Clinical, cellular and serologic analysis of selective IgA deficiency

Analysis of the Icelandic selective IgA deficiency group

Andri Leó Lemarquis

Thesis for the degree of Philosophiae Doctor

Supervisor:

Björn Rúnar Lúðvíksson

Doctoral committee:

Ásgeir Haraldsson

Ingileif Jónsdóttir

Rene Toes

Una Bjarnardóttir

Reykjavik, Iceland

2019

UNIVERSITY OF ICELAND
SCHOOL OF HEALTH SCIENCES
FACULTY OF MEDICINE

Klínísk mynd og ónæmissvar einstaklinga með sértækan IgA skort.

Rannsókn um sértækan IgA skort á Íslandi

Andri Leó Lemarquis

Ritgerð til doktorsgráðu

Umsjónarkennari:
Björn Rúnar Lúðvíksson

Doktorsnefnd:
Ásgeir Haraldsson

Ingileif Jónsdóttir,

Rene Toes

Una Bjarnardóttir

Reykjavík, Ísland

2019

UNIVERSITY OF ICELAND
SCHOOL OF HEALTH SCIENCES

FACULTY OF MEDICINE

Thesis for a doctoral degree at the University of Iceland. All right reserved. No part of this publication may be reproduced in any form without the prior permission of the copyright holder.

© Andri Leó Lemarquis 2019

ISBN 978-9935-9455-7-0

Printing by Háskólaprennt

Reykjavik, Iceland Year

Ágrip

Sértækur IgA skortur (sIgAD) er algengasti galli í sértæka ónæmiskerfinu. Gallinn er skilgreindur sem algjör skortur á IgA með eðlilegum IgG og IgM styrk í sermi. Tíðni sjúkdóma sem tengjast óeðlilegu ónæmissvari er hærri hjá sIgAD einstaklingum. Vitað er að frumubundið svar þessara einstaklinga leiðir til stöðgunar í IgA framleiðslu. Meinmyndun gallans skiljum við þó ekki fyllilega og eingena orsakir hafa ekki fundist. Í þessarri rannsókn notum við klíníska greiningu, frumugreiningu eitilfrumna, sjálfsónæmismótefna-mælingu og boðefnamælingar til betri skilgreiningar á einstaklingum með gallann. Við sjáum að hjá sIgAD einstaklingum er há tíðni sjálfsónæmis, ofnæmissjúkdóma og sýkinga. Sermis mælingar sýna fram á ofseytingu þáttanna TSLP, TWEAK, sCD40L, CCL3 og IL-18 sem tengjast B frumu þroskun. Þættirnir aðgreina sIgAD einstaklinga frá heilbrigðum og virðast einnig tengjast sjálfsónæmismyndun. Frumugreining einstaklinga bendir til stöðgunar snemma í B frumu þroska. Hinsvegar hafa þeir eðlilegar T frumur hvað varðar fjölda og virkni. Svar við örvun með T frumu óháðu svari B frumna er gallað, en það er þýðingarmikið í slímhúðum þar sem IgA er hvað mikilvægast. Fyrir utan lækkaða IL-21 svörun B frumna um pSTAT3 boðleiðina eru innanfrumuboðferlar einstaklinganna að mestu eðlilegir. mRNA tjáning einstaklinganna bendir til vanvirkrar svörunar sem tengist röskun í þroskun frá óþroskuðum B frumum til síðari þroskunarstiga. Niðurstöðurnar benda til vanstýringar ónæmissvars B frumna án T frumu galla. Þetta virðist mögulega tengjast hárri seytingu ónæmisþátta sem við teljum að leiði til ofvirkni ónæmissvars og aukinnar byrði ofnæmis og ónæmissjúkdóma. Meginniðurstöður rannsóknarinnar eru því að í sértækum IgA skorti er tenging milli klínískrar myndar sjálfsónæmis, sýkinga, ofnæmissjúkdóma og röskunar ónæmissvars tengdu B frumu þroskun með tilheyrandi ofseytingu ónæmisþátta og seytingu sjálfsónæmismótefna. Þessir þættir þarfnast frekari rannsókna en gætu orðið að gagni við greiningu og einstaklingsmiðaða meðferð einstaklinga með mótefnagalla og ónæmistengda sjúkdóma.

Lykilorð:

Sértækur ónæmisskortur, sértækur IgA skortur, B frumur, T frumur, CpG

Abstract

Selective IgA deficiency (sIgAD) is one of the most common primary antibody deficiency (PAD) characterised by an extremely low IgA with normal IgG and IgM serum levels. It is associated with an increased risk of diseases related to immune dysregulation such as autoimmunity, atopy and infections. The cellular defect is incompletely understood and the genetic mechanism responsible largely unknown. For this study various methodologic approaches were used including: clinical phenotyping with a detailed clinical questionnaire, autoantibody assessment and serum assessment of immune factors in addition to a cellular characterisation with flow cytometry, ELISA and a transcriptomic approach. The clinical phenotyping sIgAD individuals in Iceland reveals a higher prevalence of autoimmunity, atopy and infections, especially in the upper and lower respiratory tract in those with sIgAD. The serologic analysis of individuals with sIgAD without overt clinical diseases reveals a high autoantibody positivity with high serum concentrations of TSLP, TWEAK, sCD40L, IL-18 and CCL3. Interestingly these factors cluster together and may be interconnected with autoantibody positivity. The cellular characterisation reveals a defect limited to early developmental stages of B cells with a defect in transitional and class switched B. The IgA production defect is not only limited to T cell dependent stimulations but is also seen after T cell independent stimulations. While limited induction can be found after stimulation no long-lived response are seen. While multiple B cell stimuli have been shown to lead to faulty IgA production, only a lower signalling in STAT3 was seen after IL-21 stimulation. In our study, undertaking extensive T cell evaluation revealed neither phenotypic, functional nor signalling defects in sIgAD individuals. Finally, the transcriptomic signatures of sIgAD B cells seen after CpG stimulation revealed big differences in transcription after stimulation that may be linked by to defects in B cell development. Possibly, indicating a common defect in B cell development due to different polygenetic aspects or a common epigenetic component. Collectively our data shows a serologically classified disease with immune morbidities that has an early B cell developmental problem, a dysregulated serological profile related to autoantibody production and a transcriptomic signature associated with early B cell dysregulation. These may be of value in the prediction of immune burden in PADs with a possibility for the development of personalised treatment options.

Keywords:

Primary immunodeficiency, IgA deficiency, B cells, T cells, CpG

Acknowledgements

I wish to thank the Faculty of Medicine, University of Iceland, for accepting my PhD thesis for dissertation.

To start I would like to thank the **individuals with sIgAD** who participated in the study and all the researchers that have come before me and after me. I cannot, acknowledge these two grups enough. My limited work can't claim to find solutions to all evils, but I hope I tried to bring some of hope to be found within this Pandora's box.

Many people have contributed to the work presented and I would like to express my sincere gratitude to all of them for their time and support. In particular, I would like to thank my supervisor, Professor **Björn Rúnar Lúðvíksson**, for giving me the opportunity to do a PhD in his lab, for his guidance, enthusiasm and support. My doctorial committee: **Ásgeir Haraldsson** for inspiring me in science as in pediatric training**, Ingileif Jónsdóttir** for her critical, highly intellectual help and logistics. **Rene Toes** for being a role model in immunology**, Una Bjarnadóttir** as my first mentor in immunological methods, and all for their help. My co-investigators and all my hardworking friends at the department of Immunology, Landspitali – The National University Hospital of Iceland, especially **Helga Kristín Einarsdóttir** working on the project and correcting all the small things I miss in manuscripts. All the students involved directly in the project; my good friend **Fannar Theodór** for his phosphorylation work and research zeal and discussions, **Rakel Natalie** for her work on long-lived antibody production, **Ida Karnsund** with **Anna Guðrún Viðarsdóttir** and all other members of the clinical lab on their work on autoantibody production. **Berglind Eiríksdóttir** and her co-workers from þjónustumiðstöð rannsóknarverkefna for her patience and help. My dear friend **Hildur Sigurgrímsdóttir** for her help with the Luminex assay, keeping me out of research depression in the lab and listening to my complaining in Iceland and from abroad. And of course, my predecessor **Guðmundur Jorgensen**, for without his work none of this would have been possible, but also for his realistic description of what a PhD is when I was starting.

I would also like to thank my co-workers and supervisors abroad. At the department of Rheumatology at the LUMC, especially **Rene Toes** for letting me come there and have a wonderful time and my good friend **Yoann**

Rombouts which taught me to love scientific literature as much if not more than I used to like fiction, while eating, running or drinking. At the department of rheumatology and inflammatory research at the Sahlgrenska, especially **Olov Ekwall**, to have a place write my thesis and bounce ideas. And **Sanna Abrahamsson** at the Bioinformatics Core Facility of the Sahlgrenska Academy for the help with transcriptomics.

Finally, but foremost, to my family and friends. Most of all to my life partner **Una Sigtryggsdóttir** for supporting me for such a long time (can be read both in the French and English meaning of the word). And to our beautiful children **Tristan Emil** and **Elea Mía**, you are for me an inexhaustible source of inspiration and love. I want to give special thanks to my parents **Gérard** and **María**, for rationally I would be nothing without them. I think this research, and interest in research, is largely due to their caring and intellectually stimulating upbringing. My parents-in-law **Sigtryggur** and **Sigrún**, the first to help me be little bit less serious, the latter having pushed me to do a MD PhD, even before I started medicine. My incredible brothers and sister **Tómas, Þór** and **Lea** and sister in law **Eyrún**, and their families for their support and encouragement but also all the good moments when we are together. My grandfather and grandmother In France **René** et **Monique** have a very special place in my heart, they are the examples of what intellect can be and should be and my grandparents in Iceland, sadly gone to early, **Gunnar** og **Junna**, but it was in their home, while reading a book called Mannslíkaminn at the age of 8, that I decided I wanted to be a doctor and do research on cells of the human body. To my friends, **Vilhjálmur** and **Kristján** for “grill er chill”, keeping me through med school with endless good moments together, to be continued... And all my many friends in Iceland, France, Sweden, the Netherlands or wherever you all may be.

This work was supported by the Landspítali University Hospital Research Fund, The Icelandic Research Fund and European Molecular biology organization.

Contents

Ágrip iii
Abstract iv
Acknowledgements v
Contents vii
List of abbreviations xi
List of figures xiv
List of tables xvi
List of original papers xvii
Declaration of contribution xviii
1 Introduction 1
1.1 Selective IgA deficiency 2
1.1.1 Prevalence and genetics of IgA deficiency 2
1.1.2 The clinical phenotype of IgA deficiency 4
1.2 Immunoglobulin A – production, diversity and biological functions 8
1.2.1 Mucosal selectivity of IgA and its immunoregulatory role 10
1.2.2 Secretory IgA in breast milk and its role at mucosal surfaces in neonates 11
1.3 B cell maturation - From stem cell to terminally differentiated IgA secreting plasma cell. 12
1.4 The early days - B cells in the bone marrow 14
1.4.1 Peripheral maturation of B cells – To be or not to be with T cells, in lymph nodes, spleen or mucosal surfaces 16
1.4.2 Terminally differentiated antibody secreting B cells– Where they reside and how they survive 21
1.4.3 Cell signalling B cell responses and IgA production 23
1.5 Summary of introduction 24
2 Aims 25
2.1 General Aims 25
2.2 Specific aims 25
3 Materials and methods 27
3.1 Recruitment of participants 27
3.1.1 Icelandic sIgAD group 27

3.2 Analysis of immunoglobulins and autoantibodies in IgAD individuals 27
3.2.1 IgM, IgA and IgG measurement by rate nephelometry 27
3.2.2 IgG subclasses by Radial immunodiffusion (RID) 27
3.2.3 Anti-CCP by ELISA 28
3.2.4 Rheumatoid factor by ELISA 28
3.2.5 Antinuclear Abs by indirect immunofluorescence using rat tissue 29
3.2.6 Antinuclear Abs by fluorescence enzyme immunoassay 29
3.2.7 Immune factors by luminex 29
3.3 Cellular analysis by FACS and ELISA. 30
3.3.1 Isolation of PBMCs, B and T cells. 30
3.3.2 Flow cytometric analysis. 30
3.3.3 Flow cytometric analysis software 31
3.3.4 B cells culture conditions 33
3.3.5 A model for the assessment of the spontaneous production of IgA by B cells 33
3.3.6 T cell culture conditions 34
3.3.7 Measurement of IgG and cytokines by ELISA. 34
3.3.8 Phospho protein staining 34
3.4 Transcriptomic analysis. 35
3.5 Statistical analysis. 36

4 Results 39

4.1 Clinical phenotyping of the Icelandic sIgAD group 39
4.1.1 The prevalence of infections in the Icelandic sIgAD group ..39
4.1.2 Atopic and asthma manifestations in sIgAD. 40
4.1.3 Autoimmune profile of the Icelandic sIgAD group 40
4.1.4 Autoantibody production in the sIgAD group 41
4.1.5 Serologic immune profiling of sIgAD individuals 42
4.1.6 Connection between autoantibody positivity and single serum factors in sIgAD 45
Table 5. Comparison of the quantity of immune dysregulated immune factors and ANA or overall autoantibody positivity 45
4.1.7 Principal component analysis discriminates between sIgAD and HCs 45
4.1.8 Dysregulation of immune biomarkers in sIgAD and its association with IgA serum concentration 48

4.2 Prevalence of sIgAD in first degree relatives demonstrates a high prevalence of IgA defects with and without IgG and IgM defects 49
4.3 Lymphocyte populations, fractions and functions, in sIgAD...........50
4.3.1 The main B cell populations are present with defects in transitional B cells, class switched B cells and IgA expression. 50
4.3.2 CpG stimulation does not normlise transitional B cell fractions nor induce normal IL-10 production 51
4.3.3 Homing marker expression and activation of plasmablast is not altered in sIgAD 52
4.3.4 Stimulation by CpG fails to normalise IgA production in sIgAD individuals...53
4.3.5 sIgAD individuals have a normal proportion and function of classical T cell subpopulations. 54
4.3.6 Natural and induced Tregs have normal numbers and function in sIgAD. 56
4.3.7 Minimal inducibility and defective longevity of IgA secreting B cells in sIgAD 57
4.3.8 Signal transduction responses to stimulations known to induce IgA production demonstrate heterogeneous responses to different stimuli as well as between T and B cells 59
4.3.9 STAT3 activation with IL-21 is defective in sIgAD................60
4.3.10 STAT5 activation is normal with stimuli shown to induce heterogeneous IgA producing responses in sIgAD 62
4.3.11 STAT6 responses to IL- 4 are normal in sIgAD....................63
4.3.12 ERK1/2 responses to CpG are not defective in sIgAD.........64
4.3.13 B cell production of factors shown to be dysregulated in sIgAD 65
4.4 mRNA Transcriptomics of B cells from sIgAD individuals..............66
4.5 Gene transcripts significantly altered in sIgAD with and without stimulation 68
4.1 Go analysis of altered genes reveals defects related to germinal centre formation and TLR9 responses 70
4.2 Ingenuity pathway analysis of altered genes reveals defects related to B cell development, survival of memory B cells and plasma cell development. 72
4.3 The dysregulated IgA positive B cell compartment with IL-12, TWEAK and IL-18 changes do not affect MAIT cells 73

5 Discussion....75
5.1 Clinical phenotype of sIgAD individuals 75
5.1.1 Infections 76
5.1.1 Atopy 76
5.1.2 Autoimmunity....77
5.2 Serum concentrations of cytokines and autoantibodies....78
5.3 Cellular phenotype of sIgAD individuals....79
5.3.1 B cells 79
5.3.1 T cells 80
5.4 Signalling....80
5.5 Transcriptomic profile of sIgAD 81
5.6 Biases of the study....82
6 Conclusions 83
References....87
Original publications 109
Paper I 111
Paper II 127
Paper III 139

List of abbreviations

AbSC - Antibody secreting cells

Ab – Antibody

ANA – Antinuclear antibodies

APRIL – A proliferation-inducing ligand

BAFF – B-cell activating factor

BCL6 – B-cell lymphoma 6 protein

BCR – B cell receptor

CCP – Cyclic citrulinated peptides

CD40L – CD40 ligand, CD154 is an alternative name

CFSE – Fluorescent 5,6-carboxyfluorescein succinimidyl ester

CpG - CpG Oligodeoxynucleotide, TLR9 agonist.

CVID – Common variable immunodeficiency

DC – Dendritic cell

ELISA – Enzyme-linked immunosorbent assay

ENA – Extactable nuclear antigens

Fc (fragment crystallizable region)

FC – Fold change

FoxP3 – Forkhead box P3

GATA3 – Trans-acting T-cell-specific transcription factor

GMFI – Geometric mean fluorescence intensity

GI – Gastro intestinal

HLA – Human leukocyte antigen

IBD – Inflamatiory bowel disese

ICOS – Inducible T-cell costimulator

Ig – Immunoglobulin

IgH – Immunoglobulin heavy chain

IgAD – Selective IgA deficiency

IL – Interleukin

ILC – Innate lymphoid cell

IMDM – Iscove's modified dulbecco's medium

INFγ – Interferon gamma

KRECs – appa-deleting recombination excision circles

LT – lymphotoxin

n/iTreg – Natural/induced T regulatory cells

PADs – Primary antibody deficiency

PDE9A – Phosphodiesterase 9A

PIDs – Primary immune deficiency

PMA – phorbol 12-myristate 13-acetate

PB - Peripheral blood

PBMC – Peripheral blood mononuclear cells

PC – Plasma cell

PCA – Principal component analysis

PD1 – Programmed cell death protein 1

R – Receptor

RF – Rheumatic factor

RID – Radial immunodiffusion

RORγT – RAR-related orphan receptor gamma

RT – Room temperature

RUNX3 - Runt-related transcription factor

SC – Secretory component

SCID – Severe combined immune deficiency

SEA, SEB, SEE – Staphilococcal enterotoxins

SED – Subepithelial dome

SEM – standard error of the mean

SIgA – Secretory IgA

SLE – Systemic lupus erythematosus

SNP – single-nucleotide polymorphism

TACI – Transmembrane activator and calcium-modulating cyclophilin ligand

Tbet – T-box transcription factor TBX21

TCR –T cell receptor

TH – T Helper

TFH – T follicular helper cell

Tfr – T follicular regulatory cells

TGF-β: Transforming growth factor-β

TH1/2/17/22 – T helper cell 1/2/17/22

TLR – Toll like receptor

TNF – Tumor necrosis factor

TNFRSF13B – Tumor necrosis factor receptor superfamily member 13B

TRECs – T-cell receptor excision circles

Tregs – T regulatory cells

XLA – X-linked agammaglobulinemia

List of figures

Figure 1. Gut distribution of IgA in sIgAD (left) and a heatlhy control (right) 10
Figure 2. B cell developmental stages related to IgA class switching 13
Figure 3. Gating strategy for some of the main populations assessed. 32
Figure 4. PCA corrplot of immune factors from sIgAD patients and HCs 46
Figure 5. PCA of serum factors and autoantibody positivity 47
Figure 6. Correlation of dysregulated immune factors and subclasses 49
Figure 7. Distribution of serum IgA values in healthy donors and first-degree relatives of IgAD individuals. 50
Figure 8. B cell phenotyping and Ig expression in sIgAD 51
Figure 9. CD24highCD38high transitional B cells in IgAD individuals are defective in their numbers, inducibility and IL-10 secreting properties. 52
Figure 10. B cell homing and activation marker expression in sIgAD is normal. 53
Figure 11. IgA production and expression after stimulation. 54
Figure 12. The fraction of the T cell populations and inducability of T helper subsets in sIgAD is normal compared to HCs. 55
Figure 13. Tregs fractions and functions are normal in IgAD. 57
Figure 14. Longevity responses after stimulation of sIgAD B cells in PBMCs 58
Figure 15. Differences in percentage of pSTAT3, pSTAT5, pSTAT6 and ERK1/2 expression in T and B cells in HCs. 60
Figure 16. pSTAT3 expression after various stimulations in T and B cells from sIgAD and HCs. 61
Figure 17. The percentage and fold change GMFI of pSTAT5 in B (CD20+) and T cells (CD3+) after IL-21, IL-10 + IL-4 and IL-2 in sIgAD individuals and HCs. 62
Figure 18. The percentage and fold change GMFI of pSTAT6 in B (CD20+) and T cells (CD3+) after IL-4 and IL-10 + IL-4 in sIgAD individuals and HCs. 63
Figure 19. Effects of CpG on ERK1/2 expression in B and T cells from sIgAD individuals and HCs. 64

Figure 20 . Differences in gene transcription between sIgAD and HCs........ 66
Figure 21 . Effects of CpG stimulation in HCs and sIgAD............................. 67
Figure 22 . GO analysis of significantly upregulated genes in HCs.............. 71
Figure 23 . GO analysis of significantly upregulated genes in HCs. 71
Figure 24 . Ingenuity pathway analysis of transcripts in sIgAD..................... 73
Figure 25 . MAIT cells in sIgAD individuals are not significantly fewer in sIgAD compared to HCs. 74
Figure 26 . Hypothetic model of immune dysregulation based on the experimental data presented in the thesis. 84

List of tables

Table 1. Infectious profiling in sIgAD individuals.. 39
Table 2. Atopic and autoimmunity in sIgAD. 40
Table 3. Immunoglobulins and autoantibodies in sIgAD individuals. 42
Table 4. Serum concentrations of 65 immune biomarkers in sIgAD............. 44
Table 5 Comparison of the quantity of immune dysregulated immune factors and ANA or overall autoantibody positivity 45
Table 6. B cell secretion of serum dysregulated immune factors in sIgAD.. 65
Table 7. Differentially transcribed genes in sIgAD unstimulated CD19+ B cells 69
Table 8. Differentially expressed transcripts of genes in sIgAD CD19+ B cells after CpG stimulation compared to HCs 70
Table 9. Go terms enriched for significantly upregulated transcripts in sIgAD B cells after CpG stimulation. 72

List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-III)

I. Andri Leo Lemarquis, Helga Kristin Einarsdottir, Rakel Natalie Kristinsdottir, Ingileif Jónsdóttir and Bjorn Runar Ludviksson, "Transitional B cells and TLR9 responses are defective in selective IgA deficiency" Frontiers in Immunology, 2018.

II. Andri Leo Lemarquis, Fannar Teodor Palsson, Helga Kristin Einarsdottir and Bjorn Runar Ludviksson, "Mapping of signalling pathways linked to IgA production reveals a defective IL-21 driven STAT3 activation in selective IgA deficiency", Frontiers in Immunology, 2019.

III. Andri Leo Lemarquis, Ida Karnsund, Fannar Teodor Palsson, Anna G Vidarsdottir, Helga Kristin Einarsdottir, Gudmundur Jorgensen, Olov Ekwall, Ingileif Jonsdottir and Bjorn Runar Ludviksson. "Selective IgA deficient individuals have a serological and transcriptomic germinal centre associated immune dysregulation associated with autoantibody production", Manuscript.

All papers are reprinted by kind permission of the publishers.

Declaration of contribution

I participated in the formulation of the research questions and designing of the study. I prepared and processed the results presented in this thesis and had a leading part in writing the publications on which the thesis is based.

Together with my supervisor, I was responsible for the communication with the Icelandic authorities including the National Bioethics committee and the Data Protection Authority. I took a leading role in the writing of numerous grant applications of which some got large and other small amounts of funding, both to do the work presented, travel abroad to present my data at congresses or to go to other labs and learn new techniques. I participated in the recruitment of the study subjects with selective IgA deficiency, their first-degree relatives and the control individuals. Blood samples from participants were drawn by the nurses from the Icelandic Medical Research Centre.

I did the majority of the cellular and biochemical work presented, including setting up protocols, staining panels and culture methods. Intracellular phosphorylation was mainly done due to the incredible patience of Fannar Theodorsson, the analysis and writing of the article done by me. Autoantibody measurements were done by me and a student with the help of the personnel of the department of Immunology at Landspítali, The national university hospital of Iceland. RNA sequencing was done with the help of exiqon (currently qiagen). The alignment was done by exiqon. Analysis was done by me with the help of a biostatistician using R and its different libraries. Pathway analysis was done by me with the help of a biostatistician. Statistical work was done by me as well as with the help of a statistician.

Sungod, by Niki de Saint-Phalle (1930–2002)

Probably the most famous IgA deficient artist of the 20th century.

She suffered from autoimmunity and lung problems.

At the age of 72 she died from an empyema.

(Zeidler, 2013)

1 Introduction

This thesis is about the clinical, serologic and cellular alterations found in the Icelandic selective IgA deficiency (sIgAD) cohort at the University of Iceland from 2013 to 2019.

To understand the presented results, we must bring ourselves far earlier than that. Many things have evolved in our understanding of the immune system since Elie Metchnikof, Astrid Fagreus and other pioneers of immunology first described its phenomena (Fagraeus, 1947; Underhill, Gordon, Imhof, Nunez, & Bousso, 2016). Through this we see that research does bear its fruits: from immunotherapies in immune mediated diseases; should it be autoimmunity, cancer and infectious diseases to non-immune mediated diseases such as poisoning antagonism by monoclonal antibodies (mAB) (J. K. H. Liu, 2014).

These discoveries have been possible with overwhelming numbers of life years of research. The contribution of animal or monocellular models cannot be underestimated, though discrepancies in both innate and adaptive immunity, due to 65 million years of evolution, often lead to things still being poorly understood in humans (Mestas & Hughes, 2004). Over the years the immune responses of the healthy host have been highly informative, particularly related to immune homeostasis. However, various diseases related to single alterations of the immune system have also taught us about the role of key molecules. Selective IgA deficiency is an example of such an alteration. Since its existence was first described, many things have changed in our understanding in immunology. But even though several monogenic defects have been found that have enhanced our understanding of the basic immune response, the pathogenesis of sIgAD remains to be elucidated. Its pathogenesis may be due to heterogeneous factors. But its biochemical description has, at least until now, remained unchanged: with “real” patients having sIgAD many of whom have a clinical phenotype of diseases related to immune dysregulation (Chan & Behrens, 2013).

Thus, it was this potential connection between a defect in a single alteration of the immune system, a clinical phenotype and cellular abnormalities in sIgAD that made me interested in the defect. In this thesis I hope to share with the reader the keen interest I have for this primary immune deficiency. With that in mind, the first part is focused upon the pathways

responsible for IgA production, followed by results from clinical and experimental research about sIgAD closing with discussions and conclusions of how the results may come together.

1.1 Selective IgA deficiency

Selective IgA deficiency is a primary immune deficiency (PIDs) affecting the humoral adaptive immune system. The defect is characterised by a defect in serum IgA (<0.07 mg/dL) but normal titres of IgG and IgM immunoglobulin (Ig) isotypes, in individuals older than 4 of age (Al-Herz et al., 2011).

PIDs are a group of more than 300 rare disorders in which a part of the immune system is missing. Selective IgA deficiency is part of a subgroup of these PIDs limited to the humoral system of the adaptive immune system and as such classified as a primary antibody deficiency (PAD). PADs have in common that they are defective in the adaptive immune systems capacity of producing antibodies (Ab). Of these PADs, sIgAD is thought to be the most common in the Western world. PADs are clinically and biologically a heterogeneous group of defects with a common serological phenotype of an absence of serum Ig many being due to a disturbed B cell differentiation. They have an ever-enlarging group of genetic defects as perpetrators in subgroups of patients. However, the molecular and/or genetic defects responsible are still largely unknown for the majority of individuals with hypogammaglobulinemia's (van der Burg, van Zelm, Driessen, & van Dongen, 2012). None has been found to this date as the singular genetic perpetrator common to all sIgAD patients.

1.1.1 Prevalence and genetics of IgA deficiency

The prevalence of sIgAD is variable depending upon individual demographic and ethnic background. Its prevalence ranges from 1:142 in the Arabian peninsula (al-Attas & Rahi, 1998), 1:600-700 in Europeans (Gudmundur H. Jorgensen, Thorsteinsdottir, Gudmundsson, Hammarstrom, & Ludviksson, 2009) to 1:3229 in Chinese and 1:18,500 in Japanese individuals (Cunningham-Rundles, 2001; Feng et al., 2011).

