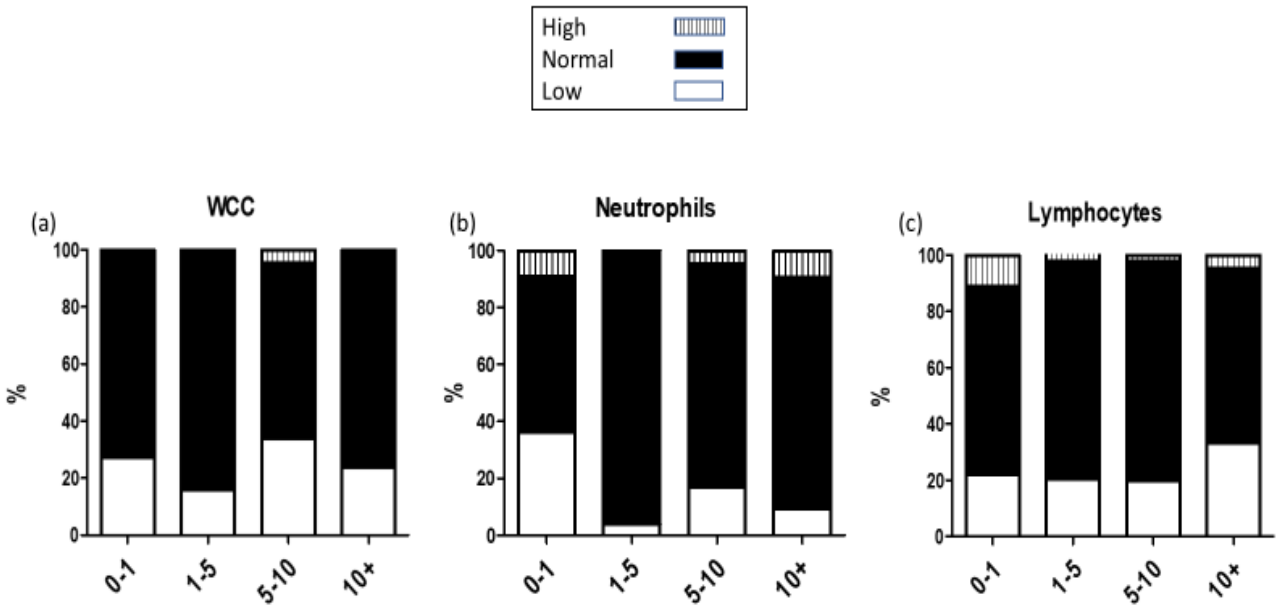


Figure 7.2. Percentage of children with DS with low, normal or high white cell, neutrophil and lymphocyte counts, stratified by age.

(WCC) (a), neutrophils (b), lymphocytes (c) stratified by age range: 0-1, 1-5, 5-10, 10-16 years old. (n=125).

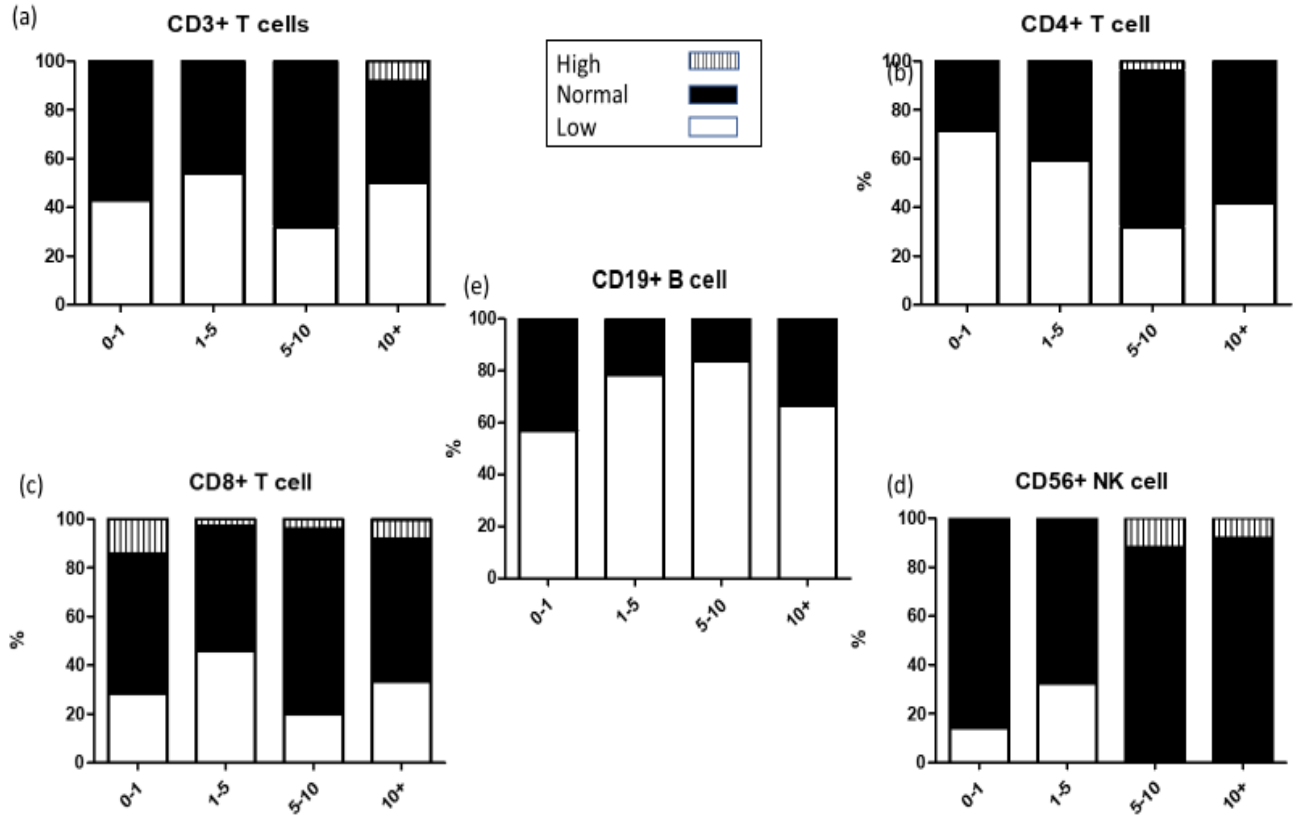


Figure 7.3. Percentage of children with DS with low, normal or high T and B cell subsets, stratified by age.

CD3+ (a), CD4+ T cell (b), CD8+ T cell (c), Natural killer (NK) cell (d), CD19+ B cell (e) stratified by age range: 0-1, 1-5, 5-10, 10-16 years old. (n=82).

7.1.4.7 Predictive markers

Possible associations between biomarkers like WCC and outcomes like RRTIs and hospital admissions with pneumonia were examined. The clinical outcome variable was split into those who were previously hospitalised (n=42) and those who have never been hospitalised (n=78) with pneumonia, and compared the mean WCC in those two groups, Table 7.1. Children who were previously hospitalised had significantly lower WCC than those who had never been admitted (p=0.009). Neutrophil counts were significantly lower in children with previous admissions with pneumonia versus those who were never hospitalised (p=0.02). There were no differences in mean lymphocyte counts between the hospitalised and non-hospitalised sub-groups (p=0.32; Table 7.1).

Table 7.1

Ever Hospitalised		n	Mean ($x10^9$/l)	Std. Deviation	Std. Error Mean	p-value
WCC	Hospital	42	5.48	1.99	0.31	**0.009**
	No Hospital	78	6.78	2.80	0.32	
Neutrophils	Hospital	42	2.53	1.31	0.20	**0.02**
	No Hospital	78	3.51	2.61	0.30	
Lymphocytes	Hospital	42	2.30	1.01	0.16	0.33
	No Hospital	78	2.47	0.91	0.10	

Table 7.1. White cell count, Neutrophils, and lymphocytes ($x10^9$/l) of children with DS who were previously hospitalised with pneumonia ("Hospital"), compared to those who were never hospitalised ("No Hospital").

WCC= white cell count; Std.= Standard. Unpaired t-test. P value <0.05 denoted in bold.

7.1.4.7 Predictive markers

There was a significant association between the white cell count and the clinical outcome of ever requiring hospitalisation, i.e. lower white cell count had a predictive association with requiring hospitalisation (p=0.015), using binary logistic regression models.

Examining this further, a WCC receiver operator curve (ROC) curve [Figure 7.4] had an area under the curve of 0.669 [95% Confidence intervals 0.563-0.774] and was significant (p=0.002). Neutrophils had similar statistical significance, with a low count more likely to have occurred on those who had been hospitalised. A ROC curve with similar stats was generated; AUC 0.668 [95% Confidence interval 0.568-0.771], (p=0.002) [Figure 7.5].

There was no association or predictive value between any biomarker and the clinical outcome measure of recurrent respiratory tract infections i.e. RRTIs were not associated with any abnormalities in immune screening tests. Those with a history of recurrent respiratory tract infections (RRTIs) also had significantly higher hospitalisation rate in the past (Odds ratio 3.6, 95% confidence interval 1.8-7.6).

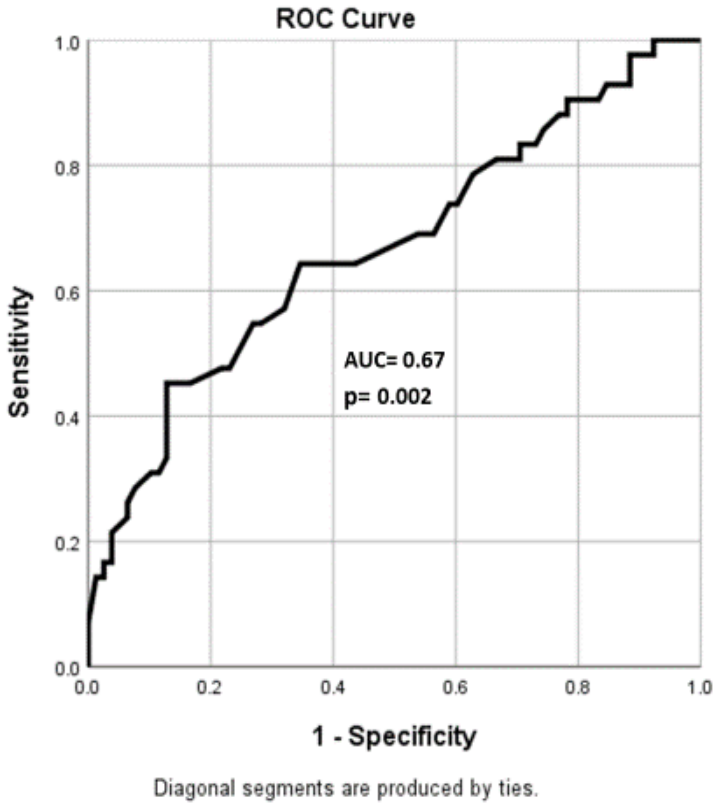


Figure 7.4. Receiver operator curve for WCC and hospitalisation in children with DS.

x axis= 1-specificty (false positive fraction), y axis= sensitivity (true positive fraction). Logistic regression. (n=120).

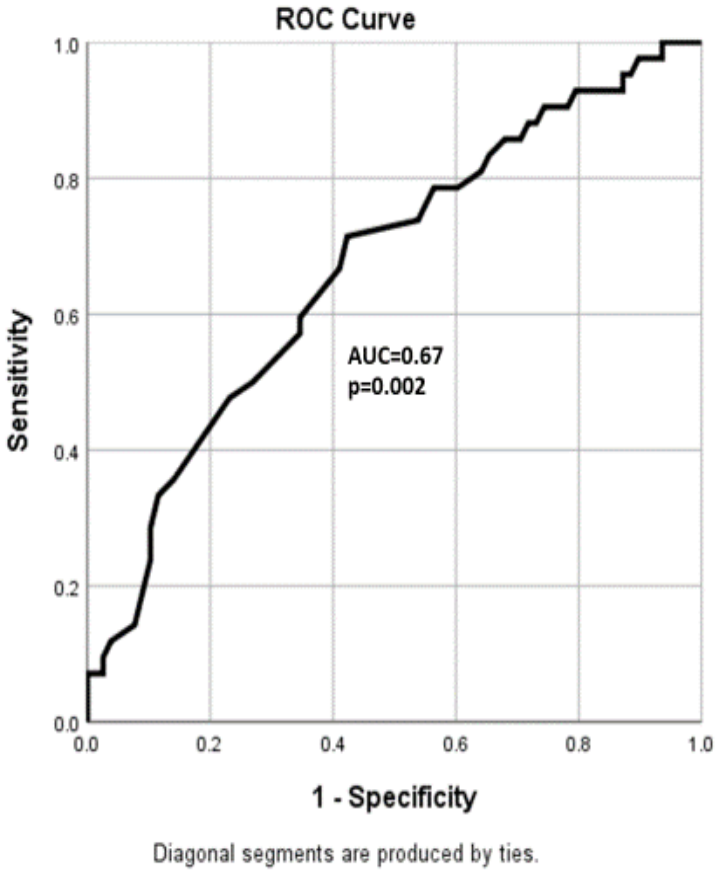


Figure 7.5. Receiver operator curve for Neutrophil count and hospitalisation in children with DS.

x axis= 1-specificty (false positive fraction), y axis= sensitivity (true positive fraction). Logistic regression. (n=120).

7.1.5 Discussion

We demonstrated significant differences in white cell count differentials and T and B cell subsets in children with DS compared to age matched normative values, in keeping with the literature (10, 16, 51). Overall, almost a quarter of children with DS had reduced WCC and lymphocyte counts, and 12% had lower neutrophil levels. The most striking deficiency in lymphocyte subsets was seen in the CD19+ B cell, with over three quarters of children with DS having reduced counts. This has been widely corroborated in other studies(27, 33). Overall T lymphocyte counts (CD3+, CD4+, CD8+) were markedly reduced in children with DS; almost half displaying low CD3+ and CD4+ counts, and over a third of the cohort showing decreased CD8+ cell numbers. Apart from the NK cells, we did not observe any significant improvements in cell subset counts as age increased. At odds with some previous reports, we found immunoglobulin levels (IgM, IgG, IgA) which were close to normal, as well as relatively robust antibody responses to pneumococcus and tetanus immunisation(127, 128).

From a clinical perspective, as expected these children have significant medical comorbidity in relation to RRTIs and hospitalisations secondary to LRTIs. We reported that over one third of children with DS have had RRTIs, and 37% of this population were hospitalised at least once due to a RTI. Furthermore, almost half of these children were admitted on more than one occasion. Evaluating the possible associations/correlations between biomarkers (WCC, neutrophils) and outcome (history of hospitalisation), we found that children with DS who had a low WCC or neutrophil count were significantly more likely to have required hospitalisation due to a RTI. Indeed, the lower the white cell count or neutrophil count the higher the probability of that patient requiring admission to hospital.

This association between WCC and previous hospitalisation could be significant, although the ROC curves do not suggest a particularly sensitive or specific test in this instance. Our AUC of 0.67 suggests this test is below the widely regarded acceptable level for diagnostic accuracy (0.7), with results greater than 0.8 considered excellent(284, 285). The clinical implications of an abnormal immune phenotype have been evaluated previously. Martinez et al(30) correlated infection related hospital admissions with abnormal immune parameters in children with DS. Retrospectively,

the numbers of Tregs, B cells and age moderately correlated with infection related hospitalisations, whilst only Treg number and age correlated with infection related admissions prospectively. Mitwalli et al performed a large (DS n=150, Controls n=100) recent prospective case control study was carried out examining the following cell surface markers by flow cytometry; T cells (CD3, CD4, CD8), B cells (CD19), Natural killer cells (CD56), and appraised whether there was any correlation between these immune parameters, recurrent infections and hospitalisation(286). Similar to our study, they acknowledged the increased infectious burden faced by these children; significantly increased infections in the DS cohort (OTM, URTIs, sinusitis, RTIs), however no clear correlation could be elucidated with the WCC or any of the T and B cell subsets. Kusters et al could also not find a significant link between reduced T cell counts and infections in this population(287). Natural killer cell aberrations in DS and links to repeated infections and hospital admissions were reviewed by Ribeiro et al(288) who did not report any significant link.

In our study the CD19+ B cell was the most markedly reduced of all the other cell lines, and as mentioned this is corroborated in several other studies on children with DS(24, 27). There is plenty of evidence that children with DS have reduced CD19+ B cells in comparison to their peers(29, 286, 289, 290), and there is also evidence that B cell function is also impaired with a reduction of switched memory B cells, a reduced capacity to respond to vaccination or a secondary pathogenic insult, leading to a less robust immune response over time(27, 33). This cell subset may have a correlation with increased infection burden and hospitalisation. Seckin et al(291) examined alterations in the CD19 complex and memory B cells in children with DS. Taking 37 patients and 39 controls they reported reduced B cell numbers for all age groups. Looking at different cell surface markers in more detail they found a link between decreased CD27+IgM+IgD+ natural effector cells and an increased risk of severe RTIs and subsequent hospital admissions. In contrast, Verstegen et al (29) looked at B cell subpopulations, immunoglobulins in DS and also concluded that although there were anomalies of the humoral immune system in DS including reduced B lymphocyte production and hypergammaglobulinaemia suggestive of defective T-lymphocytes, they could not correlate this with clinical features and increased infection risk.

Although normal immunoglobulin levels may be present perhaps it is the ability to acutely react and produce specific antibody post pathogenic insult, and or the maintenance of vigorous long-term immunity which renders these children high risk.

Regarding T lymphocyte counts in DS, and first assessing CD3+ cells, it has been reported that up until the age of 13 years children with DS have significantly fewer CD3+ cells versus controls, however, beyond this age the disparity diminishes. This supports the evidence that the gap in lymphocyte subsets between controls and children with DS narrows with age(292), something that we did not observe in our cohort up to the age of 16 years. There has been some conflicting evidence in the literature regarding CD4+ cell number in DS. Mitwalli et al(286) demonstrated a reduced number of CD4 cells in children with DS, as have several other studies(290, 293), whilst another paper quoted increased CD4 counts in this cohort(35). The latter paper also reported higher cytotoxic T cell (CD8+) percentage in children with DS, however this is also at odds with other published literature showing reduced CD8+ counts(286). Lastly, Schoch et al described no difference in CD8+ cells in this population(24).

We have reported essentially normal immunoglobulin IgM, IgG, IgA levels in children with DS. Martinez et al, reported immunoglobulin dyscrasia in DS with elevated IgA, IgG and reduced IgM(30). There have been several publications on quantitative immunoglobulins in DS, however, there are variances in the reported values and there are no definitive conclusions on the normative immunoglobulin levels in DS(294, 295). Furthermore, from a general paediatric perspective one should always consider the loss of immunoglobulin may be due to GIT, renal losses, medications or malignancy(296). Indeed, it is interesting that children with DS can produce immunoglobulins, but they seem to produce them in suboptimal quantities and may not be able to maintain long term immunity over time(27, 125).

Regarding antibody titres, we previously highlighted the vulnerability of this population in relation to vaccine preventable diseases and advocated for extra immunisations for children with DS(297). Indeed, there is evidence that the suboptimal titres may directly translate into an increased infection burden. A study looking at the infectious history, peripheral blood markers; immunoglobulins, pneumococcal

polysaccharide titres and memory B cells in 40 children with DS reported that only 5 had reached clinical protective antibody titre level to half of the serotypes evaluated. Eleven of these children were re-immunised to pneumococcus but 63% demonstrated persistently low titres after re-vaccination. Furthermore, from a clinical perspective those who had non-protective titres had 40% increased history of sinusitis and otitis and a significantly increased risk of pneumonia(298).

The clinical manifestations of immune dysregulation for adults with DS appear varied. Guffroy et al(299) looked at 37 adults with DS (mean age 27 years) and assessed for both child and adulthood history of infections, as well as performing some immune screening blood tests (T and B cell subsets, immunoglobulins and antibody titres). The authors report that although 54% reported frequent infections in childhood, only one patient was documented as having recurrent infections as an adult. In conclusion, despite persistently low total and memory B cell counts originating in childhood where recurrent infections manifest, adults with DS do not appear to succumb to this clinical complication as easily, suggesting an offsetting between the persistent anomalous immune cell markers with clinical outcomes. Interestingly, this protective timeline does not last and increased cognitive decline from the 5th decade is associated with a return of the infectious burden and morbidity(299).

Finding a biomarker which is readily available and allows effective prognostication is something that is central to much clinical research. In DS, as mentioned, there are some more specialised immune markers such as Tregs(30), and specific B cell subpopulations (CD27+IgM+IgD+)(291) which appear to have some correlation with clinical outcomes, however, these tests are not mainstream currently and further studies are required to authenticate this association. Otherwise, mannan binding lectin (MBL) deficiency in DS, which is important in opsonisation and initiation of the complement pathway, has been shown to be linked to an increased risk of recurrent infections and pneumonia(300). Again the evidence is small, this is an isolated paper involving patients with DS, and there has been other evidence questioning the clinical significance of MBL in relation to RTIs(301).

To our knowledge, this is the first study to make a link between WCC and a history of hospitalisation in children with DS. The lower the WCC the greater the probability of having a history of hospital admission due to an RTI. There was no such association between WCC or any other biomarker and RRTI. Further studies are required to corroborate our results but identifying those children with DS who are at greatest risk with a simple, ubiquitous test could be key to improving the standard of care. Consequently, the WCC and neutrophil count could serve as a predictor for poorer respiratory outcomes. Children with DS should be managed with a heightened sense of awareness and urgency in the setting of RTI.

7.2 Do children with Down syndrome benefit from extra vaccinations?

7.2.1 Introduction

We performed an evidence-based synthesis of data following the format of "Best bets" as delineated below (www.bestbets.org). This process allows "the integration of clinical information obtained from a patient with the best evidence available from clinical research and experience, and the application of this knowledge to the prevention, diagnosis or management of disease in that child"(302). Ultimately this provides a succinct summary of the best available evidence to help better inform clinical decision making.

7.2.2 Scenario

A 4-month-old baby girl with Down syndrome attended the General Paediatric clinic for routine follow up. She had commenced her routine vaccination schedule. The Registrar asked, as children with Down syndrome are at increased risk of infections, should this baby have any extra vaccinations?

7.2.3 Structured clinical question

In children with Down syndrome [population] do additional booster vaccinations [intervention] as well as routine improve outcome [outcome]?

7.2.4 Search Strategy

7.2.4.1 Primary sources

MEDLINE was searched via PubMed up to January 2018; the advanced search mode was used including the terms "Down syndrome" or "Trisomy 21" and "immunisation" or "vaccination" or "booster immunisation" or "booster vaccination" or "antibody titres" or "antibody response or "vaccine titre" or "vaccine response". The articles were selected based on the "most relevant" search mode.

7.2.4.2 Secondary sources

A search of the Cochrane Library using the search terms “Down syndrome”, “Trisomy 21” and “vaccinations”, “immunisations” were performed. No significant results were found.

7.2.5 Commentary

Children with DS are at increased risk of infections such as pneumonia and mortality from sepsis secondary to altered immune function. In Down syndrome, there are reduced numbers of T and B lymphocytes, a dysregulation of cytokines and often a sub-optimal antibody response to vaccination(1). Furthermore, there is a defect in the generation and maintenance of switched memory B cells(33), which are key in ensuring the longevity of a potent immune response over time.

Children with DS are at higher risk of admission to hospital and intensive care with respiratory tract infections (RTIs)(3), which have a significant negative impact on development, behaviour and quality of life(4). Seasonal influenza and pneumococcal vaccination have been suggested. Following the H1N1 pandemic the increased risk of hospitalisation, intubation and death in patients with DS was 16, 8 and 335-fold respectively, compared with patients without DS(303). However, the immune response in children with DS to influenza may be reduced; a study looking at this cohort reported that following two doses of Influenza A/H1N1, only 27% of patients (n=48) reached the cut off for a 50% clinical protection rate(127), compared to healthy control children in whom >90% reached the critical protection rate after one dose(304).

7.2.6 Vaccine responses

Response to immunisation against pneumococcal disease has shown varying results. Kusters et al(8) looked at antibody titres and opsonophagocytosis (n=18 DS cases) to conjugated and unconjugated pneumococcal vaccines and found that there were adequate serotype specific titres to all conjugated and nearly all unconjugated

serotypes utilised. The author suggests that children with DS do not have any significant defects in their response to polysaccharide antigen. In contrast, IgG response to inactivated influenza and pneumococcal polysaccharide vaccine (n=12) showed no differences between children with DS and controls post inactivated influenza vaccine, however the response to polysaccharide pneumococcal vaccine (PPV) was suboptimal in children with DS(9). The antibody response in Down syndrome to the capsular polysaccharide vaccine (23-valent pneumococcal vaccine) showed a significant rise, although the antibody titres post immunisation were reduced compared with controls(10).

The level of clinical protection conferred following these altered immune responses to immunisation is difficult to establish for certain. Looking at immunity to other vaccines in DS, it has been shown that specific Meningococcal C polysaccharide IgM, IgG, and IgA concentrations were significantly lower than the normal ranges but considered protective at 3 months and 1-year post immunisation (11). On the contrary, hepatitis A immunisation has been shown to be as efficacious in children with DS (n=63) as controls (n=64) (12). An appraisal of the above studies focusing on immune responses to vaccinations in Down syndrome is found in table 7.2.1.

Table 7.2.1. Immune responses to vaccinations in Down syndrome

Citation	Study group	Study type	Outcome	Key Result	Comments
Kusters et al. 2011 (a) (11)	DS n=19 5.3-17.4 years Compared with reference ranges	Prospective cohort (4) **Men C**	Specific Antibody levels at 3/12 and 12/12 to MenC conjugated vaccine	Reduced specific Antibody responses to MenC vaccine	Protective but reduced response to MenC vaccine
Kusters et al. 2012 (b) (6)	DS n=48 0.7-17 years versus unvaccinated DS n=25 0.2-19 years	Prospective cohort (4) **Influenza A/ H1N1**	Vaccination response to 2 doses of Influenza A/H1N1 At 23-267d post vaccination	92% of the children reached protective level (hemagglutination-inhibition titre >/=1:40), only 27% reached >/=1:110 which predicts 50% clinical protection rate.	Poor immune response from 2 doses influenza A/H1N1
Kusters et al. 2013 (c) (8)	DS n=18 6-24 years versus aged matched controls	Prospective cohort (4) **Pneumococcal - conjugated +unconjugated**	Titres and opsonophagocytosis to conjugated & unconjugated pneumococcal vaccine	Adequate titres to all conjugated & nearly all unconjugated pneumococcal serotypes	Despite low B cells & CVID like state, can mount a sufficient immune response to pneumococcus

Citation	Study group	Study type	Outcome	Key Result	Comments
Costa-Carvalho et al. **2006** **(10)**	DS pts n=17 6-13 years Age matched controls n=30	Prospective cohort (4) **Pneuno 23**	IgG antibodies to pneumococcal serotypes (1, 3, 6B, 9V, and 14) before and 6/52 post=immunization with a 23-valent pneumococcal vaccine (Pneumo23®)	All children with DS 4-fold increase in post-immunization in serotypes 1 and 14, in 90% of subjects for serotypes 3 and 9V, and in 65% for serotype 6B	Despite lower levels compared to normal controls, DS children showed a 7-fold mean increase in antibodies Suggests they produced pneumococcal antibodies
Joshi et al. **2011** **(9)**	DS n=12 2-18 years Control n=12 2-18 years	Prospective cohort (4) **Inactivated influenza + pneumococcal polysaccharide**	Quantitative assessment of IgG response to inactivated influenza & pneumococcal polysaccharide vaccine and lymphocyte subpopulations	No differences in the % of cases and controls who responded to inactivated influenza vaccine, Response to polysaccharide pneumococcal vaccine suboptimal in cases	Polysaccharide vaccine may have lower immunogenicity in all children possibility of using a protein conjugate vaccine may hold promise

Citation	Study group	Study type	Outcome	Key Result	Comments
Valentini et al. 2015 (2)	DS n=15 3-12 years Controls n=15 3-12 years	Prospective cohort **Influenza + pneumococcal conjugated**	Serum antibody titres and specific memory B cell numbers post influenza + pneumococcal vaccine	Impaired production of specific switched memory B cells after primary vaccination (influenza), Increase of specific switched memory B cells after booster vaccination (PCV), Normal serum antibodies response to vaccination	DS individuals respond to vaccination with antibody secretion, less able to produce and maintain switched memory B cells
Kusters et al. 2011 (d)	DS n=22 (4-9 years) Age matched controls	Cross sectional study **Tetanus**	Avidity of the antibody response post tetanus toxoid (TT) booster	Although overall anti-TT titres were protective the initial levels low at 4 years of age versus controls, and despite normalization of titres over time, the avidity maturation remained impaired at 9 years old	Partially explained by a deficiency in memory B cell selection in the germinal centres, which cannot be ameliorated by increasing age or booster immunisation in this context
Ferreira et al. 2004 (12)	DS n=63 Control =64 Age 1-12 years	Prospective cohort **HAV**	Seroconversion and anti-HAV titers were measured at months 1 and 7	Seroconversion rates at month 1 were 92% and 94% in the DS and control groups, respectively. At month 7, seroconversion rates were 100% in both groups	HAV vaccine well tolerated and highly immunogenic in children with DS.

Legend of Abbreviations: MenC = meningococcal serotype C, CVID = common variable immune deficiency, Pneumo 23 = 23 – valent pneumococcal vaccine, PCV = pneumococcal conjugate vaccine, HAV = hepatitis A virus.

7.2.7 Immunisation guidelines

Given the increased infection risk and varying immune response to vaccination, what immunisation guidelines, specific to Down syndrome, are available? Irish vaccination guidelines suggest that children with Down syndrome should receive the following extra vaccines: inactivated influenza annually, Meningococcal ACWY, Meningococcal B, and Pneumococcal polysaccharide vaccine 23 (PPV23) at ≥2 years, at least 2 months post Pneumococcal conjugate vaccine (PCV)(13). New Zealand and the DSMIG (UK) recommend influenza vaccination from 6 months, PCV13 and PPV23 from 2 years of age (14, 15). However, there are no specific guidelines form other national sources including the Center for Disease Control and Prevention (US), American Academy of Pediatrics, The UK Joint Committee on Vaccination and Immunisation (The Green Book) and Australia (16-20). The Green book does not explicitly list Down syndrome as an "at risk" condition; a systematic review appraising medical conditions at higher risk of complications of influenza was unable to draw firm conclusions regarding Down syndrome (22).

7.2.8 Conclusion

Children with DS represent an immunodeficient group and are at higher risk of infections and mortality from sepsis (21). In conclusion, DS has been described as a primary immunodeficiency, and the defective switched memory B lymphocytes offer a credible explanation for part of the immunological features described (22). Consequently, these children may not be able to maintain appropriate long-term immunity. It is important therefore to highlight to physicians the deficiencies post immunisation in Down syndrome children. Future studies assessing primary and booster response to immunisation should be considered to establish protection has been conferred, and tailored vaccination schedules can be created to ensure that the immunity in this high-risk group remains robust as they grow.

Clinical bottom line

1. All children with Down syndrome should receive the following additional vaccines: annual influenza vaccine from 6 months of age, 23 valent pneumococcal vaccine from 2 years of age, and consideration should be given to Meningococcal ACWY (and Meningococcal B if not already given).
2. Long term immunogenicity of vaccinations is unknown in this population and may require boosters.
3. Recurrent vaccine preventable diseases may have a negative impact on long-term outcome.

7.3 Pavilizumab for all children with Down syndrome?

7.3.1 Introduction

We performed an evidence-based synthesis of data following the format of "Best bets" as delineated below (www.bestbets.org). This process allows "the integration of clinical information obtained from a patient with the best evidence available from clinical research and experience, and the application of this knowledge to the prevention, diagnosis or management of disease in that child"(302). Ultimately this provides a succinct summary of the best available evidence to help better inform clinical decision making.

7.3.2 Scenario

A 4-month-old boy with Down syndrome attended the General Paediatric clinic for routine follow up. With Winter approaching the Registrar asked, as children with Down syndrome are at increased risk of respiratory tract infections, should this baby receive pavilizumab prophylaxis, even if there is no congenital heart disease?

7.3.3 Structured clinical question

Should children with Down syndrome without congenital heart disease or prematurity [population] receive pavilizumab prophylaxis [intervention] to improve outcome [outcome]?

7.3.4 Search Strategy

7.3.4.1 Primary sources

MEDLINE was searched via PubMed up to July 2018; the advanced search mode was used including the terms "Down syndrome" or "Trisomy 21" and "pavilizumab" or "synagis" or "pavilizumab vaccination" or "pavilizumab immunisation" or "synagis vaccine" or "synagis immunisation" or pavilizumab prophylaxis or synagis prophylaxis". The articles were selected based on the "most relevant" search mode.

7.3.4.2 Secondary sources

A search of the Cochrane Library using the search terms “Down syndrome”, “Trisomy 21” and “pavilizumab”, “synagis” were performed. No significant results were found.

7.3.5 Comments

Respiratory syncytial virus (RSV) is the most common cause of bronchiolitis and is responsible for significant morbidity and mortality worldwide. It primarily affects infants and approximately 1% of children of this age in Europe and the USA will be hospitalised over bronchiolitis season which typically runs from November to March(305, 306). Pavilizumab is a humanised monoclonal antibody which provides passive immunity to the recipient, and although not a cure for RSV, has shown to be effective in reducing hospitalisation rates (by 55-72%) in high risk groups(307). It is usually given prophylactically; max 5 doses one month apart starting in the Autumn to those considered at greater risk: infants who are ex premature <29 weeks, evidence of chronic lung disease (CLD), congenital heart disease (CHD), pulmonary abnormality, neuromuscular disease, significant immunosuppression(308).

Children with Down syndrome (DS) may receive pavilizumab prophylaxis due to a congenital heart lesion, prematurity or another risk factor, but they are not specifically listed as a high-risk group by the AAP, the Green Book, or the Australian immunisation handbook(308-310). Indeed, to our knowledge only Japan and parts of Canada are the only places to recommend RSV prophylaxis for DS, regardless of CHD.(311, 312).

Are children with DS, without CHD at a greater risk of RSV related hospitalisation also? Should all children with DS receive this prophylaxis regardless of cardiac history? Bloemers et al(313) performed a retrospective and prospective observational study to assess the incidence of RSV associated admissions in DS. They found that 7.6% of children with DS without CHD required admission vs 0.7% of controls (siblings of children with DS) and concluded that DS is an independent risk factor for hospitalisation due to RSV. In a national retrospective 1:2 (DS n=814) (Control n=1628) matched cohort study, Grut et al(314) found higher admission rates

in children with DS into the 2nd year of life, and surmised that DS is an individual risk factor for RSV hospitalisation even when adjusting for CHD, CLD and prematurity. A systematic review examining hospitalisation rates due to RSV in DS stated that DS constitutes a 6.8 fold increase in the relative risk of hospitalisation due to RSV, and it represents a significant independent risk factor for RSV related admissions(315).

There are several studies showing that children with DS have more severe RSV disease once admitted(7, 311, 316, 317). Stagliano et al(316) reported that children with DS were more likely to require respiratory support and that those without additional risk factors such as CHD or prematurity, were also more likely to require a longer hospital stay compared to controls. Another retrospective cohort study found a more severe clinical course in children with DS once admitted; longer admissions and an increased need for intensive care support(317). Lee et al(7) studied infants with RSV bronchiolitis requiring PICU admission, and found that the highest mortality risk was in children with DS compared to those with other at risk cohorts such as prematurity or CLD. However, further studies are needed in relation to RSV related mortality in DS to fully evaluate this link. This burden of disease will obviously have a significant economic impact; increasing the burden associated with treating these patients for longer in more expensive beds. What about the cost effectiveness of administering prophylaxis for high risk children? A UK study reported that the following high risk groups fell within the economically viable £30,000/QALY (quality adjusted life year); infants <24 months with CLD, CHD, preterm <32 weeks, preterm 33-35 weeks with risk factors(318), this was supported by the conclusions of a systematic review which stated that pavilizumab was cost effective at improving outcomes in high risk children(319). As the evidence suggests any child with DS is at risk of similar or even more severe RSV disease than others, the argument can also be made for including them from an economic perspective.

Comparing DS and other high-risk groups (prematurity, CLD etc) for RSV associated hospitalisation, it is becoming more apparent that these children are at as much risk for more severe respiratory morbidity. The authors of a retrospective cohort study concluded that DS was more robustly associated with hospitalisation due to RSV than any other risk factor apart from immunodeficiency. Furthermore, when the

estimated percent of children with RSV hospitalisation was stratified by any known risk factor and whether DS was present or not, it was found that having DS without risk factors carried a higher risk than not having DS with risk factors(316). A UK based study examining the risk factors for hospital admission with RSV bronchiolitis found that DS (with and without CHD) had a higher relative risk of hospitalisation vs prematurity or chronic lung disease (RR = 2.5, 95% CI 1.7-3.7 v RR = 1.9, 95% CI 1.8-2.0 v RR 1.6 95% CI 1.4-1.8 respectively)(320). Comparing the risk of hospitalisation between children with DS without CHD, and children without DS with CHD, the admission rate was higher in the children with DS(321). The conclusion that children with DS without CHD are at as much risk for worse RSV bronchiolitis as other high risk groups is shared by Doucette et al(322), who state that these children should be treated the same as those with CLD or CHD.

If children with DS are at greater risk of and incur greater RSV related disease, is pavilizumab efficacious in this population? Yi et al(323) in their prospective cohort study, looked at the hospitalisation rates due to RTI for children with DS treated with pavilizumab (n=532) versus those untreated (n=233). They found a 3.6-fold reduction in the RSV related hospitalisation rate in those treated with the vaccine. Children with DS receiving prophylaxis are less likely to require admission; the German synagis registry 2009-2016 reported that again, pavilizumab was effective in reducing admission rates due to RSV in DS, but also that the vaccine was safe with few adverse effects observed(324). In another observational post marketing study, Kashiwagi et al(325), examined the clinical efficacy of pavilizumab in children with DS without significant CHD and found that the intervention was well tolerated and effective in this cohort.

7.3.6 Conclusion

Although many of the studies above are either retrospective(313, 314, 316, 317) or prospective observational(313, 321, 323, 325-327) studies, and a well-designed randomised controlled trial would be desirable, a recent meta-analysis examining DS and the risk of severe RSV concluded that children with DS without additional risk

factors were at significantly greater risk of worse clinical outcomes(328). Therefore, the available evidence supports that children with DS are at increased risk of hospitalisation from RSV related RTI, have more severe disease, necessitating longer hospital stays and more often requiring ventilatory support(315-317, 327). Importantly, this appears to be independent of cardiac history(311, 313-316, 327, 328), and therefore we would advocate that every child with DS be offered prophylaxis to reduce morbidity and improve outcomes. Several studies completed show that pavilizumab is efficacious in reducing the incidence of hospitalisation due to RSV in children with DS with and without CHD(324-326). Lastly, it has been suggested that RSV related disease occurs for longer, in the second year of life and beyond in children with DS(314, 316), so should these children receive the vaccine for a second bronchiolitis season as indicated?

Clinical bottom line (Grade of recommendation CEBM):

1. DS is an independent risk factor for more severe RSV related disease. (independent of cardiac history or other risk factors) (B)
2. Increased morbidity in DS due to RSV; increased hospitalisation rates, longer length of stays and more likely to require intensive care. (B)
3. Pavilizumab is safe and effective at reducing hospitalisation rates in DS (with and without risk factors). Consideration to give prophylaxis to all children with DS. (B)

Table7.3.1. Evidence of RSV related disease and efficacy of pavilizumab in DS.

Citation	Study group	Study type (level of evidence CEBM)	Outcome	Key Result	Comments
Bloemers et al 2007	DS n=395 Age= <2y Controls= siblings of DS n= 276	A retrospective observational study & a prospective birth-cohort study of children with DS (2b)	To determine the incidence of RSV lower respiratory tract infection-associated hospitalization among children DS	7.6% of term children with DS **without CHD** v 0.7% of controls were hospitalised with RSV related LRTIs	Down syndrome (regardless of CHD) is an independent risk factor for hospitalisation due to RSV
Medrano Lopez et al 2009	DS n=279 No CHD n=105 Significant CHD n=135 Non-significant CHD n=38 Age= <24m Controls n=806 Age= <24m	Multicentre, observational prospective study (2b)	To compare hospitalisation rates for LRTI in children < 24m with significant CHD without DS to those with DS with or without CHD	Hospitalisation due to RSV was 7.8% v 3.2% in DS v non-DS cohorts. In DS hospitalisation rates were 15.9% in non-significant CHD, 9.3% in significant CHD and 3% in no CHD	In DS hospitalisation rates are higher due to RTI, especially RSV. The DS subgroup most likely to require admission were those with non-significant CHD and low immunoprophylaxis v RSV
Galleguillos et al 2016	DS n= 58 Mean age = 26.4m Control n=58 Mean age= 11.8m	Retrospective cohort study (2b)	To compare clinical characteristics of children with DS and without admitted with RSV	Children with DS had longer length of stays; 6 v 4 days (p<0.0001), more likely to need PICU (43% v 22% p=0.017)	Children with DS experience more severe RSV disease, spending longer in hospital and are more likely to require PICU

Citation	Study group	Study type (level of evidence CEBM)	Outcome	Key Result	Comments
Stagliano et al. 2015	DS n=842 0-36m Controls n=632,358 0-36m	retrospective cohort study (2b)	To assess DS as an independent risk factor for RSV hospitalization in children younger than 3 years of age and to examine severity of illness	Hospitalisation rate of 9.6% v 2.8% in DS v controls. Median length of stay was 4 days v 2 days in DS v controls (P < .001) More likely to require respiratory support in DS	DS is an independent risk factor for RSV hospitalisation and more severe disease. These children tend to be older and risk persists over 24m of age. DS without risk factors like CHD, prematurity were also more likely to be hospitalised and have longer admissions than controls
Grut et al 2017	DS n=814 0-12m= 4m 12-24m= 17.5m Controls n=1628 0-12m= 3.5m 12-24m=13m	National, retrospective 1:2 matched cohort study 2006-20011 (2b)	Hospitalisation risk due to RSV in DS under 2 years Adjust for other risk factors such as CHD, prematurity	82 children with DS (10.1%) & 22 controls (1.4%) were hospitalised for RSV Persistence of RSV hospitalisation in DS in 2nd yr. of life	DS is an individual risk factor for RSV hospitalisation, adjusted for CHD, prematurity, CLD, and older siblings.