It has also been demonstrated that familial clustering of sIgAD does exist, with prevalence in first degree relatives ranging from 7-16% (Koistinen, 1976; Soler-Palacin et al., 2016). Furthermore, it has also been linked to other PADs such as common variable immunodeficiency (CVID) where about 3% of first-degree relatives have been shown to have sIgAD (Aghamohammadi, Sedighipour, *et al.*, 2008). Interestingly the familial aggregation has also been

shown for autoimmunity and sIgAD, that is increased autoimmunity has been found in family members of sIgAD individuals without the antibody defect (Gudmundur H. Jorgensen et al., 2009). Multiple reasons are possible for these connections. There may be a common genetic basis of sIgAD, broader hypogammaglobulinemia's and autoimmunity. This may also be an indicator of the presence of a double hit therapy: where quantity of environmental factors or loss of pathways redundant to each other leads to different phenotypes. Both may then be true, depending on a case to case basis. For example the connection with variants in the HLA locus and interferon induced with helicase C domain 1 (IFIH1) (R. C. Ferreira *et al.*, 2012; R. C. Ferreira *et al.*, 2010) may indicate connection with autoimmunity, supporting the first hypothesis, while familial TACI mutations with concomitant familial sIgAD and CVID may be true for the latter (Aghamohammadi, Mohammadi, *et al.*, 2008).

Due to the familial and ethnic patterns observed, considerable effort has been done finding the gene responsible for the defect. Genetic variants leading to decreased IgA have been described, such as RUNX3 related to retinoic acid, a known inducer of IgA production (Jonsson et al., 2017). But no single gene defect responsible for sIgAD has been found. One of the biggest research efforts to find a defect responsible for sIgAD showed an association with risk genes associated with sIgAD such as variants in the HLA locus as well as interferon induced with helicase C domain 1 (IFIH1) (R. C. Ferreira *et al.*, 2012; R. C. Ferreira et al., 2010). That are, genes that may also be related to autoimmunity and may be an indication of broader immune dysregulation. The HLA association with sIgAD has in particular been with the so-called 8.1 ancestral haplotype (R. C. Ferreira et al., 2012). This ancestral haplotype is also associated with other immune mediated diseases including Graves' disease, systemic lupus erythematosus (SLE), type 1 diabetes, celiac disease and maybe myasthenia gravis and rheumatoid arthritis. Thus possibly, indicating a common predisposition to sIgAD and autoimmunity indicative of a possible common genetic background. But this has also been hypothesised to be related to effects of common differences related to the expression of TACI that is close by genome wise (N. Wang *et al.*, 2011).

While findings related to genetic defects are important, further studies are needed in order to get the whole picture (Farhadi *et al.*, 2013). Some say a cure for IgA deficiency and its comorbidities may remain unlikely as long as the genetic defects responsible for its pathogenesis remain unknown (Milner & Holland, 2013). That is since in some cases a known genetic defects in PIDs have allowed an earlier diagnosis to be made and a more precise definition of patients at risks, resulting in better treatments and long term outcomes

(Cunningham-Rundles & Ponda, 2005). It is therefore important to continue for the search for defects responsible for its pathogenesis. But one may bear in mind the changes happening in CVID where phenotype may overrule genetic basis for treatment trials, due to little power and heterogeneous presentations with even incomplete penetrance's (Wehr *et al.*, 2008). If some similarities do exist, between broader hypogammaglobulinemia's, CVID and sIgAD, the example of CVID may be the key to understanding the defect. There multiple single gene mutations with or without hypomorphisms and/or polymorphisms may lead to a common serologic phenotype with different clinical presentations (van der Burg *et al.*, 2012).

1.1.2 The clinical phenotype of IgA deficiency

The clinical presentation of individuals with PADs is heterogeneous even though similarities related to their underlying immune dysregulations may exist. sIgAD was first described in two healthy men that found the defect in themselves while doing research at the Rockefeller institute (Rockey, Hanson, Heremans, & Kunkel, 1964). Soon some patients with immune related symptoms where though found. There the example of the famous artist Niki de Saint Phalle suffering from IgAD, autoimmunity and infections has been well reviewed (Zeidler, 2013).

Some PADs may be more closely linked to certain infections, autoimmunity, atopy or malignancy depending on their mechanisms (Cunningham-Rundles, 2011; R. S. Liblau & Bach, 1992; Schmidt, Grimbacher, & Witte, 2017). Regarding sIgAD individuals their phenotype is heterogeneous, with some individuals remaining asymptomatic as the first examples described, while others suffer from various comorbidities. A classification scheme based on the literature has been proposed for clinicians to assess sIgAD (Yazdani *et al.*, 2015). Based on that, sIgAD individuals could be classified into the following groups: a) largely asymptomatic individuals, b) these with minor infections, c) atopic diseases, d) autoimmunity and finally e) sIgAD with severe concurrent complications. Thus, reflecting everything from a relatively benign course to individuals suffering from severe diseases and double mortality rates compared with the general population (Ludvigsson, Neovius, & Hammarstrom, 2013; Mellemkjaer et al., 2002).

1.1.2.1 Infections

The infectious problems related to sIgAD are mostly related to the upper respiratory tract. The main increase has been shown in pneumonia and upper respiratory recurrent infections (Aghamohammadi *et al.*, 2009; Litzman *et al.*,

2000). Depending on groups some have reported 26% of severe respiratory infections in sIgAD individuals with 16% recurrent viral respiratory infections compared to respectively 8% and 1% in control groups (Koskinen, 1996). While others with more severely affected patients have reported respiratory infections affecting from 73 - 95% of sIgAD individuals (Aghamohammadi *et al.*, 2009). One study looking at viral infections in early life showed that pseudocroup is significantly more common in infants with IgA deficiency (Janzi *et al.*, 2009).

Although one tends to think of IgA as dominating in the gut responses sIgAD individuals do not suffer from serious gastrointestinal problems. In fact, they seem to have a normal prevalence of most gastrointestinal infections (Aghamohammadi *et al.*, 2009; G. H. Jorgensen *et al.*, 2013; Koskinen, 1996; Litzman *et al.*, 2000). However, it has been shown that after vaccination with life attenuated poliovirus the virus was detectable in the stool of sIgAD individuals for a long time without a chronic carrier state development (Savilahti *et al.*, 1988). This could point towards a redundant mechanism in mucosal immunity, possibly through other isotypes. Especially since enteropathy in CVID patients does seem to be linked to their ability to produce IgA. Shown to be connected to its capacity to control bacterial gut populations (Shulzhenko *et al.*, 2018). A non-redundant mechanism may though be true for non-bacterial pathogens since a link between Giardia lamblia infections and sIgAD has been proposed (Carretero Gomez, Vera Tome, Arevalo Lorido, & Munoz Sanz, 2003; Eren, Saltik-Temizel, Yuce, Caglar, & Kocak, 2007).

The heterogenicity of infectious problems may be linked to possible genetic differences. A hypothesis strengthened by the data showing that sIgAD individuals with at least on HLA- B8 allele, when tested, had a significantly greater response to vaccination than HLA-B8 negative subjects (Edwards, Razvi, & Cunningham-Rundles, 2004).

1.1.2.2 Atopy

The connection between sIgAD and atopy is well known (Buckley & Dees 1969). Depending on population analysis it is quite variable ranging from 32-84% (Aghamohammadi et al., 2009; Jacob, Pastorino, Fahl, Carneiro-Sampaio, & Monteiro, 2008; Shkalim et al., 2010). Others have though not found any significant increase in atopic prevalence (Edwards et al., 2004; Janzi et al., 2009; Koskinen, Tolo, Hirvonen, & Koistinen, 1994).

Multiple atopic presentations have been described, from allergic rhino conjunctivitis, urticaria, atopic eczema, food allergy and asthma (Cunningham-

Rundles, 2001). In a prior study evaluating the Icelandic sIgAD cohort a significant higher prevalence of allergic diseases was observed within the sIgAD. Furthermore, an analysis of the health-related quality of life of sIgAD individuals showed that the biggest effect may be for these atopic presentations (G. H. Jorgensen et al., 2013). A rational for the screening of immunoglobulin levels in atopic diseases has been proposed and from examining the literature related to sIgAD the measurements of IgA should possibly not be excluded from such undertaking (Yazdani et al., 2015).

The relationship between IgA production defects and associated atopic risk is not known. The pathogenesis of atopic diseases is known to be linked to a Th2 skewing but no concise Th2 polarising researches have been made in sIgAD. A better characterisation of Th2 responses and other biomarkers may be especially relevant in sIgAD and other PIDs with the better classification of phenotypes and endotypes of asthma. Especially with the selectivity of treatment options having led to better control of symptoms in serious presentations (Wenzel, 2012).

1.1.2.3 Autoimmunity

Like in many other PIDs a higher prevalence of autoimmunity has been seen in sIgAD compared to the general population. Similarly, to the prevalence of atopy the prevalence differs between sIgAD cohorts, ranging from 7-36%. Its prevalence has been shown to be not only higher in sIgAD individuals but be manifold higher in first degree relatives. In a previous study of the Icelandic sIgAD group, 25% of adult SIgAD individuals where shown to suffer from autoimmune presentations. This higher prevalence was also seen in 1° relatives (10%), both higher than what was observed within a control group (4.8%) as well as estimated population prevalence in the Western world (Gudmundur H. Jorgensen et al., 2009).

The connection with autoimmunity has previously both been shown in longitudinal studies of previously healthy blood donors found to be sIgAD and children with sIgAD (Koskinen, 1996; Shkalim et al., 2010). In addition, several studies have shown an increased risk of sIgAD in various autoimmune conditions (Cassidy, Burt, Petty, & Sullivan, 1969). The augmented risk for autoimmunity has been found in both systemic and organ specific autoimmune diseases. Thus, the risk for systemic autoimmunity has been shown for both SLE (1.3% risk) and RA (0,55%) (Cassidy et al., 1969; Ning Wang et al., 2011). While for organ-specific autoimmune diseases sIgAD has associated with autoimmune thyroiditis (R. S. Liblau & Bach, 1992; Savilahti, Pelkonen, & Visakorpi, 1971), Graves' disease (G. H. Jorgensen et al., 2011) type 1

diabetes (N. Wang et al., 2011), coeliac disease (Heneghan, Stevens, Cryan, Warner, & McCarthy, 1997), myasthenia gravis (R. Liblau, Fischer, Shapiro, Morel, & Bach, 1992), psoriasis (van de Kerkhof & Steijlen, 1995), idiopathic thrombocytopenia (Ozsoylu, Karabent, & Irken, 1988) and vitiligo (Koskinen, 1996).

Autoantibodies, without overt clinical disease, have been shown to be present in many individuals with sIgAD (Barka et al., 1995). Most commonly for ANA and RF (Goshen et al., 1989; JimÉNez et al., 1991). Autoantibodies implicated in organ-specific autoimmune diseases are less commonly reported, although anti-thyroglobulin and anti-thyroid microsomal antibodies have been detected in 10-15% of sIgAD individuals (Funakoshi, Kanoh, Mizumoto, & Uchino, 1985; Koistinen & Sarna, 1975).

In addition to the above classical autoantibody profile, sIgAD individuals have been found to be seropositive for anti-IgA antibodies. This has been shown to be present in 36,8% of individuals with IgAD, compared to 17,5% in other primary immune deficiencies and 5,6% of healthy blood donors (A. Ferreira, Garcia Rodriguez, Lopez-Trascasa, Pascual Salcedo, & Fontan, 1988). Its clinical relevance is not known but they have been claimed to be important in the pathogenesis of anaphylactic reactions of IgAD individuals being treated with various blood products, including blood transfusions or IVIG (Mukherjee & Bhattacharya, 2011). However, such connection is still being debated, particularly since most patients having had serious reaction have not been found to have such anti-IgA antibodies (A. Ferreira et al., 1988). Furthermore, it has also been hypothesised that antibodies against the cytokines BAFF, APRIL and IL-21 may play a role in the pathogenesis of IgAD but even though the rate of these autoantibodies is higher its clinical relevance may be insignificant (Pott et al., 2017).

The pathological mechanisms responsible for an increased risk of autoimmune diseases in sIgAD is not known (N. Wang & Hammarstrom, 2012). The possible inflammation induced by the lack of IgA has been proposed but such a defect is also part of other immune deficiencies which do not all have increased risk of autoimmunity. In CVID, individuals have defects in multiple immunoglobulin classes in addition to IgA and an increased risk of autoimmunity. On the other hand, in X-linked agammaglobulinemia (XLA) this association has not been as strong (Behniafard et al., 2012). One plausible explanation would be a common genetic effector mechanism predisposing some individuals to both autoimmune diseases and sIgAD (Gudmundur H. Jorgensen et al., 2009). Thus, if sIgAD like CVID is a multigenetic disease with

a similar presentation it could be hypothesised that a loss in central tolerance or dysregulated feedback loops of immune homeostasis could be lost by the lack of IgA and IgA positive B cells in the periphery, tissues and bone marrow.

1.1.2.4 Malignancy

Some publications have suggested an increased risk of malignancy in selective IgA deficiency (Cunningham-Rundles, Pudifin, Armstrong, & Good, 1980). With a suggested risk especially present for lymphoma and gastric cancer (Mellemkjaer et al., 2002). Given the link between autoimmune diseases and lymphoproliferative malignancy and the link between autoimmunity and selective IgA deficiency it was hypothesised that IgA deficient individuals may be at an increased risk of any cancer. In a large study of 386 Swedish and Danish sIgAD the prevalence of cancer was not found to be increased (Mellemkjaer et al., 2002). Another study of 63 sIgAD children with a prevalence of 4.8% was found (astrocytoma and adenocarcinoma of the colon, Hodgkin´s lymphoma and neuroblastoma) (Shkalim et al., 2010). A recent Swedish study following 125 IgAD individuals found an overall HR to develop cancer of 1,31 but a HR of 2.80 a year after diagnosis. With these differences suggesting a possible degree of surveillance bias (Ludvigsson, Neovius, Ye, & Hammarstrom, 2015).

The factors responsible for lymphoproliferative malignancy in PID are complex and bring about the question of environmental factors and genetic variations (Casanova & Abel, 2013). As an example, they may not only be due to immune dysregulation but because possible susceptibility to certain infections. Epstein Barr virus is there highly implicated in the pathogenesis of lymphomas (Kim et al., 2017). The lack of IgA has not been reported to affect the response to such viral infections. It may be said that no evidence for a generalised effect of IgAD exists for all types of cancer. And for stomach cancer and lymphomas the statistical power to define few events in a restricted group has not given any conclusive responses (Mellemkjaer et al., 2002).

1.2 Immunoglobulin A – production, diversity and biological functions

IgA is the most abundant Ab isotype produced in the body, as such one of the most extensively produced protein by the immune system. It´s concentration in blood is close to that of IgG1 although detected at highest concentration at mucosal surfaces. As an example in the intestine alone it produced at a rate of 3–5 g daily (Fagarasan & Honjo, 2003).

IgA can be either serum or secretory IgA, being referred to as SIgA when secretory. An important difference between these is that the dominant serum form of IgA is monomeric while SIgA is dimeric. Thus, SIgA has a total of four binding sites for its respective antigen while the Fc (fragment crystallizable region) end has not the pleiotropic role that it is known for in other isotypes such as IgG. The SIgA undergoes transcytosis to go out to mucosal surfaces into the intestine, epithelia of bronchi, in salivary glands, in lacrimal glands, in mammary glands and in the active epithelia of fallopian tubes and uterus. A similar transport system is present in the liver, where it is involved in the release of SIgA in the bile through the epithelial cells of the bile ducts. This mechanism enables a continuous release of IgA at mucous membranes (Vo Ngoc, Krist, van Overveld, & Rijkers, 2017).

Besides being either serum or secretory, IgA can be of IgA1 or IgA2 subclass, each transcribed by one heavy chain (IgH) with selective genetic transcription (Macpherson, McCoy, Johansen, & Brandtzaeg, 2008). IgA1 in monomeric form is the dominant isotype in the circulation, whereas in saliva, the respiratory tract, mammary glands and the proximal Gastro intestinal (GI) tract it is predominantly in dimeric form. IgA2 is the subclass that does occur mostly in secretions in a dimeric form and particularly in the distal part of the intestine (Simell, Kilpi, & Kayhty, 2006). At the molecular level they are similar with slight variations at the hinge region leading with IgA1 being more flexible and therefore more prone to protease degradation by micro-organisms (Chintalacharuvu et al., 2003).

IgA like other immunoglobulins is made up of a Fab and a Fc end. In a simplified manner one can think of the Fab end as binding to the foreign without effector functions and while the Fc end to self-receptors, called Fc receptor, where effector functions can be mediated. Historically, IgA was thought of as non-inflammatory because the lack of Fc receptors at mucosal surface, making it more of a neutralising than activating antibody. However, since the description of its transmembrane receptor FcαRI (CD89), found on eosinophils, neutrophils and monocytes it is known that serum IgA may potentially trigger various activities upon binding. Where according to humanized transgenic mouse models it seems to be most important at mucosal surfaces in the upper respiratory tract (van Egmond et al., 2001).

1.2.1 Mucosal selectivity of IgA and its immunoregulatory role

The complex interdependence between IgA and the mucosal environment is not fully known. In the gut IgA-secreting B cells are dramatically reduced in germ-free animals and are virtually absent in neonates before their exposure to bacteria (Macpherson & Harris, 2004). Antibody mediated sorting has led to the identification of the bacterial taxa's preferentially binding to IgA (Palm et al., 2014) leading to functional assays. The bacteria binding to IgA have that way been assessed in *in vivo* mice models as well as in *ex vivo* human models leading to the conclusion that IgA binds to proinflammatory taxa of gut bacteria both in an autoinflammatory and non-autoinflammatory settings (Palm et al., 2014). One example is inflammatory bowel disease where IgA has been shown to have anti diseases effects by binding selectivity towards bacterial species that are symptom inducing/enhancing (Viladomiu et al., 2017). Another example is the shown protection in kwashiorkor models where IgA protects against the induced inflammation due to malnutrition (Kau et al., 2015). These studies have shown binding, which does not necessarily mean neutralizing function, directed against specific pathogens in the microbiota.

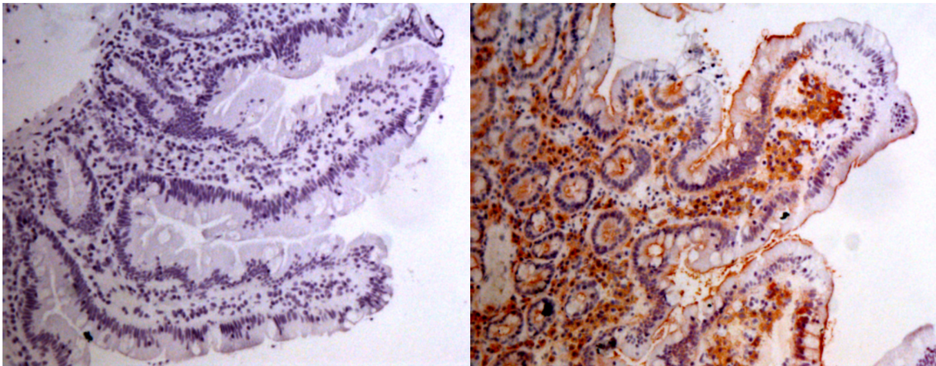

Figure 1. Gut distribution of IgA in sIgAD (left) and a heatlhy control (right)
Gut from the first sIgAD (Figure 1, left) individual described compared to a HC (Figure 1, right). Brown staining shows IgA distribution. Courtesy of Professor Lars Hansson and Professor Esbjörn Telemo, Göteborg, Sweeden.

Due to the lack of IgA one could hypothesise that alterations would be seen in sIgAD individuals at the mucosal surfaces. A study evaluating the mucosal surface of sIgAD individuals using immunohistochemistry showed increased T cell activation. Accompanied by the appearance of intraepithelial T cells with an increase in mitotic rate of crypts in sIgAD (Klemola et al., 1995).

The advent of modern faecal metagenomics has shown how IgA can be co-opted by the microbiome to engender robust host-microbial symbiosis. Being therefore not only responsible for controlling infections but being also important in shaping the microbiome (Donaldson et al., 2018). Another study analysing sIgAD individuals described only minor intestinal dysbiosis, though characterized by expansion of proinflammatory bacteria, depletion of anti-inflammatory commensals and a perturbation in the “obligatory” bacterial network (Fadlallah et al., 2018). Thus, indicating a redundant mechanism leading possibly to the not so severe gastrointestinal phenotype observed in sIgAD.

Due to restricted access with biopsies, the upper respiratory tract bacterial metagenome and its relationship to IgA is not as well understood as that of the gut. Data from transgenic humanized mouse models with FcαRI indicates that its role is most important in the upper respiratory tract (van Egmond et al., 2001). Furthermore, it is known from such models that IgA can neutralize both virus and bacteria (Mazanec, Kaetzel, Lamm, Fletcher, & Nedrud, 1992; Williams et al., 2004). In conjunction with the epidemiologic infectious clinical phenotype of sIgAD, where respiratory infections are dominating, may suppose that IgA plays an important neutralizing role in humans for the respiratory mucosa. Possibly not as redundant as the one played in the gut (G. H. Jorgensen et al., 2013).

1.2.2 Secretory IgA in breast milk and its role at mucosal surfaces in neonates

The first moments of a neonate’s life are characterised by the meeting of enteric microbiota and the innate and adaptive immune system. These are not only tightly regulated by a regulatory environment (Olin et al., 2018) but are also known to be exposure–triggered persisting immune events, shaping a life-long immune homeostasis (Torow & Hornef, 2017). The main nutrition the neonate gets is breastmilk, where IgA is one of the main antimicrobial products amongst with lactoferrin, xanthine oxidoreductase, lysozyme and a few leukocytes. Therefore, in addition to the nutritional value of breastmilk, the offspring gets an additional external adaptive and innate immune regulation (Weaver, Arthur, Bunn, & Thomas, 1998). No direct evidence exists of the effect of the breastmilk of sIgAD mothers for their children but it may be questioned if it may play a role in the effect of breastmilk for the modulation of the immune response of the neonate (Little, Blattner, & Young, 2017). Experimental mouse models have shown that actively secreted IgA and IgM can shape the long-term intestinal microbiota that is acquired by the offsprings

(Hurley & Theil, 2011). In other words, the protective effects of actively secreted polymeric immunoglobulins such as IgA are not only able to protect the early-life mucosa in the fragile postnatal period but possibly induce life long lasting effects (Torow & Hornef, 2017). Here one may also wonder about the effects of selective secretory IgA deficiency (Polosukhin et al., 2017) and the role of low titres of IgA in infancy known to be a risk for atopy (van Asperen et al., 1985).

1.3 B cell maturation - From stem cell to terminally differentiated IgA secreting plasma cell.

Since the defect in IgA production in sIgAD individuals could be due to a defect in any of the mechanisms responsible leading to IgA secretion it is important to understand its production in the healthy host. Bone marrow transplantation in individuals with sIgAD can cure the deficiency suggesting that the defect is of hematopoietic origin (Hammarström, Ringdén, Lönnqvist, Smith, & Wiebe, 1985). IgA is produced by the adaptive immune system by terminally differentiated B cells, sometimes called antibody secreting cells (AbSCs) (Federica Capolunghi et al., 2008; Radbruch et al., 2006). The mechanisms leading to the differentiation and survival of fully matured B cells to become AbSCs are dictated by a variety of control mechanisms including class switching, homing, co-stimulation and finally commitment to a plasma cell lineage (Pabst, 2012).

The bone marrow is the primary organ of B cell development. It is from there B cells emerge to gain different effector functions. V(D)J recombination in the bone marrow is essential in the maturation and affinity maturation of B cells. V(D)J recombination is when the variable region of their antibodies is recombined. Not until after that they may emerge from the bone marrow to undergo class switching in peripheral lymphoid organs. Potentially leading to the production of IgM, IgD, IgA, IgG or IgE with different effector functions. To produce their antibodies in large quantities as plasma cells they do then end their life in peripheral tissues or return to the bone marrow. To understand the mechanisms leading to this each set timepoint in their development has to been understood.

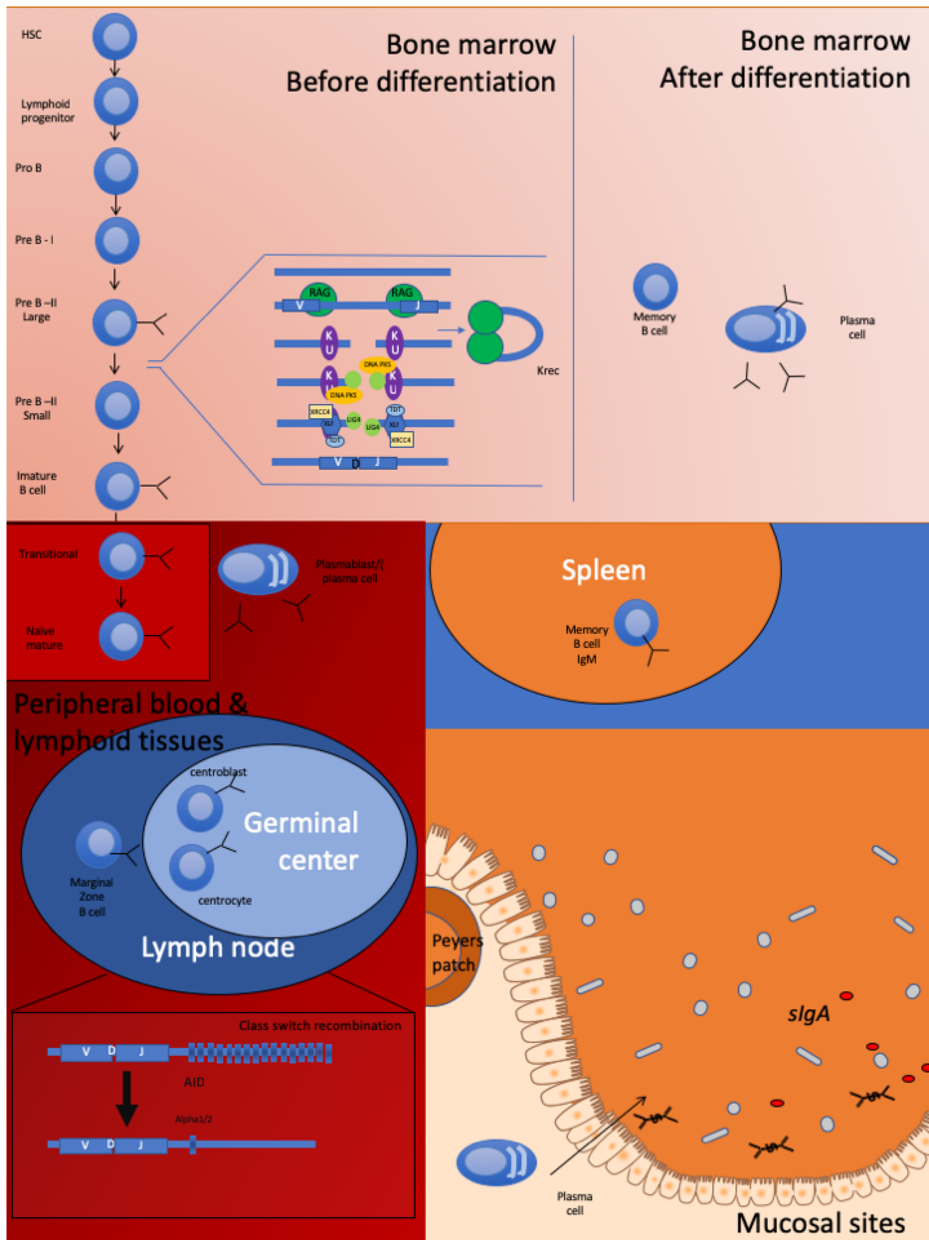

Figure 2. B cell developmental stages related to IgA class switching

The upper part of the figure demonstrates how B cells develop in the bone marrow from hematopoietic stem cell. In between the pre-B-II large and small stages V(D)J recombination happens where the different protein cut and reassemble the genetic material. In the lower part of the figure one can see the transitional and naïve B cells homing to the different compartments and maturing into memory or plasma cells which will either home back into the bone marrow or at other sites.

1.4 The early days - B cells in the bone marrow

As all other lymphoid cells, B cells develop from multipotent hematopoietic stem cells (HCS) (Eibel, 2015). HSC homeostasis, maintenance, and differentiation is controlled within the highly specialised microenvironment of the bone marrow: regulated on the one side by interactions with stroma cells of non-hematopoietic origin (Mendelson & Frenette, 2014) and by complex intrinsic programs controlled by the timed expression of multiple factors influencing epigenetic changes of the chromatin structure (Boller & Grosschedl, 2014). At the early stage of HCS receptor are expressed on the cells surface, such as Flt3 and c-kit, pattern-recognition receptors like TLRs (Toll like receptors), Rig I-like receptors (RLRs), Nod-like receptors (NLRs), and purinergic receptors. Thus, making them able to respond to the multiple signals generated in the body, such as stress, tissue damage, inflammations or infections. HSC do mature into different subgroups with one such being the common lymphoid progenitor (CLP). These differentiate preferentially into lymphoid cells that can either develop into pro B cells or into early thymic progenitors (ETPs). With the prior residing in the bone marrow and the later leaving the marrow to home into the thymus for T cell development (Eibel, 2015). A complex network of transcription factors leads to commitment to the B cell lineage of these early stages: leading the Pro-B cell to Pre-B Cell development. These early stages are interesting to hypogammaglobulinemia since all possible defects can lead to aberrant maturation.

In CVID some individuals have decreased numbers of bone marrow plasma cells due to a partial block in B cell development at the pre-B-I to pre-B-II stage that may correlate with lower transitional and mature B cells in the periphery of these patients (Ochtrop et al., 2011). Many other defects in the protein-DNA interactions can lead to serious problems, where many of these defects are not viable unless transplantation or gene therapy is initiated (Fischer, Notarangelo, Neven, Cavazzana, & Puck, 2015). The proB/pre-B cell developmental stages are regulated by the expression and activity of proteins that are essential signalling components of cytokine and antigen receptor signalling pathways. To name only few the members of the RUNX (Whiteman & Farrell, 2006) family and PAX5 genes (Medvedovic, Ebert, Tagoh, & Busslinger, 2011), IL-7, STAT5 and the IKARIOS transcription factor (Sellars, Kastner, & Chan, 2011) have been shown to be crucial to a normal early development. The description of their exact role extends beyond the scope of this thesis, but it has to be acknowledged that some of them have been described as responsible for PIDs, others for hematologic malignancies while some have never (or still) not been found to be mutated in humans (Eibel, 2015).

One of the factors important to describe is when the development of B cells comes to the stage of undergoing V(D)J recombination. In short, V(D)J recombination is a chain of events that cuts of parts of DNA, introducing point mutations at high rates into VHDJH and VLJL exons. Enabling, by that manner, the cell to be individualistic regarding its recognition of foreign material. Thus, providing the structural correlation for selection by Ag of high-affinity immunoglobulin variant (Odegard & Schatz, 2006). It starts at the genetic level with a double strand break induced by the recombination-activating gene products RAG1 and RAG2. This break produces two DNA ends. One containing the recombination signal sequence, a blunt DNA end which can be ligated directly, and another end containing the coding sequence of the Ig end for B cells and TR gene for T cells, called the coding end. This coding end is blocked by a covalent phosphodiester bond and is recognised, processed and repaired by non-homologous end joining pathway (NHEJ). For doing so a complex apparatus of multiple players have to function correctly. First Ku70/80 heterodimers form a ring around the ends of the DNA that migrate into DNA and attract DNA-dependent protein kinase catalytic subunit (DNA-PKCS), which acquires protein kinase activity upon DNA end binding (Smith & Jackson, 1999; Weterings & van Gent, 2004). This does induce conformational changes in DNA enabling artemis to open a DNA hairpin. The ends can then, if compatible, be ligated by ligase IV forming a stable complex with XRCC4 which can be called “Cernunnos”. Before ligation non-templated (N) nucleotides can be inserted by TdT or deleted by exonuclease activity. The activity of these multiple factors has been used for the analysis of the diversity of the total T and B cell receptors. For example, the different combinations of V, (D) and J genes, the analysis (N) nucleotide insertions that are introduced by the enzyme TdT and/or the presence of palindromic (P) nucleotides that arise due to asymmetric hairpin opening by Artemis can be used for the analysis of junctional diversity in PID (Fischer et al., 2015). The biological outcome of this process can though be used to understand the pathogenesis of PADs and autoimmunity. The naïve repertoire is present in the transitional and naïve mature B-cells while cells that have undergone later developmental stages have more complex diversity. This was used to demonstrate that the naïve repertoire of most CVID is normal in most patients, indicating a defect after V(D)J recombination (van Schouwenburg et al., 2018). Other relevant processes occurring during V(D)J recombination is the maturation of kappa-deleting recombination excision circles (KRECs) in B cells and T-cell receptor excision circles (TRECs) in T cells. With KRECs being a by-product of the excision of genetic material during the recombination, they can be used for the detection of early B cell defects such as CVID; where individuals with low Trec

and Krec or only low Krec numbers have worse clinical outcome compared to those with only low Trec or normal Trec and Krec (Kamae et al., 2013).

As B cells have undergone the V(D)J process, they can enter the developmental stage of pre-B cells where the heavy chain is presented on the surface of the cell with a surrogate light chain for the selection of a productive heavy chain. Cells without a productive heavy chain gene rearrangement are then removed from the repertoire while these with aproductive heavy chain undergo a few rounds of proliferation and are then defined as “large” pre-B cells (Hardy, Carmack, Shinton, Kemp, & Hayakawa, 1991). The surface expression of a complete Ab leads to the checkpoint of self-tolerance so that these with receptors with too high an affinity for self-antigens undergo receptor editing to change the light chains. Up to half of early immature B cells carry a polyreactive Ig gene. But after undergoing receptor editing or deletion from the repertoire only 7% are left bearing polyreactive antibodies (Wardemann et al., 2003).

1.4.1 Peripheral maturation of B cells – To be or not to be with T cells, in lymph nodes, spleen or mucosal surfaces

After having undergone their initial developmental stages within the bone marrow, B cells emigrate into the periphery. The maturation steps residing in between the time point when B cells have recently emerged from the bone marrow and until they have gained survival signals to undergo further maturation is called the transitional maturation stage. As such, these B cells have been described as transitional B cells (Agrawal, Smith, Tangye, & Sewell, 2013). A classification has been proposed of various developmental stages of these transitional cells in mouse T1, T2 and T3, where these cells develop in their numerical chronologic order. These are not as clearly defined in humans, but they have been described as either CD24hiCD38hi (Martin et al., 2016) or as CD38hiIgM+ (Nechvatalova et al., 2012). Most of these transitional B cells are precursors of mature-naive B cells. That is, they will mature into naive follicular B cells, which continue to circulate to the follicles of the spleen and lymph nodes through the bone marrow until they die or encounter their cognate antigen and BAFF signalisation (Martin et al., 2016). Some of the transitional B cells may though differentiate directly into switched and IgM memory B cells by homing to the marginal zone of the spleen and remain there as naive non-circulating B cells. The cells that do so may be the equivalent of B1 cells in mouse that can respond with IgM production. As such they may be the remnants of a primitive adaptive splenic system that we have kept due to its importance in keeping variability and fighting senescence (F. Capolunghi,

Rosado, Sinibaldi, Aranburu, & Carsetti, 2013).

An increased interest has been in the non-antibody dependent immune functions of B cells especially through IL-10 and IL-35 secretion (Rosser & Mauri, 2015). While the regulatory capacities of B cells have been shown to be related to the secretion of IL-10 (Simon et al., 2016) no specific transcription factor has been found for the cells secreting these cytokines. It has even been shown that their effector functions may be responsive to environmental factors. For example in different inflammatory states the transitional B cells may be capable of producing anti-inflammatory cytokines such as IL-10 as well as pro-inflammatory cytokines such as TNF-α (van der Vlugt, Haeberlein, de Graaf, Martha, & Smits, 2014).

Transitional B cells have also been of increased interest due to their potential sensitivity towards various immune alterations. For example a significantly increase in transitional B cells has been seen in IgM deficiency (Louis, Agrawal, & Gupta, 2016), SLE (Szabo, Papp, Szanto, Tarr, & Zeher, 2016) (Henriques et al., 2016) and XLA (X linked agammaglobulinemia) (Cuss et al., 2006). While lower numbers have been shown in CVID (Barsotti et al., 2016), APECEED (Perri et al.) and GATA2 deficiency (Collin, Dickinson, & Bigley, 2015). Furthermore, immunomodulation has also been found to significantly affect their numbers. Treatment with calcineurin inhibitors following renal transplantation has been linked to lower numbers of transitional B cells (Svachova et al., 2016; Tebbe et al., 2016). In infectious diseases this has also been observed; e.g. a higher number of immature transitional B cells at birth is seen in hepatitis B surface Ag positive infants (Shrivastava, TrehanPati, Kottilil, & Sarin, 2013). With similar observations found in neonatal sepsis (Pan, Ji, & Xue, 2016). Related to a PID linked to sIgAD, CVID analysis of bone marrow has revealed a subgroup of patients with a partial block in the pre-B-I to pre-B-II stage correlating with lower transitional and mature B-cell counts in the periphery (Ochtrop et al., 2011). Creating a link with the possibility of an early maturation stop and isolated hypogammaglobulinemia.

Related to IgA production it is known that transitional B cells will enter into the next maturational stage following encounter with their cognate Ag, required for their survival (Eibel, 2015). If transitional B cells manage to get the correct signal for survival, they have the possibility to undergo isotype class switching such as IgA. Class switching is the substitution of the IgH constant region μ (Cμ) and Cδ genes encoding for IgM and IgD with Cγ, Cα or Cε genes encoding for IgG, IgA or IgE through class-switch recombination (CSR) with the same specificity but different effector functions (Chaudhuri & Alt, 2004).

These crucial steps can occur through two different mechanisms; T cell dependent and T cell independent isotype class switching.

In T cell dependent reactions, B cells meet T cells and present Ags to them, providing them the necessary help to migrate and make germinal centre reactions (Pape, Catron, Itano, & Jenkins, 2007). B cells then home into the follicles in secondary lymphoid organs where they are fostered for proliferation, AID expression and Ab gene diversification through CSR and somatic hypermutation (Muramatsu, Nagaoka, Shinkura, Begum, & Honjo, 2007). Within the mucosal immune system in the gut, homing of B cells to Peyer's patches is thought to be essential for such reactions leading to their migration to lamina propria and mesenteric lymph nodes (Spencer & Sollid, 2016).

The T cell dependent germinal centre mechanism leading to IgA class switching is highly dependent on interactions with Ag specific B and T cells through essential CD40L-CD40 interactions (Marasco et al., 2017; Zan, Cerutti, Dramitinos, Schaffer, & Casali, 1998). These stimulations are strongly biased towards IgA in the gut with an important role for transforming growth factor-β1 (TGFβ1) and RUNX3 (Watanabe et al., 2010). Other cytokines that are important in inducing IgA class switching in a T cell dependent manner with CD40L include IL-2, IL-4, IL-5, IL-6, IL-10, IL21 and vasoactive intestinal peptide (Borte et al., 2009; Cerutti, 2008; Fujieda, Waschek, Zhang, & Saxon, 1996). In germinal centres both dendritic cells (DCs) and follicular T helper cells (TFH) play essential roles for the CSR process (Crotty, 2014). A subset of these TFH cells have a T regulatory like phenotype that has been described as being T follicular regulatory cells (Tfr). In mice these have been shown to be responsible for the diversification and selection of IgA and in that way controlling microbial balance (Kawamoto et al., 2014).

T regulatory cells are known to be important for IgA production at mucosal surfaces. Especially for the induction of production outside of Peyers patches. There the Tregs (T regulatory cells) reside in the mucosa and are thought to maintain commensalism with the microbiota (Cong, Feng, Fujihashi, Schoeb, & Elson, 2009). In sIgAD Tregs have been shown to be of decreased proportions in a subgroup of IgAD individuals with chronic inflammation (Soheili et al., 2013). However, their they did not include a stringent gating strategy including $CD4^{+}CD25^{hi}CD127^{-}FoxP3^{hi}$ Tregs nor did the study evaluate their functional capacities.

The lamina propria is thought to be the main site of action for T cell independent stimulations at mucosal sites (Cerutti, 2008). Where B cell maturation happens both through antigen specific and non-specific recall. One

example is though TLR stimulation after or before maturation (Nadia L. Bernasconi, Elisabetta Traggiai, & Antonio Lanzavecchia, 2002). Without maturation this mechanism may induce transitional B cells to mature and respond to TLR9 stimulation, first acquiring the phenotype of IgM memory B cells and then terminally differentiating into plasma cells that produce antibacterial Abs (Federica Capolunghi et al., 2008). There may also be a maturational relationship between IgM memory B cells and IgA producing memory B cells (Magri et al., 2017). It is though unknown to witch subtype of IgM memory B cell it is related since they seem to have separate origins (Bagnara et al., 2015). IgM memory B cells may be related to splenic B maturation and may be induced through T cell independent mechanism from transitional phenotype to a plasma cell phenotype (Federica Capolunghi et al., 2008). But it may also be that the population is heterogeneous originating from different maturation pathways (F. Capolunghi et al., 2013).

In mice, T cell independent responses have mainly been thought to be due to B1 cell responses. These cells originate from peritoneal cavities and have rapid responses to secrete natural antibodies from early life. They have though not been shown to exist as such in other species such as in in rat models or humans. Research has looked extensively for a human analog, but it has been difficult to characterise. Complex and controversial gating strategies for such a population in humans has been used without revealing any differences in the analysis of CVID patients (Quach et al., 2016; Suchanek, Sadler, Bateman, Patel, & Ferry, 2012). In humans the B cells responsible for T cell independent maturation might be slightly different cells, including IgM+ memory B cell (Weller et al., 2004).

The equilibrium between the amount of each maturation pathway on each isotype is difficult to assess but studies from T cell-deficient mice show only about a 75% reduction in intestinal IgA, indicating a substantial amount of T cell independent maturation for IgA (Bemark, Boysen, & Lycke, 2012). Furthermore, CD40L defective mice have been shown to have normal IgA producing plasma cell populations in mice (Bergqvist, Gardby, Stensson, Bemark, & Lycke, 2006), whereas hyper IgM patients with absent CD40L have severely low serum levels of IgA but are found to retain intestinal IgA class switching (He et al., 2007).

For what is known about IgA maturation both these pathways may be biased towards the maturation of mucosal resident cells which may though not be true for all IgA secreting cells. For example, bone marrow plasma cells (BMPC) are largely expressing IgA (28- 56%), with only 40% originating from

mucosal surfaces based on migration marker expression. Moreover a group of IgA+ plasma blasts circulating in the periphery are present in healthy individuals, as of a fraction of 60%, not affected by vaccination induced re-emergence of plasma blasts (Henrik E. Mei et al., 2009) nor rituximab treatment (Mei et al., 2010), possibly affecting to these medical interventions.

The heterogenicity of pathways enabling IgA maturation makes it difficult to predict where a defect can be found in sIgAD. Furthermore, it has now been known for quite some time that most sIgAD individuals do have very few B cells with surface IgA expression. Thus, indicating a functional IgA class switching with dysfunctional maturation and/or an intrinsic survival defects in only IgA positive B cells (Scott, Bryant, Webster, & Farrant, 1994). Regarding these mechanisms phenotypic analysis of B cells have shown lower numbers of switched memory B cells, which inversely correlate with disease severity in sIgAD (Aghamohammadi et al., 2011; Nechvatalova et al., 2012) pointing towards a CSR defect. Furthermore, sIgAD individuals have been shown to be able to produce IgA after various stimulations: CD40L, TGF-β, IL-10, IL-4, IL-21 even though at lower rates than healthy individuals (Asano et al., 2004; Borte et al., 2009; Briere et al., 1994; Hummelshoj, Ryder, Nielsen, Nielsen, & Poulsen, 2006; Marconi et al., 1998; Zielen, Bauscher, Hofmann, & Meuer, 1993), possibly pointing towards some remaining normal T cell dependent reactivity. Many of these pathways use similar intracellular signalling pathways in different ways but do though demonstrate heterogeneous responsivity (Borte et al., 2009). However, such IgA induction has not been shown to result in a long-lived Ab response. In addition, T cell independent pathways in sIgAD in adults are not known.

A defect in T cell help could be imagined base on the known importance of T cell dependent responses. In sIgAD individuals the role of T cells has been controversial. In the early days of its description some sIgAD individuals seemed to be unable to induce normal IgA production in healthy B cells while others were shown to point towards a certain diversity in the function of T cells (De la Concha et al., 1982). Recent investigations seem though to point towards a B cell defect. Possibly, corresponding with changes seen in the classification of CVID, where individuals with a T cell defect are increasingly being classified as CID with different phenotypes and prognosis (Malphettes et al., 2009; MD et al., 2015). Current data on T cell subpopulations in sIgAD is though until now somewhat limited (N. Wang & Hammarstrom, 2012). Some groups have shown specific defects in sIgAD T cells (Aghamohammadi et al., 2011; Litzman, Vlková, Pikulová, Štikarovská, & Lokaj, 2007; Rakhmanov et al., 2009). Three studies have demonstrated that IgAD individuals have

reduced numbers of total T cells (Nechvatalova, Pavlik, Litzman, & Vlkova, 2017; Nechvatalova et al., 2012). In addition, a recent study with individuals with sIgAD with and without autoimmunity showed slight variations in Th17 and Th22 polarisation (Fadlallah et al., 2018). Similarly, autoimmunity in sIgAD has been linked to lower frequency Tregs (Soheili et al., 2013).

1.4.2 Terminally differentiated antibody secreting B cells– Where they reside and how they survive

After having gone through peripheral maturation steps, B cells can further differentiate into two major types of memory cells, namely memory B cells or plasma cells.

Memory B cells are fully matured cells that do not have Ab secreting functions in their resting stage. They can though get re-stimulated depending on the Ag encountered or the immediate immune microenvironment, to then secrete their Abs. It is believed they account for about 1/3 -1/2 of the peripheral blood residing B cells (Mei et al., 2007).

Plasma cells are the B cells which provide humoral immunity through their secretion of Igs. At the intermediate stage between memory and plasma cells there are plasma blasts. They have acquired some secretory functions but can migrate from their initial antigen encounter site to their final peripheral site of residency. The mechanisms behind longevity of immunological memory related to microbial responsiveness as well as other clinical states such as autoimmunity and atopic diseases are still under intense research.

Three main theories about the live of plasma cells exist. The first theory is that plasma cell memory may arise when plasma cells develop from re-stimulated memory B cells from an Ag specific rection. That is, they encounter an antigen and mature to become plasma cells producing Abs and disappear after clearance of the specific antigen. This theory cannot though explain the residing titres for some vaccines for decades but may explain the surge of specific Abs with reinfections of infectious agents (Rolf M. Zinkernagel et al., 1996). The second theory claims that memory might arise from memory B cells re-stimulated without Ags. That is with cytokines and/or TLRs in a non-specific manner and develop into plasma cells. The danger of this model is the potential development of auto-Abs. The positive part would be a polyclonal bystander activation of memory B cells to maintain serum Ab levels high (N. L. Bernasconi, E. Traggiai, & A. Lanzavecchia, 2002). The third theory is that memory might arise from the survival of plasma cells driven from T cell dependent or independent induction in survival niches in the bone marrow.

(Manz, Thiel, & Radbruch, 1997). Experimental data exists though pointing towards the possible co-existence of all theories depending on responses.

Related to the IgA-secreting plasma blasts the ones that are generated in mucosal immune responses have been shown to express CCR9 and CCR10 in addition to CXCR4, which would allow them to home to the mucosa rather than to the bone marrow (Kunkel & Butcher, 2003). But not all IgA plasma cells in the bone marrow express homing markers indicating mucosal origins (H. E. Mei et al., 2009). There is evidence that bone-marrow plasma cells are more stringently affinity selected than memory B cells are, indicating that germinal centres have a crucial role in the process of bone marrow plasma cell generation (Radbruch et al., 2006). What is the specificity of IgA at mucosal surfaces and do the plasma cells there need to be long-lived? The functional role of mucosal IgA is different from other isotypes and it may therefore not be relevant for them. In the intestine the T cell independent pathway has been shown to be sufficient to coat intestinal microbes in a specific way (Bunker et al., 2015). Probably due the uptake of microbes into Peyer´s patches by IgA driving induction where B cells get re-stimulated (Fransen et al., 2015).

B cell activating factor (BAFF), APRIL and their receptor TACI may be important for plasma cell survival (Mackay & Schneider, 2009). These factors have been used as new therapeutic targets in autoimmune diseases such as Lupus and Sjögrens disease (Mariette et al., 2013; Vincent, Morand, Schneider, & Mackay, 2014). The quantification of BAFF and APRIL levels in serum of sIgAD individuals could give valuable information due to its importance as survival factor for B cells (Mackay & Schneider, 2009). In addition, families with TNFRSF13B mutations have been shown to have sIgAD and CVID phenotypes (Aghamohammadi, Mohammadi, et al., 2008) and the link with the ancestral HLA haplotype 8.1 has been hypothesised to be due to its proximity to TNFRSF13B (N. Wang et al., 2011). This connection with survival and B cells could hypothetically indicate a role for BAFF treatment as a potential therapeutic target for sIgAD individuals suffering of autoimmune diseases. Even though other cytokines linked to B cell maturation may not be forgotten. IL-21 for example has been of special interest in sIgAD and CVID due to its role in inducing normal productions of Ig´s (Borte et al., 2009). So far, the affect of IL-21 has only been assessed in isolated B cells. The systemic effect of IL-21 is not known but it has an affect on various cell types of the immune system (Zotos et al., 2010). But the link between specific immune biomarkers and sIgAD may not exclude the analysis of other B cell related factors.

1.4.3 Cell signalling B cell responses and IgA production

Through development and maturation, a B cell needs multiple external signals mediated by signal transduction. When a cytokine binds to a receptor it induces phosphorylation of key cellular proteins which relocate to the nucleus and effect gene expression. Their receptors are made up of a specific subunits, of witch multiple cytokines may use similar subunits (Jatiani, Baker, Silverman, & Reddy, 2010). The T and B cell receptors (TCRs and BCRs) have highly specific recognition capacities but need other proteins they assemble with to make up their functional receptor complex. These proteins are the ones that do lead to signal transduction when the receptors bind to an Ag (Murphy, 2014).

Downstream of receptors, multiple pathways may co-exist and be shared by different stimulants. Protein kinases are enzymes that catalyse the bonding of a phosphate group on serine, threonine or tyrosine of certain proteins inside cells. This phosphorylation can modulate their activity, interaction with other molecules and proteins, and their location within the cell (Johnson & Lapadat, 2002).

The JAK-STAT pathway, Janus kinase and signal transducer and activator of transcription, is an extensively used signalling pathway widely responsible for cytokine signalling transduction. JAKs get activated, phosphorylate STAT monomer proteins in the cytoplasm, dimerising and translocating to the nucleus and binding to DNA. STATs then act as transcription factors and effectors of transcription for some genes. Over 50 cytokines and growth factors use the four JAKs (Jak1, Jak2, Jak3, TYK2) and seven STAT (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6) isoforms. These affect one or more protein at different degrees, possibly inducing varying degrees of intensity and duration of response (Villarino, Kanno, Ferdinand, & O'Shea, 2015). Defects in these pathways have serious consequences and are being increasingly described in the different pathways with various genotype/phenotype correlations (Villarino et al., 2014).

A few drugs are already on the market targeting these pathways. Such as ruxolitinib, tofacitinib, and oclacitinib used in the treatments of autoimmune presentations (Villarino et al., 2015) or specific defects of these specific components (Eren Akarcan et al., 2017; Meesilpavikkai et al., 2018).

JAK STAT signalling pathways are important in the activation of different cytokines involved in the induction of IgA production. A lot of phenotypic defects have been shown in sIgAD with defects in responsiveness to multiple

cytokines using similar signalling pathways. Similarities are also found between the B cell phenotypes present in sIgAD individuals (Nechvatalova et al., 2012) and children with STAT3-deficiency and other defects with signalling affection (Berglund et al., 2013).

1.5 Summary of introduction

Having revised some of the main aspects of the current knowledge related to sIgAD. Particularly in relation to a healthy immune response and the key questions unanswered in the defect, I will present the aims of this research.

However, the beauty of science may be obscured by its complexity. Thus, as Eva Lantsoght has described in a concrete manner: "Imagine you are at a family event, and your family members would like to know what your research is about. You may find it hard to answer this question directly and in an understandable way" (Lantsoght, 2018). For that sake I would like to sum up the introduction in the simplest way to prepare readers for the chapters describing the results:

1. Selective IgA deficiency is a common defect in the production one of the most broadly produced factors of the immune system.
2. Affected individuals have problems related to immune regulation, namely: autoimmunity, atopy and infections.
3. Today the exact cellular mechanisms responsible or underlying genes are not known but they are related to B cells.
4. It is important to understand immune defects since they can help us understanding immune responses. Potentially leading to the discovery of better means to control them in individuals suffering from immune related diseases.

2 Aims

2.1 General Aims

The main aim of this study was to evaluate the cellular phenotype and transcriptomic abnormalities leading to sIgAD based on the previous literature and to define better the cellular mechanisms responsible as well as the clinical phenotype of affected individuals.

2.2 Specific aims

The specific aims of this study may be divided in two:

1. Evaluate the clinical and serologic phenotype of sIgAD
2. Evaluate cellular phenotype and functions of lymphocytes in sIgAD
    a. B cell fractions, numbers and functions. With a special emphasis on T cell independent responses, cytokine production, migration markers and longevity.
    b. T cell fractions, numbers and functions. With a special emphasis on T helper subsets and T regulatory cell functions.
    c. Signal transduction in T and B cells in sIgAD. With a special emphasis on known altered stimuli in B and T cells in sIgAD.
    d. Transcriptomic signatures of B cells in sIgAD. With a special emphasis on T cell independent responses.

3 Materials and methods

3.1 Recruitment of participants

3.1.1 Icelandic sIgAD group

A total of 136 individuals have been found to have sIgAD in Iceland. At the time of the study, 100 individuals were alive and 31 were under 18 years of age. Of the 69 individuals alive and over 17 years of age, 61 individuals (88%) accepted to participate.

3.1.1.1 Study population

61 participated in the clinical evaluation, 24 where harvested for the evaluation of serological markers and autoantibodies, 15 where evaluated for cellular analysis, 10 for transcriptomic analysis and 10 for the intracellular signalisation analysis.

3.2 Analysis of immunoglobulins and autoantibodies in IgAD individuals

3.2.1 IgM, IgA and IgG measurement by rate nephelometry

For measurements of serum IgA, IgG and IgM the IMMAGE® 800 Immunochemistry System (Beckman Coulter) was used. The machine uses rate nephelometry to determine the concentrations of proteins. An Ag-Ab reaction forms complexes, and as a result of this light scatters from particles suspended in solution and the rate of increase in light is measured. The assays were performed according to the instructions of the manufacturer.

3.2.2 IgG subclasses by Radial immunodiffusion (RID)

Measurements of IgG-subclasses were carried out using radial immunodiffusion technique. Agar films were prepared using TRIS-buffer, PEG 6000 (Sigma), agar and PeliClass anti-human IgG subclasses 1-4 (Sanquin Reagents). One agar film contained one of the subclasses. Holes were made in the film and standards and samples diluted in TRIS-buffer were added to the holes. The samples were all from adults and therefore all samples were diluted 1:20, and samples that had been high in total IgG measurements were diluted 1:30. Samples and standards floated out in the gel for 24-72 hours or until the least diluted standard reached 70 mm. Afterwards, the agar films were put in

salt water bath for 24 hours, the films dried, pressed and coloured in coomassie blue. The diameter of the circles was read manually with an electronic RID plate reader and the concentration was calculated by the program Titer (locally designed). The procedures are according to local protocol of the Department of Immunology, Landspítali University hospital, Iceland.

3.2.3 Anti-CCP by ELISA

For the determination of IgG antibodies to cyclic citrullinated peptides (CCP) in human sera the immunoscan CCplus® test kit (Euro Diagnostica AB) was used. The test kit is an enzyme-linked immunosorbent assay (ELISA). The assays were performed according to the instructions from the manufacturer.

3.2.4 Rheumatoid factor by ELISA

The screening for rheumatoid factor (RF) was performed using ELISA technique. The preparation of wells was made with rabbit IgG diluted in coating buffer. The plates stood in 4°C over night and were then washed by microplate washer (Thermo Fisher Scientific) 2 times with PBS-tween. The plates were then dried and stored in the fridge with plastic film on top until use. Samples were diluted 1/40 in serum dilutions buffer (SDB). The plate was washed once before running the test. Standard, blank and control were run in every plate (negative, low, high). Dilution (standards, control, and samples) were put in every well in duplicates and stood for 2 hours at room temperature (RT). The plates were then washed 3 times and dried after last wash. Diluted monoclonal anti-human kappa light chain (Sigma) were added to all wells and the same procedure as after adding the dilutions was repeated. Diluted polyclonal rabbit anti-mouse immunoglobulins/AP (Dako) was then added to the wells and the same procedures as described earlier, after adding the dilutions, was repeated. Lastly, substrate solution was added to every well and the plate stood at RT until the highest standard had reached around 1500 nm. The plate was read with microplate photometer multiskan EX (Thermo Fisher Scientific, Massachussetts, USA) with wave length 405 nm. For calculating the results, the average of the blanks was subtracted from the average of the reading and the standard curve evaluated.

For the isotyping of RF, samples were heated to 56°C to inactivate other factors. A similar procedure as for the RF-screen was then performed. Monoclonal anti-human kappa light chain (bound and free) Ab produced in mouse (Sigma), Monoclonal anti-human IgM-alkaline phosphatase Ab produced in mouse (Sigma) and AKP mouse anti-human IgA1/IgA2 (BD Pharmingen™, BD Biosciences) were used.