Citation	Study group	Study type (level of evidence CEBM)	Outcome	Key Result	Comments
Mori et al. 2014	N/A	Review article (5)	Review of the literature on RSV infections in children with immunodeficiencies or Down's syndrome To establish the risks, pathophysiology of severe RSV disease	DS itself has been shown to be a risk factor for worse RSV infection, regardless of CHD status	Pavilizumab has been approved in immunodeficiencies and Down syndrome (regardless of CHD status)
Beckhaus et al. 2018	DS and the risk of severe RSV disease	Meta-analysis (2a)	Systematic review and meta-analysis from 12 studies over 10 countries	9 times higher mortality and 8.7 times the risk of hospitalisation due to RSV in DS	DS even without RFs (CHD, prematurity) are also at significantly increased risk of worse RSV disease
Chan et al. 2017	Systematic review Children with DS all <2 years	Systematic review; 1995 – 2017 5 studies eligible for analysis (2a)	Hospitalisation rate due to RSV in children with DS	DS contributes to a 6.8 (95% CI 5.5-8.4) fold increase in the relative risk of hospitalisation due to RSV	DS is a significant independent risk factor for the development of severe RSV infection requiring hospitalisation

Citation	Study group	Study type (level of evidence CEBM)	Outcome	Key Result	Comments
Sanchez-Luna et al. **2017**	DS with no other RFs n= 93 Controls with no RFs n=68	Prospective multicentre cohort (2b)	Hospitalisation rates due to RSV in DS V Controls	Hospitalizations were significantly increased in the DS cohort v the non-DS cohort (9.7% vs 1.5%, P=.03)	Worse RSV disease in DS (without sig. CHD) v controls Consider prophylaxis in all children with DS
Yi et al. **2014**	DS treated c pavilizumab n=532 DS not treated n=233 Age: Up to 2 years	Prospective cohort (2b) Age: Up to 2 years	To compare hospitalisation rates due to RTI in children with DS treated with pavilizumab v those untreated	Palivizumab is associated with a 3.6-fold reduction in RSV-related hospitalization in children with DS	Pavilizumab appears to have good efficacy in DS
Paes et al. **2014**	DS received pavilizumab n=600 Age <2y Non- DS received pavilizumab for standard indications n=11,081 & other medical illnesses n=1629 Age= <2y	Prospective multicentre cohort study 2006-2012 (2b)	To compare RSV hospitalization (RSVH) hazard ratios in DS aged <2 years who received palivizumab versus children who received prophylaxis for standard indications (SI) and for other medical illnesses (MI)	RSVH incidence rates were: 1.53%, 1.45% and 2.27% for DS, SI and MI, respectively.	Pavilizumab is equally effective at reducing admission to hospital due to RSV in DS as other groups requiring the vaccine

Citation	Study group	Study type (level of evidence CEBM)	Outcome	Key Result	Comments
Simon et al. 2018	DS n=249 Median GA= ex 37/40 1st vaccine= 3.4 mths Without DS n=12,480 Median GA 31/40 1ST vaccine= 3.2m	Prospective observational German synagis registry 2009-2016 (2b)	Risk factors for RSV, Indications for synagis, No. of doses, Clinical outcomes, Adverse event	Low RSV related hospitalisation rate (1.2%) in those with DS vaccinated Pavilizumab is safe and effective in DS	High risk population for RTIs and admission Pavilizumab reduces admission rates in DS
Kashiwagi et al. 2018	DS n=138 **(without CHD)** & Immunocompromised n=167 Median GA= 38/40 1st vaccine= 11.5m	Prospective observational post marketing study 2013-2015 (2b)	Indications for vaccination, Adverse drug reactions, Clinical efficacy of vaccine	5 developed RSV 2 were hospitalised Both had DS= 2/138= 1.5% Safe well tolerated	Pavilizumab is safe and effective at reducing LRTIs caused by RSV in Down syndrome

Citation	Study group	Study type (level of evidence CEBM)	Outcome	Key Result	Comments
Pignotti et al. 2016	N/A	Consensus review (5)	Multidisciplinary consensus conference to evaluate the appropriateness of palivizumab prophylaxis in respiratory syncytial virus disease	In general, not candidates for prophylaxis Could be considered if concomitant CHD	Authors feel the increase in hospitalisation rate is due to co-existing CHD
American Academy of Paediatrics: Bronchiolitis Guidelines Committee 2014	N/A	Consensus review and policy statement (2a)	Guidance for palivizumab prophylaxis among young children at increased risk of hospitalization for RSV infection	Insufficient data available to recommend palivizumab prophylaxis for children with DS	Do acknowledge slight increase risk of hospitalisation due to RSV in DS but recommend prophylaxis only if another risk factor present

Legend of Abbreviations: DS= Down syndrome; RSV= respiratory syncytial virus; CHD= congenital heart disease; RTI= respiratory tract infection; PICU= paediatric intensive care; CLD= chronic lung disease; GA= gestational age; LRTI= lower respiratory tract infection; RFs= risk factors

Conclusion

Children with Down syndrome are a high-risk group at increased risk of respiratory tract infections and admission to hospital with pneumonia(6). There are several potential measures which may allow us to mitigate this risk, pick out high risk individuals and decrease the burden of disease. Firstly, from an immune screening perspective simple laboratory investigation like the white cell and neutrophil count may be predictive of respiratory morbidity. Lower counts of both these biomarkers was associated with an increased risk of hospitalisation with pneumonia. Further investigation is required but highlighting these routine test results could allow us to treat and follow high risk individuals more aggressively.

Prevention is better than cure and there are several vaccinations which if administered universally for children with DS could reduce hospital admissions and improve outcomes. RSV bronchiolitis is more severe for infants with DS independent of cardiac history or other risk factors(329). Providing pavilizumab prophylaxis to all infants with DS would lessen the burden of this disease for this cohort. Influenza is another virus which can cause increased morbidity and mortality in DS(303). Therefore, annual vaccination against this pathogen could deliver significant health benefits. Lastly, invasive pneumococcal disease (IPD) is a serious infection which can cause pneumonia, sepsis and meningitis(330). Children with DS are prone to pneumonia and more likely to die from sepsis, consequently booster immunisation against pneumococcus for all children over 2 years of age should be highlighted, encouraged and administered.

Chapter 8: Conclusion

8.1 Introduction

Down syndrome is the commonest chromosomal abnormality, and is associated with a multitude of medical co-morbidities(331). Although much of the associated health problems like developmental delay, congenital heart disease (CHD), arthropathy, thyroid and coeliac disease are well known and highlighted among the medical profession, the extent and the clinical significance of Down syndrome as the most prevalent syndromic immunodeficiency is perhaps more understated, and requires further examination, research and publication. Ireland has one of the highest prevalence rates of DS worldwide, with 1 in 550 births affected annually(137), therefore we have a unique opportunity to be a world leader in developing our research and understanding of the immune system of children with DS.

There is longstanding evidence about immune differences of children with DS with lower white cell counts, T and B cell subsets, smaller thymus glands, immunoglobulin dyscrasias, and cytokine dysregulation(10, 16). However, there also appeared to be significant gaps in the literature on the innate immune response in this population. Furthermore, limited research has focused on the clinical relevance and significance of specific immune deficiencies and highlighting the vulnerability of children with DS. Indeed, one of the reasons we chose to examine the CD11b, TLR2 and TLR4 responses on neutrophils and monocytes is that these cells have significant clinical relevance in day to day practice. The Full blood count is one of the most readily available and widely utilised laboratory tests and it provides quantitative data on neutrophil and monocytes. Studies have demonstrated that anomalous numbers of these cells can lead to poorer outcomes in sepsis(332, 333), and we were keen to examine if the function of neutrophils and monocytes was also impaired. Although other front line innate responders like macrophages and dendritic cells were also of interest, and are under researched in DS, to date they are not routinely used by clinicians.

An evolving picture of profound immune dysregulation and the serious clinical sequelae as a result were the main drivers for our research in this area. Children with

DS are prone to increased infections; both viral and bacterial, especially of the respiratory tract. They are more likely to be hospitalised, and for a longer duration with pneumonia than other children(5). Recurrent otitis media with effusion (OME) leads to high rates of conductive hearing loss in children with DS(187). Their ability to communicate may be potentially compromised further impacting on their speech and language skills. The increased burden of disease also has an impact on quality of life and on development which may be overlooked given the impaired cognitive abilities at baseline(278). Further research is needed to ascertain the impact of recurrent infections on development, behaviour and school performance. In infancy bronchiolitis occurs more commonly and with increased severity in DS, regardless of CHD status. These infants have prolonged hospitalisations, require PICU more frequently and an increased mortality(327, 328). A higher risk of death has also been reported in children with DS who have sepsis(8), further highlighting the poorer outcomes and stark clinical significance relating to a compromised immunity. The dedicated DS clinic provided us an ideal opportunity to delve deeper and evaluate facets and responses, to date unexplored, of their immune system. We focused mainly on innate immunity in DS by appraising cell surface markers on neutrophils and monocytes, TLR signalling pathways, cytokine responses, and the inflammasome in vitro using pro and anti-inflammatory immunomodulatory agents (LPS, Pam3Csk4, Melatonin, SsnB), highlighting immune screening tests, establishing if there are any biomarkers that could help predict prognosis.

8.2 Altered endotoxin responsiveness in healthy children with Down syndrome

Chapter 3 focused on neutrophil and monocyte function by examining CD11b and TLR4 expression at baseline and in response to LPS endotoxin and melatonin in children with DS and paediatric controls. As children with DS have a higher mortality from sepsis(8), and increased autoimmunity we wanted to see if these key mediators involved in the innate response were different in DS.

We discovered significantly greater CD11b responses to LPS stimulation on neutrophils and intermediate monocytes on children with DS compared to controls. Furthermore, non-classical monocytes exhibited increased TLR4 in children with DS at baseline versus controls. These differences in the innate immune response may in part explain the poorer outcomes in sepsis described in this cohort. In the context of SIRS and sepsis upregulation of both CD11b and TLR4 has been linked to a dysregulation of inflammatory cytokine release and less favourable outcomes(80, 157). Autoimmunity is also more prevalent in DS, and as the non-classical monocyte and TLR4 have been implicated in chronic disease, the excess TLR4 demonstrated on this monocyte subtype lead us to postulate that this anomaly could be a contributory factor for persistent inflammation in DS(141).

Melatonin was used as an immunomodulator in an anti-inflammatory capacity and was found to have significant effects on both CD11b and TLR4. Neutrophil and total monocyte CD11b was reduced, and TLR4 was decreased on total monocytes. This suggests melatonin may have a therapeutic role as an adjunct in the treatment of sepsis by attenuating neutrophil and monocyte activation and TLR4 signalling. Further clinical trials are necessary to establish any potential benefits of melatonin in this context.

As sepsis is such an important clinical sequela for children with DS, future studies could concentrate on elucidating why there is such an apparent variance in outcomes. This could be partly achieved by characterising other key cell surface markers involved in acute inflammation and sepsis. Indeed, due to the suboptimal sensitivity and specificity of routine lab investigations such as differential leucocyte count and CRP in suspected sepsis, research has focused on other biomarkers for increasing the diagnostic yield in a timelier manner. CD64 is a high affinity Fc receptor normally displayed on monocytes but expressed at low levels on resting neutrophils. In response to a pathogenic insult there is rapid upregulation of CD64 expression on neutrophils and this change in cell surface expression in this context makes CD64 a potential biomarker with diagnostic utility in suspected sepsis in DS(334). Human leucocyte antigen-DR (HLA-DR) is a cell surface marker found on monocytes and is involved in antigen processing and presentation. If reduced on the surface of

monocytes it has been associated with immunosuppression, and an increased risk of sepsis and infection, there is potential for their use as biomarkers in this context and for children with DS(335, 336).

8.3 Altered Toll-Like Receptor Signalling in Children with Down Syndrome

In chapter 4 TLR pathways were explored. TLRs are crucial in initiating and propagating the immune response, whilst also linking the innate and adaptive immune systems. We focused on TLR2 as this receptor is charged with the recognition of gram-positive bacteria and implicated in chronic disease(173). Children with DS are more likely to develop autoimmune conditions and are at greater risk of infections from this type of bacteria(10). The TLR pathway was evaluated in three ways. Firstly, at the cell surface we measured TLR2 expression on neutrophils and monocytes. Next, downstream of the receptor, we studied expression of 3 key genes involved in inflammatory signal propagation, MyD88, IRAK4, TRIF. Thirdly, pro- and anti-inflammatory cytokine levels were measured at baseline and following treatment with pro inflammatory agents (LPS, Pam3Csk4), and the immunomodulator Sparstolonin B (SsnB).

We discovered anomalous TLR signalling in children with DS; excess TLR2 at the cell surface of neutrophils and monocytes, and abnormal signalling distally with decreased MyD88 and excess TRIF, the latter possibly due to a compensatory increase in MyD88 independent signalling. This could be clinically relevant for children with DS as several infections more prevalent in DS like pneumonia and recurrent otitis media are caused by gram positive bacteria (*Strep. pneumoniae and Staphylococcus aureus)*, components of which bind to TLR2, which we found to be upregulated. There are also links to autoimmune conditions seen commonly in DS, such as arthropathy and coeliac disease, and dysregulated TLR pathways(141). The potential reasons for the greater TLR2 expression in children with DS could be due to an inherent upregulation from birth or perhaps due to greater exposure to gram positive infection over time leading to increased TLR2 at the cell surface. Overall, the aberrant TLR signalling in children with DS could partly explain chronic inflammation in DS.

Immunomodulation of TLR pathways was another key objective of this research. SsnB was used as the anti-inflammatory agent, a TLR antagonist known to reduce pro-inflammatory cytokines, and improve outcomes in sepsis(123). For the first time we established that SsnB attenuates TLR2 and CD11b expression on neutrophils and monocytes of both paediatric cohorts. SsnB was also effective at decreasing pro inflammatory cytokine levels in our populations. SsnB appears to have several mechanisms of action as an abrogator of inflammation. Therefore, future clinical trials of SsnB are warranted to ascertain the potential clinical benefits that could be extremely useful for children with DS.

Another avenue worth pursuing to improve our understanding of TLR pathways in DS would be to evaluate other key adaptor proteins downstream of the TLR receptor. We have established abnormal TLR2 expression at the cell surface, along with variances in MyD88 and TRIF. Given the significantly elevated levels of inflammatory cytokines produced at the end of these pathways, it stands to reason that other TLR signalling proteins may also be dysregulated; ones that promote inflammation and negative regulators that prevent it. Some worth considering could be MyD88-adaptor-like protein (MAL), which recruits and binds directly with MyD88 to promote inflammatory signalling or TRAF6 which engages downstream of MyD88(73). Another protein of interest is TRAM which is involved in the recruitment of TRIF and could be worth evaluating given our discovery of significant increases in the latter. To prevent persistent inflammation TLR signalling is tightly controlled with many negative regulators interacting at several levels of these complex pathways to maintain a balance. For example, MAL is under regulatory control by two further proteins; Bruton's tyrosine kinase (BTK) and suppressor of cytokine signalling 1 (SOCS1) which serve to degrade MAL(337) and abrogate TLR signalling. MicroRNAs are now also described as key controllers of signals from TLR receptors(83). O'Neill et al(84), in their review of Micro-RNAs and their influence on fine tuning TLR responses, described several key mi-RNAs that attenuate signalling. The following have been implicated in the control and reduction of TLR responses; MAL - miR-145, MyD88 – miR-155, and TLR2 – miR-105.

8.4 Increased Systemic Inflammation in children with Down Syndrome

We examined a comprehensive panel of pro- and anti- inflammatory cytokines in children with DS and controls. Although anomalous cytokine levels have been documented in DS previously(90, 139), our research represents the largest study of paediatric patients to date. Secondly, the cytokine profiles previously documented did not include some of the mediators we explored in this population like Epo, VEGF, GM-CSF and the anti-inflammatory cytokine IL-1ra. This represents previously unreported work in children with DS. Furthermore, we examined the possible clinical relevance of the extensive cytokine profile through a couple of ways; evaluating how children with DS and control samples reacted to LPS (endotoxin) in vitro, in effect mimicking gram-negative sepsis, significant given the increased mortality from sepsis in children with DS(8). Secondly, as congenital heart disease is seen in around 50% of children with DS, we explored whether significant congenital heart disease influences circulating cytokines. Again, this clinical association is underreported in children with DS. Another clinical objective was to see given the increased incidence of pulmonary hypertension in children with DS, whether cytokines implicated in this disorder were different in this population.

Children with DS demonstrated increased plasma levels of pro- and anti-inflammatory cytokines (IL-2, IL-6, IL-10, IL-1ra) as well as increased of Epo, VEGF, GM-CSF at baseline in comparison to controls. This excess cytokine profile adds to the evidence surrounding elevated inflammatory mediators in DS(35, 171, 338). However, the concomitant increase in anti-inflammatory signals, IL-10 and IL-1ra, add to the complexity of Down's immunity. Perhaps these are elevated to counteract the elevated pro-inflammatory cytokines, and/or do they result in shifting the overall balance from a pro- to an anti-inflammatory state which could be clinically significant in the setting of sepsis, where continuous anti-inflammatory state (CARS) could lead to a poorer outcome(88).

In response to LPS endotoxin stimulation children with DS demonstrated a robust response equivalent to controls. This suggests that an appropriate cytokine reaction to this pathogenic insult is not deficient or indeed hyper-responsive as we found with neutrophil CD11b(182). Beyond the acute post-operative phase, children with DS

requiring CHD surgery did not show any differences in their cytokine levels compared to other cohorts. VEGF and Epo are implicated in the development of vascular changes associated with pulmonary hypertension(PH)(207), and this disorder is more common in children with DS even without overt CHD(214). We demonstrated greater levels of both Epo and VEGF in DS and hypothesise that this may be a possible contributory factor in the pathogenesis of pulmonary hypertension in DS.

In the future further work could examine in more detail the possible role cardiovascular mediators like VEGF and Epo play in pulmonary hypertension by taking samples from patients with DS and active (PH) and comparing with patients with DS without PH. Another interesting clinical study would be to further evaluate the role of pro and especially anti-inflammatory cytokines in children with DS who have acute inflammation e.g. sepsis and chronic disease, tracking their inflammatory mediators over time to see if and when a shift occurs. Lastly, the above research may yield a biomarker which could predict prognosis thus improving clinical decision making. In addition, this could aid in individualising sepsis management in children with DS.

8.5 Melatonin as an immunomodulator in Down syndrome

One of the principal aims of this research was to examine potential therapeutic agents which could potentially improve clinical outcomes. Chapter 6 discusses melatonin as an immunomodulatory agent in children with DS. Melatonin has a favourable safety profile and could be especially useful in a population prone to sleep disturbance, possible anomalous endogenous melatonin levels, and increased oxidative stress(339, 340). Given its varied repertoire of immune functions we wanted to evaluate additional effects of melatonin; neutrophil and monocyte activation (CD11b, TLR4 expression on the cell surface), fold change expression of genes implicated in TLR signalling (TRIF, Myd88, IRAK4), inflammatory cytokine levels, and the inflammasome (IL-1β, ASC, NLRP3). These experiments included LPS endotoxin as a pro-inflammatory mediator to further examine the potential anti-inflammatory properties of melatonin.

We reported that melatonin has several important immunomodulatory effects; reduction of CD11b on neutrophils and monocytes, TLR4 on monocytes, and decreasing pro- and anti-inflammatory cytokine levels. The above changes were seen in both controls and children with DS, however, more significant results were demonstrated in the latter cohort; greater overall cytokine abrogation, attenuation of gene expression in TLR signalling (decreased MyD88 with LPS) and the inflammasome (reduced IL-1β, NLRP3 with LPS). A possible reason for these differences was a potential variance in endogenous melatonin levels due to abnormal sleep patterns and circadian rhythm in children with DS. However, evidence has shown that despite altered sleep patterns the circadian rhythm is intact in children with DS, and their melatonin levels may not be any different either(234, 272). Work from our lab demonstrated that genes involved in regulating circadian rhythm; CLOCK, CRY, BMAL, REV-ERBα were equivalent between controls and children with DS initially and post treatment with LPS and melatonin. Further studies are required to fully elucidate the role of endogenous melatonin in DS.

The potential clinical benefits of melatonin are numerous, although its effects appear dose and context dependant, with pro- and anti-inflammatory actions demonstrated(265, 266). Clinically children with DS are more likely to die from sepsis, develop chronic inflammation and autoimmune conditions, and although further clinical trials are needed, several studies report beneficial effects of melatonin in these contexts such as; sepsis via reduction of CD11b and TLR4 pathway activation and optimisation of cytokine profile (reduced pro- and increased IL-10)(268), and chronic inflammation (e.g. arthropathy) via inflammasome deactivation(256, 260).

As alluded to above immunomodulation of the inflammasome could be an important focus in the future. Inflammasomes are multi-protein complexes that generate IL-1 family cytokines. Their activation results in an innate inflammatory cascade involving caspases and the cleavage of pro IL-1β and IL-18 to their active forms(94). The NLRP3 inflammasome has been well characterised and is associated with several medical conditions such as metabolic disorders, inflammatory bowel disease, multiple sclerosis and other autoimmune diseases(95). As children with DS are reported to have significantly elevated levels of IL-1β(97), and are more prone to

autoimmune conditions the inflammasome and its potential immunomodulation is potentially important target for further research in DS. Therefore, examining the effect of specific inflammasome inhibitors in vitro may be useful to modulate the immune response without decreasing the ability to combat infection.

Children with DS appear to have an inferior immune response to vaccination, which may make the vulnerable to vaccine preventable infection(341). Melatonin has been shown to ameliorate the humoral response and immunoglobulin titres in animals if given with vaccination(276). Future trials co-administering melatonin alongside immunisations could yield improved outcomes in conferring better long-term immunity for children with DS.

8.6 Clinical Immunology

In the previous chapter we reviewed some of the more mainstream clinically relevant aspects pertaining to the immune system of children with DS. Baseline immune screening investigations (full blood count, T and B cell subsets, immunoglobulins and antibody titres) are frequently ordered due to RRTIs or other infections. We found that over a third of our group had a history of RRTIs and 37% were hospitalised due to pneumonia. Our cohort of children with DS demonstrated several deficiencies in WCC, T and B cell subsets in line with other literature(25, 286). Interestingly, appraisal of potential biomarkers with an association to a clinical outcome (RRTIs, previous hospitalisations), yielded the WCC to be significantly associated with hospitalisation due to pneumonia in children with DS. The lower the WCC the greater the probability of a history of hospital admission ($p=0.015$). Previous studies have linked other cell subtypes (regulatory T cells, CD27+IgM+IgD+ natural effector cells) and severe RTIs and hospitalisation(30, 291), although, currently these tests are not readily available to aid in clinical decision making. More research is required to confirm our results, and to establish if with greater numbers there are any other cellular links to comorbidities, multi-organ dysfunction and development. However, the WCC may potentially help us to predict who is at greater risk of

respiratory morbidity and allow us to treat the most vulnerable with increased insight and urgency.