3.2.5 Antinuclear Abs by indirect immunofluorescence using rat tissue

Antinuclear antibodies (ANA) by indirect immunofluorescence (IIF) using rat tissue is a screening for non-organ-specific Abs. For this study, preparations were made with male rat tissue from Keldur (Icelandic Institute for Experimental Pathology). Liver, kidney and stomach tissue with esophagus were cut in small cubes and frozen in liquid nitrogen and ISO-pentan, and then kept at –70°C.

The blocks were casted in OCT (cryo-embedding media) and cut in 3-6 μm thick slices and put on glass. After cutting, the samples dried in cold air for 1.5-2 hours. The glasses were put in storage box with blue gel and kept in minus 20°C. Glasses dried in cold wind for 15 minutes before usage. Sera was diluted 1/40 and put on slide and put in a moisture box for 30 minutes. After drained off, the slide stood in PBS 30 minutes with a switch of PBS in the middle. Diluted polyclonal rabbit anti-human IgA, IgG, IgM, kappa, lambda/FITC (Dako) was put on every slide, then the slides were stored for 30 minutes in moisture box. The FITC anti-serum is then drained off and the same PBS rinsing as earlier was performed. After drying empty area on glass with paper, one drop of diluted glycerol buffer with DABCO was added to every slide. Cover glass was sealed with nail polish.

The ANA was read with optical microscope DM 2500 LED (Leica Microsystems). Positive samples were recognised as diffuse, speckled, membrane or nucleolar pattern.

3.2.6 Antinuclear Abs by fluorescence enzyme immunoassay

Antinuclear IgG Abs in sera were detected by fluorescence enzyme immunoassay on the Phadia® 250 system using EliA™ CTD screen (Thermo Fisher Scientific, Massachussetts, USA) The wells are coated with U1RNP (RNP70, A, C), SS-A/Ro52, SS-A/RO60, SS-B/La, Centromere B, Scl-70, Jo-1, fibrillarin, RNA Pol III, Rib-P, PM-Scl, PCNA, Mi-2 proteins, Sm proteins and native purified DNA.

In addition to this screening assay, Phadia EliA™ assays were used to measure Abs to individual Ags. Abs to individual Ags were determined in all individuals that tested positive with the EliA CTD screen assay. The assays were performed according to the instructions of the manufacturer.

3.2.7 Immune factors by luminex

Serum samples and supernatants from CpG (TLR9 agonist, ODN 2009, Invivogen, 1 mg/ml) stimulated CD19 iusolated B cells (isolated and stimulated

as described in chapter 3.3.4) where analysed with luminex. Chemokines and cytokines were measured using a magnetic immune monitoring 65-Plex human procartaPlex™ luminex assay (R&D systems) and analysed in Bio-Plex 200 system (Bio-Rad Laboratories).

3.3 Cellular analysis by FACS and ELISA

3.3.1 Isolation of PBMCs, B and T cells.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood using Ficoll–Paque gradient centrifugation (Sigma-Aldrich, St. Louis, MO, USA). CD19+ B cells were isolated by magnetic bead-based positive selection using dynabeads and DETACHaBEAD CD19 (Invitrogen). CD4+ T cells were isolated with dynabeads CD4 (Invitrogen) and CD25+ T cells where depleted from the CD4+ T cell population using dynabeads CD25 (Invitrogen). All isolations where done according to the manufacturer's instructions. The purity of isolated CD19+ B cells was consistently >95% as analysed by flow cytometry. For intracellular phosphorylation analysis: after isolation the cells were suspended in X-vivo 20 serum free medium (Lonza; Walkersville, Maryland, USA). Cells frozen in X-vivo were supplemented with ProFreeze chemically defined freeze medium (Lonza; Verviers, Belgium). Cell suspensions were mixed with DMSO (Sigma Aldrich) to prevent damage and then cryopreserved in liquid nitrogen.

3.3.2 Flow cytometric analysis.

For extracellular staining single-cell suspensions were prepared and surface molecules were stained for 20 min at 4°C with a stain for viable cells using LIVE/DEAD fixable yellow stain kit (Invitrogen) and optimal dilutions of Abs for the following anti-human mAb: CD3 PE-Cy7 (UCHT1), CD3 PerCP-Cy5.5 (OKT3), CD4 eFluor 450 (RPA-T4), CD8a APC-eFluor 780 (RPA-T8), CD19 PerCP-Cy5.5 (HIB19), CD20 APC-eFluor 780 (2H7), CD24 PE-Cy7 (eBioSN3), CD25 PE (BC96), CD27 eFluor 450 (eBioSN3), CD38 PE-eFluor 610 (HIT2), CD45RO PE (UCHL1), CD45RA (JS-83), CD127 FITC (eBioRDR5), CD194(CCR4), PerCP-eFluor 710 (D8SEE), CD278(ICOS) APC (ISA-3), CD279 PD-1 (EH12.2H7), IgM FITC (SA-DA4), IgG PE (JDC-10), HLA-DR eFluor 450 (L243), CCR10 PE (6588-5), CCR7 (3D12), β7 (FIB504), CCR4 (D8SEE), CD185(CXCR5) APC (MU5UBEE) all from affymetrix eBioscience; CD3 Brilliant Violet 510 (OKT3), CD14 Brilliant Violet 510 (M5E2), CD62L FITC (DREG-56), IgD Alexa Fluor 700 (IA6-2), CXCR4 (12G5) all from Biolegend and IgA APC (IS11-8E10) from Miltenyi-Biotec.

For intracellular staining single-cell suspensions were prepared and surface molecules were stained as described above. They were subsequently fixed with paraformaldehyde and permeabilised with either 0.5% saponine or FoxP3/transcription factor staining set (eBio-sciences), stained according to the manufacturer's instructions for the following anti-human mAb: FoxP3 APC (236A/E7), T-bet PerCP-Cy5.5 (eBio4B10), BCL-6 PerCP-eFluor 710 (BCL-UP), GATA3 eFluor 660 (TWAJ), TNF alpha PE (MAb11), IL-4 PE (8D4-8), IL-6 Alexa Fluor 700 (MQ2-13A5), IL-10 Alexa Fluor 488 (JES3-9D7), IL-17 PE (eBio64CAP17), IL-22 PE-Cy7 (22URTI) all from affymetrix eBioscience, INFγ APC (4S.B3) from Biolegend. To assess proliferation fluorescent 5,6-carboxyfluorescein succinimidyl ester (CFSE, Life Technologies) labelling was used according to manufacturer's instructions. Stained cells were subsequently analysed using 10 colors Beckman Coulter Navios flow cytometer.

3.3.3 Flow cytometric analysis software

Proliferation calculations were done using Flow Jo Tree star V7.6.5. Expression of cell surface markers was measured by flow cytometry and analysed using Flow Jo Tree star VX.0.7. Basis of gates was determined with the use of fluorescence minus one (FMO) together with appropriate isotype controls. Results are presented as average with standard error of the mean (SEM).

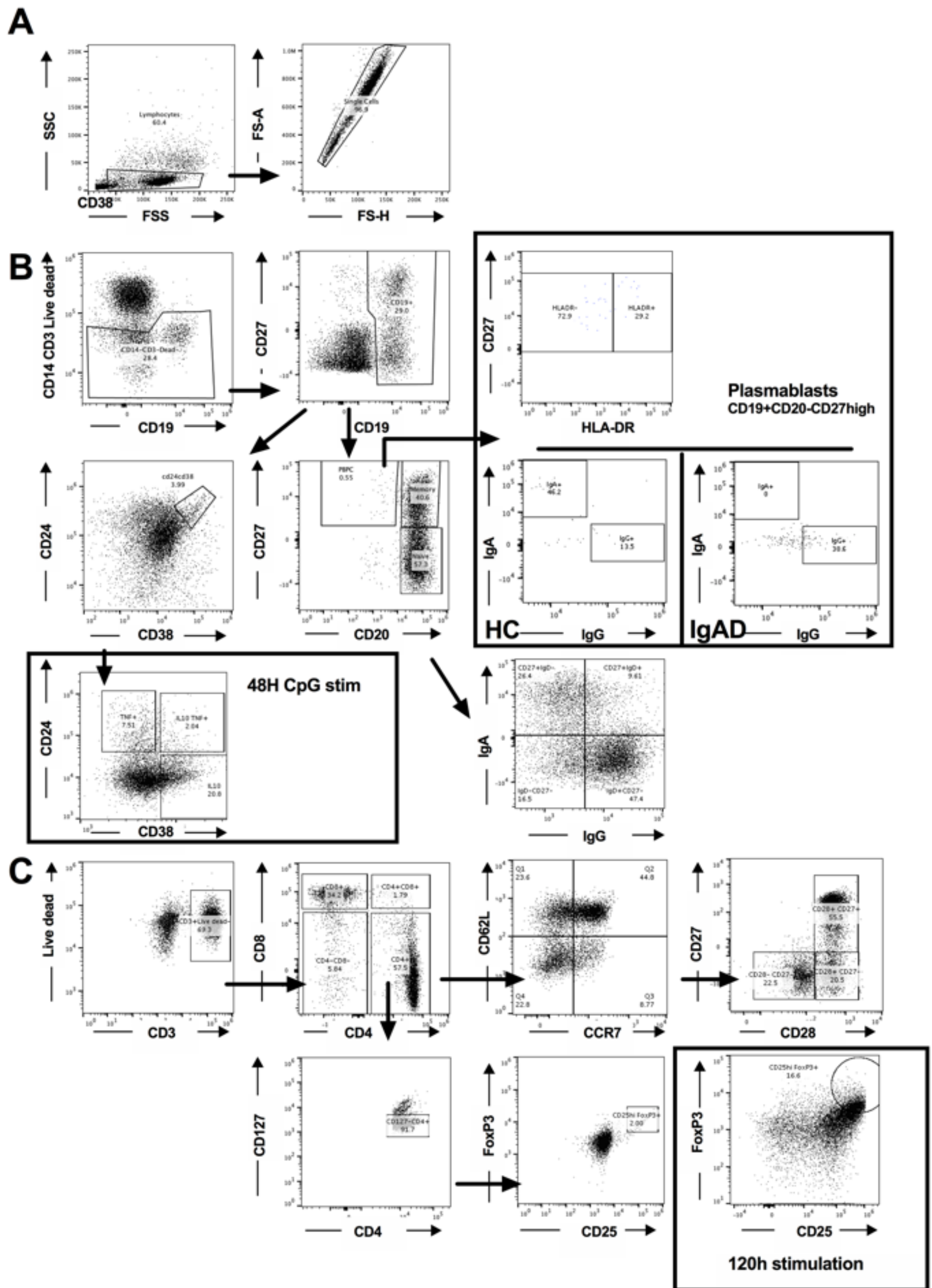
A
SSC
FSS
CD38
Lymphocytes
60.4
FS-A
FS-H
B
CD14 CD3 Live dead
CD19
CD14-CD3-Dead-
28.4
CD27
CD19
CD19+
29.0
CD27
HLA-DR
HLADR-
72.9
HLADR+
29.2
Plasmablasts
CD19+CD20-CD27high
CD24
CD38
cd24cd38
3.99
CD27
CD20
PBPC
0.55
IgA
IgG
IgA+
46.2
IgG+
13.5
HC
IgA
IgG
IgAD
IgG+
38.6
48H CpG stim
CD24
CD38
TNF+
7.51
IL10 TNF+
2.04
IgA
IgG
IgD-CD27-
16.5
IgD+CD27-
47.4
C
Live dead
CD3
CD8
CD4
CD4+CD8+
1.79
CD4-CD8-
5.84
CD62L
CCR7
Q1
23.6
Q2
44.8
Q4
22.8
Q3
8.77
CD27
CD28
CD28+ CD27+
55.5
CD28- CD27-
22.5
CD28+ CD27-
20.5
CD127
CD4
CD127-CD4+
91.7
FoxP3
CD25
CD25hi FoxP3+
2.00
FoxP3
CD25
CD25hi FoxP3+
16.6
120h stimulation

Figure 3. Gating strategy for some of the main populations assessed.

Gating strategy described in the methods for T and B cells. (A) Cells were first identified based on forward scatter (FSC) and side scatter (SSC) and doublets excluded by forward area (FA) and forward height properties (FH) (B) B cells where identified based on their expression of CD19 while excluding CD14+ and CD3+ positive cells and dead cells. Then a gating was done based on CD19 and CD27 to get CD19 low CD27hi B cells. B cells where further analysed based on their different expression of CD20 and CD27 for naïve, memory and plasmablasts/plasma cells; CD24 and CD38 for transitional B cells, IgD and CD27 for class switched memory B cells. A gating is shown for HLADR expression on plasmablasts/plasma cells and for the expression of IgA and IgG in both healthy controls (HC) and IgAD individuals on plasmablasts/plasma cells. (C) T cells where identified based on their expression of CD3 and furthermore evaluated for their expression of CD4 and CD8. The subsets where subsequently analysed for their expression of CD28 vs CD27 and CCD62L vs CCR7. nTregs and iTregs cells where assessed based on their expression of CD4, CD127 negativity, CD25high and FoxP3high expression. As can be seen the expression is greatly enhanced by stimulation for 5 days with IL-2 and TGF-β.

3.3.4 B cells culture conditions

Isolated CD19+ B cells were cultured in iscove's modified dulbecco's medium (IMDM) supplemented with 10% heat-inactivated foetal calf serum (FCS), penicillin/streptomycin (100 U/mL), and 2 mM glutamax in 96-well round bottom plates at a density of 50×10^4 cells/well. T cell independent mimicking stimulations were performed in 96 well round bottom plates in the presence of CpG-ODN2006 (1ug/mL TLR9 agonist, InvivoGen). Assessment of the induced transitional CD24highCD38high phenotype was done on B cells harvested after 48 h CpG stimulation. They were further re-stimulated with brefeldin A for 6 hours and PMA (phorbol 12-myristate 13-acetate) and ionomycin for 4 hours for the assessment of cytokine producing B cells by intracellular flow cytometry or cultured futher for the assessment of surface expression of Ab isotypes and the production of immunoglobulins for 7 days before harvesting. Supernatant were harvested and cells stained for flow cytometric analysis.

3.3.5 A model for the assessment of the spontaneous production of IgA by B cells

To assess spontaneous IgA production, PBMC were cultured at a density of 2×10^5 cells/well in IMDM supplemented with 10% heat-inactivated FCS, penicillin/streptomycin (100 U/mL), and 2 mM Glutamax with and without the

exogenous addition of IL-10.

3.3.6 T cell culture conditions

T helper cell subsets were assessed by intracellular flow cytometry on PBMCs stimulated in 96 well round bottom plates with PMA and ionomycin for 4 h. T regulatory cell induction was done by culturing CD4+CD25- T cells in serum free medium (AimV, Invitrogen) in 96 well round bottomed cell culture plates with plate bound anti-CD3ε (UCHT) with soluble anti-CD28 for 120 hrs with IL-2 (100 IU) and TGF-β1 (10 ng/mL) (Abs and cytokines used are from R&D Systems, Abingdon, UK) and then harvested to measure their maturation as induced T regulatory cells (iTregs) by flow cytometry. Their suppressive capacity was assessed after harvest by co-culturing them with CFSE labeled allogeneic PBMCs and superantigen pulsated Epstein–Barr transformed B cells (EB-B cells) in AimV. Staphilococcal enterotoxins (SEA, SEB and SEE, Toxin Technologies, 1 µg/mL of each) were used for for 2 h pulses before washing 3 times with PBS. The PBMCs: EB-B cells and iTregs: EB-B cells ratio was constant at 10:1 while the iTregs: PBMCs ratio varied from 1:1 to 1:32.

3.3.7 Measurement of IgG and cytokines by ELISA

IgG, IgA and IgM concentrations from cultures were quantified in culture supernatants by standard ELISA (Bethyl Laboratories).

3.3.8 Phospho protein staining

A phosphoflow stimulation and staining protocol was established and optimised which was based on methods previously published (Krutzik, Clutter, Trejo, & Nolan, 2011; Montag & Lotze, 2006). Briefly, cryotubes were taken out of liquid nitrogen storage and thawed quickly in a 37°C water bath. They were then gently pipetted using Pasteur pipettes into 50 mL conical tube containing 25 mL of IMDM medium. Cells were then centrifuged at RT at 300 g for 10 minutes and the supernatant discarded. Washing was repeated with 25 mL of IMDM medium and the cell pellet was re-suspended in medium and counted. $5x10^5$ cells per well were aliquoted into sterile 96 well plates (#163320 Thermo fisher, Waltham, Massachusetts, USA). The cells were then incubated for one hour at 37°C in a 5% CO_2 incubator to let the cells reach a basal phosphorylation. Cells were stimulated with various concentrations and combinations of cytokines for 15 minutes at 37°C and then fixed in 1.8% formaldehyde for 10 minutes at RT. Cells were then pelleted and centrifuged at 1600 RPM at 4°C for 10 minutes. Supernatants were then discarded, and cells washed with 200 µL of staining buffer. Cells were then permeabilised with

150 µL of ice-cold 95% methanol. This was followed by washes, first with 80 µL of staining buffer and then repeated with 200 µL of staining buffer. Cell pellet was re-suspended in the residual volume and 50 µL of that was transferred to fresh 96-well plate. Staining with fluor conjugated Abs was carried out in the dark for 30 minutes at RT. One more wash was performed with 100 µL of staining buffer and then the samples were put on ice until analysis with Beckman Coulter Navios flow cytometer. For each analysis a minimum of 50.000 events were collected into a defined lymphocyte gate. Abs used were Alexa flour 488 anti-human ERK1/2 (T202/Y204) (Ebioscience), PE anti-human pSTAT1(Y701) (Ebioscience), APC anti-human pSTAT3 (Y705) (Ebioscience), PE-Cyanine 7 anti-human pSTAT5 (Y694) (Ebioscience), Pacific Blue anti-human p38 (T180/Y182) BD biosciences), PerCp Cy 5.5 anti human CD20 clone H1 cytoplasmic tail (#55802 1 BD biosciences), Brilliant violet 510 anti-human CD3 (clone UCHT1 Biolegend). Stimulations used were IL-2 (50ng/ml), IL-4 (100ng/ml), IL-10 (100ng/ml), IL-21(50 ng/ml) (all from R&D systems) and CpG-ODN2006 (1 µg/mL TLR9 agonist, InvivoGen).

3.4 Transcriptomic analysis.

3.4.1.1 RNA sampling and processing

Total RNA was isolated from 40 samples using RNeasy micro kit (Qiagen Inc. (cat. no. 74004)) according to the manufacturer’s instructions (including a DNAse treatment). 10 samples where unstimulated B cells from HC, 10 samples unstimulated B cells from IgAD individuals, 10 sample CpG stimulated B cells from HC and 10 sample CpG stimulated B cells from IgAD individuals. RNA was eluded with 14 µL RNAse free water and stored at -80°C. We quantified and QC-ed the RNA samples by qPCR using Exilerate LNA^{TM} qPCR cDNA synthesis kit from Exiqon AS (Cat no.303301) and an endogenous control qPCR assay for the housekeeping gene GAPDH (Cat No 308005) and ACTB (β-actin) (Cat.no.308001). The amplification was performed in a LightCycler® 480 real-time PCR System (Roche) in 384 well plates. The amplification curves were analysed using the Roche LC software, for the determination of Cq (by the 2nd derivative method). We used 2 µL of the total RNA in the cDNA synthesis and diluted the cDNA 10-fold in water before performing qPCR in duplicate per sample.

3.4.1.2 Library preparation and next generation sequencing

The double stranded cDNA synthesis was performed using the SMARTer® UltraTM Low Input RNA Kit for Sequencing (Cat. No. 634891, Clontech

Laboratories, version 4) as by the manufacturer using 12 cycles of pre-PCR cDNA amplification.

The cDNA was purified using AmpureXP beads using 1.8 x bead volume and washed twice in 80% EtOH. cDNA was re-suspended in 17 µL water (Beckman Coulter). The cDNA size distribution was validated, and quality inspected on a bioanalyzer high sensitivity DNA chip (Agilent Technologies). The size distribution analysis showed a smear of about 2000 – 9000 bp in size (peaked at approx. 2500-3000 bp). Samples were quantified by calculating the concentration on the bioanalyser/tapestation (size range 500 - 8000 bp.) using the smear analysis feature.

3.4.1.3 NGS library generation and sequencing

The libraries made using the Nextera^{XT} transposon-based library generation kit (Illumina Inc.) using 250 pg input material from the cDNA amplification, according to the manufacturer's instructions. The DNA was post-PCRed for 12 cycles and purified using Ampure^{XP} beads as described above and eluated in 30 µL water.

Finally, the libraries size distribution was validated, and quality inspected on a bioanalyser high sensitivity DNA chip (Agilent Technologies). The library peak is broad in the size range between 200-500 bp (and peaked at approx. 350 bp). The nextera libraries were quantified by calculating the concentration on the bioanalyser (size range 150 - 1000 bp) using the smear analysis feature and pooled accordingly.

The library pool was quantified with qPCR (KAPA qPCR library quantification kit) and optimal concentration of the library pool used to generate the clusters on the surface of NextSeq500 flowcells (4 flowcells/4 nextseq runs) before sequencing using v2 chemistry according to the manufacturer instructions (Illumina Inc.) using paired end 2x51 specifications.

3.4.1.4 Pathway analysis

To study how stimulation with CpG affects B cells and wich pathways are differentially expressed between sIgAD individuals and HCs, ingenuity pathway analysis (IPA, Rewood City, California, USA; http://www.ingenuity.com/), which is a knowledge-based software package, was used.

3.5 Statistical analysis.

Statistical analyses and graphs were made using GraphPad Prism (V 8.0.1

GraphPadSoftware Inc, for Windows, La Jolla, CA, USA) or R version 3.5.1 with RStudio desktop version 1.1.463. For cellular testing ANOVA was used for multiple comparisons, Mann-Whitney or Student's t-test when the data was normally distributed for smaller groups. Results are expressed as mean ± SEM (standard error of the mean). Probability values <0.05 were considered significant. The chi-squared or the Fisher's exact tests were applied for categorical variables. Correlations between continuous variables were assessed with Pearson's or Spearman's correlation coefficients depending on normality of the data and a Bonferroni correction was classically used for multiple measurments.

4 Results

4.1 Clinical phenotyping of the Icelandic sIgAD group

4.1.1 The prevalence of infections in the Icelandic sIgAD group

Of the 136 individuals found to have sIgAD in Iceland, 100 where alive at the time of enrolment and 31 where under 18 years of age. Of the 69 individuals alive and over 17 years of age, 61 individuals (88%) participated in the study and answered our previously described online questionnaire and where compared to a previously described control group (G. H. Jorgensen et al., 2013). As shown in Table 1, sIgAD individuals had higher prevalence of serious respiratory tract infections with a significant difference seen for sinusitis (90% sIgAD v.s. 36% HCs with symptoms, $p = 0,00015$) and pneumonia (33% sIgAD v.s. 15% HC without symptoms, $p < 0,0001$).

Table 1. Infectious profiling in sIgAD individuals.

Comparison of self-reported data regarding infections in 61 individuals with sIgAD and 73 matched controls. Unpaired t tests were performed with significant p values marked as * = $p < 0.05$, ** = $p < 0.01$ *** = $p < 0.001$ and non-significant (n.s.). Values were normalised before unpaired t test was performed.

	HCs without symptoms		**SIgAD without symptoms**			
Upper respiratory tract infections	n	%	n	%	p	
Infectious conjunctivitis	52	71%	42	69%	0,7002	n.s.
Common viral cold (1 year)	10	14%	13	21%	0,3582	n.s.
Pharyngitis	17	23%	42	69%	0,4338	n.s.
Laryngitis	57	78%	42	69%	0,1596	n.s.
Sinusitis	47	64%	6	10%	0,0015	**
Acute otitis media	47	84%	55	90%	0,0792	n.s.
Lower respiratory tract infections	n	%	n	%	p	
Bronchitis SIgAD controls	50	68%	36	59%	0,2014	n.s.
Pneumonia	62	85%	41	67%	<0,0001	****
Gastrointestinal infections	n	%	n	%	p	
Campylobacter	62	85%	55	90%	0,7845	n.s.
Salmonella	62	85%	58	90%	0,1416	n.s.

Infectious gastroenteritis nos	60	82%	53	87%	0,8057	n.s.
Protozoal- or worm infections	63	86%	47	77%	0,1006	n.s.
Urogenital infections	n	%	n	%	p	
Cystitis	59	81%	42	69%	0,0652	n.s.
Pyelonephritis	61	84%	54	89%	0,796	n.s.
Skin infections	n	%	n	%	p	
Bacterial skin infections	51	70%	47	77%	0,7002	n.s.

4.1.2 Atopic and asthma manifestations in sIgAD.

After infections atopic and autoimmune presentation are the most likely to affect sIgAD individuals and are therefore important to evaluate. In our group when evaluating the prevalence of atopic manifestations; we see a higher prevalence of general eczema (16 sIgAD v.s. 3 HCs, p <0,0001) and allergic urticarial (5 sIgAD v.s. 1 HCs, p = 0,0015).

4.1.3 Autoimmune profile of the Icelandic sIgAD group

A higher prevalence of autoimmunity is seen in sIgAD individuals (12 IgAD v.s. 3 HCs, p = 0,0031). Both systemic and organ specific manifestations are seen in sIgAD individuals. For systemic presentations one had psoriasis, two had RA, one had sarcoidosis and one had polymyalgia rheumatica. For organ specific presentations; two had primary hypothyroidism, one had pernicious anaemia, one had pemphigus, one had PLEVA skin disease (Pityriasis lichenoides et varioliformis acuta), one had interstitial cystitis, one had multiple sclerosis (MS) and one had pemphigus.

Table 2. Atopic and autoimmunity in sIgAD.

Comparison of self-reported data regarding atopy and autoimmunity in 61 individuals with sIgAD and 73 matched controls. Mean, standard deviation (SD) in HC and sIgAD. Unpaired t tests were performed with significant p values marked as * = p < 0.05, ** = p < 0.01, and *** = p < 0.001. Values were normalised before unpaired t test was performed.

	Healthy controls with symptoms		sIgAD with symptoms			
Atopic diseases	**n**	**%**	**n**	**%**		
Asthma	8	13%	9	15%	0,4338	n.s.
Allergic rhinoconjunctivitis	12	19%	9	15%	0,1596	n.s.
Allergic urticaria	1	2%	5	8%	0,0015	**
Food allergy	1	2%	3	5%	0,0792	n.s.
Allergic anaphylaxis	0	0%	3	5%	0,2014	n.s.
General eczema	3	5%	16	26%	<0,0001	****
Contact dermatitis	0	0%	6	10%	0,7002	n.s.
Autoimmunity						
Diagniosed with an autoimmune disease	3	4%	12	16%	0,0031	**

4.1.4 Autoantibody production in the sIgAD group

Given the high prevalence of autoimmunity in sIgAD and its association with B cell dysfunction we evaluated autoimmune serologic positivity in sIgAD. Not to evaluate the effect of inflammation from associated disorders we chose to focus on individuals without overt clinical autoinflammatory disease. We were able to obtain serum from 24 sIgAD individuals and compared them to healthy donors. As shown in Table 3, sIgAD individuals without known clinical autoimmunity were more likely to be ANA positive (>1:40) than our age matched control group (ANA seropositivity: sIgAD = 26% v.s. HCs = 0%; p = 0,0021. In addition, this was also true regarding ENA positivity (ENA: sIgAD = 5% v.s. HCs = 0%, p = 0,0336). Of the ones who were ENA positive two had a positive anti dsDNA, two had a positive anti SSA/RO, one had a positive anti RNP and one had a positive anti GPC. This increase in autoantibody seropositive individuals was not seen not for RF (sIgAD = 15% v.s. HCs = 9%, p = 1) or CCP (sIgAD = 15% v.s. HCs = 6%, p = 0,2446).

Regarding the immunoglobulin production not only was IgA found to be deficient, but was IgG also found to be different. When looking at subclasses; IgG1 was significantly higher and IgG4 lower in sIgAD individuals compared to HCs (Table 4).

Table 3. Immunoglobulins and autoantibodies in sIgAD individuals.

Mean, SD in HC and IgAD. Unpaired t tests were performed with significant p values marked as * = p < 0.05, ** = p < 0.01, and *** = p < 0.001. Values were normalised before unpaired t test was performed.