Increased severity of respiratory infections in children with DS is a key outcome measure and has significant impacts on development, long term health and mortality. It is imperative therefore to raise awareness and implement change which can improve current results and deliver an improved prognosis for this population. One of the potential reasons for the greater infectious burden is sub-optimal vaccine responses and a deficiency in maintaining appropriate long term immunity, partly due to a paucity of switched memory B cells – imperative in conferring lasting immune memory(27). Through our dedicated DS clinic, we highlighted this vulnerability, sent out information leaflets on vaccine preventable diseases like pneumococcus and influenza, and actively immunised children with PPV from 2 years and annual influenza vaccine from 6 months. We believe these simple measures will have significant effects on ameliorating health outcomes for this cohort.

Another vaccine preventable illness which disproportionately affects infants with DS is respiratory syncytial virus (RSV) bronchiolitis. This pathogen is the primary cause of this illness, which accounts for significant number of hospital admissions and deaths throughout the world each year(342). Infants with DS are at increased risk for more severe disease independent of congenital heart disease status. Beckhaus et al in a systematic review and meta-analysis reported a 9 fold increased mortality and 8.7 fold increase in risk of hospitalisation(343) for infants with DS. Mitra et al corroborated this finding reporting significantly increased admissions, length of stay and ventilatory requirement, again independent of CHD(344). A passive from of immunisation is available via a monoclonal antibody to RSV, pavilizumab, which reduces burden of disease and admission rate by 55-72%(345). Currently, most countries, including Ireland, do not offer this prophylaxis against RSV for infants with DS on a routine basis unless they have concomitant risk factors like CHD or prematurity. However, given the greater burden of RSV disease in this cohort; higher hospitalisation rates, increased length of stay and requirement for PICU admission, regardless of CHD status, the evidence is growing to support universal vaccination for infants with DS(315-317, 327).

This evidence has been used in discussion with the Irish Health Service and Immunisation Board to potentially introduce pavilizumab for all children with DS.

In the future novel immunomodulators like melatonin, SsnB, and Broncho-Vaxom; an oral therapy which is composed of lyophilised bacterial lysate from 8 bacteria causing RTIs (*Haemophilus influenzae, Streptococcus pneumoniae, Klebsiella pneumoniae, Klebsiella ozaenae, Staphylococcus aureus, Streptococcus pyogenes, Streptococcus viridans, and Moraxella catarrhalis*) may prove beneficial for high risk groups like DS. The latter promotes the immune response by increasing serum IgA and IgG as well as improving T lymphocyte stimulatory signalling(346). A meta-analysis appraising efficacy in paediatric populations in 53 RCTs (n=4851) reported that Broncho-Vaxom was efficacious in reducing recurrent RTIs in children and more large scale trials are required to evaluate its efficacy and safety further(347). Azithromycin is increasingly used in children with DS as prophylaxis against recurrent RTIs. To our knowledge no clinical trial solely examining children with DS and this agent has been undertaken to date, however, an RCT found that early treatment with Azithromycin in preschool children with a background of severe LRTIs, resulted in a significant decrease in the likelihood of severe LRTIs(348). This raises the prospect of the potential clinical benefits for children with DS in this context, and an RCT in this cohort would indeed be welcome to further assess this.

Another key goal is to continue to highlight the implications of immune dysregulation in DS, and to promote and advocate for extra immunisations including pavilizumab prophylaxis for all children with DS. We are developing a national clinical guideline, available online for clinicians consisting of a checklist on multi-organ follow up would allow an evidence-based standard of care to be delivered. Lastly, the success of our dedicated DS clinic where medical, allied health, audiology and phlebotomy come together is a framework to try and replicate in the new children's hospital and in regional centres. This will ensure equity of care; every child with DS receives access to the same treatment wherever they may live in Ireland. We would hope that the ongoing national registry of children with DS could facilitate the inception and regionalisation of this outpatient service.

8.7 Conclusion and Future Directions

Due to the myriad of immune deficits of children with DS, it is difficult to attribute one biomarker to a single clinical outcome or medical co-morbidity. However, it is likely that a complex and profound immune dysregulation involving multiple facets of innate and adaptive immunity contribute to the phenotype observed in DS. However, there is potential for laboratory investigations like the WCC to augment clinical decision making by helping predict prognosis in respiratory disease. Future research may prove other biomarkers to have therapeutic benefit. Through a wider collaboration of research on children with DS in the department of paediatrics Trinity College and RCSI, there is work ongoing examining the important impact of multi-organ dysfunction, (including recurrent infections) on neurodevelopmental outcomes. It has already been established the link between quality of life and behaviour and repeat infections in this cohort, and it will be interesting and could be highly significant if proven that an overall increased burden of disease negatively impacts cognitive ability and development of children with DS.

In conclusion, the growing evidence of immune dysregulation, and the significant clinical co-morbidities faced by children with DS, makes them a vulnerable population that requires a pro-active and aggressive approach in the setting of severe infection or suspected sepsis. Greater education, optimisation of vaccinations, and early antimicrobial intervention could reduce the burden of disease. Long term, regular and systematic follow up of chronic conditions in DS would ensure an improved level of care and equity of service for all children with DS on our island. Lastly, future research on other novel components of their immunity and on promising immunomodulators could help target high risk individuals, augment therapeutic options, and ameliorate outcomes.

9. References

1. Parker SE, Mai CT, Canfield MA, Rickard R, Wang Y, Meyer RE, et al. Updated National Birth Prevalence estimates for selected birth defects in the United States, 2004-2006. Birth defects research Part A, Clinical and molecular teratology. 2010;88(12):1008-16.

2. Ni She R, Filan PM. Trisomy 21--incidence and outcomes in the first year, in Ireland today. Irish medical journal. 2014;107(8):248-9.

3. van Trotsenburg AS, Heymans HS, Tijssen JG, de Vijlder JJ, Vulsma T. Comorbidity, hospitalization, and medication use and their influence on mental and motor development of young infants with Down syndrome. Pediatrics. 2006;118(4):1633-9.

4. Cruz NV, Mahmoud SA, Chen H, Lowery-Nordberg M, Berlin K, Bahna SL. Follow-up study of immune defects in patients with dysmorphic disorders. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology. 2009;102(5):426-31.

5. Hilton JM, Fitzgerald DA, Cooper DM. Respiratory morbidity of hospitalized children with Trisomy 21. Journal of paediatrics and child health. 1999;35(4):383-6.

6. Fitzgerald P, Leonard H, Pikora TJ, Bourke J, Hammond G. Hospital admissions in children with down syndrome: experience of a population-based cohort followed from birth. PLoS One. 2013;8(8):e70401.

7. Lee YI, Peng CC, Chiu NC, Huang DT, Huang FY, Chi H. Risk factors associated with death in patients with severe respiratory syncytial virus infection. Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi. 2016;49(5):737-42.

8. Garrison MM, Jeffries H, Christakis DA. Risk of death for children with down syndrome and sepsis. The Journal of pediatrics. 2005;147(6):748-52.

9. Joshi AY, Abraham RS, Snyder MR, Boyce TG. Immune evaluation and vaccine responses in Down syndrome: evidence of immunodeficiency? Vaccine. 2011;29(31):5040-6.

10. Ram G, Chinen J. Infections and immunodeficiency in Down syndrome. Clinical and experimental immunology. 2011;164(1):9-16.

11. Da Rosa Utiyama SR, Nisihara RM, Nass FR, Oliveira NP, Fiedler PT, De Messias-Reason IT. Autoantibodies in patients with Down Syndrome: Early senescence of the immune system or precocious markers for immunological diseases? Journal of paediatrics and child health. 2008;44(4):182-6.

12. Ballard C, Mobley W, Hardy J, Williams G, Corbett A. Dementia in Down's syndrome. The Lancet Neurology. 2016;15(6):622-36.

13. Perluigi M, Di Domenico F, Buttterfield DA. Unraveling the complexity of neurodegeneration in brains of subjects with Down syndrome: insights from proteomics. Proteomics Clin Appl. 2014;8(1-2):73-85.

14. Kamer AR, Fortea JO, Videla S, Mayoral A, Janal M, Carmona-Iragui M, et al. Periodontal disease's contribution to Alzheimer's disease progression in Down syndrome. Alzheimers Dement (Amst). 2016;2:49-57.

15. Overgaard NH, Jung JW, Steptoe RJ, Wells JW. CD4+/CD8+ double-positive T cells: more than just a developmental stage? Journal of leukocyte biology. 2015;97(1):31-8.

16. Kusters MA, Verstegen RH, Gemen EF, de Vries E. Intrinsic defect of the immune system in children with Down syndrome: a review. Clinical and experimental immunology. 2009;156(2):189-93.

17. de Hingh YC, van der Vossen PW, Gemen EF, Mulder AB, Hop WC, Brus F, et al. Intrinsic abnormalities of lymphocyte counts in children with down syndrome. The Journal of pediatrics. 2005;147(6):744-7.

18. Roat E, Prada N, Lugli E, Nasi M, Ferraresi R, Troiano L, et al. Homeostatic cytokines and expansion of regulatory T cells accompany thymic impairment in children with Down syndrome. Rejuvenation research. 2008;11(3):573-83.

19. Kondelkova K, Vokurkova D, Krejsek J, Borska L, Fiala Z, Ctirad A. Regulatory T cells (TREG) and their roles in immune system with respect to immunopathological disorders. Acta medica (Hradec Kralove). 2010;53(2):73-7.

20. Marcovecchio GE, Bortolomai I, Ferrua F, Fontana E, Imberti L, Conforti E, et al. Thymic Epithelium Abnormalities in DiGeorge and Down Syndrome Patients Contribute to Dysregulation in T Cell Development. Frontiers in immunology. 2019;10:447.

21. Cocchi G, Mastrocola M, Capelli M, Bastelli A, Vitali F, Corvaglia L. Immunological patterns in young children with Down syndrome: is there a temporal trend? Acta Paediatr. 2007;96(10):1479-82.

22. Ferreira CT, Leite JC, Taniguchi A, Vieira SM, Pereira-Lima J, da Silveira TR. Immunogenicity and safety of an inactivated hepatitis A vaccine in children with Down syndrome. Journal of pediatric gastroenterology and nutrition. 2004;39(4):337-40.

23. Rigas DA, Elsasser P, Hecht F. Impaired in vitro response of circulating lymphocytes to phytohemagglutinin in Down's syndrome: dose- and time-response curves and relation to cellular immunity. International archives of allergy and applied immunology. 1970;39(5-6):587-608.

24. Schoch J, Rohrer TR, Kaestner M, Abdul-Khaliq H, Gortner L, Sester U, et al. Quantitative, Phenotypical, and Functional Characterization of Cellular Immunity in Children and Adolescents With Down Syndrome. The Journal of infectious diseases. 2017;215(10):1619-28.

25. Noble RL, Warren RP. Altered T-cell subsets and defective T-cell function in young children with Down syndrome (trisomy-21). Immunological investigations. 1987;16(5):371-82.

26. Liang B, Eaton-Bassiri A, Bugelski PJ. B cells and beyond: therapeutic opportunities targeting inflammation. Inflammation & allergy drug targets. 2007;6(3):142-9.

27. Carsetti R, Valentini D, Marcellini V, Scarsella M, Marasco E, Giustini F, et al. Reduced numbers of switched memory B cells with high terminal differentiation potential in Down syndrome. European Journal of Immunology. 2015;45(3):903-14.

28. Capolunghi F, Rosado MM, Sinibaldi M, Aranburu A, Carsetti R. Why do we need IgM memory B cells? Immunology letters. 2013;152(2):114-20.

29. Verstegen RH, Kusters MA, Gemen EF, E DEV. Down syndrome B-lymphocyte subpopulations, intrinsic defect or decreased T-lymphocyte help. Pediatric research. 2010;67(5):563-9.

30. Martinez E, Castaneda D, Jaramillo S, Iregui A, Quinonez T, Rodriguez JA, et al. Altered immune parameters correlate with infection-related hospitalizations in children with Down syndrome. Hum Immunol. 2016;77(7):594-9.

31. Hogg N, Patzak I, Willenbrock F. The insider's guide to leukocyte integrin signalling and function. Nature reviews Immunology. 2011;11(6):416-26.

32. Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. Immunity. 2013;38(3):596-605.

33. Valentini D, Marcellini V, Bianchi S, Villani A, Facchini M, Donatelli I, et al. Generation of switched memory B cells in response to vaccination in Down syndrome children and their siblings. Vaccine. 2015;33(48):6689-96.

34. Chaushu S, Yefenof E, Becker A, Shapira J, Chaushu G. Severe impairment of secretory Ig production in parotid saliva of Down Syndrome individuals. Journal of dental research. 2002;81(5):308-12.

35. Cetiner S, Demirhan O, Inal TC, Tastemir D, Sertdemir Y. Analysis of peripheral blood T-cell subsets, natural killer cells and serum levels of cytokines in children with Down syndrome. International journal of immunogenetics. 2010;37(4):233-7.

36. Nespoli L, Burgio GR, Ugazio AG, Maccario R. Immunological features of Down's syndrome: a review. Journal of intellectual disability research : JIDR. 1993;37 (Pt 6):543-51.

37. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood. 1994;84(5):1415-20.

38. Gemen EF, Verstegen RH, Leuvenink J, de Vries E. Increased circulating apoptotic lymphocytes in children with Down syndrome. Pediatr Blood Cancer. 2012;59(7):1310-2.

39. Elsayed SM, Elsayed GM. Phenotype of apoptotic lymphocytes in children with Down syndrome. Immunity & ageing : I & A. 2009;6:2.

40. Teng TS, Ji AL, Ji XY, Li YZ. Neutrophils and Immunity: From Bactericidal Action to Being Conquered. Journal of immunology research. 2017;2017:9671604.

41. Izumi Y, Sugiyama S, Shinozuka O, Yamazaki T, Ohyama T, Ishikawa I. Defective neutrophil chemotaxis in Down's syndrome patients and its relationship to periodontal destruction. Journal of periodontology. 1989;60(5):238-42.

42. Licastro F, Melotti C, Parente R, Davis LJ, Chiricolo M, Zannotti M, et al. Derangement of non-specific immunity in Down syndrome subjects: low leukocyte chemiluminescence activity after phagocytic activation. American journal of medical genetics Supplement. 1990;7:242-6.

43. Maiguel D, Faridi MH, Wei C, Kuwano Y, Balla KM, Hernandez D, et al. Small molecule-mediated activation of the integrin CD11b/CD18 reduces inflammatory disease. Science signaling. 2011;4(189):ra57.

44. Novo E, Garcia MI, Lavergne J. Nonspecific immunity in Down syndrome: a study of chemotaxis, phagocytosis, oxidative metabolism, and cell surface marker expression of polymorphonuclear cells. Am J Med Genet. 1993;46(4):384-91.

45. Khocht A, Russell B, Cannon JG, Turner B, Janal M. Oxidative burst intensity of peripheral phagocytic cells and periodontitis in Down syndrome. J Periodontal Res. 2014;49(1):29-35.

46. Boyette LB, Macedo C, Hadi K, Elinoff BD, Walters JT, Ramaswami B, et al. Phenotype, function, and differentiation potential of human monocyte subsets. PLoS One. 2017;12(4):e0176460.

47. Wong KL, Yeap WH, Tai JJ, Ong SM, Dang TM, Wong SC. The three human monocyte subsets: implications for health and disease. Immunologic research. 2012;53(1-3):41-57.

48. Ziegler-Heitbrock HW. Heterogeneity of human blood monocytes: the CD14+ CD16+ subpopulation. Immunology today. 1996;17(9):424-8.

49. Mukherjee R, Kanti Barman P, Kumar Thatoi P, Tripathy R, Kumar Das B, Ravindran B. Non-Classical monocytes display inflammatory features: Validation in Sepsis and Systemic Lupus Erythematous. Scientific reports. 2015;5:13886.

50. Ong SM, Hadadi E, Dang TM, Yeap WH, Tan CT, Ng TP, et al. The pro-inflammatory phenotype of the human non-classical monocyte subset is attributed to senescence. Cell Death Dis. 2018;9(3):266.

51. Bloemers BL, van Bleek GM, Kimpen JL, Bont L. Distinct abnormalities in the innate immune system of children with Down syndrome. The Journal of pediatrics. 2010;156(5):804-9, 9.e1-9.e5.

52. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. Journal of leukocyte biology. 2007;81(3):584-92.

53. Barroeta O, Nungaray L, Lopez-Osuna M, Armendares S, Salamanca F, Kretschmer RR. Defective monocyte chemotaxis in children with Down's syndrome. Pediatric research. 1983;17(4):292-5.

54. Zhang W, Li M, Xiong S, Wang H, Xiong Y, Li M, et al. Decreased myeloid dendritic cells indicate a poor prognosis in patients with severe fever with thrombocytopenia syndrome. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases. 2017;54:113-20.

55. Herberman RB, Nunn ME, Holden HT, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int J Cancer. 1975;16(2):230-9.

56. Maccario R, Ugazio AG, Nespoli L, Alberini C, Montagna D, Porta F, et al. Lymphocyte subpopulations in Down's syndrome: high percentage of circulating HNK-1+, Leu 2a+ cells. Clinical and experimental immunology. 1984;57(1):220-6.

57. Cossarizza A, Ortolani C, Forti E, Montagnani G, Paganelli R, Zannotti M, et al. Age-related expansion of functionally inefficient cells with markers of natural killer activity in Down's syndrome. Blood. 1991;77(6):1263-70.

58. Nurmi T, Huttunen K, Lassila O, Henttonen M, Sakkinen A, Linna SL, et al. Natural killer cell function in trisomy-21 (Down's syndrome). Clinical and experimental immunology. 1982;47(3):735-41.

59. Nair MP, Schwartz SA. Association of decreased T-cell-mediated natural cytotoxicity and interferon production in Down's syndrome. Clinical immunology and immunopathology. 1984;33(3):412-24.

60. Noble RL, Warren RP. Analysis of blood cell populations, plasma zinc and natural killer cell activity in young children with Down's syndrome. J Ment Defic Res. 1988;32 (Pt 3):193-201.

61. Holtmeier W, Kabelitz D. gammadelta T cells link innate and adaptive immune responses. Chemical immunology and allergy. 2005;86:151-83.

62. Paul S, Shilpi, Lal G. Role of gamma-delta (gammadelta) T cells in autoimmunity. Journal of leukocyte biology. 2015;97(2):259-71.

63. Duhindan N, Farley AJ, Humphreys S, Parker C, Rossiter B, Brooks CG. Patterns of lymphokine secretion amongst mouse gamma delta T cell clones. Eur J Immunol. 1997;27(7):1704-12.

64. Ashour HM, Niederkorn JY. Gammadelta T cells promote anterior chamber-associated immune deviation and immune privilege through their production of IL-10. Journal of immunology (Baltimore, Md : 1950). 2006;177(12):8331-7.

65. Bertotto A, Scalise F, Gerli R, Castellucci G, Fabietti GM, Spinozzi F, et al. Lymphocytes bearing the gamma/delta T-cell receptors in Down's syndrome. Scandinavian journal of immunology. 1992;35(3):275-8.

66. Carr R. Neutrophil production and function in newborn infants. British journal of haematology. 2000;110(1):18-28.

67. Romero R, Chaiworapongsa T, Espinoza J. Micronutrients and intrauterine infection, preterm birth and the fetal inflammatory response syndrome. The Journal of nutrition. 2003;133(5 Suppl 2):1668s-73s.

68. Delanghe JR, Speeckaert MM. Translational research and biomarkers in neonatal sepsis. Clinica chimica acta; international journal of clinical chemistry. 2015;451(Pt A):46-64.

69. O'Hare FM, Watson RW, O'Neill A, Segurado R, Sweetman D, Downey P, et al. Serial cytokine alterations and abnormal neuroimaging in newborn infants with encephalopathy. Acta Paediatr. 2017;106(4):561-7.

70. Khan SQ, Khan I, Gupta V. CD11b Activity Modulates Pathogenesis of Lupus Nephritis. Frontiers in medicine. 2018;5:52.

71. Ding C, Ma Y, Chen X, Liu M, Cai Y, Hu X, et al. Integrin CD11b negatively regulates BCR signalling to maintain autoreactive B cell tolerance. Nature communications. 2013;4:2813.

72. O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. Immunological reviews. 2008;226:10-8.

73. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nature reviews Immunology. 2007;7(5):353-64.

74. Redondo AC, Ceccon ME, Silveira-Lessa AL, Quinello C, Palmeira P, Carvalho WB, et al. TLR-2 and TLR-4 expression in monocytes of newborns with late-onset sepsis. Jornal de pediatria. 2014;90(5):472-8.

75. Meng G, Rutz M, Schiemann M, Metzger J, Grabiec A, Schwandner R, et al. Antagonistic antibody prevents toll-like receptor 2-driven lethal shock-like syndromes. J Clin Invest. 2004;113(10):1473-81.

76. Williams DL, Ha T, Li C, Kalbfleisch JH, Schweitzer J, Vogt W, et al. Modulation of tissue Toll-like receptor 2 and 4 during the early phases of polymicrobial sepsis correlates with mortality. Critical care medicine. 2003;31(6):1808-18.

77. Huggard D, Koay WJ, Kelly L, McGrane F, Ryan E, Lagan N, et al. Altered Toll-Like Receptor Signalling in Children with Down Syndrome. Mediators of inflammation. 2019;2019:4068734.

78. Drexler SK, Foxwell BM. The role of toll-like receptors in chronic inflammation. The international journal of biochemistry & cell biology. 2010;42(4):506-18.