	HC		sIgAD			
Age						
	Mean	SD	Mean	SD	p value	
Age	48,7	15.10	49,9	14.90	0,84	Ns
Immunoglobulins						
	Mean	SD	Mean	SD	p value	
IgM	0.93	0.44	0.93	0.43	0.850	Ns
IgA	2.22	0.78	0.05	0.03	< 0.0001	****
IgG	10.62	2.33	13.66	3.33	0.0004	***
IgG1	7.76	1.95	10.59	3.08	0.0004	***
IgG2	3.53	1.60	3.66	1.54	0.776	Ns
IgG3	0.39	0.23	0.44	0.29	0.516	Ns
IgG4	0.50	0.42	0.22	0.21	0.0008	***
Autoantibodies						
	n	%	n	%	p	
ANA	0	0%	8	28%	0,0021	**
ENA	0	0%	5	17%	0,0336	*
RF	4	11%	5	17%	1	Ns
CCP	3	9%	5	17%	0,2446	Ns
All together	7	20%	12	41%	0,0714	Ns

4.1.5 Serologic immune profiling of sIgAD individuals

Given the above clinical and autoantibody profiling we also evaluated a detailed serological immune profiling in sIgAD. During that study 65 immunological factors from majority of the potential key pathways associated with the immune-pathogenesis of sIgAD were analysed. As shown in Table 5, a significantly higher production of CCL3, sCD40L, TSLP and TWEAK (p=0.0001) was found (Bonferroni correction). In addition, IL-18 (p=0.0006), APRIL (p=0.0107), VEGF-α (p=0.0037), IL-20 (p=0.0019), and IL-22 (0.0046) was also found to be higher but did not pass the Bonnferroni correction. However, factors which have been linked to sIgAD such as BAFF and IL-10 did not seem to vary significantly in sIgAD individuals compared to HCs.

	Mean ± SEM, HC	Mean ± SEM, IgAD	Diff between means	P value	P
CCL3	45,99 ± 17,51	194,3 ± 18,17	-148,4 ± 27,43	<0,0001	*
sCD40L	230,7 ± 104,2	995,9 ± 103	-765,2 ± 162,2	<0,0001	*
TSLP	25,94 ± 5,072	71,59 ± 5,826	-45,64 ± 8,361	<0,0001	*
TWEAK	1097 ± 266,7	3547 ± 331,6	-2450 ± 467,7	<0,0001	*
IL-18	70,99 ± 11,1	196,6 ± 26,31	-125,6 ± 34,25	0,0006	*
April	1833 ± 350	4980 ± 816,1	-3147 ± 1186	0,0107	ns
CXCL13	176,3 ± 25,72	291,9 ± 35,11	-115,6 ± 49,96	0,0247	ns
G-CSF	37,99 ± 5,627	57,86 ± 6,553	-19,87 ± 9,82	0,0485	ns
IL-15	28,59 ± 6,52	52,07 ± 7,053	-23,47 ± 10,27	0,0264	ns
IL-21	247,5 ± 93,57	574,1 ± 74,19	-326,5 ± 148,3	0,0345	ns
IL-23	90,52 ± 16,83	476,6 ± 109,2	-386,1 ± 145,1	0,0105	ns
IL-27	95,49 ± 21,16	229,9 ± 38	-134,4 ± 62,87	0,0385	ns
IL-30	263,1 ± 58,5	1049 ± 235,7	-785,6 ± 302,7	0,0124	ns
IL-4	68,81 ± 12,57	193,5 ± 38,78	-124,7 ± 52,77	0,0222	ns
IL-7	1,825 ± 0,2674	4,35 ± 0,9466	-2,526 ± 1,213	0,0428	ns
IL-8	20,44 ± 4,472,	86,8 ± 21,91	-66,35 ± 30,84	0,0365	ns
MIP3-alpha	65,15 ± 12,7	166 ± 30,81	-100,9 ± 40,93	0,0172	ns
SCF	23,22 ± 3,808	37,47 ± 5,016	-14,25 ± 6,929	0,0449	ns
INF-alpha	5,626 ± 0,4103	93,79 ± 22,35	-88,16 ± 28,82	0,0035	ns
IL-20	88,47 ± 36,9,	260,8 ± 27,33	-172,3 ± 52,04	0,0019	ns
IL-22	141,2 ± 52,29	508,6 ± 80,24	-367,4 ± 123,1	0,0046	ns
LIF	22,86 ± 6,543	65,42 ± 9,993	-42,56 ± 14,75	0,0058	ns
MDC CCL22	121,9 ± 12,83	189,9 ± 14,15	-68,05 ± 20,51	0,0017	ns
VEGF-a	52,07 ± 15,8	131,1 ± 18,12	-79,01 ± 26,01	0,0037	ns
BAFF	34,63 ± 8,597	32,07 ± 3,704	2,566 ± 7,962	0,7487	ns
bNGF	107 ± 28,04	169,5 ± 29,88	-62,45 ± 45,8	0,1802	ns
CD30	274,2 ± 54,01	423,5 ± 57,89	-149,3 ± 85,12	0,0853	ns
ENA78	248,7 ± 37,93,	180,2 ± 25,22	68,49 ± 43,71	0,1233	ns
Eotaxin	80,08 ± 18,35	98,61 ± 13,05	-18,52 ± 21,98	0,4033	ns
Eotaxin2	89,79 ± 11,14	100,6 ± 17,72	-10,78 ± 23,98	0,6549	ns
Eotaxin3	16,21 ± 3,774	26,76 ± 4,411	-10,55 ± 7,885	0,1882	ns
FGF2	130,8 ± 52,54	288,3 ± 62,51	-157,5 ± 110,1	0,1605	ns
Fraktalkine	19,39 ± 10,77	24,06 ± 4,306	-4,666 ± 9,69	0,6328	ns

GM-CSF	63,48 ± 13	139,4 ± 33,14	-75,88 ± 41,73	0,0765	ns
Gro alpha	14,3 ± 2,707	30,08 ± 5,29	-15,78 ± 7,863	0,0506	ns
HGF	149,8 ± 28,13	261,1 ± 113,7	-111,3 ± 146,9	0,452	ns
IL-1-beta	17,37 ± 2,305	29,9 ± 5,478	-12,53 ± 7,223	0,0886	ns
IL-10	22,66 ± 10,35	28,02 ± 9,54	-5,368 ± 19,41	0,7868	ns
IL-12p70	12,56 ± 2,467	18,52 ± 2,048	-5,959 ± 5,327	0,2765	ns
IL-13	11,51 ± 2,61	31,89 ± 8,507	-20,38 ± 13,53	0,1429	ns
IL-16	645,6 ± 53,71	791,2 ± 55,69	-145,6 ± 81,99	0,0817	ns
IL-17A	30,29 ± 3,571	39,66 ± 4,086	-9,375 ± 8,008	0,2588	ns
IL-2	24,72 ± 2,745	36,27 ± 3,967	-11,56 ± 8,367	0,2045	ns
IL-2R	2630 ± 506,4	3726 ± 428,7	-1096 ± 672,5	0,1091	ns
IL-3	99,95 ± 31,01	310,2 ± 89,32,	-210,2 ± 121,8	0,0909	ns
IL-5	53,93 ± 12,29	113 ± 21,35	-59,12 ± 35,37	0,1022	ns
IL-6	69,5 ± 19,46	186,2 ± 40,45	-116,7 ± 61,01	0,0627	ns
IL-9	45,34 ± 9,208	119,7 ± 29	-74,38 ± 48,74	0,1343	ns
IP10	25,54 ± 2,786	44,91 ± 11,53	-19,37 ± 14,67	0,1926	ns
I-TAC	408,9 ± 182,7	567,9 ± 96,79	-159 ± 194,1	0,4176	ns
MCP1	125,6 ± 17,45	182,3 ± 20,94	-56,68 ± 29,76	0,0624	ns
MCP-2	10,03 ± 1,039	10,57 ± 1,131	-0,539 ± 1,645	0,7444	ns
MCP3	40,7 ± 5,568	56,34 ± 7,168	-15,64 ± 10,92	0,1606	ns
M-CSF	135,3 ± 21,99	257,9 ± 41,24	-122,6 ± 79,65	0,1343	ns
MIF	94,56 ± 20,41	148,5 ± 23,73	-53,89 ± 33,96	0,1186	ns
MIG	99,21 ± 37,61	178,1 ± 28,3	-78,89 ± 50,09	0,1228	ns
MIP1-beta	69,69 ± 35,75	136,4 ± 37,89	-66,75 ± 59,41	0,2675	ns
MMP1	103,2 ± 28,16	218,7 ± 47,43	-115,5 ± 64,93	0,0812	ns
SDF-1alpha	2157 ± 727,3	1977 ± 232,7	179,8 ± 584	0,7598	ns
TNF-alpha	14,68 ± 2	24,61 ± 3,785	-9,931 ± 5,487	0,0766	ns
TNF-beta	52,43 ± 20,82	292,1 ± 59,35	-239,7 ± 120,7	0,0569	ns
TNF-RII	131,4 ± 29,14	170,1 ± 21,17	-38,69 ± 35,26	0,2776	ns
TRAIL	72,98 ± 35,37	107,7 ± 13,71	-34,73 ± 30,98	0,269	ns

Table 4. Serum concentrations of 65 immune biomarkers in sIgAD

This table shows the biomarkers from sIgAD individuals with the highest difference in serum concentration compared to HCs (n=23 from sIgAD v.s. 21 HCs). The data is from the analysis of 65-Plex human ProcartaPlex™ luminex assay (R&D systems) and analysed in Bio-Plex 200 system (Bio-Rad Laboratories, California, USA). Significance

was calculated in relation to control group determined by one-way ANOVA with Bonferroni correction, with significant p values marked as * = p < 0.00076.

4.1.6 Connection between autoantibody positivity and single serum factors in sIgAD

Having seen such big alterations in serum concentrations we wondered if these could predict the autoantibody positivity seen in our group. To do so we started off looking for the differences in ANA positivity in the individuals with sIgAD with the highest production of immune factors using a cut-off of 2 SD over the median. No differences are seen in autoantibody seropositivity for individual with higher titres of the found dysregulated factors.

Table 5 Comparison of the quantity of immune dysregulated immune factors and ANA or overall autoantibody positivity

Chi square for numbers of individuals with ANA positivity based on their high or low production of APRIL, IL-18, CCL3, sCD40L, TSLP and TWEAK. Significant p values are above 0,05 with none being significantly different.

Upper 95%	ANA+	ANA-	p	Aab +	Aab -	p
APRIL	50%	58%	1.00	50%	60%	0.70
IL-18	83%	53%	0.34	60%	60%	1.00
CCL3/MIP1a	83%	63%	0.62	70%	67%	1.00
sCD40L	67%	63%	1.00	60%	67%	1.00
TSLP	83%	68%	0.64	70%	73%	1.00
Tweak	67%	68%	1.00	60%	73%	0.67

4.1.7 Principal component analysis discriminates between sIgAD and HCs

Since it has been shown that immune serologic biomarkers may correlate together in human serum, we evaluated its potential correlation in corrplots. As shown in Figure 3 a high correlation is seen between multiple factors in both IgAD and HCs. Previous publications have shown that multivariate statistical methods are needed to explore such complex associations between immune serologic biomarkers and clinically relevant endpoints (Evans, Esnault, Denlinger, & Jarjour, 2018). Therefore, we next analysed the association of these biomarkers to antinuclear autoantibody positivity.

Figure 4. PCA corrplot of immune factors from sIgAD patients and HCs
Correlation plot of serum cytokines, chemoattractants, growth factors and immunoglobulins with principal component correlation based variable ordering in sIgAD and HCs. Visualisation of the correlations between each pair of variables. Variables are grouped based on correlation of first factor of principal component analysis so that similar variables are placed adjacently using correlation-based variable ordering. Stronger correlations are represented by darker colours and larger circles. Blue indicates positive correlation and red indicates negative correlation.

As seen in Figure 4 a PCA (Principal component analysis) predicted up to 90% of variance with the factors with highest differences between sIgAD and HCs, demonstrating a difference between sIgAD and HCs. Furthermore, the cytokines have similar vectoral weights in the 2D projection accounting for 90% of variance, indicating interconnected influences. It is though noted that the individuals strongly positive for ANA (ANA over 1:40) or weakly positive (under 1:40) do not cluster together only in the direction of one cytokine even though many do go in the direction of common factors such as CCL3, sCD40L and TWEAK.

Correlation plot of serum cytokines, chemoattractants and growth factors and immunoglobulins with PCA based variable ordering in sIgAD and HCs. Visualisation of the correlations between each pair of variables. Variables are grouped based on correlation of first factor of principal component analysis so that similar variables are placed adjacently using correlation-based variable

ordering. Stronger correlations are represented by darker colours and larger circles. Blue indicates positive correlation and red indicates negative correlation.

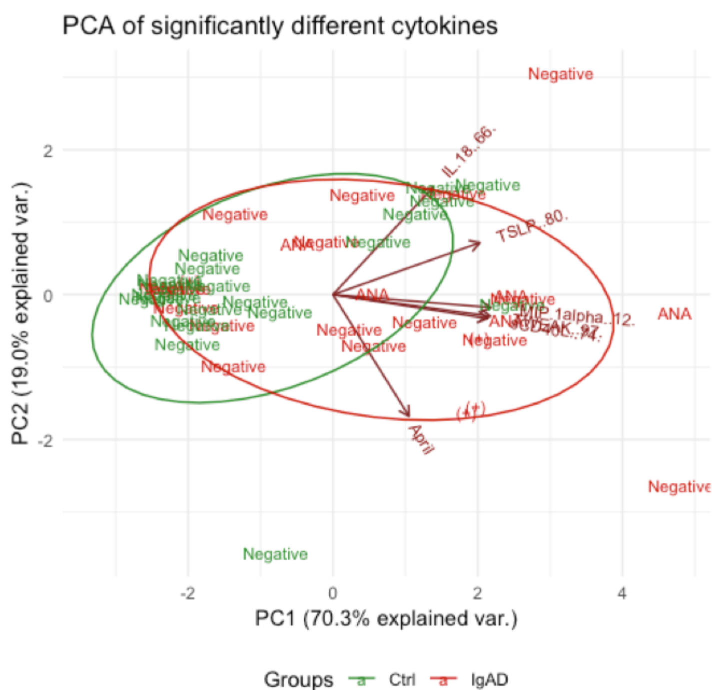

Figure 5. PCA of serum factors and autoantibody positivity

Loadings plot from PCA for the two orthogonal latent components. Structure of the components of the PCA regression analysis of serum measurements of dysregulated immune factors. TSLP, sCD40L, TWEAK, CCL3, IL-18 and APRIL vis-à-vis Ab positivity with X and Y loadings for the first component (horizontal axis) and second component (vertical axis). The sIgAD grouping is depicted in red while controls are green.

4.1.8 Dysregulation of immune biomarkers in sIgAD and its association with IgA serum concentration

Given the above dysregulation of biomarkers in sIgAD and autoantibody positivity, we next analysed its correlation with immunoglobulin production. As shown in Figure 6, sCD40L, TWEAK and CCL3 correlated inversely with IgA.

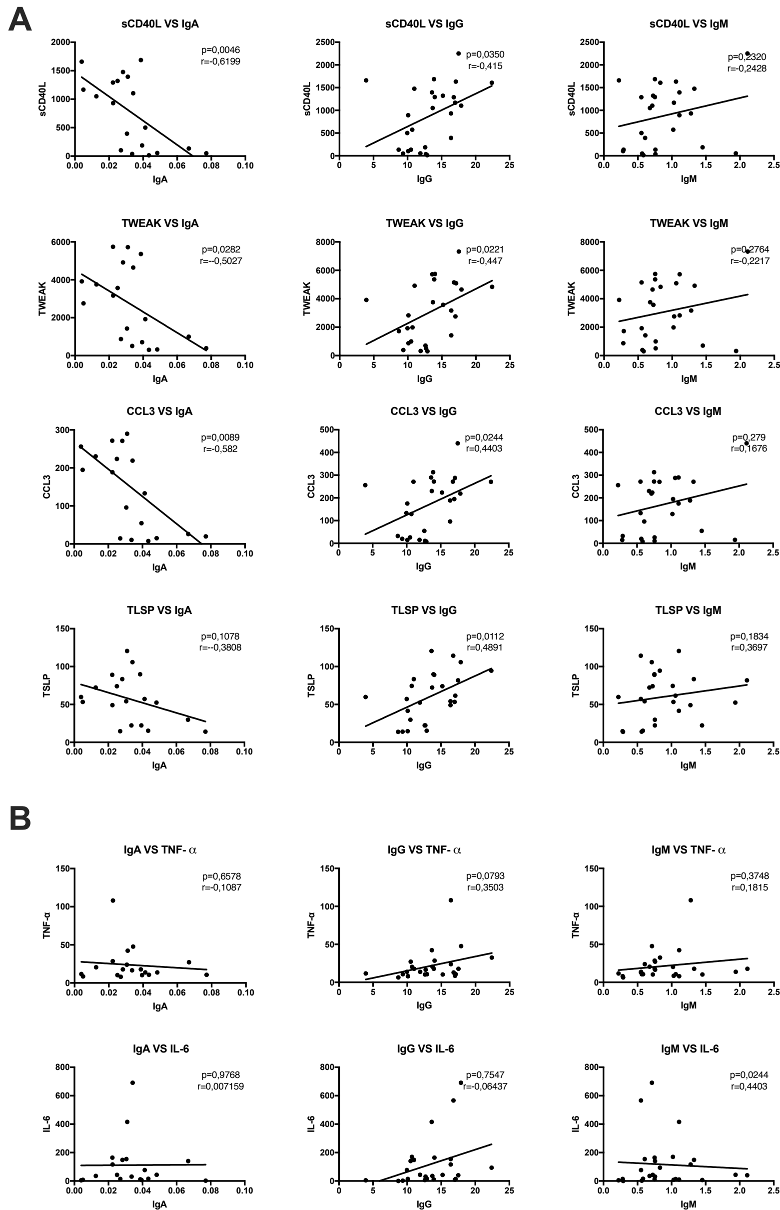

Figure 6. Correlation of dysregulated immune factors and subclasses
Correlation of serum biomarkers and IgA, IgG and IgM in sIgAD individuals. (A) The correlation of observed dysregulated factors, sCD40L, TWEAK, CCL3 and TSLP, with the isotypes IgA, IgM and IgG. (B) T cell associated biomarkers, TNFα and IL-6, not seen to be dysregulated in sIgAD and their correlation with IgA, IgG and IGM.

However, all three dysregulated biomarkers had a positive correlation with IgG in sIgAD individuals while no correlation was seen for serum IgM concentration and these factors (Figure 6A). For non-dysregulated T cell related biomarkers, no correlation was seen for IgA, IgG or IgM (Figure 6B).

4.2 Prevalence of sIgAD in first degree relatives demonstrates a high prevalence of IgA defects with and without IgG and IgM defects

It has been shown that sIgAD has a familial pattern. Thus, we were interested to analyse the association of IgA with other Ig in the sIgAD first degree relatives (n=169) compared to HC (n=100). As shown in Figure 7, the prevalence of individuals with IgA above or below the set 2 SD above and below the median from healthy individuals was significantly higher in first degree relatives compared to healthy controls (14,3% vs 5%, p < 0,02). A significantly higher number of individuals in the low range was also observed (7,7% vs 2%, p < 0,05). Of individuals with low IgA levels, three had a complete deficiency, <0,07 g/L, and where therefore eligible for the subsequent evaluation of sIgAD. Of these one had a sIgAD, while two had also low IgM.

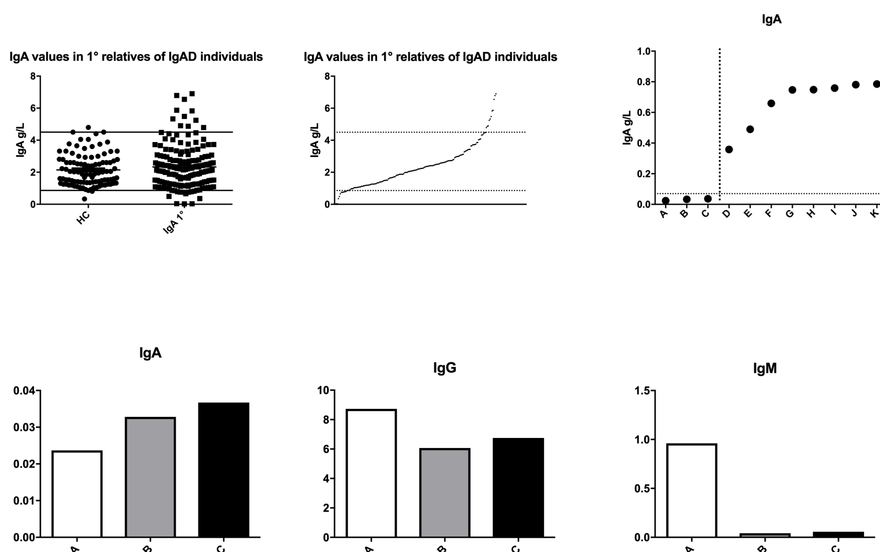

Figure 7. Distribution of serum IgA values in healthy donors and first-degree relatives of IgAD individuals.

Distribution of serum IgA values in the healthy controls (n=100 individuals) and first-degree relatives of IgA deficient individuals (n=156 individuals). Lines demarcate upper and lower limit of normal values of human serum IgA. Of the assessed individuals 11 individuals (A-K) had low IgA. Of these three where deficient (A-C). Of these Only A had a normal IgG and IgM.

4.3 Lymphocyte populations, fractions and functions, in sIgAD

4.3.1 The main B cell populations are present with defects in transitional B cells, class switched B cells and IgA expression.

In order to elucidate the main B cell population multiple B cell populations where evaluated in peripheral blood from sIgAD individuals and compared to HC. These include transitional (D20+/CD38^{hi}/CD24^{hi}/CD27-), mature-naïve (CD20/CD27-), memory (CD20+/CD27+), and Ab secreting B cells (CD20-/CD27^{hi}). IgM, IgG, IgD and IgA were assessed as well.

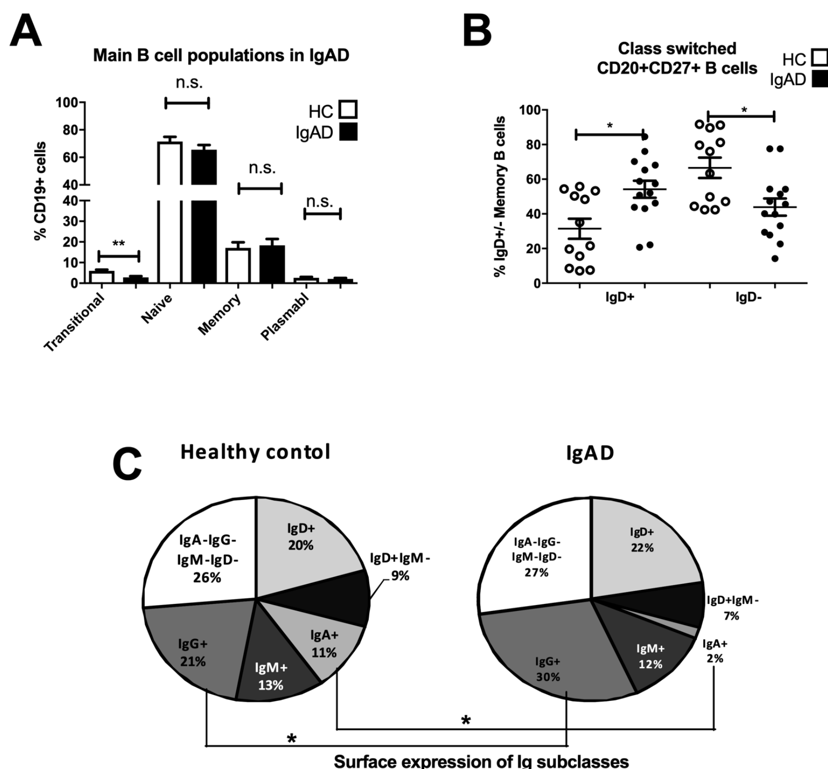

Figure 8. B cell phenotyping and Ig expression in sIgAD

B cell phenotyping and Ig expression in sIgAD was evaluated by flow cytometry. Figure 8 shows B cell populations as measured by flow cytometry comparing sIgAD donors to HCs. (A) Percentage of transitional (CD20+CD27-) naïve (CD20+CD27-), memory (CD20+CD27+) and plasmablasts (CD20-CD27+) as % of CD19+ B cells are shown. (B) Representative pie chart for frequencies of Ig isotypes expressed in total CD19+ B cells in sIgAD and HC donors. The fraction is reduced for IgA ($p<0,0009$) positive B cells and increased for IgG ($p<0,029$) positive B cells in sIgAD compared to HC. (C) The percentages of class switched (CD27+IgD+) memory CD19+ B cells in sIgAD individuals and HC. sIgAD donors have significantly less class switched memory B cells.

As expected, the fraction of naïve, memory and Ab secreting populations were found to be normal in sIgAD. The analysis of IgA, IgG, IgM and IgD expression in total B cells demonstrates a defect in IgA expressing B cells together with a significantly increased expression of IgG+ B cells within the IgAD group. As could be expected and has been previously shown by others, class switched memory B cells (CD20+CD27+IgD-) were significantly decreased in our sIgAD study group.

4.3.2 CpG stimulation does not normlise transitional B cell fractions nor induce normal IL-10 production

Since the transitional B cell compartment showed alterations both in frequency and total numbers (transitional B cell total numbers: IgAD; 564,9 ± 312,6 vs. HC; 2183 ± 583,7; $p<0,02$) it was important to assess their functionality further. TLR9 stimulation is linked to transitional B cells both due to its inducible capacities for IL-10 production of transitional B cells and due to the expansivity of the population with its stimulation. Therefore, CD19+ B cells isolated from IgAD individuals were stimulated with CpG for 48 hours, with the addition of brefeldin A, PMA and ionomycin for intracellular cytokine assessment. As shown in Figure 9B, even under such strong inducing transitional B-cell differentiation conditions, this phenotype remained significantly reduced in the IgAD group. In addition, the fraction of IL-10+ expressing CD20+CD24^{hi}CD38^{hi} transitional B cells *in vitro* was decreased in IgAD individuals (CD20+CD24^{hi}CD38^{hi}IL-10+), Figure 9C. This was not due to an overall decrease in cytokine production since the fraction of TNF α+ transitional B cells was normal (CD20+CD24^{hi}CD38^{hi}TNFα+).

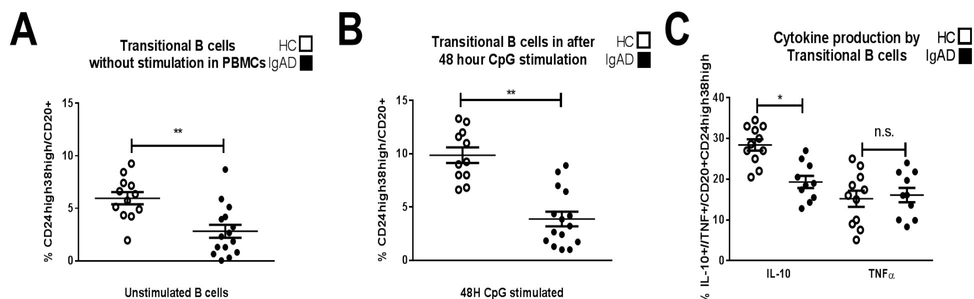

Figure 9. CD24highCD38high transitional B cells in IgAD individuals are defective in their numbers, inducibility and IL-10 secreting properties.

Figure 9 shows transitional B cells as measured in PBMCs and after *in vitro* stimulation of B cells by CpG for 48 hours measured by flow cytometry comparing IgAD and controls. (A) B cells with a transitional B cell phenotype (CD24highCD38high) are of lower fraction in IgAD individuals compared to HCs before stimulation and (B) after stimulation. Both IgAD and HC do though respond to CpG, as the fractions of the transitional population are increased after stimulation. (C) Evaluation of IL-10 secreting function demonstrates a lower fraction of IL-10+ transitional cells (CD24highCD38hiIL-10+) in IgAD compared to HCs. A measurement of intracellular TNFα in transitional B cells shows no difference between the two groups. HCs are depicted as white while IgAD individuals are depicted as black dots. Data is shown as mean ± SEM of all IgAD and HC assessed. All data is from 5 independent experiments, no duplicates. Significance was calculated in relation to the control group. *p < 0.05, **p < 0.01, ***p < 0.001 (one sided Student's t test).

4.3.3 Homing marker expression and activation of plasmablast is not altered in sIgAD

Homing of B cells to the different compartments after stimulation is essential for their survival. We therefore hypothesised if the homing of B cells could be altered in sIgAD. However, the expression of tested markers CCR10, CD62L, CXCR4 and α4β7 did not differ in sIgAD compared to HCs.

The activation of plasmabalst has been of special interest in autonflammatory diseases dominated by autoantibody production. Furthermore, the numbers of plasmablast in the periphery tend to be IgA expressing. As shown in Figure 10, the expression of HLA-DR on plasmablasts in our sIgAD group was found to be similar to HCs.

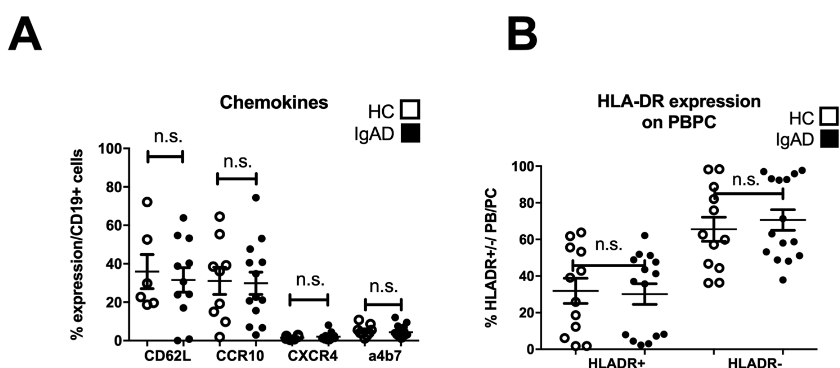

Figure 10. B cell homing and activation marker expression in sIgAD is normal
The figure shows homing markers of B cells and activation of plasmablasts. (A) The surface expression of the homing markers CD62L, CCR10, CXCR4 and $\alpha 4\beta 7$ on CD19+ B cells as measured in PBMCs (B) The activation of plasmablasts/plasma cells in IgAD, evaluated by percentage of HLA-DR expression, shows comparable activation in sIgAD and HCs. Data is shown as mean ± SEM of all IgAD and HCs assessed. All data is from 5 independent experiments, no duplicates. Significance was calculated in relation to the control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (one sided Student's t test).

4.3.4 Stimulation by CpG fails to normalise IgA production in sIgAD individuals.

Little is known about T cell independent responses in sIgAD while T cell dependent stimulations have been shown to be defective. We looked at CpG dependent responses with or without IL-10 stimulation in B cells from sIgAD individuals. In our model CpG induced B cell stimulation led to a significant IgA secretion and surface expression in HCs but only to very limited IgA production in sIgAD individuals. Exogenous IL-10 administration did not reverse this defect. On the other hand, IgG surface expression and secretion remained similar in IgAD and HCs, even after additional IL-10 and CpG driven stimulation.

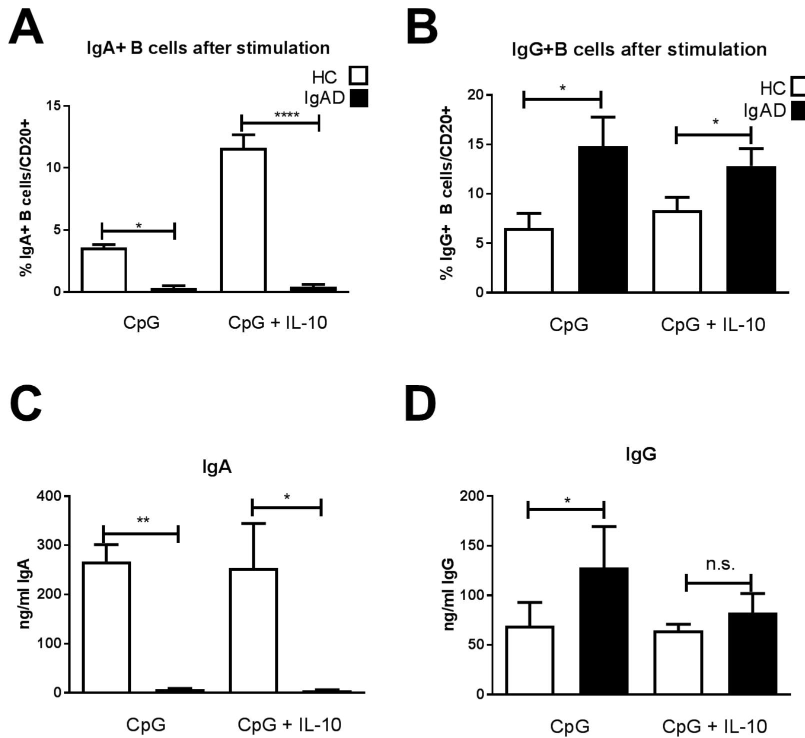

Figure 11. IgA production and expression after stimulation.
The stimulation of isolated CD19+ B cells from IgAD individuals and HCs after T cell independent stimulations with CpG only or CpG + IL-10 reveals (A) an increased surface expression of IgA with the addition of IL-10 to CpG in HCs but not in IgAD individuals. (B) The expression of IgG is significantly higher in IgAD individuals compared to HCs. (C) After stimulation with CpG the production of IgA is not equalised in IgAD compared to HC (D) but the production of IgG after stimulation remains similar in sIgAD individuals compared to HCs after stimulation. Data are shown as mean ± SEM of all IgAD and HCs assessed. All data are from 5 independent experiments. Significance is shown as *p < 0.05, **p < 0.01, ***p < 0.001 (as determined by one-way ANOVA).

4.3.5 sIgAD individuals have a normal proportion and function of classical T cell subpopulations.

Antibody production can be heavily influenced by T cell signalling with subpopulations of T cells influencing different isotype switching as well as

being important in germinal centre B cell maturation. Therefore, a detailed phenotypic analysis of the various subpopulations of T cells was performed. Our results suggest that proportions of CD3+, CD4+ and CD8+ T cells are comparable in sIgAD individuals compared to HCs. Additionally, no differences were seen between the two groups for any of the measured CD4+ and CD8+ T cell subpopulations. The expression of surface markers CD27, CD28, CD62L and CCR7 was used to define the following T cell subpopulations (flow cytometry gating depicted in supplementary Figure 1C); naïve (CD62L+CCR7+), central memory (CD62L-CCR7+), effector memory (CD62L-CCR7-), terminally differentiated effector memory (CD62L+CCR7-), early differentiated (CD27+CD28+), intermediately differentiated (CD27+CD28-) or late differentiated (CD27-CD28-) T cells. T effector functions were further assessed by the stimulation of PBMCs with PMA and Ionomycin in the presence of brefeldin A. This was followed by staining for detection of intracellular cytokines and associated transcription factors. Such stimulation did not reveal any significant difference of the various effector T cell subpopulations between sIgAD and HCs in the following T effector cell types: TFH (CD4+CXCR5+ICOS+PD1+BCL6+), TH1 (CD4+Tbet+CXCR3+IL-17-IFNγ+), TH2 (CD4+CCR4+ GATA3+IL-10-IL-4+), TH17 (CD4+CCR4+ RORγT+IL-17+) and TH22 (CD4+IL-22+).

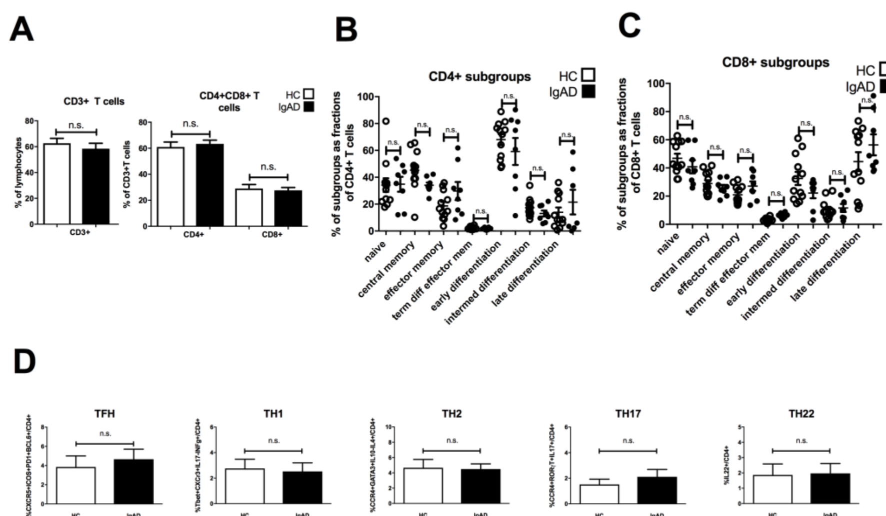

Figure 12. The fraction of the T cell populations and inducability of T helper subsets in sIgAD is normal compared to HCs.

T cell populations as measured by flow cytometry on PBMCs for extracellular markers and in PBMCs stimulated with PMA and ionomycin for 4 hours comparing IgAD and HCs. No significant differences were found for the proportions of T cells. (A) The fraction of CD3+ T cells as proportions of lymphocytes and CD4+ and CD8+ T cells as proportions of CD3+ T cells in IgAD vs. healthy controls. (B) Fractions of the different T cell subpopulations of CD4+ and (C) CD8+ T cells. The subpopulation gating is based on their classification as naïve (CD62L+CCR7+), central memory (CD62L-CCR7+), effector memory (CD62L-CCR7-), terminally differentiated effector memory (CD62L+CCR7-), early differentiated (CD27+CD28+), intermediately differentiated (CD27+CD28-) or lately differentiated (CD27-CD28-). (D) The proportions of T helper cell populations as a fraction of CD4 T cells is shown. No significant differences were seen between TFH (CD4+CXCR5+ICOS+PD1+BCL6+), TH1 (CD4+Tbet+CXCR3+IL-17-IFNγ+), TH2 (CD4+CCR4+GATA3+IL-10-IL-4+), TH17 (CD4+CCR4+RORγT+IL-17+) and TH22 (CD4+IL-22+) cells between IgAD and HCs. All data is from 5 independent experiments, no duplicates. Significance was calculated in relation to the control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (as determined by one-way ANOVA).

4.3.6 Natural and induced Tregs have normal numbers and function in sIgAD.

Previous studies have shown an interaction between IL-10+ from B cells and Tregs (Rosser & Mauri, 2015). Since IL-10+ transitional B cells were shown to be abrogated in sIgAD, Tregs have been shown to be of limited numbers in sIgAD with ongoing autoimmunity and they are known to be important at mucosa surfaces for IgA, a phenotypic analysis of Tregs was undertaken. To fully understand the role of Tregs in the pathophysiology of diseases and defects one needs to look both at the natural Tregs (nTregs), induced Tregs (iTregs) and their function. nTregs were measured in PBMCs stimulated *in vitro* for 4 hours with PMA and ionomycin and in the presence of brefeldin A. iTregs were induced from isolated CD4+CD25- T cells by stimulating them with anti-CD28, IL-2 and TGF-β1 on anti-CD3ε coated plates. No difference was found between IgAD individuals and HCs for either population. We further assessed the functional capacity of these iTregs in a previously published model using proliferation of CFSE stained CD8+ T cells (from HC) in a co-culture with iTregs (from IgAD or HC) and superantigen pulsed EBV-B cells. The iTregs from IgAD individuals showed normal suppressive capacity compared to iTregs from healthy controls at the different dilutions of iTregs: EBV from 1:1 to 1:32.

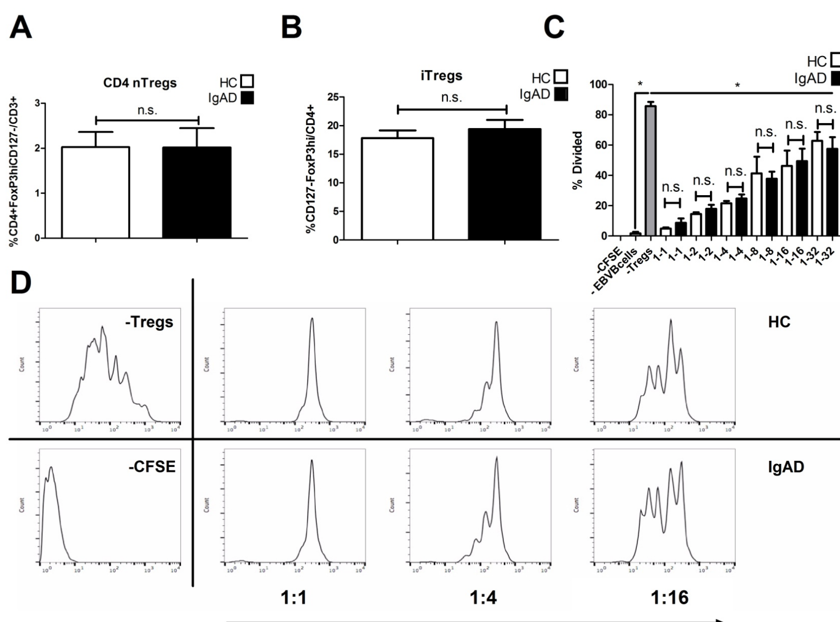

Figure 13. Tregs fractions and functions are normal in IgAD.
(A) The proportion of cells expressing a CD4 nTreg phenotype characterised by CD4+CD127-CD25hiFoxP3hi expression was not found to differ in IgAD from HCs. (B) Neither did *in vitro* induced CD4+ iTregs after 120h of stimulation. (C) The suppressive capacity of the iTregs from IgAD is intact and comparable to HCs at different ratios when comparing proliferation of CFSE stained CD8+ T cells in allogeneic co-cultured PBMCs stimulated by super Ag pulsated EBV-B cells. (D) A representative suppressive assay experiment showing a representative HC and an IgAD individual, -CFSE is a negative control with no CFSE staining, -Tregs shows a positive control of proliferation without Tregs. All data is from 5 independent experiments, no duplicates. Significance was calculated in relation to the control group. *p < 0.05, **p < 0.01, ***p < 0.001 (as determined by one-way ANOVA).

4.3.7 Minimal inducibility and defective longevity of IgA secreting B cells in sIgAD

IL-10 as a model for T cell dependent stimulation has previously been shown to induce a short-livedi IgA production in IgAD (Briere et al., 1994). We chose to explore this stimulation to evaluate the effect of exogenous cytokine stimulation on longuevity since its usage has been proposed for the treatment of PIDs such as IgAD and CVID (Borte et al., 2009). In a model mimicking a

long lived *in vivo* mucosal IgA (previously described in [24]), we explored whether B cells cultured together with other PBMCs and exogenous IL-10 could be forced into class switching and sustaining long-lived *ex vivo* IgA production. Robust IgA levels were detected in healthy donors 3-5 weeks following stimulation, but only weak IgA levels were detected in IgAD donor cultures. This was not detected after 2 weeks, indicating only defective short-lived responses following stimulation in IgAD (Figure 14A). The opposite was found for IgG production in IgAD where a long-lived response was seen for up to 3 weeks and a significantly higher production in week two following IL-10 stimulation (Figure 14B). The difference between the two groups of donors reached significance for the first three weeks for conditions with and without IL-10. This model demonstrated that IL-10 stimulation in the presence of other immune cells induces only a modest, short lived IgA production in IgAD individuals but leads instead to robust IgG responses, in stark contrast to the response observed in all HCs.

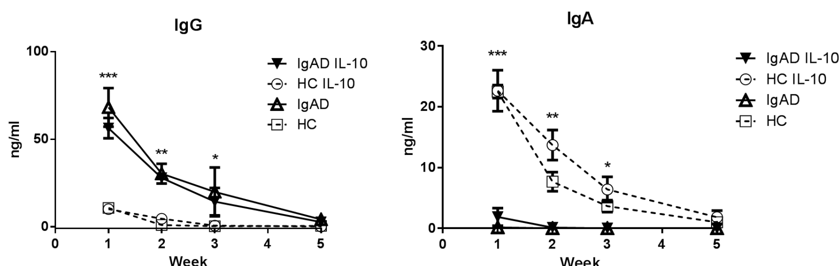

Figure 14. Longevity responses after stimulation of sIgAD B cells in PBMCs

The Ab response of B cells in PBMCs from IgAD and HCs with or without IL-10 stimulation as measured by ELISA. (A) IgG production is statistically higher in IgAD individuals compared to HCs at week one with production seen up to 5 weeks. (B) A small amount of IgA production is seen at week 1 in PBMCs from IgAD individuals with the exogenous addition of IL-10 (with - ▼/ or without IL-10 ∆), it remains significantly lower compared to HC (with - ○ / or without IL-10) and short lived, in contrast to HCs where production was seen up to the 5th week. Significance was calculated in relation to the control group. *p < 0.05, **p < 0.01, ***p < 0.001 (as determined by one-way ANOVA).

4.3.8 Signal transduction responses to stimulations known to induce IgA production demonstrate heterogeneous responses to different stimuli as well as between T and B cells

Lymphocytes from HCs demonstrate a variable pattern of pSTAT3, pSTAT5, pSTAT6 and ERK1/2 depending on the given underlying stimulatory conditions (Figure 15). In our model it was important to assess the stimulations previously shown in the literature to be defective in sIgAD, that is IL-2, IL-4, IL-10, IL-21 and CpG. Furthermore, it was important to try the concomitant stimulation of IL-10 and either IL-2 and/or IL-4 since it has been shown that these cytokines improve IgA secretion in sIgAD (Marconi et al., 1998).

In exploring the responses of these above-mentioned stimuli in pSTAT3 expression a good response was seen after IL-10 and IL-21 separately in both T and B cells (Figure 15A). However, neither antagonistic nor synergistic effects were found in any of the conditions tested (Figure 15A, 16A and 16C). The mapping of STAT5 signalling following various T cell dependent and independent mimicking stimulations revealed that significant phosphorylation was confined to T cells. Of the stimulations tested IL-2 alone induced phosphorylation in T cells. Dual stimulation with IL-2 and IL-10 induced phosphorylation in both T and B cells while IL-4 and IL-10 alone did not induce significant STAT5 phosphorylation. Regarding pSTAT6 expression, expression was seen after IL-4 stimulation in both T and B cells (Figure 15C). For T cell independent responses, the evaluation ERK1/2 expression was done following CpG stimulation and where expression was seen following stimulation in a small proportion of B cells in both HCs and IgAD (Figure 19A, HCs B cells 17,7% +/- 1,0 v.s. sIgAD B cells 13,8% +/- 2,6).

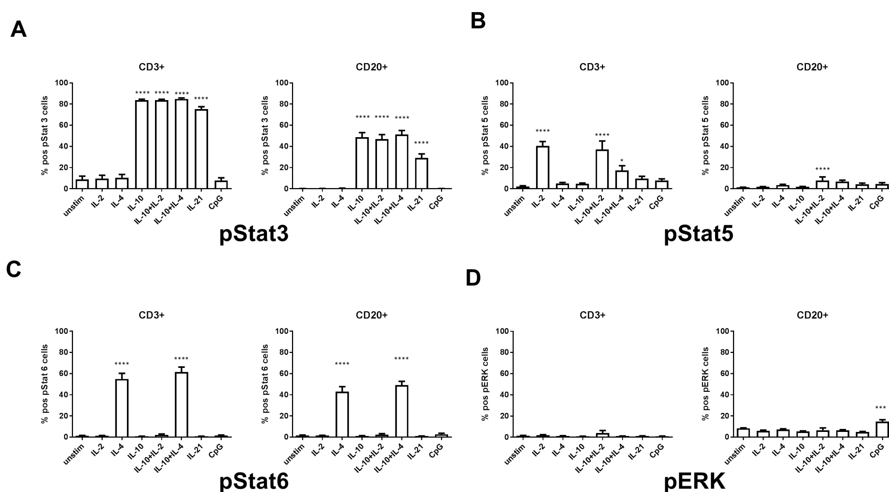

Figure 15. Differences in percentage of pSTAT3, pSTAT5, pSTAT6 and ERK1/2 expression in T and B cells in HCs.

Expression of pSTAT3 (S1A), pSTAT5 (S1B), pSTAT6 (S1C) and ERK1/2 (S1D) as measured *ex vivo* in PBMCs after 15 minute stimulation of IL-2 (50 ng/mL), IL4 (100 ng/mL), IL-10 (100 ng/mL), IL-21 (50 ng/mL) or CpG ODN 2006 (1 µg/mL TLR9 agonist) alone or in combinations in CD3+ T and CD20+ B cells of healthy individuals. Figure A shows the percentage of pSTAT3 positive T (CD3+) and B cells (CD20+). A positive response is seen with IL-10, IL-10 + IL-2, IL-10 + IL-4 and IL-21 stimulation but none after IL-2, IL-4 alone or CpG. Figure B shows the percentage of pSTAT5 positive T (CD3+) and B cells (CD20+). A positive response is seen with IL-2, IL-10 + IL-2 and IL-10 + IL-4 in T cells but IL-10 + IL-2 in B cells, but no activation in neither T nor B cells after IL-4, IL-10, IL-21 or CpG. Figure C shows the percentage of pSTAT6 positive T (CD3+) and B cells (CD20+). A positive response is seen with IL-4 and IL-10 + IL-4 in T and B cells, but no activation in neither T nor B cells after IL-2, IL-10, IL-10 + IL-2, IL-21 or CpG. Figure D shows the percentage of ERK1/2 positive T (CD3+) and B cells (CD20+). A positive response is seen with CpG stimulation in B cells but not in T cells. Furthermore, no activation is seen in either T nor B cells after IL-2, IL+4, IL-10, IL-10 + IL-2, IL-10 + IL-4, IL-21 or CpG. Significance was calculated in relation to the control group. *p < 0.05, **p < 0.01, ***p < 0.001 (as determined by one-way ANOVA).

4.3.9 STAT3 activation with IL-21 is defective in sIgAD

When looking at pSTAT3 a good response was seen after IL-10 and IL-21 stimulation separately (Figure 15). These stimulations where therefore tested in sIgAD. Proportionally fewer B cells responded to IL-21 driven STAT3 phosphorylation in sIgAD compared to HCs (Fraction of HCs B cells = 30,4% +/-11,5 vs. sIgAD B cells 16,1% +/- 7,5; p=0,03; Figure 16A and 16C) but not

in GMFI (Geometric mean fluorescence intensity) fold change (HC B cells = 2,3+/-0,3 vs. sIgAD B cells = 1,6 +/- 0,3; p<0,14; Figure 16B and 16D).

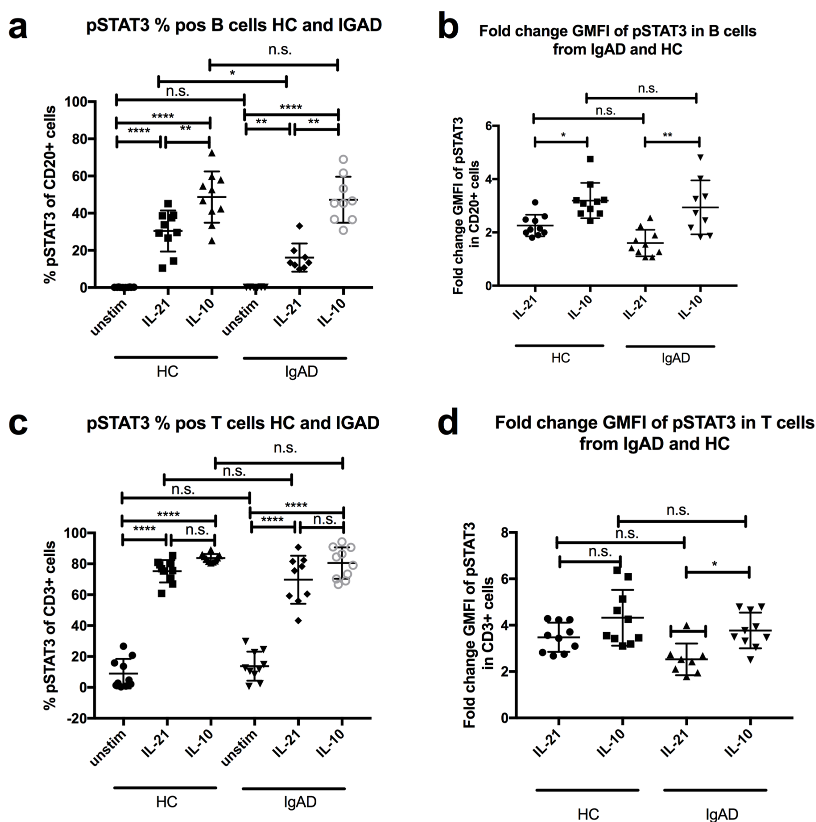

Figure 16. pSTAT3 expression after various stimulations in T and B cells from sIgAD and HCs.

STAT3 phosphorylation and fold change GMFI as measured *ex vivo* in PBMCs after 15 minute stimulation with IL-21 (50 ng/mL), IL-10 (100 ng/mL) + IL4 (100 ng/mL) and IL-2 (50 ng/mL). Analysis of (A) % pSTAT3 positive B cells (CD20+) and (B) fold change GMFI shows a significant lower percentage of B cells expressing pSTAT3 after IL-21 stimulation but no differences between phosphorylation responses after IL10 in sIgAD and HCs. Analysis of (C) % pSTAT3 positive T cells (CD3+) and (D) fold change GMFI shows no differences between phosphorylation responses after IL-21 and in sIgAD compared to HCs. Significance was calculated in relation to the control group. *p < 0.05, **p < 0.01, ***p < 0.001 (as determined by one-way ANOVA).

4.3.10 STAT5 activation is normal with stimuli shown to induce heterogeneous IgA producing responses in sIgAD

STAT5 signalling in HCs revealed that significant phosphorylation was confined to T cells with IL-2 alone inducing phosphorylation in T cells (Figure 15B). In sIgAD it has been shown that the addition of IL-2 to IL-10 stimulation leads to higher "rescue production" of IgA after stimulation compared to IL-10 alone (Marconi et al., 1998). In our setup a difference was seen in percentage of phosphorylation in STAT5 in B cells with IL-2 + IL-10 compared to IL-2 alone for both HCs and sIgAD (Figure 15B). However, we did not observe any differences between the two groups in cell proportions or fold change GMFI (Figure 17A-D) following dual IL-2 and Il-10 stimulation.

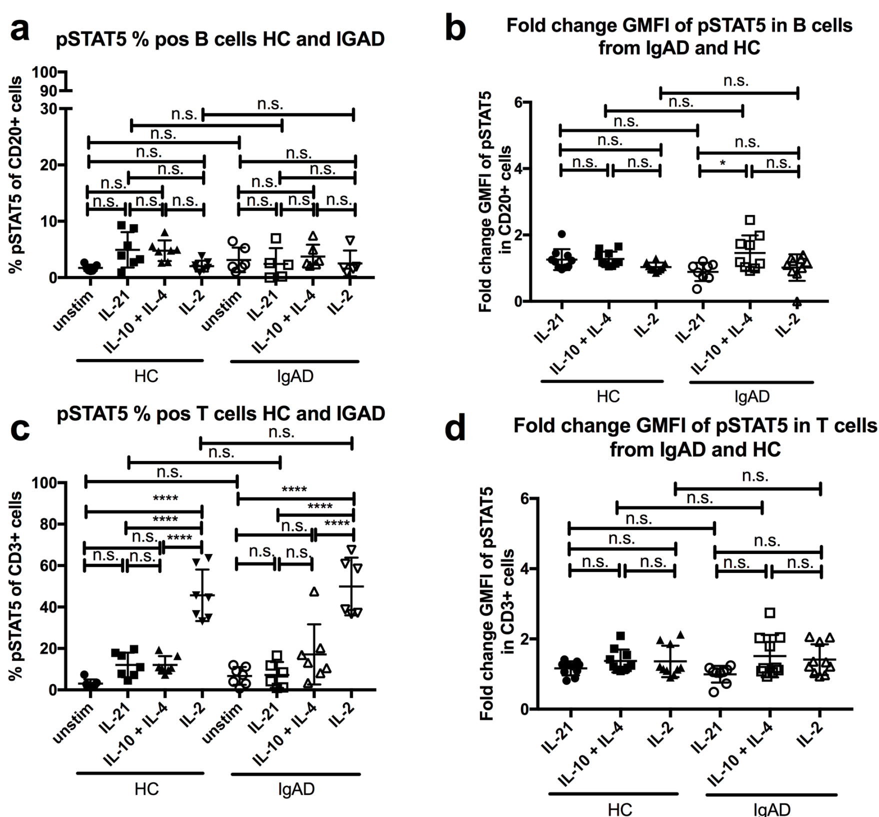

Figure 17. The percentage and fold change GMFI of pSTAT5 in B (CD20+) and T cells (CD3+) after IL-21, IL-10 + IL-4 and IL-2 in sIgAD individuals and HCs.
STAT5 phosphorylation and fold change GMFI as measured *ex vivo* in PBMCs after 15 minute stimulation of IL-21 (50 ng/mL), IL-10 (100 ng/mL) + IL4 (100 ng/mL) and IL-2

(50 ng/mL). Analysis of (A) % pSTAT5 positive B cells (CD20+) and (B) fold change GMFI shows no significant difference in the percentage or fold change GMFI of B cells expressing pSTAT5 after IL-21, IL-10 + IL-4 and IL-2 stimulation in sIgAD and HCs. Analysis of (C) % pSTAT5 positive T cells (CD3+) and (D) fold change GMFI shows neither differences between phosphorylation responses in T cells after IL-21, IL-10 + IL-4 and IL-2 stimulation in sIgAD and HCs. Significance was calculated in relation to the control group. *p < 0.05, **p < 0.01, ***p < 0.001 (as determined by one-way ANOVA).