79. Tsukamoto H, Fukudome K, Takao S, Tsuneyoshi N, Ihara H, Ikeda Y, et al. Multiple potential regulatory sites of TLR4 activation induced by LPS as revealed by novel inhibitory human TLR4 mAbs. International immunology. 2012;24(8):495-506.

80. Rosadini CV, Kagan JC. Early innate immune responses to bacterial LPS. Current opinion in immunology. 2017;44:14-9.

81. He Z, Chen X, Wang S, Zou Z. Toll-like receptor 4 monoclonal antibody attenuates lipopolysaccharide-induced acute lung injury in mice. Experimental and therapeutic medicine. 2014;8(3):871-6.

82. Oomori Y, Imamura Y, Fujigaki Y, Hosaka K, Miyazawa H, Kasahara H, et al. Analysis of mutations of inflammatory cytokine and Toll-like receptor genes in periodontitis in Down syndrome patients. Pediatric Dental Journal. 2007;17(1):19-26.

83. Caplan IF, Maguire-Zeiss KA. Toll-Like Receptor 2 Signaling and Current Approaches for Therapeutic Modulation in Synucleinopathies. Front Pharmacol. 2018;9:417.

84. O'Neill LA, Sheedy FJ, McCoy CE. MicroRNAs: the fine-tuners of Toll-like receptor signalling. Nature reviews Immunology. 2011;11(3):163-75.

85. Kondo T, Kawai T, Akira S. Dissecting negative regulation of Toll-like receptor signaling. Trends in immunology. 2012;33(9):449-58.

86. Zhang JM, An J. Cytokines, inflammation, and pain. International anesthesiology clinics. 2007;45(2):27-37.

87. Surbatovic M, Popovic N, Vojvodic D, Milosevic I, Acimovic G, Stojicic M, et al. Cytokine profile in severe Gram-positive and Gram-negative abdominal sepsis. Scientific reports. 2015;5:11355.

88. Osuchowski MF, Welch K, Siddiqui J, Remick DG. Circulating cytokine/inhibitor profiles reshape the understanding of the SIRS/CARS continuum in sepsis and predict mortality. Journal of immunology (Baltimore, Md : 1950). 2006;177(3):1967-74.

89. Hall MW, Geyer SM, Guo CY, Panoskaltsis-Mortari A, Jouvet P, Ferdinands J, et al. Innate immune function and mortality in critically ill children with influenza: a multicenter study. Critical care medicine. 2013;41(1):224-36.

90. Zhang Y, Che M, Yuan J, Yu Y, Cao C, Qin XY, et al. Aberrations in circulating inflammatory cytokine levels in patients with Down syndrome: a meta-analysis. Oncotarget. 2017;8(48):84489-96.

91. Tanaka T, Kishimoto T. Targeting interleukin-6: all the way to treat autoimmune and inflammatory diseases. International journal of biological sciences. 2012;8(9):1227-36.

92. Licastro F, Chiappelli M, Ruscica M, Carnelli V, Corsi MM. Altered cytokine and acute phase response protein levels in the blood of children with Downs syndrome: relationship with dementia of Alzheimer's type. International journal of immunopathology and pharmacology. 2005;18(1):165-72.

93. Broers CJ, Gemke RJ, Morre SA, Weijerman ME, van Furth AM. Increased production of interleukin-10 in children with Down syndrome upon ex vivo stimulation with Streptococcus pneumoniae. Pediatric research. 2014;75(1-1):109-13.

94. Gross O, Thomas CJ, Guarda G, Tschopp J. The inflammasome: an integrated view. Immunological reviews. 2011;243(1):136-51.

95. Shao BZ, Xu ZQ, Han BZ, Su DF, Liu C. NLRP3 inflammasome and its inhibitors: a review. Front Pharmacol. 2015;6:262.

96. Guarda G, Zenger M, Yazdi AS, Schroder K, Ferrero I, Menu P, et al. Differential expression of NLRP3 among hematopoietic cells. Journal of immunology (Baltimore, Md : 1950). 2011;186(4):2529-34.

97. Broers CJ, Gemke RJ, Weijerman ME, van der Sluijs KF, van Furth AM. Increased pro-inflammatory cytokine production in Down Syndrome children upon stimulation with live influenza A virus. Journal of clinical immunology. 2012;32(2):323-9.

98. Griffiths MR, Neal JW, Fontaine M, Das T, Gasque P. Complement factor H, a marker of self protects against experimental autoimmune encephalomyelitis. Journal of immunology (Baltimore, Md : 1950). 2009;182(7):4368-77.

99. Li YY, Alexandrov PN, Pogue AI, Zhao Y, Bhattacharjee S, Lukiw WJ. miRNA-155 upregulation and complement factor H deficits in Down's syndrome. Neuroreport. 2012;23(3):168-73.

100. Stoltzner SE, Grenfell TJ, Mori C, Wisniewski KE, Wisniewski TM, Selkoe DJ, et al. Temporal accrual of complement proteins in amyloid plaques in Down's syndrome with Alzheimer's disease. The American journal of pathology. 2000;156(2):489-99.

101. Head E, Azizeh BY, Lott IT, Tenner AJ, Cotman CW, Cribbs DH. Complement association with neurons and beta-amyloid deposition in the brains of aged individuals with Down Syndrome. Neurobiology of disease. 2001;8(2):252-65.

102. Sullivan KD, Evans D, Pandey A, Hraha TH, Smith KP, Markham N, et al. Trisomy 21 causes changes in the circulating proteome indicative of chronic autoinflammation. Scientific reports. 2017;7(1):14818.

103. Volpi S, Picco P, Caorsi R, Candotti F, Gattorno M. Type I interferonopathies in pediatric rheumatology. Pediatric rheumatology online journal. 2016;14(1):35.

104. Sullivan KD, Lewis HC, Hill AA, Pandey A, Jackson LP, Cabral JM, et al. Trisomy 21 consistently activates the interferon response. eLife. 2016;5.

105. Gross GN, Rehm SR, Pierce AK. The effect of complement depletion on lung clearance of bacteria. J Clin Invest. 1978;62(2):373-8.

106. Andreani TS, Itoh TQ, Yildirim E, Hwangbo DS, Allada R. Genetics of Circadian Rhythms. Sleep medicine clinics. 2015;10(4):413-21.

107. Harfmann BD, Schroder EA, Esser KA. Circadian rhythms, the molecular clock, and skeletal muscle. Journal of biological rhythms. 2015;30(2):84-94.

108. Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P, et al. A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. Neuron. 2004;43(4):527-37.

109. Xie Y, Tang Q, Chen G, Xie M, Yu S, Zhao J, et al. New Insights Into the Circadian Rhythm and Its Related Diseases. Frontiers in physiology. 2019;10:682.

110. Cermakian N, Lange T, Golombek D, Sarkar D, Nakao A, Shibata S, et al. Crosstalk between the circadian clock circuitry and the immune system. Chronobiology international. 2013;30(7):870-88.

111. Labrecque N, Cermakian N. Circadian Clocks in the Immune System. Journal of biological rhythms. 2015;30(4):277-90.

112. Carlson DE, Chiu WC. The absence of circadian cues during recovery from sepsis modifies pituitary-adrenocortical function and impairs survival. Shock (Augusta, Ga). 2008;29(1):127-32.

113. Gimenez S, Videla L, Romero S, Benejam B, Clos S, Fernandez S, et al. Prevalence of Sleep Disorders in Adults With Down Syndrome: A Comparative Study of Self-Reported,

Actigraphic, and Polysomnographic Findings. Journal of clinical sleep medicine : JCSM : official publication of the American Academy of Sleep Medicine. 2018;14(10):1725-33.

114. Acuna-Castroviejo D, Escames G, Venegas C, Diaz-Casado ME, Lima-Cabello E, Lopez LC, et al. Extrapineal melatonin: sources, regulation, and potential functions. Cellular and molecular life sciences : CMLS. 2014;71(16):2997-3025.

115. Bourne RS, Mills GH. Melatonin: possible implications for the postoperative and critically ill patient. Intensive care medicine. 2006;32(3):371-9.

116. Abdelgadir IS, Gordon MA, Akobeng AK. Melatonin for the management of sleep problems in children with neurodevelopmental disorders: a systematic review and meta-analysis. Archives of disease in childhood. 2018.

117. Sanchez-Barcelo EJ, Mediavilla MD, Reiter RJ. Clinical uses of melatonin in pediatrics. International journal of pediatrics. 2011;2011:892624.

118. Tarocco A, Caroccia N, Morciano G, Wieckowski MR, Ancora G, Garani G, et al. Melatonin as a master regulator of cell death and inflammation: molecular mechanisms and clinical implications for newborn care. Cell Death & Disease. 2019;10(4):317.

119. Stankov B, Fraschini F, Reiter RJ. Melatonin binding sites in the central nervous system. Brain research Brain research reviews. 1991;16(3):245-56.

120. Jockers R, Delagrange P, Dubocovich ML, Markus RP, Renault N, Tosini G, et al. Update on melatonin receptors: IUPHAR Review 20. British journal of pharmacology. 2016;173(18):2702-25.

121. Wang M, Xiu L, Diao J, Wei L, Sun J. Sparstolonin B inhibits lipopolysaccharide-induced inflammation in 3T3-L1 adipocytes. European journal of pharmacology. 2015;769:79-85.

122. Liang Q, Wu Q, Jiang J, Duan J, Wang C, Smith MD, et al. Characterization of sparstolonin B, a Chinese herb-derived compound, as a selective Toll-like receptor antagonist with potent anti-inflammatory properties. The Journal of biological chemistry. 2011;286(30):26470-9.

123. Liang Q, Dong S, Lei L, Liu J, Zhang J, Li J, et al. Protective effects of Sparstolonin B, a selective TLR2 and TLR4 antagonist, on mouse endotoxin shock. Cytokine. 2015;75(2):302-9.

124. Li P, Liang QL, Cui XD, Li J, Zou NS, Wu QN, et al. Protective effects of the active fraction from the tuber of Scirpus yagara in mouse endotoxin shock model. Journal of ethnopharmacology. 2014;158 Pt A:331-7.

125. Kusters MA, Jol-Van Der Zijde EC, Gijsbers RH, de Vries E. Decreased response after conjugated meningococcal serogroup C vaccination in children with Down syndrome. The Pediatric infectious disease journal. 2011;30(9):818-9.

126. Kusters MA, Manders NC, de Jong BA, van Hout RW, Rijkers GT, de Vries E. Functionality of the pneumococcal antibody response in Down syndrome subjects. Vaccine. 2013;31(52):6261-5.

127. Kusters MA, Bok VL, Bolz WE, Huijskens EG, Peeters MF, de Vries E. Influenza A/H1N1 vaccination response is inadequate in down syndrome children when the latest cut-off values are used. The Pediatric infectious disease journal. 2012;31(12):1284-5.

128. Kusters MA, Jol-van der Zijde CM, van Tol MJ, Bolz WE, Bok LA, Visser M, et al. Impaired avidity maturation after tetanus toxoid booster in children with Down syndrome. The Pediatric infectious disease journal. 2011;30(4):357-9.

129. Favero G, Franceschetti L, Bonomini F, Rodella LF, Rezzani R. Melatonin as an Anti-Inflammatory Agent Modulating Inflammasome Activation. International Journal of Endocrinology. 2017;2017:1835195.

130. Escames G, Acuna-Castroviejo D, Lopez LC, Tan DX, Maldonado MD, Sanchez-Hidalgo M, et al. Pharmacological utility of melatonin in the treatment of septic shock: experimental and clinical evidence. The Journal of pharmacy and pharmacology. 2006;58(9):1153-65.

131. Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: basic principles and applications. Critical reviews in biotechnology. 2017;37(2):163-76.

132. Molloy EJ, O'Neill AJ, Grantham JJ, Sheridan-Pereira M, Fitzpatrick JM, Webb DW, et al. Labor promotes neonatal neutrophil survival and lipopolysaccharide responsiveness. Pediatric research. 2004;56(1):99-103.

133. Deepak S, Kottapalli K, Rakwal R, Oros G, Rangappa K, Iwahashi H, et al. Real-Time PCR: Revolutionizing Detection and Expression Analysis of Genes. Curr Genomics. 2007;8(4):234-51.

134. Chen FH, Samson KT, Chen H, Pan SN, He ZX, Iikura Y, et al. Clinical applications of real-time PCR for diagnosis and treatment of human cytomegalovirus infection in children. Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology. 2004;15(3):210-5.

135. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif). 2001;25(4):402-8.

136. Leng SX, McElhaney JE, Walston JD, Xie D, Fedarko NS, Kuchel GA. ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. J Gerontol A Biol Sci Med Sci. 2008;63(8):879-84.

137. Down's Syndrome Medical Interest Group (DSMIG) (UK & Ireland), Department of Paediatrics University of Dublin TCTNCsH, Tallaght Hospital. Medical Management of Children & Adolescents with Down Syndrome in Ireland 2015.

138. Murphy M, Epstein LB. Down syndrome (trisomy 21) thymuses have a decreased proportion of cells expressing high levels of TCR alpha, beta and CD3. A possible mechanism for diminished T cell function in Down syndrome. Clinical immunology and immunopathology. 1990;55(3):453-67.

139. Nateghi Rostami M, Douraghi M, Miramin Mohammadi A, Nikmanesh B. Altered serum pro-inflammatory cytokines in children with Down's syndrome. European cytokine network. 2012;23(2):64-7.

140. O'Hare FM, Watson W, O'Neill A, Grant T, Onwuneme C, Donoghue V, et al. Neutrophil and monocyte toll-like receptor 4, CD11b and reactive oxygen intermediates, and neuroimaging outcomes in preterm infants. Pediatric research. 2015;78(1):82-90.

141. Liu Y, Yin H, Zhao M, Lu Q. TLR2 and TLR4 in autoimmune diseases: a comprehensive review. Clin Rev Allergy Immunol. 2014;47(2):136-47.

142. Escames G, Leon J, Macias M, Khaldy H, Acuna-Castroviejo D. Melatonin counteracts lipopolysaccharide-induced expression and activity of mitochondrial nitric oxide synthase in rats. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2003;17(8):932-4.

143. El Frargy M, El-Sharkawy HM, Attia GF. Use of melatonin as an adjuvant therapy in neonatal sepsis. Journal of neonatal-perinatal medicine. 2015;8(3):227-32.

144. Hu W, Deng C, Ma Z, Wang D, Fan C, Li T, et al. Utilizing melatonin to combat bacterial infections and septic injury. British journal of pharmacology. 2017;174(9):754-68.

145. Rinder CS, Fontes M, Mathew JP, Rinder HM, Smith BR. Neutrophil CD11b upregulation during cardiopulmonary bypass is associated with postoperative renal injury. The Annals of Thoracic Surgery. 2003;75(3):899-905.

146. Asaduzzaman M, Zhang S, Lavasani S, Wang Y, Thorlacius H. LFA-1 and MAC-1 mediate pulmonary recruitment of neutrophils and tissue damage in abdominal sepsis. Shock. 2008;30(3):254-9.

147. Maekawa K, Futami S, Nishida M, Terada T, Inagawa H, Suzuki S, et al. Effects of trauma and sepsis on soluble L-selectin and cell surface expression of L-selectin and CD11b. J Trauma. 1998;44(3):460-8.

148. O'Hare FM, Watson RW, O'Neill A, Blanco A, Donoghue V, Molloy EJ. Persistent systemic monocyte and neutrophil activation in neonatal encephalopathy. J Matern Fetal Neonatal Med. 2016;29(4):582-9.

149. Eliwan HO, Watson RWG, Aslam S, Regan I, Philbin B, O'Hare FM, et al. Neonatal brain injury and systemic inflammation: modulation by activated protein C ex vivo. Clinical and experimental immunology. 2015;179(3):477-84.

150. Zhou X, Gao XP, Fan J, Liu Q, Anwar KN, Frey RS, et al. LPS activation of Toll-like receptor 4 signals CD11b/CD18 expression in neutrophils. American journal of physiology Lung cellular and molecular physiology. 2005;288(4):L655-62.

151. Ling GS, Bennett J, Woollard KJ, Szajna M, Fossati-Jimack L, Taylor PR, et al. Integrin CD11b positively regulates TLR4-induced signalling pathways in dendritic cells but not in macrophages. Nature communications. 2014;5:3039.

152. Zhang Q, Lee W-B, Kang J-S, Kim LK, Kim Y-J. Integrin CD11b negatively regulates Mincle-induced signaling via the Lyn–SIRPα–SHP1 complex. Experimental & Molecular Medicine. 2018;50(2):e439.

153. Faridi MH, Khan SQ, Zhao W, Lee HW, Altintas MM, Zhang K, et al. CD11b activation suppresses TLR-dependent inflammation and autoimmunity in systemic lupus erythematosus. The Journal of Clinical Investigation. 2017;127(4):1271-83.

154. Muller Kobold AC, Tulleken JE, Zijlstra JG, Sluiter W, Hermans J, Kallenberg CG, et al. Leukocyte activation in sepsis; correlations with disease state and mortality. Intensive care medicine. 2000;26(7):883-92.

155. Chishti AD, Shenton BK, Kirby JA, Baudouin SV. Neutrophil chemotaxis and receptor expression in clinical septic shock. Intensive care medicine. 2004;30(4):605-11.

156. Pellegrini FP, Marinoni M, Frangione V, Tedeschi A, Gandini V, Ciglia F, et al. Down syndrome, autoimmunity and T regulatory cells. Clinical and experimental immunology. 2012;169(3):238-43.

157. Rosenbloom AJ, Pinsky MR, Napolitano C, Nguyen TS, Levann D, Pencosky N, et al. Suppression of cytokine-mediated beta2-integrin activation on circulating neutrophils in critically ill patients. Journal of leukocyte biology. 1999;66(1):83-9.

158. Tak T, van Groenendael R, Pickkers P, Koenderman L. Monocyte Subsets Are Differentially Lost from the Circulation during Acute Inflammation Induced by Human Experimental Endotoxemia. J Innate Immun. 2017;9(5):464-74.

159. Thaler B, Hohensinner PJ, Krychtiuk KA, Matzneller P, Koller L, Brekalo M, et al. Differential in vivo activation of monocyte subsets during low-grade inflammation through experimental endotoxemia in humans. Scientific reports. 2016;6:30162.

160. Skrzeczynska J, Kobylarz K, Hartwich Z, Zembala M, Pryjma J. CD14+CD16+ monocytes in the course of sepsis in neonates and small children: monitoring and functional studies. Scandinavian journal of immunology. 2002;55(6):629-38.

161. Skinner NA, MacIsaac CM, Hamilton JA, Visvanathan K. Regulation of Toll-like receptor (TLR)2 and TLR4 on CD14dimCD16+ monocytes in response to sepsis-related antigens. Clinical and experimental immunology. 2005;141(2):270-8.

162. Alvarez-Sanchez N, Cruz-Chamorro I, Lopez-Gonzalez A, Utrilla JC, Fernandez-Santos JM, Martinez-Lopez A, et al. Melatonin controls experimental autoimmune encephalomyelitis by altering the T effector/regulatory balance. Brain, behavior, and immunity. 2015;50:101-14.

163. Xia MZ, Liang YL, Wang H, Chen X, Huang YY, Zhang ZH, et al. Melatonin modulates TLR4-mediated inflammatory genes through MyD88- and TRIF-dependent signaling pathways in lipopolysaccharide-stimulated RAW264.7 cells. Journal of pineal research. 2012;53(4):325-34.

164. Reiter RJ, Mayo JC, Tan DX, Sainz RM, Alatorre-Jimenez M, Qin L. Melatonin as an antioxidant: under promises but over delivers. Journal of pineal research. 2016;61(3):253-78.

165. Manchester LC, Coto-Montes A, Boga JA, Andersen LP, Zhou Z, Galano A, et al. Melatonin: an ancient molecule that makes oxygen metabolically tolerable. Journal of pineal research. 2015;59(4):403-19.

166. Volt H, Garcia JA, Doerrier C, Diaz-Casado ME, Guerra-Librero A, Lopez LC, et al. Same molecule but different expression: aging and sepsis trigger NLRP3 inflammasome activation, a target of melatonin. Journal of pineal research. 2016;60(2):193-205.

167. Gitto E, Karbownik M, Reiter RJ, Tan DX, Cuzzocrea S, Chiurazzi P, et al. Effects of melatonin treatment in septic newborns. Pediatric research. 2001;50(6):756-60.

168. El-Gendy FM, El-Hawy MA, Hassan MG. Beneficial effect of melatonin in the treatment of neonatal sepsis. J Matern Fetal Neonatal Med. 2018;31(17):2299-303.

169. Effenberger-Neidnicht K, Brencher L, Broecker-Preuss M, Hamburger T, Petrat F, de Groot H. Immune stimulation by exogenous melatonin during experimental endotoxemia. Inflammation. 2014;37(3):738-44.

170. Tipping PG. Toll-like receptors: the interface between innate and adaptive immunity. Journal of the American Society of Nephrology : JASN. 2006;17(7):1769-71.

171. Zaki ME, El-Bassyouni HT, Tosson AM, Youness E, Hussein J. Coenzyme Q10 and pro-inflammatory markers in children with Down syndrome: clinical and biochemical aspects. Jornal de pediatria. 2017;93(1):100-4.

172. Shi B, Huang Q, Tak PP, Vervoordeldonk MJ, Huang CC, Dorfleutner A, et al. SNAPIN: an endogenous Toll-like receptor ligand in rheumatoid arthritis. Annals of the rheumatic diseases. 2012;71(8):1411-7.

173. Sampaio NG, Kocan M, Schofield L, Pfleger KDG, Eriksson EM. Investigation of interactions between TLR2, MyD88 and TIRAP by bioluminescence resonance energy transfer is hampered by artefacts of protein overexpression. PLoS One. 2018;13(8):e0202408.