4.3.11 STAT6 responses to IL- 4 are normal in sIgAD

pSTAT6 expression was only seen after IL-4 stimulation in both T and B cells. No differences were seen in the fraction nor fold change GMFI between the two cohorts after IL-4 or IL-4 + IL-10 stimulation.

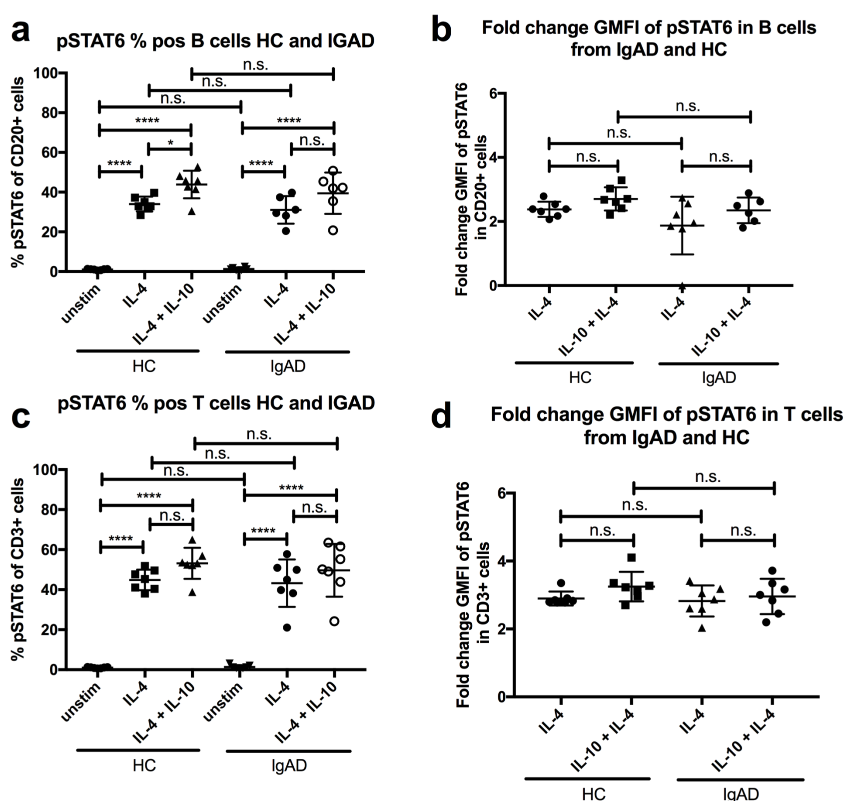

Figure 18. The percentage and fold change GMFI of pSTAT6 in B (CD20+) and T cells (CD3+) after IL-4 and IL-10 + IL-4 in sIgAD individuals and HCs.
pSTAT6 expression was only seen after IL-4 stimulation in both T- and B cells, with slight but noticeable synergistic effects following dual IL-4 and IL-10 stimulations in B

cells only. Analysis of (Figure 18C) % pSTAT6 positive T cells (CD3+) and (Figure 18D) fold change GMFI shows neither differences between phosphorylation responses in T cells after IL-4 nor IL-10 + IL-4 stimulation in sIgAD and HCs. Significance was calculated in relation to the control group. *p < 0.05, **p < 0.01, ***p < 0.001 (as determined by one-way ANOVA).

4.3.12 ERK1/2 responses to CpG are not defective in sIgAD

Expression of ERK1/2 was only seen following T cell independent stimulation in HCs. Even though stimulation with CpG leads to defective IgA production in sIgAD we saw no differences in phosphorylation of ERK1/2 between T or B cells, in sIgAD or HCs (Figure 19).

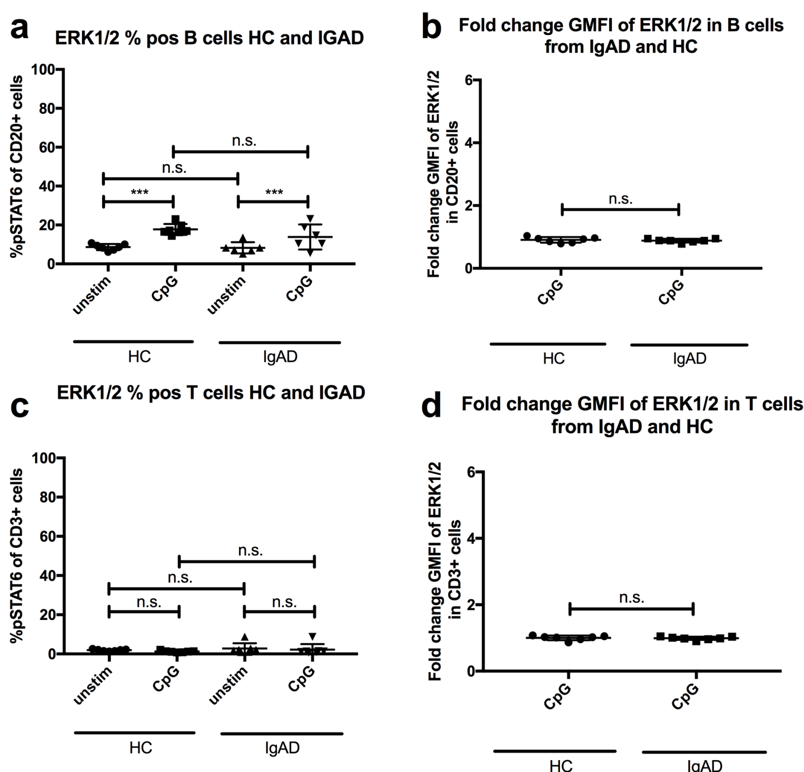

Figure 19. Effects of CpG on ERK1/2 expression in B and T cells from sIgAD individuals and HCs.

ERK1/2 expression and fold change GMFI as measured *ex vivo* in PBMCs after 15 minute stimulation of CpG (1 µg/mL, ODN2006, TLR9 agonist). Analysis of (A) % ERK1/2 positive B cells (CD20+) and (B) fold change GMFI shows no significant

difference in the percentage or fold change GMFI of B cells expressing ERK1/2 after CpG stimulation in sIgAD and HCs. Analysis of (C) % CpG positive T cells (CD3+) and (D) fold change GMFI shows neither differences between phosphorylation responses in T cells after CpG stimulation in sIgAD and HCs. Significance was calculated in relation to the control group. *p < 0.05, **p < 0.01, ***p < 0.001 (as determined by one-way ANOVA).

4.3.13 B cell production of factors shown to be dysregulated in sIgAD

Since a concomitant dysregulation in serum factors and B cell defects are seen in sIgAD it is important to assess the secretion of B cells of these factors. We therefore measured the factors found to be significantly higher in CpG stimulated B cells and the ones which did not pass significance tests but where trending to be higher in the serum from sIgAD individuals. No differences were seen in supernatant from *in vitro* cultured CpG stimulated CD19 B cells for IL-18, CCL3, sCD40L, TSLP, TWEAK and April.

Table 6. B cell secretion of serum dysregulated immune factors in sIgAD
The table shows the biomarkers from sIgAD B cells after CpG stimulation. N=10, in 3 separate experiments. The data is from the analysis by 65-plex human ProcartaPlex™ luminex assay (R&D systems) and analysed in Bio-Plex 200 system (Bio-Rad Laboratories, California, USA). Significance was calculated in relation to control group determined by one-way ANOVA with bonnferroni correction. No significant difference is marked as ns.

	Mean, HC	**Mean, IgAD**	**Diff between means**	**P value**	
IL-18	157,2	69,51	87,69	>0,9999	ns
CCL3	113,8	99,21	14,64	>0,9999	ns
sCD40L	231,1	173,8	57,28	>0,9999	ns
TSLP	31,52	21,25	10,27	>0,9999	ns
TWEAK	1651	950,7	700,2	>0,9999	ns
April	105 604	111 532	5927	>0,9999	ns

4.4 mRNA Transcriptomics of B cells from sIgAD individuals

Given the above dysregulated serologic biomarkers and B cell alteration in sIgAD there might be an underlying B cell intrinsic transcriptional defect. For this purpose, it was decided to focus upon T independent pathways due to its relatively relationship to the seen transitional defect and alteration predominantly seen in B cell dysregulation in sIgAD.

As predicted the stimulation with CpG induced transcriptomic upregulation in B cells (Figure 20). By comparing the upregulated transcripts paired to unstimulated B cells numerous transcripts were found to differ between HCs and sIgAD.

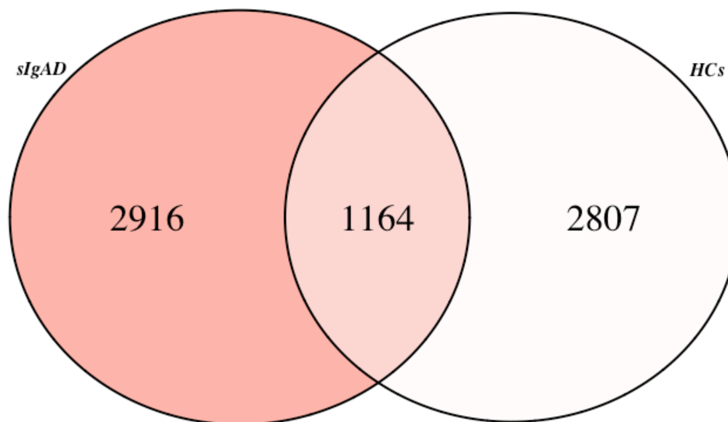

Figure 20 . Differences in gene transcription between sIgAD and HCs
Venn diagram depicting the differences in significantly altered ($p<0.05$) transcripts in a paired analysis of sIgAD and HCs B cells after CpG stimulation for 48h.

Some of the genes differenctially expressed are directly linked to IgA such as Immunoglobulin J chain (IGJ), coding for the joining chain of IgA with a fold change of 1.74 for IGLC1, IGLJ1, IGLL5 encoding for immunoglobulin parts. Interestingly, the one with the most significant enhanced exprerssion was the pseudogene ANKRD36BP2, which has unknown function. However, it may be related to plasmacell development and found in the gut mucosa of patients with inlammatory bowel disease (Fagerberg et al., 2014; Jourdan et al., 2009). Thus, potentially indicating its involvement in mucosal B cell maturation.

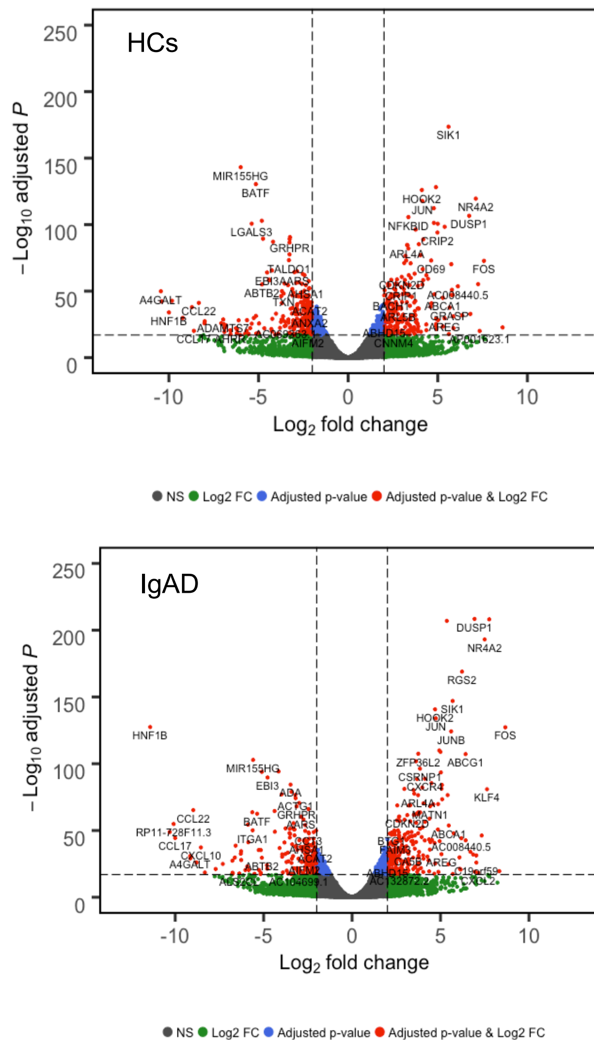

Figure 21 . Effects of CpG stimulation in HCs and sIgAD.

(A) The figure shows a volcano plot for the expression of up- and downregulated transcripts in CD19+ B cells after TLR9 agonist (CpG ODN 2006, 1 µg/mL) stimulation for 48. Red transcripts are these with a – log10(p-value) over 2.

4.5 Gene transcripts significantly altered in sIgAD with and without stimulation

When analysing the differences in gene expression in sIgAD individuals five genes had at least 2 fold log increase; PDE9A (Phosphodiesterase 9A), PRLR (Prolactin Receptor), PIGR (Polymeric immunoglobulin receptor), ANKRD36BP2 and RPGR-Interacting Protein 1 (RPGRIP1). PDE9A is related to the hydrolysis of cAMP and cGMP. PRLR is from the type I cytokine receptor family. In B cells it has been shown to be able to affect Ab responses but is not needed for a classical T cell dependent Ag response of IgA production (Bouchard, Ormandy, Di Santo, & Kelly, 1999). PIGR is a member of the immunoglobulin superfamily. ANKRD36BP2 is a pseudogene wich has only been shown to be up-regulated in B cell maturation and in the gut mucosa of individuals undergoing treatment for Inflamatiory bowel disese (IBD) (Fagerberg et al., 2014; Jourdan et al., 2009).

When analysing the differences in gene expression in sIgAD individuals after stimulation with CpG only three genes had a significant change (a log 2 fold change), that is; AC095064.1, CALB1 and ANKRD36BP2. Nothing is known about AC095064.1, CALB1 is part of the calcium-binding protein superfamily, while ANKRD36BP2 remains significantly different after stimulation, previously seen in the unstimulated analysis.

Table 7. Differentially transcribed genes in sIgAD unstimulated CD19+ B cells
Results from the analysis of isolated B cells from sIgAD compared to HCs. Results from B cells stimulated for 48 h and paired to unstimulated B cells from the same sIgAD subjects, compared to Hcs.

Gene name	Log 2-fold change	Adjusted p value
PDE9A	3.19899	0.0441962
PRLR	3.15845	0.019279
PIGR	2.62082	0.0441962
ANKRD36BP2	2.27402	0.019279
RPGRIP1	2.11536	0.0441962
IL18R1, IL1RL1	-1.92858	0.019279
DAPK1	1.73704	0.0441962
CCDC58	1.69023	0.019279
ITGA2B	-1.58361	0.0441962
RPL39L	1.5192	0.0441962
PTCH1	1.50969	0.019279
IGLC1, IGLJ1, IGLL5	1.45462	0.019279
CRIP2	1.3259	0.019279
MYB	1.2229	0.019279
IGKV2D-29	1.18697	0.019279
IGLC3	1.03824	0.019279
IL32	1.0343	0.0441962
LGALS1	0.890071	0.019279
SOCS3	-0.872763	0.019279
KLF10	-0.829425	0.0335828

Table 8. Differentially expressed transcripts of genes in sIgAD CD19+ B cells after CpG stimulation compared to HCs
Results from the comparison of the analysis of isolated B cells from sIgAD compared to HCs. Results from B cells stimulated for 48 h and paired to unstimulated B cells from the same sIgAD subjects, compared to HCs.

Gene name	Log 2-fold change	Adjusted p value
AC095064.1	-6.89966	0.0243744
CALB1	-2.35429	0.0331174
ANKRD36BP2	2.12477	0.0147903
IGJ	1.75019	0.0376148
CCDC58	1.65037	0.0147903
RP5-1043L13.1	-1.58263	0.0331174
SORBS2	1.52571	0.0147903
RANBP17	-1.47973	0.0147903
RPL39L	1.21791	0.0147903
IGLC1, IGLJ1, IGLL5	1.12941	0.0376148
IGHV6-1	0.955847	0.0376148
LGALS1	0.776264	0.0147903

4.1 Go analysis of altered genes reveals defects related to germinal centre formation and TLR9 responses

Differences are seen in significantly upregulated genes by paired analysis of stimulated and unstimulated B cells from sIgAD and HCs. By looking at the genes with an expression above an adjusted p value of 0.05 by GO analysis we observe a certain paucity of pathways affected in sIgAD individuals compared to HCs. In sIgAD, by looking at the most significantly changed genes and their affection on pathways linked to B cells using ingenuity pathway analysis, pathways of genes related to B cell longevity and plasma cell development came up. Abnormal morphology of plasma cells and survival of memory B cells with a delay in their division had increased measurement. No decreased measurement was detected with significance in sIgAD compared to HCs. In IgAD individuals genes related to negative regualtion of germinal center formation and defective TLR7 and TLR9 are seen to be upregulated.

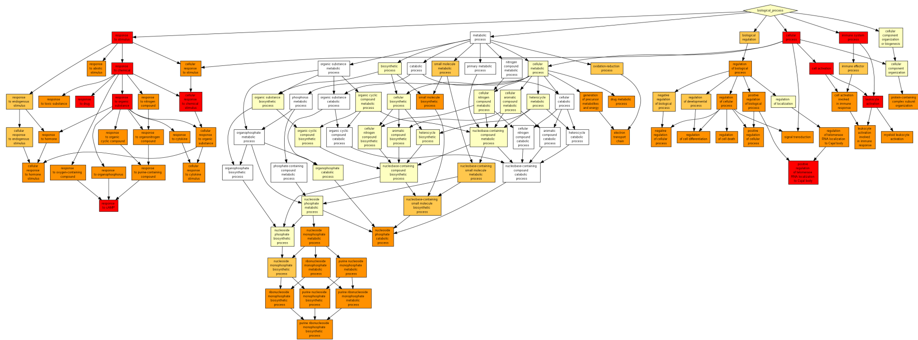

Figure 22 . GO analysis of significantly upregulated genes in HCs

GO enrichment for biological processess in HCs where multiple activation processes are seen to be significantly affected. Most significant are responses to cAMP, cell processess, immune system processes, leukocyte activation and positive regulation of telomerase RNA localisation to cajal bodies.

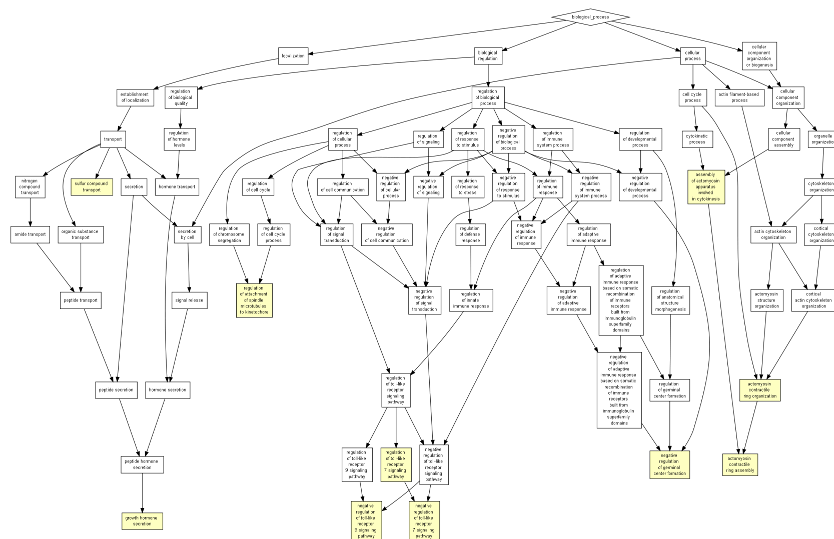

Figure 23 . GO analysis of significantly upregulated genes in HCs.

GO enrichment for biological processess in IgAD individuals where it can be visually seen that not so many processess are activated as in HCs. The main processess related to B cells are negative regulation of TLR7 and TLR9 as well as negative regulation of germinal center formation.

Table 9. Go terms enriched for significantly upregulated transcripts in sIgAD B cells after CpG stimulation.
Go enrichment terms gotten from the analysis of significantly upregulated transcripts from sIgAD B cells after 48 h CpG stimulation.

GO term	Description	P-value	FDR q-value	Enrichment (N, B, n, b)
GO:0034156	negative regulation of toll-like receptor 7 signaling pathway	1.49E-4	1E0	6,706.00 (6706,1,1,1)
GO:0030252	growth hormone secretion	2.57E-4	1E0	19.61 (6706,3,342,3)
GO:0072348	sulfur compound transport	3.28E-4	1E0	5.28 (6706,18,565,8)
GO:0002635	negative regulation of germinal center formation	3.97E-4	1E0	70.59 (6706,2,95,2)
GO:0034164	negative regulation of toll-like receptor 9 signaling pathway	5.9E-4	1E0	3,353.00 (6706,2,1,1)
GO:0034155	regulation of toll-like receptor 7 signaling pathway	5.9E-4	1E0	3,353.00 (6706,2,1,1)
GO:0051988	regulation of attachment of spindle microtubules to kinetochore	6.79E-4	1E0	18.84 (6706,4,267,3)
GO:0000915	actomyosin contractile ring assembly	6.89E-4	1E0	49.67 (6706,2,135,2)
GO:0000912	assembly of actomyosin apparatus involved in cytokinesis	6.89E-4	9.27E-1	49.67 (6706,2,135,2)
GO:0044837	actomyosin contractile ring organisation	6.89E-4	8.34E-1	49.67 (6706,2,135,2)

4.2 Ingenuity pathway analysis of altered genes reveals defects related to B cell development, survival of memory B cells and plasma cell development.

By looking at the most significantly changed genes and their affection on pathways linked to B cells, using Ingenuity pathway analysis, pathways of genes related to B cell longevity and plasma cell development came up. Abnormal morphology of plasma cells and survival of memory B cells with a delay in their division had increased measurement.

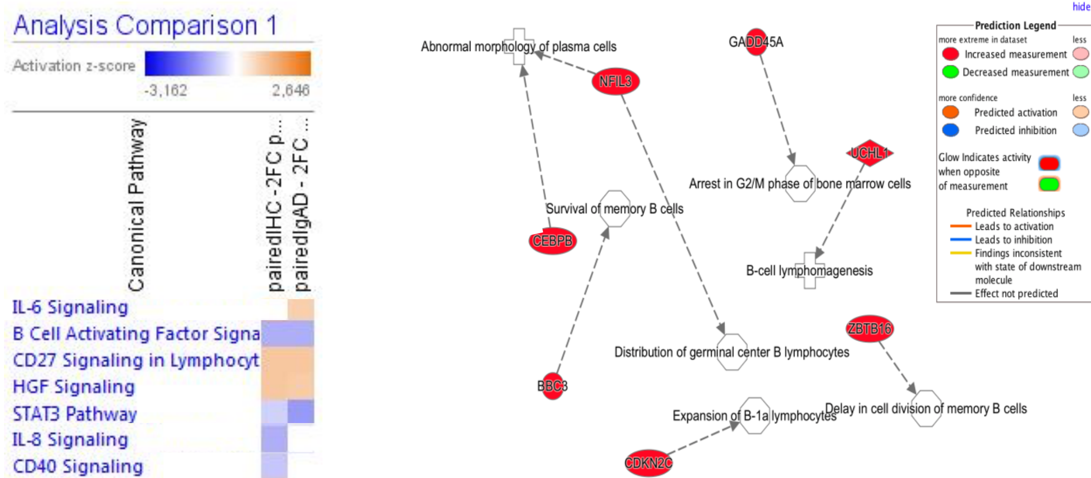

Figure 24 . Ingenuity pathway analysis of transcripts in sIgAD.
Differentially enriched pathways by Ingenuity pathway analysis of CpG stimulated B cells from sIgAD individuals compared to HCs. The pathays linked to B cell development shows defects in pathways associated with the survival and maturation of Ab secreting cells; e.g. paythways associated with delay in cell division of memory B cells, distribution of germinal centers, survival of memory B cells and abnormal morphology of plasma cells.

4.3 The dysregulated IgA positive B cell compartment with IL-12, TWEAK and IL-18 changes do not affect MAIT cells

Mucosal invariant T cells (MAIT) cells are important at mucosal sites where the defective IgA secreting B cells are dominant. Furthermore, they rely on IL-18, with IL18R shown to be differentially transcribed in B cells in sIgAD as well as IL-18 and TWEAK are shown to be dysregulated in serum of sIgAD individuals. Therefore, we assessed their fractions in sIgAD. No significant changes were seen in MAIT cells in sIgAD individuals pointing towards a cytokine redundant mechanism for their induction and their possible non-dependence of IgA positive B cells for their maintenance.

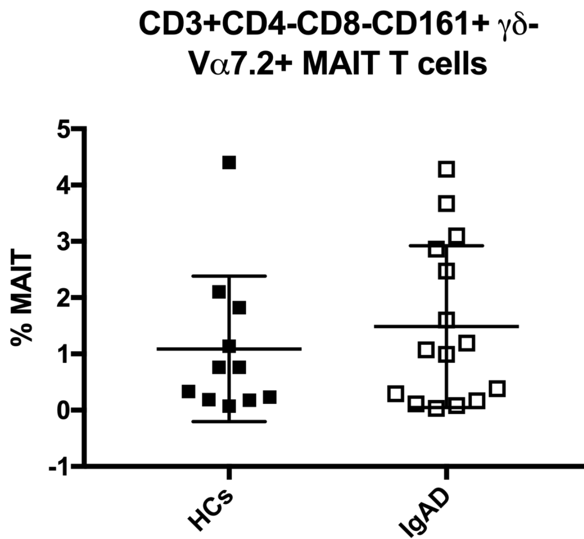

Figure 25 . MAIT cells in sIgAD individuals are not significantly fewer in sIgAD compared to HCs.

The analysis of MAIT cells from PBMCs from sIgAD individuals and HCs are shown. MAIT cells are defined by their expression of CD3+CD4-CD8-CD161+gammadelta-Balpha7.2+. Data compares 10 HCs and 15 sIgAD individuals. No significance was found.

5 Discussion

In this study the clinical, serologic and cellular phenotype of sIgAD has been evaluated. This has been done with the analysis of the clinical and immunological profile of sIgAD individuals. Including a detailed clinical questionnaire, extensive cellular functional and phenotyping analyses, with particular focus on IgA isotype switching and secretion, serological immune biomarkers in sIgAD and finally transcriptomic analyses linked to IgA.

The main findings of our study are:

a) As previously shown, sIgAD individuals are more prone to severe respiratory infections, atopy, asthma and autoimmunity.

b) Novel linking of key serological immune biomarkers to sIgAD was identified and included; CCL3, TWEAK, TLSP and sCD40L that were linked to autoantibody seropositivity.

c) T cell independent dysregulation of IgA production involving transitional B cell function

d) Dysregulation of B cell signalling linked to IL-21 driven Stat-3 phosphorylation

e) B cell driven transcriptomic analyses identified several potentially important transcripts with direct connection to autoimmunity, B cell maturation, IgA production, cytokine secretion and Stat-3 signalling.

5.1 Clinical phenotype of sIgAD individuals

The clinical phenotype of sIgAD individuals is characterised by a higher prevalence of upper respiratory infections, atopic manifestations and autoimmunity similar to what has been previously described by our group and others (G. H. Jorgensen et al., 2013). However, the significance of this study is that our current sIgAD group is larger and has a more stringent inclusion criterion. The description is done in a retrospective way and therefore has the caveats involved in such an approach. However, this retrospective approach enabled us to investigate the association with autoimmune seropositivity to the established clinical findings. Our description of clinical symptoms does underline the importance of following individuals with Ab deficiencies. Furthermore, it encourages to find the mechanisms responsible to find better

targets for personalised treatments of their comorbidities (Yazdani et al., 2015). That classification does assume that autoimmunity is always worse than atopy, which is challenged by the quality of life data in sIgAD (G. H. Jorgensen et al., 2014).

One of the possible future steps to take would be to do a prospective study of Ab deficiencies. One could then assess the baseline of immunological biomarkers and cellular phenotypes with various degrees of IgA levels and follow the clinical course of specific subgroups. This could be technically challenging since not all individuals with lower immunoglobulins have clinical symptoms and are therefore not found nor followed by health care personnel.

5.1.1 Infections

The self-reported infection rate in sIgAD individuals in the study showed more frequent upper and lower respiratory tract infections. The predisposition to pneumonias is especially striking with a prevalence of 33% compared to 15% in HCs.

Related to other upper respiratory tract infections, an increase in sinusitis was seen in our expanded group not previously seen by prior analysis on a bigger group where only a trend was seen.