174. Marsik C, Mayr F, Cardona F, Derhaschnig U, Wagner OF, Jilma B. Endotoxaemia modulates Toll-like receptors on leucocytes in humans. British journal of haematology. 2003;121(4):653-6.

175. Muthukuru M, Jotwani R, Cutler CW. Oral mucosal endotoxin tolerance induction in chronic periodontitis. Infection and immunity. 2005;73(2):687-94.

176. Ferreira R, Michel RC, Greghi SL, Resende ML, Sant'Ana AC, Damante CA, et al. Prevention and Periodontal Treatment in Down Syndrome Patients: A Systematic Review. PLoS One. 2016;11(6):e0158339.

177. D'Souza RS, Bhat KG, Sailaja D, Babji DV, Bandiwadekar TK, Katgalkar RM. Analysis of Expression and Localization of TLR-2 by Immunofluorescent Technique in Healthy and Inflammed Oral Tissues. Journal of clinical and diagnostic research : JCDR. 2013;7(12):2780-683.

178. Wara-aswapati N, Chayasadom A, Surarit R, Pitiphat W, Boch JA, Nagasawa T, et al. Induction of toll-like receptor expression by Porphyromonas gingivalis. Journal of periodontology. 2013;84(7):1010-8.

179. Foley C, Killeen OG. Musculoskeletal anomalies in children with Down syndrome: an observational study. Archives of disease in childhood. 2018.

180. Mele T, Madrenas J. TLR2 signalling: At the crossroads of commensalism, invasive infections and toxic shock syndrome by Staphylococcus aureus. The international journal of biochemistry & cell biology. 2010;42(7):1066-71.

181. Lacerte P, Brunet A, Egarnes B, Duchene B, Brown JP, Gosselin J. Overexpression of TLR2 and TLR9 on monocyte subsets of active rheumatoid arthritis patients contributes to enhance responsiveness to TLR agonists. Arthritis research & therapy. 2016;18:10.

182. Huggard D, McGrane F, Lagan N, Roche E, Balfe J, Leahy TR, et al. Altered endotoxin responsiveness in healthy children with Down syndrome. BMC immunology. 2018;19(1):31.

183. Tang L, Li Q, Bai J, Zhang H, Lu Y, Ma S. Severe pneumonia mortality in elderly patients is associated with downregulation of Toll-like receptors 2 and 4 on monocytes. The American journal of the medical sciences. 2014;347(1):34-41.

184. Bloemers BL, Broers CJ, Bont L, Weijerman ME, Gemke RJ, van Furth AM. Increased risk of respiratory tract infections in children with Down syndrome: the consequence of an altered immune system. Microbes and infection. 2010;12(11):799-808.

185. Takeuchi O, Kaufmann A, Grote K, Kawai T, Hoshino K, Morr M, et al. Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. Journal of immunology (Baltimore, Md : 1950). 2000;164(2):554-7.

186. Han F, Yu H, Tian C, Li S, Jacobs MR, Benedict-Alderfer C, et al. Role for Toll-like receptor 2 in the immune response to Streptococcus pneumoniae infection in mouse otitis media. Infection and immunity. 2009;77(7):3100-8.

187. Maris M, Wojciechowski M, Van de Heyning P, Boudewyns A. A cross-sectional analysis of otitis media with effusion in children with Down syndrome. European journal of pediatrics. 2014;173(10):1319-25.

188. Jagannathan R, Lavu V, Rao SR. Comparison of the proportion of non-classic (CD14+CD16+) monocytes/macrophages in peripheral blood and gingiva of healthy individuals and patients with chronic periodontitis. Journal of periodontology. 2014;85(6):852-8.

189. Bloemers BL, van Bleek GM, Kimpen JL, Bont L. Distinct abnormalities in the innate immune system of children with Down syndrome. The Journal of pediatrics. 2010;156(5):804-9, 9 e1-9 e5.

190. Olson JC, Bender JC, Levinson JE, Oestreich A, Lovell DJ. Arthropathy of Down syndrome. Pediatrics. 1990;86(6):931-6.

191. Juj H, Emery H. The arthropathy of Down syndrome: an underdiagnosed and under-recognized condition. The Journal of pediatrics. 2009;154(2):234-8.

192. Marild K, Stephansson O, Grahnquist L, Cnattingius S, Soderman G, Ludvigsson JF. Down syndrome is associated with elevated risk of celiac disease: a nationwide case-control study. The Journal of pediatrics. 2013;163(1):237-42.

193. Du Y, Shan LF, Cao ZZ, Feng JC, Cheng Y. Prevalence of celiac disease in patients with Down syndrome: a meta-analysis. Oncotarget. 2018;9(4):5387-96.

194. Peng S, Li C, Wang X, Liu X, Han C, Jin T, et al. Increased Toll-Like Receptors Activity and TLR Ligands in Patients with Autoimmune Thyroid Diseases. Frontiers in immunology. 2016;7:578.

195. Bagchi A, Herrup EA, Warren HS, Trigilio J, Shin H-S, Valentine C, et al. MyD88-Dependent and MyD88-Independent Pathways in Synergy, Priming, and Tolerance between TLR Agonists. The Journal of Immunology. 2007;178(2):1164-71.

196. Takeuchi O, Hoshino K, Akira S. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. Journal of immunology (Baltimore, Md : 1950). 2000;165(10):5392-6.

197. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, et al. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity. 1998;9(1):143-50.

198. Santos-Sierra S, Golenbock DT, Henneke P. Toll-like receptor-dependent discrimination of streptococci. Journal of endotoxin research. 2006;12(5):307-12.

199. Ullah MO, Sweet MJ, Mansell A, Kellie S, Kobe B. TRIF-dependent TLR signaling, its functions in host defense and inflammation, and its potential as a therapeutic target. Journal of leukocyte biology. 2016;100(1 PATHWAYS!!!):27-45.

200. Gupta N, Richter R, Robert S, Kong M. Viral Sepsis in Children. Front Pediatr. 2018;6:252.

201. Broad A, Kirby JA, Jones DEJ, Applied I, Transplantation Research G. Toll-like receptor interactions: tolerance of MyD88-dependent cytokines but enhancement of MyD88-independent interferon-beta production. Immunology. 2007;120(1):103-11.

202. Dominguez-Nieto A, Zentella A, Moreno J, Ventura JL, Pedraza S, Velazquez JR. Human endotoxin tolerance is associated with enrichment of the CD14+ CD16+ monocyte subset. Immunobiology. 2015;220(1):147-53.

203. Mathias B, Szpila BE, Moore FA, Efron PA, Moldawer LL. A Review of GM-CSF Therapy in Sepsis. Medicine. 2015;94(50):e2044.

204. Faria PF, Nicolau JA, Melek MZ, de Oliveira Nde S, Bermudez BE, Nisihara RM. Association between congenital heart defects and severe infections in children with Down syndrome. Rev Port Cardiol. 2014;33(1):15-8.

205. Evans JM, Dharmar M, Meierhenry E, Marcin JP, Raff GW. Association between Down syndrome and in-hospital death among children undergoing surgery for congenital heart disease: a US population-based study. Circulation Cardiovascular quality and outcomes. 2014;7(3):445-52.

206. Martin T, Smith A, Breatnach CR, Kent E, Shanahan I, Boyle M, et al. Infants Born with Down Syndrome: Burden of Disease in the Early Neonatal Period. The Journal of pediatrics. 2018;193:21-6.

207. Karamanian VA, Harhay M, Grant GR, Palevsky HI, Grizzle WE, Zamanian RT, et al. Erythropoietin upregulation in pulmonary arterial hypertension. Pulmonary circulation. 2014;4(2):269-79.

208. Pako J, Bikov A, Karlocai K, Csosza G, Kunos L, Losonczy G, et al. Plasma VEGF levels and their relation to right ventricular function in pulmonary hypertension. Clinical and experimental hypertension (New York, NY : 1993). 2015;37(4):340-4.

209. Miyauchi J, Kawaguchi H. Fetal liver stromal cells support blast growth in transient abnormal myelopoiesis in Down syndrome through GM-CSF. Journal of cellular biochemistry. 2014;115(6):1176-86.

210. Tozser J, Benko S. Natural Compounds as Regulators of NLRP3 Inflammasome-Mediated IL-1beta Production. Mediators of inflammation. 2016;2016:5460302.

211. French C. Erythropoietin in Critical Illness and Trauma. Critical care clinics. 2019;35(2):277-87.

212. Widness JA, Pueschel SM, Pezzullo JC, Clemons GK. Elevated erythropoietin levels in cord blood of newborns with Down's syndrome. Biology of the neonate. 1994;66(1):50-5.

213. Lemus-Varela ML, Flores-Soto ME, Cervantes-Munguia R, Torres-Mendoza BM, Gudino-Cabrera G, Chaparro-Huerta V, et al. Expression of HIF-1 alpha, VEGF and EPO in peripheral blood from patients with two cardiac abnormalities associated with hypoxia. Clinical biochemistry. 2010;43(3):234-9.

214. Shah PS, Hellmann J, Adatia I. Clinical characteristics and follow up of Down syndrome infants without congenital heart disease who presented with persistent pulmonary hypertension of newborn. Journal of perinatal medicine. 2004;32(2):168-70.

215. Zbucka-Kretowska M, Charkiewicz K, Goscik J, Wolczynski S, Laudanski P. Maternal plasma angiogenic and inflammatory factor profiling in foetal Down syndrome. PLoS One. 2017;12(12):e0189762.

216. Hesser BA, Liang XH, Camenisch G, Yang S, Lewin DA, Scheller R, et al. Down syndrome critical region protein 1 (DSCR1), a novel VEGF target gene that regulates expression of inflammatory markers on activated endothelial cells. Blood. 2004;104(1):149-58.

217. Ackerman C, Locke AE, Feingold E, Reshey B, Espana K, Thusberg J, et al. An excess of deleterious variants in VEGF-A pathway genes in Down-syndrome-associated atrioventricular septal defects. American journal of human genetics. 2012;91(4):646-59.

218. Sánchez O, Domínguez C, Ruiz A, Ribera I, Alijotas J, Cabero L, et al. Angiogenic Gene Expression in Down Syndrome Fetal Hearts. Fetal Diagnosis and Therapy. 2016;40(1):21-7.

219. Gordan JD, Bertout JA, Hu CJ, Diehl JA, Simon MC. HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. Cancer cell. 2007;11(4):335-47.

220. Druyan S, Cahaner A, Ashwell CM. The expression patterns of hypoxia-inducing factor subunit alpha-1, heme oxygenase, hypoxia upregulated protein 1, and cardiac troponin T during development of the chicken heart. Poultry science. 2007;86(11):2384-9.

221. Marlow N, Morris T, Brocklehurst P, Carr R, Cowan F, Patel N, et al. A randomised trial of granulocyte-macrophage colony-stimulating factor for neonatal sepsis: childhood outcomes at 5 years. Archives of disease in childhood Fetal and neonatal edition. 2015;100(4):F320-6.

222. Becher B, Tugues S, Greter M. GM-CSF: From Growth Factor to Central Mediator of Tissue Inflammation. Immunity. 2016;45(5):963-73.

223. Zhao J, Shang H, Cao X, Huang Y, Fang X, Zhang S, et al. Association of polymorphisms in TLR2 and TLR4 with asthma risk: An update meta-analysis. Medicine. 2017;96(35):e7909.

224. Guazzarotti L, Trabattoni D, Castelletti E, Boldrighini B, Piacentini L, Duca P, et al. T lymphocyte maturation is impaired in healthy young individuals carrying trisomy 21 (Down syndrome). American journal on intellectual and developmental disabilities. 2009;114(2):100-9.

225. Iulita MF, Ower A, Barone C, Pentz R, Gubert P, Romano C, et al. An inflammatory and trophic disconnect biomarker profile revealed in Down syndrome plasma: Relation to cognitive decline and longitudinal evaluation. Alzheimer's & dementia : the journal of the Alzheimer's Association. 2016;12(11):1132-48.

226. Thompson C, Davies R, Choy E. Anti cytokine therapy in chronic inflammatory arthritis. Cytokine. 2016;86:92-9.

227. Ali A, Na M, Svensson MN, Magnusson M, Welin A, Schwarze JC, et al. IL-1 Receptor Antagonist Treatment Aggravates Staphylococcal Septic Arthritis and Sepsis in Mice. PLoS One. 2015;10(7):e0131645.

228. Ramirez-Perez S, De la Cruz-Mosso U, Hernandez-Bello J, Martinez-Bonilla GE, Ramirez-Duenas MG, Pereira-Suarez AL, et al. High expression of interleukine-1 receptor antagonist in rheumatoid arthritis: Association with IL1RN*2/2 genotype. Autoimmunity. 2017;50(8):468-75.

229. Rose-John S, Winthrop K, Calabrese L. The role of IL-6 in host defence against infections: immunobiology and clinical implications. Nature reviews Rheumatology. 2017;13(7):399-409.

230. Carta MG, Serra P, Ghiani A, Manca E, Hardoy MC, Del Giacco GS, et al. Chemokines and pro-inflammatory cytokines in Down's syndrome: an early marker for Alzheimer-type dementia? Psychotherapy and psychosomatics. 2002;71(4):233-6.

231. Trotta MB, Serro Azul JB, Wajngarten M, Fonseca SG, Goldberg AC, Kalil JE. Inflammatory and Immunological parameters in adults with Down syndrome. Immunity & ageing : I & A. 2011;8(1):4.

232. Abbas AK, Trotta E, D RS, Marson A, Bluestone JA. Revisiting IL-2: Biology and therapeutic prospects. Science immunology. 2018;3(25).

233. Greenwald AC, Licht T, Kumar S, Oladipupo SS, Iyer S, Grunewald M, et al. VEGF expands erythropoiesis via hypoxia-independent induction of erythropoietin in noncanonical perivascular stromal cells. The Journal of experimental medicine. 2019;216(1):215-30.

234. Reiter RJ, Barlow-Walden L, Poeggeler B, Heiden SM, Clayton RJ. Twenty-four hour urinary excretion of 6-hydroxymelatonin sulfate in Down syndrome subjects. Journal of pineal research. 1996;20(1):45-50.

235. Corrales A, Vidal R, Garcia S, Vidal V, Martinez P, Garcia E, et al. Chronic melatonin treatment rescues electrophysiological and neuromorphological deficits in a mouse model of Down syndrome. Journal of pineal research. 2014;56(1):51-61.

236. Parisotto EB, Vidal V, Garcia-Cerro S, Lantigua S, Wilhelm Filho D, Sanchez-Barcelo EJ, et al. Chronic Melatonin Administration Reduced Oxidative Damage and Cellular Senescence in the Hippocampus of a Mouse Model of Down Syndrome. Neurochemical research. 2016;41(11):2904-13.

237. Uberos J, Romero J, Molina-Carballo A, Munoz-Hoyos A. Melatonin and elimination of kynurenines in children with Down's syndrome. Journal of pediatric endocrinology & metabolism : JPEM. 2010;23(3):277-82.

238. Amaral FGD, Cipolla-Neto J. A brief review about melatonin, a pineal hormone. Archives of endocrinology and metabolism. 2018;62(4):472-9.

239. Brom J, Koller M, Schluter B, Muller-Lange P, Ulrich Steinau H, Konig W. Expression of the adhesion molecule CD11b and polymerization of actin by polymorphonuclear granulocytes of patients endangered by sepsis. Burns : journal of the International Society for Burn Injuries. 1995;21(6):427-31.

240. Lorenz E, Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. Archives of internal medicine. 2002;162(9):1028-32.

241. Chuffa LG, Fioruci-Fontanelli BA, Mendes LO, Ferreira Seiva FR, Martinez M, Favaro WJ, et al. Melatonin attenuates the TLR4-mediated inflammatory response through MyD88- and TRIF-dependent signaling pathways in an in vivo model of ovarian cancer. BMC cancer. 2015;15:34.

242. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). Jama. 2016;315(8):801-10.

243. Iskander KN, Osuchowski MF, Stearns-Kurosawa DJ, Kurosawa S, Stepien D, Valentine C, et al. Sepsis: multiple abnormalities, heterogeneous responses, and evolving understanding. Physiological reviews. 2013;93(3):1247-88.

244. Lowes DA, Webster NR, Murphy MP, Galley HF. Antioxidants that protect mitochondria reduce interleukin-6 and oxidative stress, improve mitochondrial function, and reduce biochemical markers of organ dysfunction in a rat model of acute sepsis. British journal of anaesthesia. 2013;110(3):472-80.

245. Crespo E, Macias M, Pozo D, Escames G, Martin M, Vives F, et al. Melatonin inhibits expression of the inducible NO synthase II in liver and lung and prevents endotoxemia in lipopolysaccharide-induced multiple organ dysfunction syndrome in rats. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1999;13(12):1537-46.

246. Wu JY, Tsou MY, Chen TH, Chen SJ, Tsao CM, Wu CC. Therapeutic effects of melatonin on peritonitis-induced septic shock with multiple organ dysfunction syndrome in rats. Journal of pineal research. 2008;45(1):106-16.

247. Yu GM, Kubota H, Okita M, Maeda T. The anti-inflammatory and antioxidant effects of melatonin on LPS-stimulated bovine mammary epithelial cells. PLoS One. 2017;12(5):e0178525.

248. Yu GM, Tan W. Melatonin Inhibits Lipopolysaccharide-Induced Inflammation and Oxidative Stress in Cultured Mouse Mammary Tissue. Mediators of inflammation. 2019;2019:8597159.

249. Deng WG, Tang ST, Tseng HP, Wu KK. Melatonin suppresses macrophage cyclooxygenase-2 and inducible nitric oxide synthase expression by inhibiting p52 acetylation and binding. Blood. 2006;108(2):518-24.

250. Wang H, Xu DX, Lv JW, Ning H, Wei W. Melatonin attenuates lipopolysaccharide (LPS)-induced apoptotic liver damage in D-galactosamine-sensitized mice. Toxicology. 2007;237(1-3):49-57.

251. Xu L, Zhang W, Kwak M, Zhang L, Lee PCW, Jin JO. Protective Effect of Melatonin Against Polymicrobial Sepsis Is Mediated by the Anti-bacterial Effect of Neutrophils. Frontiers in immunology. 2019;10:1371.

252. Kang JW, Koh EJ, Lee SM. Melatonin protects liver against ischemia and reperfusion injury through inhibition of toll-like receptor signaling pathway. Journal of pineal research. 2011;50(4):403-11.

253. Fu J, Xia X, Liu Z, Wang Y, Wang Y, Shi Q, et al. The acute exposure of tetrachloro-p-benzoquinone (a.k.a. chloranil) triggers inflammation and neurological dysfunction via Toll-like receptor 4 signaling: The protective role of melatonin preconditioning. Toxicology. 2017;381:39-50.

254. Tamtaji OR, Mobini M, Reiter RJ, Azami A, Gholami MS, Asemi Z. Melatonin, a toll-like receptor inhibitor: Current status and future perspectives. Journal of Cellular Physiology. 2019;234(6):7788-95.

255. Liu Y, Yao W, Xu J, Qiu Y, Cao F, Li S, et al. The anti-inflammatory effects of acetaminophen and N-acetylcysteine through suppression of the NLRP3 inflammasome pathway in LPS-challenged piglet mononuclear phagocytes. Innate immunity. 2015;21(6):587-97.

256. Rahim I, Djerdjouri B, Sayed RK, Fernandez-Ortiz M, Fernandez-Gil B, Hidalgo-Gutierrez A, et al. Melatonin administration to wild-type mice and nontreated NLRP3 mutant mice share similar inhibition of the inflammatory response during sepsis. Journal of pineal research. 2017;63(1).

257. Carrillo-Vico A, Lardone PJ, Alvarez-Sanchez N, Rodriguez-Rodriguez A, Guerrero JM. Melatonin: buffering the immune system. Int J Mol Sci. 2013;14(4):8638-83.

258. Cutolo M, Masi AT. Circadian rhythms and arthritis. Rheumatic diseases clinics of North America. 2005;31(1):115-29, ix-x.

259. El-Awady HM, El-Wakkad AS, Saleh MT, Muhammad SI, Ghaniema EM. Serum melatonin in juvenile rheumatoid arthritis: correlation with disease activity. Pakistan journal of biological sciences : PJBS. 2007;10(9):1471-6.

260. Chen Q, Wei W. Effects and mechanisms of melatonin on inflammatory and immune responses of adjuvant arthritis rat. International immunopharmacology. 2002;2(10):1443-9.

261. Forrest CM, Mackay GM, Stoy N, Stone TW, Darlington LG. Inflammatory status and kynurenine metabolism in rheumatoid arthritis treated with melatonin. British journal of clinical pharmacology. 2007;64(4):517-26.

262. Morrey KM, McLachlan JA, Serkin CD, Bakouche O. Activation of human monocytes by the pineal hormone melatonin. Journal of immunology (Baltimore, Md : 1950). 1994;153(6):2671-80.

263. Jaworek J, Szklarczyk J, Jaworek AK, Nawrot-Porabka K, Leja-Szpak A, Bonior J, et al. Protective effect of melatonin on acute pancreatitis. International journal of inflammation. 2012;2012:173675.

264. Chen CF, Wang D, Reiter RJ, Yeh DY. Oral melatonin attenuates lung inflammation and airway hyperreactivity induced by inhalation of aerosolized pancreatic fluid in rats. Journal of pineal research. 2011;50(1):46-53.

265. Pieri C, Recchioni R, Moroni F, Marcheselli F, Marra M, Marinoni S, et al. Melatonin regulates the respiratory burst of human neutrophils and their depolarization. Journal of pineal research. 1998;24(1):43-9.

266. Carrillo-Vico A, Lardone PJ, Naji L, Fernandez-Santos JM, Martin-Lacave I, Guerrero JM, et al. Beneficial pleiotropic actions of melatonin in an experimental model of septic shock in mice: regulation of pro-/anti-inflammatory cytokine network, protection against oxidative damage and anti-apoptotic effects. Journal of pineal research. 2005;39(4):400-8.

267. Shang Y, Xu SP, Wu Y, Jiang YX, Wu ZY, Yuan SY, et al. Melatonin reduces acute lung injury in endotoxemic rats. Chinese medical journal. 2009;122(12):1388-93.

268. Xu DX, Wang H, Ning H, Zhao L, Chen YH. Maternally administered melatonin differentially regulates lipopolysaccharide-induced proinflammatory and anti-inflammatory cytokines in maternal serum, amniotic fluid, fetal liver, and fetal brain. Journal of pineal research. 2007;43(1):74-9.

269. Garcia-Maurino S, Gonzalez-Haba MG, Calvo JR, Rafii-El-Idrissi M, Sanchez-Margalet V, Goberna R, et al. Melatonin enhances IL-2, IL-6, and IFN-gamma production by human circulating CD4+ cells: a possible nuclear receptor-mediated mechanism involving T helper type 1 lymphocytes and monocytes. Journal of immunology (Baltimore, Md : 1950). 1997;159(2):574-81.

270. Maestroni GJ, Covacci V, Conti A. Hematopoietic rescue via T-cell-dependent, endogenous granulocyte-macrophage colony-stimulating factor induced by the pineal neurohormone melatonin in tumor-bearing mice. Cancer research. 1994;54(9):2429-32.

271. Heise I, Fisher SP, Banks GT, Wells S, Peirson SN, Foster RG, et al. Sleep-like behavior and 24-h rhythm disruption in the Tc1 mouse model of Down syndrome. Genes, brain, and behavior. 2015;14(2):209-16.

272. Fernandez F, Nyhuis CC, Anand P, Demara BI, Ruby NF, Spano G, et al. Young children with Down syndrome show normal development of circadian rhythms, but poor sleep efficiency: a cross-sectional study across the first 60 months of life. Sleep medicine. 2017;33:134-44.

273. Kasai T, Tatebe H, Kondo M, Ishii R, Ohmichi T, Yeung WTE, et al. Increased levels of plasma total tau in adult Down syndrome. PLoS One. 2017;12(11):e0188802.

274. Corrales A, Parisotto EB, Vidal V, Garcia-Cerro S, Lantigua S, Diego M, et al. Pre- and post-natal melatonin administration partially regulates brain oxidative stress but does not improve cognitive or histological alterations in the Ts65Dn mouse model of Down syndrome. Behavioural brain research. 2017;334:142-54.

275. Regodon S, Ramos A, Morgado S, Tarazona R, Martin-Palomino P, Rosado JA, et al. Melatonin enhances the immune response to vaccination against A1 and C strains of Dichelobacter nodosus. Vaccine. 2009;27(10):1566-70.

276. Regodon S, Martin-Palomino P, Fernandez-Montesinos R, Herrera JL, Carrascosa-Salmoral MP, Piriz S, et al. The use of melatonin as a vaccine agent. Vaccine. 2005;23(46-47):5321-7.

277. 2019 [Available from: www.clinicaltrialsregister.eu.

278. Verstegen RH, van Gameren-Oosterom HB, Fekkes M, Dusseldorp E, de Vries E, van Wouwe JP. Significant impact of recurrent respiratory tract infections in children with Down syndrome. Child: care, health and development. 2013;39(6):801-9.

279. Kanariou M, Petridou E, Liatsis M, Revinthi K, Mandalenaki-Lambrou K, Trichopoulos D. Age patterns of immunoglobulins G, A & M in healthy children and the influence of breast feeding and vaccination status. Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology. 1995;6(1):24-9.

280. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: The pediatric AIDS clinical trials group P1009 study. Journal of Allergy and Clinical Immunology. 2003;112(5):973-80.

281. Milford Ward A, Sheldon J, Rowbottom A, Wild G. Handbook of clinical immunochemistry. Protein Reference Units, Sheffield: PRU Publications. 2007.

282. Schauer U, Stemberg F, Rieger CH, Buttner W, Borte M, Schubert S, et al. Levels of antibodies specific to tetanus toxoid, Haemophilus influenzae type b, and pneumococcal capsular polysaccharide in healthy children and adults. Clinical and diagnostic laboratory immunology. 2003;10(2):202-7.

283. de Benedictis FM, Bush A. Recurrent lower respiratory tract infections in children. BMJ. 2018;362:k2698.

284. Obuchowski NA, Bullen JA. Receiver operating characteristic (ROC) curves: review of methods with applications in diagnostic medicine. Physics in medicine and biology. 2018;63(7):07tr1.

285. Schulzer M. Diagnostic tests: a statistical review. Muscle & nerve. 1994;17(7):815-9.

286. Mitwalli M, Wahba Y, Shaltout A, Gouida M. Lymphocyte subgroups and recurrent infections in children with Down syndrome - a prospective case control study. Cent Eur J Immunol. 2018;43(3):248-54.

287. Kusters MA, Gemen EF, Verstegen RH, Wever PC, E DEV. Both normal memory counts and decreased naive cells favor intrinsic defect over early senescence of Down syndrome T lymphocytes. Pediatric research. 2010;67(5):557-62.

288. Ribeiro LM, Jacob CM, Pastorino AC, Kim CA, Fomin AB, Castro AP. [Evaluation of factors associated with recurrent and/or severe infections in patients with Down's syndrome]. Jornal de pediatria. 2003;79(2):141-8.

289. Verstegen RHJ, Driessen GJ, Bartol SJW, van Noesel CJM, Boon L, van der Burg M, et al. Defective B-cell memory in patients with Down syndrome. The Journal of allergy and clinical immunology. 2014;134(6):1346-53.e9.

290. Yilmaz C, Dogan M, Basarslan F, Yilmaz N, Yuca S, Bulan K, et al. Evaluation of Lymphocyte Subgroups in Children With Down Syndrome. Clinical and applied thrombosis/hemostasis : official journal of the International Academy of Clinical and Applied Thrombosis/Hemostasis. 2015;21(6):546-9.

291. Seckin AN, Ozdemir H, Ceylan A, Artac H. Age-related alterations of the CD19 complex and memory B cells in children with Down syndrome. Clin Exp Med. 2018;18(1):125-31.

292. Abu Faddan N, Sayed D, Ghaleb F. T lymphocytes apoptosis and mitochondrial membrane potential in Down's syndrome. Fetal and pediatric pathology. 2011;30(1):45-52.

293. Broers CJ, Gemke RJ, Weijerman ME, Kuik DJ, van Hoogstraten IM, van Furth AM. Frequency of lower respiratory tract infections in relation to adaptive immunity in children with Down syndrome compared to their healthy siblings. Acta Paediatr. 2012;101(8):862-7.

294. Avanzini MA, Monafo V, De Amici M, Maccario R, Burgio GR, Plebani A, et al. Humoral immunodeficiencies in Down syndrome: serum IgG subclass and antibody response to hepatitis B vaccine. American journal of medical genetics Supplement. 1990;7:231-3.

295. Deepa C, Chand P, Vishnu Bhat B, Negi VS, Ramachandra Rao K. Serum immunoglobulin levels and lower respiratory tract infections in children with Down syndrome. Current Pediatric Research. 2012;16(1):53-6.

296. Azar AE, Ballas ZK. Evaluation of the adult with suspected immunodeficiency. The American journal of medicine. 2007;120(9):764-8.

297. Huggard D, Molloy EJ. Question 1: Do children with Down syndrome benefit from extra vaccinations? Archives of disease in childhood. 2018;103(11):1085-7.

298. Shvarts A, Ballow M, Senn K, Lehman H. Impaired Pneumococcal Antibody Titers in Children with Down Syndrome. Journal of Allergy and Clinical Immunology. 2011;127(2):AB11.

299. Guffroy A, Dieudonné Y, Uring-Lambert B, Goetz J, Alembik Y, Korganow A-S. Infection risk among adults with down syndrome: a two group series of 101 patients in a tertiary center. Orphanet journal of rare diseases. 2019;14(1):15-.

300. Nisihara RM, Utiyama SR, Oliveira NP, Messias-Reason IJ. Mannan-binding lectin deficiency increases the risk of recurrent infections in children with Down's syndrome. Hum Immunol. 2010;71(1):63-6.

301. Heitzeneder S, Seidel M, Forster-Waldl E, Heitger A. Mannan-binding lectin deficiency - Good news, bad news, doesn't matter? Clinical immunology (Orlando, Fla). 2012;143(1):22-38.

302. Phillips R. Evidence Based Pediatrics and Child Health: Edited by VA Moyer, EJ Elliott, RL Davies, et al. London: BMJ Books, 2000,£ 65.00 (HB), pp 404. ISBN 0727914243. BMJ Publishing Group Ltd; 2002.

303. Perez-Padilla R, Fernandez R, Garcia-Sancho C, Franco-Marina F, Aburto O, Lopez-Gatell H, et al. Pandemic (H1N1) 2009 virus and Down syndrome patients. Emerging infectious diseases. 2010;16(8):1312-4.

304. Meier S, Bel M, L'Huillier A, Crisinel PA, Combescure C, Kaiser L, et al. Antibody responses to natural influenza A/H1N1/09 disease or following immunization with adjuvanted vaccines, in immunocompetent and immunocompromised children. Vaccine. 2011;29(19):3548-57.

305. Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, et al. The Burden of Respiratory Syncytial Virus Infection in Young Children. The New England journal of medicine. 2009;360(6):588-98.

306. Bollani L, Baraldi E, Chirico G, Dotta A, Lanari M, Del Vecchio A, et al. Revised recommendations concerning palivizumab prophylaxis for respiratory syncytial virus (RSV). Italian journal of pediatrics. 2015;41:97.

307. Gutfraind A, Galvani AP, Meyers LA. Efficacy and Optimization of Palivizumab Injection Regimens Against Respiratory Syncytial Virus Infection. JAMA pediatrics. 2015;169(4):341-8.

308. Updated guidance for palivizumab prophylaxis among infants and young children at increased risk of hospitalization for respiratory syncytial virus infection. Pediatrics. 2014;134(2):e620-38.

309. The Australian Immunisation handbook 10th edition 10th ed2015.

310. The Green Book. Chapter 27a Respiratory syncytial virus. . 2015 ed2015.

311. Mori M, Morio T, Ito S, Morimoto A, Ota S, Mizuta K, et al. Risks and prevention of severe RS virus infection among children with immunodeficiency and Down's syndrome. Journal of Infection and Chemotherapy. 2014;20(8):455-9.

312. Ministry of Health and Long Term Care O, Canada. Respiratory Syncytial Virus (RSV) Prophylaxis for High-Risk Infants Program (Program) 2017-2018 [Available from: www.health.gov.on.ca/en/pro/programs/drugs/funded_drug/pdf/rsv_info.pdf.

313. Bloemers BL, van Furth AM, Weijerman ME, Gemke RJ, Broers CJ, van den Ende K, et al. Down syndrome: a novel risk factor for respiratory syncytial virus bronchiolitis--a prospective birth-cohort study. Pediatrics. 2007;120(4):e1076-81.

314. Grut V, Soderstrom L, Naumburg E. National cohort study showed that infants with Down's syndrome faced a high risk of hospitalisation for the respiratory syncytial virus. Acta Paediatr. 2017;106(9):1519-24.

315. Chan M, Park JJ, Shi T, Martinon-Torres F, Bont L, Nair H. The burden of respiratory syncytial virus (RSV) associated acute lower respiratory infections in children with Down syndrome: A systematic review and meta-analysis. Journal of global health. 2017;7(2):020413.

316. Stagliano DR, Nylund CM, Eide MB, Eberly MD. Children with Down Syndrome Are High-Risk for Severe Respiratory Syncytial Virus Disease. The Journal of pediatrics. 2015;166(3):703-9.e2.

317. Galleguillos C, Galleguillos B, Larios G, Menchaca G, Bont L, Castro-Rodriguez JA. Down's syndrome is a risk factor for severe lower respiratory tract infection due to respiratory syncytial virus. Acta Paediatr. 2016;105(11):e531-e5.

318. Bentley A, Filipovic I, Gooch K, Büsch K. A cost-effectiveness analysis of respiratory syncytial virus (RSV) prophylaxis in infants in the United Kingdom. Health Economics Review. 2013;3:18-.

319. Díez-Domingo J, Pérez-Yarza EG, Melero JA, Sánchez-Luna M, Aguilar MD, Blasco AJ, et al. Social, economic, and health impact of the respiratory syncytial virus: a systematic search. BMC Infectious Diseases. 2014;14:544.

320. Murray J, Bottle A, Sharland M, Modi N, Aylin P, Majeed A, et al. Risk factors for hospital admission with RSV bronchiolitis in England: a population-based birth cohort study. PLoS One. 2014;9(2):e89186.

321. Medrano Lopez C, Garcia-Guereta Silva L, Lirio Casero J, Garcia Perez J. [Respiratory infections, Down's syndrome and congenital heart disease: the CIVIC 21 study]. Anales de pediatria (Barcelona, Spain : 2003). 2009;71(1):38-46.

322. Doucette A, Jiang X, Fryzek J, Coalson J, McLaurin K, Ambrose CS. Trends in Respiratory Syncytial Virus and Bronchiolitis Hospitalization Rates in High-Risk Infants in a United States Nationally Representative Database, 1997-2012. PLoS One. 2016;11(4):e0152208.

323. Yi H, Lanctôt KL, Bont L, Bloemers BLP, Weijerman M, Broers C, et al. Respiratory Syncytial Virus Prophylaxis in Down Syndrome: A Prospective Cohort Study. Pediatrics. 2014.

324. Simon A, Gehrmann S, Wagenpfeil G, Wagenpfeil S. Palivizumab use in infants with Down syndrome—report from the German Synagis™ Registry 2009–2016. European journal of pediatrics. 2018;177(6):903-11.

325. Kashiwagi T, Okada Y, Nomoto K. Palivizumab Prophylaxis Against Respiratory Syncytial Virus Infection in Children with Immunocompromised Conditions or Down Syndrome: A Multicenter, Post-Marketing Surveillance in Japan. Paediatric Drugs. 2018;20(1):97-104.

326. Paes B, Mitchell I, Yi H, Li A, Lanctot KL. Hospitalization for respiratory syncytial virus illness in Down syndrome following prophylaxis with palivizumab. The Pediatric infectious disease journal. 2014;33(2):e29-33.

327. Sanchez-Luna M, Medrano C, Lirio J. Down syndrome as risk factor for respiratory syncytial virus hospitalization: A prospective multicenter epidemiological study. Influenza and other respiratory viruses. 2017;11(2):157-64.

328. Beckhaus AA, Castro-Rodriguez JA. Down Syndrome and the Risk of Severe RSV Infection: A Meta-analysis. Pediatrics. 2018.

329. Huggard D, Molloy EJ. Palivizumab for all children with Down syndrome? Archives of disease in childhood. 2018.

330. Papadatou I, Tzovara I, Licciardi PV. The Role of Serotype-Specific Immunological Memory in Pneumococcal Vaccination: Current Knowledge and Future Prospects. Vaccines. 2019;7(1):13.

331. Patil S, Rao RS, Majumdar B. Chromosomal and multifactorial genetic disorders with oral manifestations. J Int Oral Health. 2014;6(5):118-25.

332. Chung H, Lee JH, Jo YH, Hwang JE, Kim J. Circulating Monocyte Counts and its Impact on Outcomes in Patients With Severe Sepsis Including Septic Shock. Shock (Augusta, Ga). 2019;51(4):423-9.

333. Djordjevic D, Rondovic G, Surbatovic M, Stanojevic I, Udovicic I, Andjelic T, et al. Neutrophil-to-Lymphocyte Ratio, Monocyte-to-Lymphocyte Ratio, Platelet-to-Lymphocyte Ratio, and Mean Platelet Volume-to-Platelet Count Ratio as Biomarkers in Critically Ill and Injured Patients: Which Ratio to Choose to Predict Outcome and Nature of Bacteremia? Mediators of inflammation. 2018;2018:3758068.

334. van der Meer W, Pickkers P, Scott CS, van der Hoeven JG, Gunnewiek JK. Hematological indices, inflammatory markers and neutrophil CD64 expression: comparative trends during experimental human endotoxemia. Journal of endotoxin research. 2007;13(2):94-100.

335. Genel F, Atlihan F, Ozsu E, Ozbek E. Monocyte HLA-DR expression as predictor of poor outcome in neonates with late onset neonatal sepsis. The Journal of infection. 2010;60(3):224-8.

336. Juskewitch JE, Abraham RS, League SC, Jenkins SM, Smith CY, Enders FT, et al. Monocyte HLA-DR expression and neutrophil CD64 expression as biomarkers of infection in critically ill neonates and infants. Pediatric research. 2015;78(6):683-90.

337. Kagan JC, Medzhitov R. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell. 2006;125(5):943-55.

338. Kalman J, Juhasz A, Laird G, Dickens P, Jardanhazy T, Rimanoczy A, et al. Serum interleukin-6 levels correlate with the severity of dementia in Down syndrome and in Alzheimer's disease. Acta neurologica Scandinavica. 1997;96(4):236-40.

339. Andersen LP, Gogenur I, Rosenberg J, Reiter RJ. The Safety of Melatonin in Humans. Clinical drug investigation. 2016;36(3):169-75.

340. Barone E, Arena A, Head E, Butterfield DA, Perluigi M. Disturbance of redox homeostasis in Down Syndrome: Role of iron dysmetabolism. Free radical biology & medicine. 2018;114:84-93.

341. Eijsvoogel NB, Hollegien MI, Bok VLA, Derksen Lubsen AG, Dikken FPJ, Leenders S, et al. Declining antibody levels after hepatitis B vaccination in Down syndrome: A need for booster vaccination? Journal of medical virology. 2017;89(9):1682-5.

342. Diaz-Diaz A, Garcia-Maurino C, Jordan-Villegas A, Naples J, Ramilo O, Mejias A. Viral Bacterial Interactions in Children: Impact on Clinical Outcomes. The Pediatric infectious disease journal. 2019;38(6S Suppl 1):S14-s9.

343. Beckhaus AA, Castro-Rodriguez JA. Down Syndrome and the Risk of Severe RSV Infection: A Meta-analysis. Pediatrics. 2018;142(3).

344. Mitra S, El Azrak M, McCord H, Paes BA. Hospitalization for Respiratory Syncytial Virus in Children with Down Syndrome Less than 2 Years of Age: A Systematic Review and Meta-Analysis. The Journal of pediatrics. 2018;203:92-100.e3.

345. Gutfraind A, Galvani AP, Meyers LA. Efficacy and optimization of palivizumab injection regimens against respiratory syncytial virus infection. JAMA pediatrics. 2015;169(4):341-8.

346. Koatz AM, Coe NA, Cicerán A, Alter AJ. Clinical and Immunological Benefits of OM-85 Bacterial Lysate in Patients with Allergic Rhinitis, Asthma, and COPD and Recurrent Respiratory Infections. Lung. 2016;194(4):687-97.

347. Yin J, Xu B, Zeng X, Shen K. Broncho-Vaxom in pediatric recurrent respiratory tract infections: A systematic review and meta-analysis. International immunopharmacology. 2018;54:198-209.

348. Bacharier LB, Guilbert TW, Mauger DT, Boehmer S, Beigelman A, Fitzpatrick AM, et al. Early Administration of Azithromycin and Prevention of Severe Lower Respiratory Tract Illnesses in Preschool Children With a History of Such Illnesses: A Randomized Clinical Trial. Jama. 2015;314(19):2034-44.

10. Appendix

Appendix i

- Ethical approval

Appendix ii

- Patient information leaflet
- Control information leaflet
- Patient Assent form
- Consent form

Appendix iii – Clinical proforma

Appendix iv – Publications in PDF

Appendix i – Ethical approval letter

Not for prescription purposes

Ospidéal Mhuire na Leanaí, Cromghlinn
Our Lady's Children's Hospital, Crumlin

ETHICS (MEDICAL RESEARCH) COMMITTEE OFFICE
Tel: + 353 (01) 409 6307/6243

Dr Dean Huggard
Paediatric SpR
Endocrine Department
Our Lady's Children's hospital
Crumlin
Dublin 12

14th September 2017

REC Reference: GEN/565/17

Down Syndrome and Inflammation and Clinical Outcomes – DISCO
Principal Investigators: Dr. Dean Huggard, Professor Eleanor Molloy.

Dear Dr Huggard

Further to our previous correspondence in relation to the above dated 30th May 2017 and 17th July 2017.

This is to acknowledge and thank you for the amended documentation which you submitted on 14th September 2017, as requested by the Ethics (Medical Research) Committee.

The amended documentation was approved by Professor Andrew Green, Chairperson, Ethics (Medical Research) Committee.

This completes our records.

Yours Sincerely

Claire Rice
Secretary
Ethics (Medical Research) Committee

CC: Professor Eleanor Molloy, Neonatology, Our Lady's Children's Hospital, Crumlin, Dublin 12.

Appendix ii – Patient information leaflet

Patient Information Leaflet

DIsCO study – Down syndrome and Inflammation and Clinical Outcomes

Principal investigator's name:	Professor Eleanor Molly
Principal investigator's title:	Professor of Paediatrics in Trinity Consultant Neonatologist
Telephone number of principal investigator:	01 873 3763
Data Controller's/joint Controller's Identity:	Dr Niamh Lagan, Dr Dean Huggard Professor Molloy, Trinity College Dublin
Data Controller's/joint Controller's Contact Details:	Paediatrics, Academic Centre, Tallaght Hospital Trinity College, The University of Dublin, Ireland
Data Protection Officer's Contact Details:	dataprotection@tcd.ie

You and your child are being invited to take part in a research study to be carried out at Tallaght University Hospital by Professor Molloy, Trinity College Dublin.

Before you decide whether or not you wish to take part, you should read the information provided below carefully and, if you wish, discuss it with your family, friends or GP (doctor). Take time to ask questions – don't feel rushed and don't feel under pressure to make a quick decision.

You should clearly understand the risks and benefits of taking part in this study so that you can make a decision that is right for you. This process is known as 'Informed Consent'.

You don't have to take part in this study. If you decide not to take part, it won't affect your future medical care.

You can change your mind about taking part in the study any time you like. Even if the study has started, you can still opt out. You don't have to give us a reason. If you do opt out, rest assured it won't affect the quality of treatment you get in the future.

Why is this study being done?

Children with Down syndrome have a higher risk of infection and inflammatory conditions. This project will allow further understanding of the immune system in children with Down syndrome and their development.

Plan: We want to assess this further by talking to the children and their parents, examining the child and taking a blood test. We will evaluate any medical problems and also assess the child's development. To look at the immune system and its role in fighting infection we will take a blood sample from each patient and examine the key regulators involved in upgrading and downgrading the response to inflammation. Furthermore, other factors that influence the immune system will be assessed in the laboratory, as possible methods to improve persistent inflammation. Some children may have further extensive developmental assessments with psychology in the Trinity College Dublin Infant and Child Research Lab.

Conclusion: This research will improve the understanding of the immune system and development in children with Down syndrome and the potential for newer therapies. In addition, these immune markers may assist in predicting which children might have less health problems in the future.

Who is organising and funding this study?

This research is being carried out by the DISCO research project group which consists of a number of postgraduate researchers under the supervision of Prof. Molloy. The

research is funded by the National Children's Hospital Research Fund and the National Children's Research Centre. No commercial funding has or will be obtained. Ethical approval has been obtained from the Our Lady's children Hospital Crumlin and Tallaght University Hospital Ethical committees in 2017.

Why am I being asked to take part?

Your child has been asked to partake in this study as they have diagnosis of Down syndrome.

How will the study be carried out?

Your child will have a detailed check-up involving a medical history, physical examination, and a single blood test which will be taken in addition to their routine bloods, if the blood is flowing freely and they are not too distressed. This sample will be used to assess the immune system further. You may be asked to complete a couple of questionnaires in relation to your child's sleep habits and developmental progress. Further detailed developmental assessment may be offered with psychology in the Trinity College Dublin- Child and Infant Research Lab.

What are the benefits?

Children with Down Syndrome have a higher risk of infection and developmental issues. This project will allow further understanding of the immune system. Knowing more about it in these children may allow, in the future, the creation of new treatments involving the immune system and improve outcomes. Your child can help us achieve our aims.

What are the risks?

No! This study does not involve taking any medication or substance. The sampling of blood can hurt a little however this will be taken along with your child's routine bloods.

Is the study confidential?

Records:

Information on their birth details and any history of medical issues which are associated with DS will be collected, as well as bloods results, and the results of the completed questionnaires on sleep and behaviour. Identifiable data is required initially to ensure all data is required to review the blood results and medical notes of your child. A Research Patient number will be assigned, and any identifiers will be removed. A master key will be stored on an encrypted locked computer in a locked room in the Hospital grounds.

Data will be obtained directly from you the parent in clinic and from the medical chart. Blood results will be obtained from the laboratory. We ensure the data will only be processed to achieve the aim of the project and will not be processed in a way that damage or distress will be caused to you or your child. We aim to keep the personal data until completion of the project and until publication of results. We hope this will be within five years. During this time period data will remain stored in an encrypted locked computer in a locked room.

Samples:

A small sample of blood will be taken with your child's annual bloods if the blood is running freely and they are not too distressed. A small urine small maybe requested. The blood and urine will be stored in the laboratory in Taillight University Hospital. It will subsequently be brought to Trinity Translation Medicine Institute, St James' Hospital. The sample will be stored until completion of the project and until publication of results. It will be stored under the Research Patient number.

Results:

This study will take up to 5 years to complete, you will not be notified of any results specific to your child, however you will be updated on the final overall results of this research after its completion. The results of this research project will be published in

medical journals and presented at medical conferences. No identifying factors will be used.

Data Protection

1. We will be using your child's information to learn about the clinical outcomes in children with Down syndrome.

2. The legal basis for the processing of your data is that it is in the public interest and for scientific research which fall under Articles six and nine of the General Data Protection Regulations 2016 (GDPR).

3. The research group will have access to the data. All persons carrying out the research are bound by a professional code of secrecy to ensure confidentiality.

4. The data will be stored until completion of the project and until publication of results. We hope this will be within five years. During this time period data will remain stored in an encrypted locked computer in a locked room.

5. While every effort will be made to protect your data, however if a breach of data to a third party occurred, we would inform you immediately.

6. You have every right to withdraw for the research project. You can do so by contacting one of the research team.

7. You have a right to lodge a complaint with the Data Protection Commissioner.

8. You have a right to request access to your data and a copy of it, unless your request would make it impossible or make it very difficult to conduct the research.

9. You have a right to restrict or object to processing, unless your request would make it impossible or make it very difficult to conduct the research e.g. you do not want their data shared but do not mind having it collected and stored.

10. You have a right to have any inaccurate information about you corrected or deleted, unless your request would make it impossible or make it very difficult to conduct the research.

11. You have a right to have your personal data deleted, unless your request would make it impossible or make it very difficult to conduct the research. e.g. if want your data deleted before the end of a research project just before it is due to be published.

12. You have a right to data portability, meaning you have a right to move their data from one controller to another in a readable format.

13. There will be no automated decision making, including profiling. Profiling is any form of automated processing of personal data consisting of the use of personal data to evaluate certain personal aspects relating to the person, in particular to analyse or predict aspects of their performance at work, health or behaviour.

14. You have a right to object to automated processing including profiling if you wish.

15. You will be informed if we intend to further process your personal data and will provide you with information on that other purpose.

16. We may transfer your anonymised data to a country outside of the EU or an international organisation for further testing which is not available in Ireland, however if the occasion arose, we would inform you.

Consent to Future Uses

Data will be held until completion of the project and until publication of results. We hope this will be within five years. During this time period data will remain stored in an encrypted locked computer in a locked room. Biological samples will be held in the Trinity College laboratory at the St James' site, Trinity Translation Medicine Institute, St James' Hospital.

We would like to obtain your permission to the use your child's data/biological material to be used for the current research and that we could store the data/biological material for possible future uses in research. This would likely be research undertaken by the Paediatric group in Trinity College Dublin. Any such research must remain in

keeping with recognised ethical standards for scientific research. Participation is voluntary. No data will be shared with commercial entities. All data will be pseudo-anonymised.

If you wish to withdraw from partaking in future studies, please contact Dr Niamh Lagan or Dr Dean Huggard via email or phone. Details of how to contact them are at the end of this document.

Where can I get further information?

If you have any further questions about the study or if you want to opt out of the study, you can rest assured it won't affect the quality of treatment you get in the future.

If you need any further information now or at any time in the future, please contact:

Name: Dr. Niamh Lagan & Dr. Dean Huggard

Address: Paediatrics, Academic Centre, Tallaght Hospital, Trinity College, The University of Dublin, Ireland

Phone No: 01 896 3763

Email: laganni@tcd.ie, dean.huggard@tcd.ie

Appendix ii – Control information leaflet

Patient Information Leaflet for Controls

DIsCO study – Down syndrome and Inflammation and Clinical Outcomes

Principal investigator's name:	Professor Eleanor Molly
Principal investigator's title:	Professor of Paediatrics in Trinity Consultant Neonatologist
Telephone number of principal investigator:	01 873 3763
Data Controller's/joint Controller's Identity:	Dr Niamh Lagan, Dr Dean Huggard Professor Molloy**,** Trinity College Dublin
Data Controller's/joint Controller's Contact Details:	Paediatrics, Academic Centre, Tallaght Hospital Trinity College, The University of Dublin, Ireland
Data Protection Officer's Identity:	
Data Protection Officer's Contact Details:	

You are being invited to take part in a research study to be carried out at Tallaght University Hospital by Professor Molloy, Trinity College Dublin.

Before you decide whether or not you wish to take part, you should read the information provided below carefully and, if you wish, discuss it with your family, friends or GP (doctor). Take time to ask questions – don't feel rushed and don't feel under pressure to make a quick decision.

You should clearly understand the risks and benefits of taking part in this study so that you can make a decision that is right for you. This process is known as 'Informed Consent'.

You don't have to take part in this study. If you decide not to take part, it won't affect your future medical care.

You can change your mind about taking part in the study any time you like. Even if the study has started, you can still opt out. You don't have to give us a reason. If you do opt out, rest assured it won't affect the quality of treatment you get in the future.

Why is this study being done?

Children with Down syndrome have a higher risk of infection and inflammatory conditions. This project will allow further understanding of the immune system in children with Down syndrome and their development.

Plan: We want to assess this further by talking to the children and their parents, examining the child and taking a blood test. We will evaluate any medical problems and also assess the child's development. To look at the immune system and its role in fighting infection we will take a blood sample from each patient and examine the key regulators involved in upgrading and downgrading the response to inflammation. Furthermore, other factors that influence the immune system will be assessed in the laboratory, as possible methods to improve persistent inflammation. Some children may have further extensive developmental assessments with psychology in the Trinity College Dublin Infant and Child Research Lab.

Conclusion: This research will improve the understanding of the immune system and development in children with Down syndrome and the potential for newer therapies. In addition, these immune markers may assist in predicting which children might have less health problems in the future.

Who is organising and funding this study?

This research is being carried out by the DISCO research project group which consists of a number of postgraduate researchers under the supervision of Prof. Molloy. The research is funded by the National Children's Hospital Research Fund and the National Children's Research Centre. No commercial funding has or will be obtained. Ethical approval has been obtained from the Our Lady's children Hospital Crumlin and Tallaght University Hospital Ethical committees in 2017.

Why am I being asked to take part?

Your child has been asked to partake in this study as they although they do not have Down Syndrome, we need children without Down syndrome ("the controls") that we can compare to our patients with Down syndrome, and see what the differences are, if any, between these two groups.

How will the study be carried out?

Your child will have a brief assessment involving a medical history, physical examination to assess for any medical issues and a single blood test which will be taken in addition to the bloods they are already having, if the blood is flowing freely and they are not too distressed. This sample will be used to assess the immune system further. You may be asked to complete a couple of questionnaires in relation to your child's sleep habits and developmental progress. Further detailed developmental assessment may be offered with psychology in the Trinity College Dublin- Child and Infant Research Lab.

What are the benefits?

Children with Down Syndrome have a higher risk of infection and developmental issues. This project will allow further understanding of the immune system and development of children with Down Syndrome. Knowing more about it in these children may allow, in

the future, the creation of new treatments involving the immune system and improve outcomes. Your child can help us achieve our aims.

What are the risks?

No! This study does not involve taking any medication or substance. The sampling of blood can hurt a little however this will be taken along with your child's requested blood sample.

Is the study confidential?

Records:

Information on their birth details and any history of medical issues which are associated with DS will be collected, as well as bloods results, and the results of the completed questionnaires on sleep and behaviour. Identifiable data is required initially to ensure all data is required to review the blood results and medical notes of your child. A Research Patient number will be assigned, and any identifiers will be removed. A master key will be stored on an encrypted locked computer in a locked room in the Hospital grounds.

Data will be obtained directly from you the parent in clinic and from the medical chart. Blood results will be obtained from the laboratory. We ensure the data will only be processed to achieve the aim of the project and will not be processed in a way that damage or distress will be caused to you or your child. We aim to keep the personal data until completion of the project and until publication of results. We hope this will be within five years. During this time period data will remain stored in an encrypted locked computer in a locked room.

Samples:

A small sample of blood will be taken with your child's bloods if the blood is running freely and they are not too distressed. A small urine small maybe requested. The blood and urine will be stored in the laboratory in Tallaght University Hospital. It will subsequently be brought to Trinity Translation Medicine Institute, St James' Hospital.

The sample will be stored until completion of the project and until publication of results. It will be stored under the Research Patient number.

Results:

This study will take up to 5 years to complete, you will not be notified of any results specific to your child, however you will be updated on the final overall results of this research after its completion. The results of this research project will be published in medical journals and presented at medical conferences. No identifying factors will be used.

Data Protection

1. We will be using your child's information to learn about the clinical outcomes in children with Down syndrome.

2. The legal basis for the processing of your data is that it is in the public interest and for scientific research which fall under Articles six and nine of the General Data Protection Regulations 2016 (GDPR).

3. The research group will have access to the data. All persons carrying out the research are bound by a professional code of secrecy to ensure confidentiality.

4. The data will be stored until completion of the project and until publication of results. We hope this will be within five years. During this time period data will remain stored in an encrypted locked computer in a locked room.

5. While every effort will be made to protect your data, however if a breach of data to a third party occurred, we would inform you immediately.

6. You have every right to withdraw for the research project. You can do so by contacting one of the research team.

7. You have a right to lodge a complaint with the Data Protection Commissioner.

8. You have a right to request access to your data and a copy of it, unless your request would make it impossible or make it very difficult to conduct the research.

9. You have a right to restrict or object to processing, unless your request would make it impossible or make it very difficult to conduct the research e.g. you do not want their data shared but do not mind having it collected and stored.

10. You have a right to have any inaccurate information about you corrected or deleted, unless your request would make it impossible or make it very difficult to conduct the research.

11. You have a right to have your personal data deleted, unless your request would make it impossible or make it very difficult to conduct the research. e.g. if want your data deleted before the end of a research project just before it is due to be published.

12. You have a right to data portability, meaning you have a right to move their data from one controller to another in a readable format.

13. There will be no automated decision making, including profiling. Profiling is any form of automated processing of personal data consisting of the use of personal data to evaluate certain personal aspects relating to the person, in particular to analyse or predict aspects of their performance at work, health or behaviour.

14. You have a right to object to automated processing including profiling if you wish.

15. You will be informed if we intend to further process your personal data and will provide you with information on that other purpose.

16. We may transfer your anonymised data to a country outside of the EU or an international organisation for further testing which is not available in Ireland, however if the occasion arose, we would inform you.

Consent to Future Uses

Data will be held until completion of the project and until publication of results. We hope this will be within five years. During this time period data will remain stored in an encrypted locked computer in a locked room. Biological samples will be held in the Trinity College laboratory at the St James' site, Trinity Translation Medicine Institute, St James' Hospital.

We would like to obtain your permission to the use your child's data/biological material to be used for the current research and that we could store the data/biological material for possible future uses in research. This would likely be research undertaken by the Paediatric group in Trinity College Dublin. Any such research must remain in keeping with recognised ethical standards for scientific research. Participation is voluntary. No data will be shared with commercial entities. All data will be pseudo-anonymised.

If you wish to withdraw from partaking in future studies, please contact Dr Niamh Lagan or Dr Dean Huggard via email or phone. Details of how to contact them are at the end of this document.

Where can I get further information?

If you have any further questions about the study or if you want to opt out of the study, you can rest assured it won't affect the quality of treatment you get in the future.

If you need any further information now or at any time in the future, please contact:

Name: Dr. Niamh Lagan & Dr. Dean Huggard

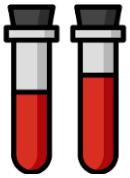
Address: Paediatrics, Academic Centre, Tallaght Hospital, Trinity College, The University of Dublin, Ireland

Phone No: 01 896 3763

Email: laganni@tcd.ie, huggardd@tcd.ie

Appendix ii – Patient Assent form

Participant Assent Form DISCO Study <12 yrs

You are getting your blood checked today	
If it's going ok… I can have an extra sample	
It will help me with my work	
This will help other children	
You will get a sticker to show how brave you have been after the blood test.	

Appendix ii – Parent Consent form

PARENT'S CONSENT FORM

Reference Number:

Title of Study: DISCO study – Down syndrome and Inflammation and Clinical Outcomes

Research Principal Investigator: Prof Eleanor Molloy, Prof of Paediatrics in Trinity, Consultant Neonatologist.

I have read and understood the **Information Leaflet** about this research project. The information has been fully explained to me and I have been able to ask questions, all of which have been answered to my satisfaction.	**Yes** ☐	**No** ☐
I understand that I don't have my child to partake in this study and that I can opt out at any time. I understand that I don't have to give a reason for opting out and I understand that opting out won't affect my future medical care.	**Yes** ☐	**No** ☐
I am aware of the potential risks and benefits of this research study.	**Yes** ☐	**No** ☐
I give permission for researchers to look at my child's medical records to get information. I have been assured that information about my child will be kept private and confidential.	**Yes** ☐	**No** ☐
I have been given a copy of the Information Leaflet and this completed consent form for my records.	**Yes** ☐	**No** ☐
I consent for my child to part in this research study having been fully informed of the risks and benefits.	**Yes** ☐	**No** ☐

I give informed explicit consent to have my child's data processed as part of this research study.	**Yes** ☐	**No** ☐
I consent to be contacted by researchers as part of this research study, including a psychology PhD student to contact me to organize an additional extensive developmental assessment in the TCD Child and Infant lab.	**Yes** ☐	**No** ☐

FUTURE CONTACT		
*I consent to be re-contacted by researchers about possible future research **related** to the current study for which I may be eligible.*	***Yes*** ☐	***No*** ☐

STORAGE AND FUTURE USE OF INFORMATION		
RETENTION OF RESEARCH MATERIAL IN THE FUTURE		
*I give permission for material/data to be stored for <u>possible future research **related** </u>to the current study **<u>without further consent being required</u>** but only if the research is approved by a Research Ethics Committee.*	*Yes* ☐	*No* ☐

Patient Name (block Capitals): _____

Date: _____

Parent/ Guardian Name: _____

Tel: _____

Email: _____

Parent/ Guardian Signature: _____

Investigator Name: _____

Investigator Signature: _____

Appendix iii – Clinical proforma

Down Syndrome Health Surveillance Clinic:

Date of Assessment:

Time:

Name:			
Address:			
DoB:		Current Age:	
Parents Names & DOB	M D	Who present	

Background:	

Medications	

Early history:	
Antenatal Hx: **Diagnosis: Postnatal Antenatal** **If Antenatal- CVS Amino Harmony** **Birth hx :** Gestation: BW: Mode of delivery: Reason: APGARS: Resus required: Admission to SCBU/PICU-	**Feeding:** Method Breast Bottle Combination Duration of NG feeding Time of Breastmilk Exposure

Reason for admission- Length of stay- Transfer to diff hospital-	RSV Prophylaxis
Parental Concerns:	
General Health	

Feeding:
Disability Service/ Intervention:
Developmental Progress:

Motor Gross Fine Communication Verbal Nonverbal Comprehension Play skills Self Care Behaviour Sensory
School/Creche:
Family/Social hx

Systems Review:	
Cardiology **Assessment before 6 weeks, outcome**	Surgery? Date of surgery? PICU length of stay?
Thyroid Function Testing **TFT neonatal screen followed by rpt at 1yr and every 2yrs(irish)** **AAP-yearly**	
Ophthalmology **Eye exam 6weeks,** **18month to 2yr formal ophthalomology assessment, 4yrs rpt formal assessment (irish)** **Aap- annual**	
Hearing: **Neonatal screening programme, audiology 6-10month, 18/12 2nd review, rpt yearly until 5, thereafter 2yearly (Irish guidelines)** **AAP- 6/12ly till 4-5yr**	Hearing loss? Aids? s/b ENT
Blood Disorder **Neonatal fbc, symptoms** **Aap- fbc annually**	

Sleep Disordered Breathing: **Symptoms OSA low threshold for sleep study Dsmig** **AAP- sleep study at 4yrs annual review of symptoms**	Symptoms? Sleep study? If OSA+, - ?Ts&As - ?NIV - ?Both
Respiratory Symptoms:	Symptoms Number chest infections per year (recently vs past): Admissions: PICU? Grommits? Tonsillectomy? Prophylaxis? Attend resp:
Immunisations: **Annual flu, PPV 23, MenC ACWY** **Extras**	Routine Flu Men b varicella PPV 23 Men ACWY
GIT	

Cervical Spine Instability: **Assess symptoms of atlantoaxial instability**	
Rheum **Include symptoms and pals (DMISG)**	
Dental: **Annual r/v**	
Exam:	Wgt: Hgt: OFC:
Impression:	
Plan:	

Next r/v:	
Results/Follow up	
Bloods: **FBC/ smear** **RLB profile** **Vit D** **Ferritin/Fe studies** **Coeliac screen** **TFT incld TPO** **Immunoglobulins** **Vaccination titres (pneumococcal, hib, tetanus)** **T/B cell subsets**	
Name **Signature** **IMC**	

Appendix iv – Publications in PDF

- **Huggard D**, McGrane F, Lagan N, Roche E, Balfe J, Leahy TR, et al. *Altered endotoxin responsiveness in healthy children with Down syndrome.* BMC immunology. 2018;19(1):31.

- **Huggard D**, Kaoy WJ, Kelly L, McGrane F, Ryan E, Lagan N, Roche E, Balfe J, Leahy TR, Franklin O, Moreno-Oliveira A, Melo AM, Doherty DG, Molloy EJ. *Altered TLR signalling in children with Down syndrome.* Mediators of Inflammation 2019 Sep 12; 2019:4068734.

- **Huggard D,** Kelly L, Ryan E, McGrane F, Lagan N, Roche E, Balfe J, Leahy TR, Franklin O, Doherty DG, Molloy EJ. *Increased systemic inflammation in children with Down syndrome.* Cytokine. 2019 Nov 27;127:154938.

- **Huggard D**, Molloy EJ. *Do children with Down syndrome benefit from extra vaccinations?* Archives of disease in childhood. 2018;103(11):1085-7.

- **Huggard D**, Molloy EJ. *Palivizumab for all children with Down syndrome?* Archives of disease in childhood. 2018.