The same was not seen for gastrointestinal infections or urogenital infections. For these, no increase was seen in our group. This is in concordance with what was previously seen in a smaller group and by others (G. H. Jorgensen et al., 2013). This is especially interesting due to the extreme abundance of IgA at the mucosal surface. The exact reason for why gastrointestinal infections is not a major problem is not fully understood but the recent advances in faecal metagenomics from sIgAD individuals and CVID individuals do though shed some light on the matter. In sIgAD, no major changes are seen in faecal bacterial taxa while in CVID no enteropathy is seen unless IgM is also affected. Thus, strengthening the prior redundancy by IgM hypothesised to protect sIgAD individuals from gastrointestinal problems. This is interesting in relation to the amount of upper respiratory problems encountered in sIgAD and does point towards the need to better assess the metagenomics of the upper respiratory tract.

5.1.1 Atopy

The prevalence of allergic diseases in our sIgAD group was high. In total 26% had a self-reported eczema. These results strengthen the previous study about sIgAD in Iceland (G. H. Jorgensen et al., 2013). This is also interesting seeing

the heterogeneity of prior studies related to the different atopic and asthmatic presentations. The fact that the dominant atopic manifestations are related to skin, but not mucosal surfaces, may possibly indicate a relationship between a breach in immune homeostasis related to the evaluated immune biomarkers seen to be dysregulated in this study but not directly to IgA itself. Related to biological mechanisms this may point towards a non-classical atopic presentation related to TH2 polarisation since no T helper cell skewing was seen in our cellular analysis. In the analysis of serologic immune biomarkers a potential connection is though seen with TSLP (Indra, 2013). Potentially opening the possibility of personalised treatment options for PAD and atopic presentations inside the tumult of novel treatments related to the phenotype/endotype classifications of asthma (Wenzel, 2012)

5.1.2 Autoimmunity

In our enlarged group the differences seen with HCS do still indicate a higher prevalence of autoimmune presentation with about a fourth of the individuals suffering from some type of autoimmune presentations. This is higher than what was seen in the control group and is estimated to be the prevalence in the western world. The association with autoimmunity has been previously demonstrated by our group and by others but never in such a fashion or on such a large scale. The reason for the increase in autoimmunity in sIgAD is not fully known. Some have hypothesised that a defect in clearance at mucosal surfaces could lead to inflammation. Others have linked it to the survival of potentially autoreactive clones because of cytokine dysregulation and T cell regulation (Jacob et al., 2008). Some have also linked sIgAD to a common genetic background for autoimmunity and sIgAD. The link to HLA regions common to autoimmunity, namely HLAB8, DR3, DQ2 (8.1), has been especially strong. But about 45% of sIgAD have at least one copy of these compared to 10% in the normal population (Mohammadi et al., 2010; Olerup, Smith, & Hammarstrom, 1990).

Besides wanting to understand the pathogenesis, the main message from our clinical evaluation is that clinicians should keep in mind not to forget the possibility of an underlying PID when evaluating individuals with autoimmunity. Those with a patient with a PID should not to forget to inquire about autoimmunity, both directly as for family history and for the evaluation of the need of a follow-up.

The heterogenous phenotypic clinical presentations made us want to evaluate individuals without clinical symptoms in a more biological fashion. Our hypothesis in that manner was that we would not have the interference of

inflammation mediated by inflammatory diseases but also possibly find better tools to evaluate biomarkers related to the pathogenesis of comorbidities associated with sIgAD.

From a biological standpoint related to the clinical phenotype characterised by immune dysregulation, we describe no increase in Th2 polarisation known to be related to atopy, nor Th1/th17 polarisation known to be important in autoimmunity, or dampened function of Tregs important for example in the resolution of inflammation. Though implementing the role of other pathways possibly linked to B cell driven autoinflammation (Minguet et al., 2017; Tsubata, 2017) and the role of other isotypes than IgE in atopic diseases (Chen et al., 2009).

5.2 Serum concentrations of cytokines and autoantibodies

The high prevalence of immune mediated diseases in our group and the phenotypic defects in B cell lymphocyte populations described make it likely that a dysregulation in B cells may be implicated in the pathogenesis. Furthermore, previous publications have shown higher autoantibody production in sIgAD (G. H. Jorgensen et al., 2011; Gudmundur H. Jorgensen et al., 2009; Ning Wang et al., 2011). The evaluation of such phenomenon can be biased due to the measurement of individuals with ongoing disease. For example, with the interference of chronic inflammation or immunomodulatory drugs. However, it has been described that individuals with asymptomatic sIgAD have higher prevalence of autoantibody production (Barka et al., 1995). By using our analysis of autoantibodies and immune biomarkers we identified key immune serologic biomarkers linked to sIgAD and B cell development. The raised concentration was true for TWEAK, TSLP, sCD40 and CCL3. Their concentrations could be related to autoreactivity or provide a potential link to lymphocyte alterations as well as autoimmune and atopic dysregulation in these individuals. Related to TSLP prior animal models have shown a link between its production and B cell autoreactivity. Thus, autoantibody production has been associated with serum levels of TSLP were it may have a protective role in autoantibody formation (Astrakhan et al., 2007). In addition, sCD40L, CCL3 and TWEAK (Benet, Wu, Marthi, Turner, & Grigorova, 2016; Min et al., 2016) are influential at the germinal centre level for autoreactivity. Furthermore, CD40L may have a dual role during autoantibody formation both as a tolerogenic molecule, but also in autoimmune reactivity (Arpin et al., 1995b). This is of special interest in connection to signalling strength and duration following CD40L stimulation during different B cell maturational stages (Arpin et al., 1995a). In contrast, CCL3 does bring about questions related to

the homing of B cells during their different maturational stages. This links to the surface homing markers that where shown to be normal in B cells from sIgAD in this study.

In using our mathematical PCA modelling, we see a clear differentiation between the sIgA and HC group. The dissociation indicates a potential link between these factors and autoantibody positivity. That is, that seropositive individuals may have higher serum concentrations of the evaluated cytokines., but a clear causal relationship has not been demonstrated. It must be acknowledged though that some individuals had autoantibodies without being the highest producers of these serum factors.

5.3 Cellular phenotype of sIgAD individuals

The cellular analysis of our sIgAD group does to a large extent confirm a phenotype with no big alterations in T cell populations, restricting sIgAD mainly to a B cell defect.

5.3.1 B cells

Our results related to the phenotyping of B cells show alterations in the early transitional maturation stages of B cells and in class switched B cells. The transitional defect is seen in peripheral blood before stimulation but is also seen after TLR9 agonist stimulation. Furthermore, IL-10 production is known to be sensitive to immune alterations in this populations is diminished. The induction of IgA production by TLR9 agonist stimulation did furthermore not induces normal production. The addition of IL-10, previously shown to induce IgA production in sIgAD did not reverse the production defect, even though such stimulation did induce the phenotypic defect in IL-10 producing cells.

The hypothesis that defective production could be related to a homing defect does not seem to be true based on surface expression of homing markers on B cells.

The autoimmunity in B cell mediated autoimmunity has been linked to hyperactivation of plasma blasts. The assessment of HLA-DR did not show any differences in sIgAD in our analysis. Thus, leaving us with a relatively benign B cell phenotype with a non-hyperreactive but dysregulated B cell phenotype with early maturation defects.

The defect in transitional B cells is interesting. The defect is also seen in many pathologies related to immune dysregulation possibly indicating responses to immune dysregulation. That said, the lack of homogeneity of the

population must be acknowledged. Some have even proposed to revisit the paradigm of transitional B cells (Martin et al., 2016). As is evident though from post Hematopoietic cell transplantation, rituximab treated patients or new-born children (Marasco et al., 2017) the transitional B cell compartment of CD24highCD38high is mainly representative of B cells that have recently emerged from the bone marrow. As such, they are following a classical trajectory to get survival signals (Ikemiyagi et al., 2017). Furthermore, it has been shown that the transitional B cell compartment has not undergone somatic hypermutation (Berkowska et al., 2011). One question here may therefore be why the transitional B cells are so sensitive, are they comprised of cells with an already determined fate of IgG, IgA and IgM and how can these cells be differentiating? In infants some transitional B cells are dedicated to an IgM phenotype going to splenic sites to mature but in adults it may be that such a population may rather be inclined to become IgA secreting after stimulation at mucosal sites (Weller et al., 2004).

5.3.1 T cells

In sIgAD the main T cell subtypes are of normal fractions with equal production of cytokines and surface expression of chemokines related to the different T Helper (TH) subtypes. The fraction’s inducibility and suppressive capacity of Tregs are furthermore normal. This is in conjunction with recently published data about TH cells and somewhat in relation to prior described studies (Fadlallah et al., 2018). But we did not see the in CD4+ or CD8+ populations, nor in Tregs alterations that a prior study has described (Soheili et al., 2013). This could be explained by our stringent gating methods with CD4+CD25highCD127−(FoxP3high) Tregs for both nTregs and iTregs and that we chose not only sIgAD individuals with serious autoimmune presentations. But autoimmunity is known to affect Tregs, due to cytokines overexpressed in autoimmunity and inflammation. We furthermore evaluated their suppressive functions in an induced inflammation. That is important, since the sole use of nTregs in groups prone to inflammation, and immune dysregulation could be misleading. Also since it has been shown that numbers may not represent function in Tregs (Morgan et al., 2005). Tregs and T – B cell interactions for IgA induction may be mostly important at mucosal surfaces, not tested in our study, which is also true for Th and TFH cell subsets.

5.4 Signalling

In our experimental setup estimating intracellular signalling, B and T cells responded in heterogenous ways. Furthermore, sIgAD individuals responded

in all pathways shown to be defective in relationship to IgA production in defective individuals. Significantly lower signalling was seen though in pSTAT3 signalling in B cells only after IL-21 stimulation. In the functional assessment of sIgAD individuals, B cells are less reactive to T cell independent stimulations than T cell dependent stimulations. It is therefore interesting that in our experimental setup the signal transduction related to CpG is normal while the one related to IL-21 is abrogated. Though it is known that TLR9 may predispose stronger STAT3 activation by IL-21 in B cells, a link not assessed by this study (B.-S. Liu, Stoop, Huizinga, & Toes, 2013). It could also be that the differences are related to the dysregulated environment from which these cells are extracted. Thus, related to the high serum concentration of sCD40L and others serologic biomarkers seen to be dysregulated. It can also be an inherent intracellular defect in the cells. The latter being less likely since other stimulations lead to normal pSTAT3 activation. In addition, this can be due to a specific maturation defect seen in the phenotyping of B cells from the sIgAD individuals.

5.5 Transcriptomic profile of sIgAD

Our transcriptomic analysis of sIgAD individuals does indicate differences related to B cell maturation, both before and after activation with CpG. The results are very much in conjunction with the functional assays analysing cells using flow cytometric analysis and analysis of serum from sIgAD individuals. In this thesis, the variation is shown implicating the heterogeneity of B cell responses related to T cell independent stimulation in the sIgAD individuals. By pathway and GO analysis one can furthermore see how these different responses do lead to a paucity in pathways used, possibly indicating maturational halt. The GO analysis does furthermore indicate a defect linked to T cell independent responses that need further assessment. Of the differentially expressed genes, the pseudogene ANKRD36BP2 may be of the highest interest. It has been shown to be one of the 150 genes upregulated with a Fold change (FC) of 4.8 from plasma blasts compared to naïve or memory B cells in a project characterising the changes in expression in these populations (Jourdan et al., 2009). While its expression is widely expressed in lymph nodes and thymus in transcriptome atlases it is not so highly expressed in peripheral blood (Fagerberg et al., 2014). Therefore, its relevance to IgA class switching needs to be evaluated further with functional assays. Interestingly, other genes related to immune dysregulation such as PIGR have an increased expression. PIGR is a poly-Ig receptor that binds polymeric immunoglobulin molecules at the basolateral surface of epithelial cells to

transport them across the cell to be secreted at the apical surface. Interestingly a significant association has been found between immunoglobulin A nephropathy and several single-nucleotide polymorphism (SNPs) in this gene (Narita et al., 2001) where abnormal transitional B cell numbers are also seen (Y. Y. Wang et al., 2014).

5.6 Biases of the study

Working with defect or a disease affecting a rather small population does always induce the biases of power that are difficult to overcome in our study. The serological assessment also suffers from power that must be weighed against the potential benefit of not including individuals with many defects in immune dysregulations with immunomodulating drugs. This might be a problem though when analysing slighter differences e.g. in BAFF or APRIL, which one could possibly have detected with bigger numbers of individuals and/or a more restricted multiplex panel. We chose to measure more factors and rather get the factors that may be greatly differing, since we think it may be more biologically relevant to get larger than smaller alterations. Again, the cellular analysis could have seen slighter variations in a bigger group but should not affect the null to full reaction in relationship to the TLR9 induction of the group. With regards to the signalling pathways a broader proteomic analysis could have given more information but again could have been more difficult for slighter variations and with a possible limitation of the number of stimulant possible to use due to means available. Overall, we are aware of the limitations of studying a small group and the limitation of each technique but try to our best to draw conclusions taking these into consideration.

6 Conclusions

In this study we present results demonstrating alterations related to the clinical phenotype, cellular dysregulation and non-responsiveness of B cells to specific stimulations in sIgAD. They all indicate, through different mechanisms, a link to dysfunctional peripheral maturation of B cells.

With the presented results we hypothesise a mechanism related to the autoimmunity and immune dysregulation seen in sIgAD. A hypothesis where some underlying epi- or genetic abnormality or variance may be present that has not been found. Where a profound T cell dependent and T cell independent defect is seen in B cells leading to developmental halt with abrogated transitional B cells and class switched B cells pointing towards abnormal germinal centre reactions. The hypothesis that a germinal centre defect is present is strengthened by immune biomarkers found in serum. The defect is though without abnormal induction of T helper subsets or Tregs. Together, this leads to a defect in IgA production and IgA positive B cells without altered homing or plasma blast activation. At the end, this potentially leads to a dysregulated immune environment, leading to a loss in central tolerance and/or dysregulated feedback loops of immune homeostasis loss due to the lack of IgA and IgA+ B cells in the periphery, tissues and bone marrow.

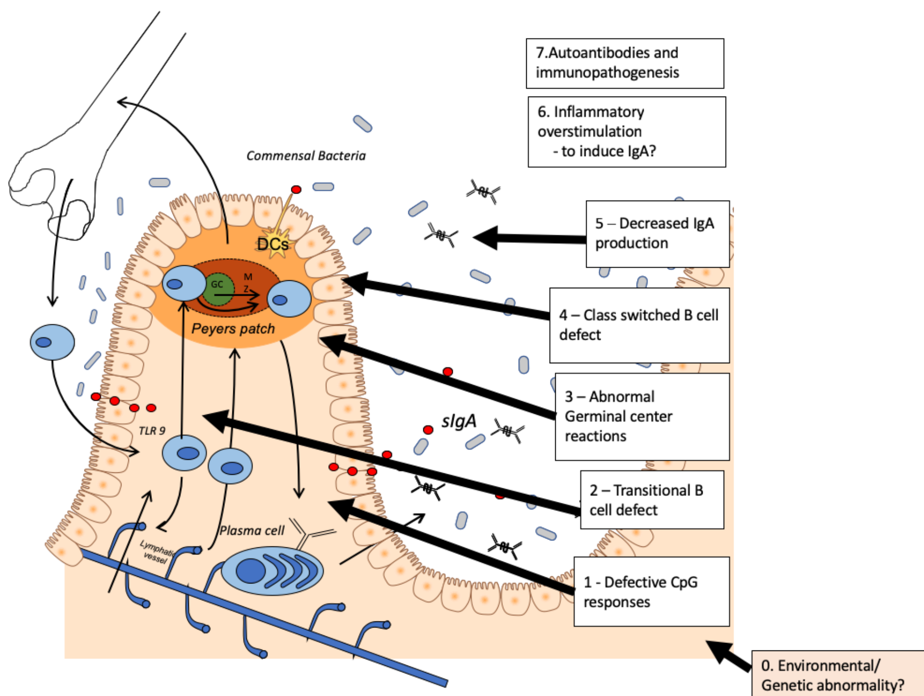

Figure 26 . Hypothetic model of immune dysregulation based on the experimental data presented in the thesis.

The schematic figure shows the maturation of B cells recently emerged from the bone marrow. (1) sIgAD B cells do not react normally to T cell independent stimulations. (2) They have a transitional B cell defect. (3) There is an abnormal germinal centre reaction reflected by (4) low numbers of class switched B cells leading to abnormal IgA production (5) with high serum concentrations of immune factors (6) and development of autoimmunity (7).

This dysregulation may enable the activation and survival of autoreactive lymphocytes possibly with changed reactivity in certain intracellular signalling pathways enabling the error of self-targeting, autoreactivity and hypersensitivity.

This model has limitations, leaving a lot of things left to explain and experimental and observational research to be done. We do though think it may help lead the way for future research related to the role of IgA and/or immune dysregulation. Eventually opening the possibility of a link between

serologic immune biomarkers in autoantibody deficiencies and personalised treatment option.

In a more specific manner conclusions can be drawn from each part of the study.

Regarding the clinical phenotyping of our group we strengthen the known alterations in immune surveillance and autoreactivity in sIgAD individuals. The data both indicates the need of better monitoring of hypogammaglobulinemia's, potentially using novel technological advances for better evaluation and personalised treatments. The findings related to the increased risk of respiratory problems with a relatively benign gastrointestinal (GI) course may be due to our control of GI infections with western hygienic standards but poor control of respiratory infections. It may also indicate the need for further explorations of the role of IgA at mucosal surfaces. Potentially evaluating the interaction of new or old players related to infections and atopic problems (Chen et al., 2009; Toussaint et al., 2017). This is especially interesting due to CVID where enteropathy has been shown to have redundancy through IgM, but CVID patients are known to be especially prone to serious respiratory infections with the development of bronchiectasis (Shulzhenko et al., 2018).

The serological abnormalities found in the sIgAD group do indicate wide alterations in immune reactivity in factors not expected to be altered while BAFF and APRIL, which hypothetically one could think to be altered were normal. The fact that the dysregulation is not seen after B cell stimulation indicates the need to think about the effects of the gap present when no IgA is there but also when no IgA positive B cells are present, possibly on T cells, Innate lymphoid cells (ILCs) or other non-lymphoid cells. This does need further investigation in other PADs with and without autoimmunity and in HCs, for the effect of IgA inducing stimulations. One possibility is that these alterations could affect the seen abrogated transitional cell compartment or class switched B cells.

The cellular differences in B cells with no visible defect in T cells points towards an isolated B cell defect, concomitant with the findings related to the newest diagnostic criteria for CVID (Al-Herz et al., 2011; Wehr et al., 2008). The described defect in responsiveness related to TLR9 induction does bring up questions related to the different pathways responsible for B cell maturation after emergence from the bone marrow. A lot has been done for the understanding of the maturation of IgM producing memory B cells but the exact differences between the different groups of IgA producing B cells is still poorly

understood. Having a better understanding of these different pathways may be needed before understanding the meaning of defects in specific subpopulations of B cells. The advent of multicolour flow cytometry and help of automatic gating possibilities may be of use there to understand beyond the scope of two-dimensional gating. The problem with heterogeneous diseases is though that it may be difficult link the biological to a relevant approach in clinical practice. Clinical trials, even of low power, may be indicative such scenarios of which way to go for the individualised approach to the patient (Wehr et al., 2008).

The transcriptomic analysis brings up many questions related to the effect of singular defects on the maturation of antibodies. The pathways and molecules found are important to assess further by functional assays, e.g. by looking at the variation of their protein level expression after different IgA inducing stimulation. This is especially true in relation to the genes related to faulty TLR activation, ANKRD36BP2 related to mucosal inflammation and plasma cells and PIGR related to IgA nephropathy and IgA transport.

The strength of this study is an expansion of a one of a kind case control study about sIgAD with the addition of extensive immunological investigations related to cellular and serological evaluation. I believe that it highlights the importance of continuing investigations of clinical, cellular, genetic and pathophysiological features of PADs. With more clinical data available, different genetic phenotypes of sIgAD are likely to be discovered. With better cellular and biochemical understanding, better treatments can be developed. However, in order to achieve this, the focus may be on larger cohorts extending prior boundaries with wider Ab deficiencies while at the same time zooming in on smaller subgroups with specific phenotypes (Janssen et al., 2018; Moschese et al., 2018). The newest knowledge in the field of basic immunology and biotechnology must be used to investigate the different compartments related to the production of Abs and their mechanisms. Last but not least friendships in research and creative zeal must have their place in the path to new discoveries (Davis, 2018).

References

Aghamohammadi, A., Abolhassani, H., Biglari, M., Abolmaali, S., Moazzami, K., Tabatabaeiyan, M., . . . Rezaei, N. (2011). Analysis of switched memory B cells in patients with IgA deficiency. *Int Arch Allergy Immunol, 156*(4), 462-468. doi:10.1159/000323903

Aghamohammadi, A., Cheraghi, T., Gharagozlou, M., Movahedi, M., Rezaei, N., Yeganeh, M., . . . Moin, M. (2009). IgA deficiency: correlation between clinical and immunological phenotypes. *J Clin Immunol, 29*(1), 130-136. doi:10.1007/s10875-008-9229-9

Aghamohammadi, A., Mohammadi, J., Parvaneh, N., Rezaei, N., Moin, M., Espanol, T., & Hammarstrom, L. (2008). Progression of selective IgA deficiency to common variable immunodeficiency. *Int Arch Allergy Immunol, 147*(2), 87-92. doi:10.1159/000135694

Aghamohammadi, A., Sedighipour, L., Saeed, S. E., Kouhkan, A., Heydarzadeh, M., & Pourpak, Z. (2008). Alterations in humoral immunity in relatives of patients with common variable immunodeficiency. *J Investig Allergol Clin Immunol, 18*(4), 266-271.

Agrawal, S., Smith, S. A., Tangye, S. G., & Sewell, W. A. (2013). Transitional B cell subsets in human bone marrow. *Clin Exp Immunol, 174*(1), 53-59. doi:10.1111/cei.12149

al-Attas, R. A., & Rahi, A. H. (1998). Primary antibody deficiency in Arabs: first report from eastern Saudi Arabia. *J Clin Immunol, 18*(5), 368-371.

Al-Herz, W., Bousfiha, A., Casanova, J. L., Chapel, H., Conley, M. E., Cunningham-Rundles, C., . . . Tang, M. L. (2011). Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. *Front Immunol, 2*, 54. doi:10.3389/fimmu.2011.00054

Arpin, C., Dechanet, J., Van Kooten, C., Merville, P., Grouard, G., Briere, F., . . . Liu, Y. J. (1995a). Generation of memory B cells and plasma cells in vitro. *Science, 268*(5211), 720-722.

Arpin, C., Dechanet, J., Van Kooten, C., Merville, P., Grouard, G., Briere, F., . . . Liu, Y. J. (1995b). Generation of memory B cells and plasma cells in vitro. *Science, 268*(5211), 720.

Asano, T., Kaneko, H., Terada, T., Kasahara, Y., Fukao, T., Kasahara, K., & Kondo, N. (2004). Molecular analysis of B-cell differentiation in selective or partial IgA deficiency. *Clin Exp Immunol, 136*(2), 284-290. doi:10.1111/j.1365-2249.2004.02440.x

Astrakhan, A., Omori, M., Nguyen, T., Becker-Herman, S., Iseki, M., Aye, T., . . . Rawlings, D. J. (2007). Local increase in thymic stromal lymphopoietin induces systemic alterations in B cell development. *Nat Immunol, 8*(5), 522-531. doi:10.1038/ni1452

Bagnara, D., Squillario, M., Kipling, D., Mora, T., Walczak, A. M., Da Silva, L., . . . Reynaud, C. A. (2015). A Reassessment of IgM Memory Subsets in Humans. *J Immunol, 195*(8), 3716-3724. doi:10.4049/jimmunol.1500753

Barka, N., Shen, G. Q., Shoenfeld, Y., Alosachie, I. J., Gershwin, M. E., Reyes, H., & Peter, J. B. (1995). Multireactive pattern of serum autoantibodies in asymptomatic individuals with immunoglobulin A deficiency. *Clin Diagn Lab Immunol, 2*(4), 469-472.

Barsotti, Nathalia Silveira, Almeida, Rafael Ribeiro, Costa, Priscilla Ramos, Barros, Myrthes Toledo, Kalil, Jorge, & Kokron, Cristina Maria. (2016). IL-10-Producing Regulatory B Cells Are Decreased in Patients with Common Variable Immunodeficiency. *PLoS ONE, 11*(3), e0151761. doi:10.1371/journal.pone.0151761

Behniafard, N., Aghamohammadi, A., Abolhassani, H., Pourjabbar, S., Sabouni, F., & Rezaei, N. (2012). Autoimmunity in X-linked agammaglobulinemia: Kawasaki disease and review of the literature. *Expert Rev Clin Immunol, 8*(2), 155-159. doi:10.1586/eci.11.94

Bemark, M., Boysen, P., & Lycke, N. Y. (2012). Induction of gut IgA production through T cell-dependent and T cell-independent pathways. *Ann N Y Acad Sci, 1247*, 97-116. doi:10.1111/j.1749-6632.2011.06378.x

Benet, Zachary, Wu, Rita, Marthi, Matangi, Turner, Jackson, & Grigorova, Irina. (2016). B cell-intrinsic production of CCL3/4 promotes direct sampling of Germinal Center B cells by T follicular regulatory cells and limits non-foreign antigen specific B cell response. *The Journal of Immunology, 196*(1 Supplement), 195.113-195.113.

Berglund, L. J., Avery, D. T., Ma, C. S., Moens, L., Deenick, E. K., Bustamante, J., . . . Tangye, S. G. (2013). IL-21 signalling via STAT3 primes human naive B cells to respond to IL-2 to enhance their differentiation into plasmablasts. *Blood, 122*(24), 3940-3950. doi:10.1182/blood-2013-06-506865

Bergqvist, P., Gardby, E., Stensson, A., Bemark, M., & Lycke, N. Y. (2006). Gut IgA class switch recombination in the absence of CD40 does not occur in the lamina propria and is independent of germinal centers. *J Immunol, 177*(11), 7772-7783.

Berkowska, M. A., Driessen, G. J., Bikos, V., Grosserichter-Wagener, C., Stamatopoulos, K., Cerutti, A., . . . van Zelm, M. C. (2011). Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood, 118*(8), 2150-2158. doi:10.1182/blood-2011-04-345579

Bernasconi, N. L., Traggiai, E., & Lanzavecchia, A. (2002). Maintenance of serological memory by polyclonal activation of human memory B cells. *Science, 298*(5601), 2199-2202. doi:10.1126/science.1076071

Bernasconi, Nadia L., Traggiai, Elisabetta, & Lanzavecchia, Antonio. (2002). Maintenance of Serological Memory by Polyclonal Activation of Human Memory B Cells. *Science, 298*(5601), 2199-2202. doi:10.1126/science.1076071

Boller, S., & Grosschedl, R. (2014). The regulatory network of B-cell differentiation: a focused view of early B-cell factor 1 function. *Immunol Rev, 261*(1), 102-115. doi:10.1111/imr.12206

Borte, S., Pan-Hammarstrom, Q., Liu, C., Sack, U., Borte, M., Wagner, U., . . . Hammarstrom, L. (2009). Interleukin-21 restores immunoglobulin production ex vivo in patients with common variable immunodeficiency and selective IgA deficiency. *Blood, 114*(19), 4089-4098. doi:10.1182/blood-2009-02-207423

Bouchard, B., Ormandy, C. J., Di Santo, J. P., & Kelly, P. A. (1999). Immune system development and function in prolactin receptor-deficient mice. *J Immunol, 163*(2), 576-582.

Briere, F., Bridon, J. M., Chevet, D., Souillet, G., Bienvenu, F., Guret, C., . . . Banchereau, J. (1994). Interleukin 10 induces B lymphocytes from IgA-deficient patients to secrete IgA. *J Clin Invest, 94*(1), 97-104. doi:10.1172/jci117354

Buckley , R. H., & Dees , S. C. (1969). Correlation of Milk Precipitins with IgA Deficiency. *New England Journal of Medicine, 281*(9), 465-469. doi:10.1056/nejm196908282810903

Bunker, Jeffrey J, Flynn, Theodore M, Koval, Jason C, Shaw, Dustin G, Meisel, Marlies, McDonald, Benjamin D, . . . Bendelac, Albert. (2015). Innate and Adaptive Humoral Responses Coat Distinct Commensal Bacteria with Immunoglobulin A. *Immunity, 43*(3), 541-553. doi:https://doi.org/10.1016/j.immuni.2015.08.007

Capolunghi, F., Rosado, M. M., Sinibaldi, M., Aranburu, A., & Carsetti, R. (2013). Why do we need IgM memory B cells? *Immunol Lett, 152*(2), 114-120. doi:10.1016/j.imlet.2013.04.007

Capolunghi, Federica, Cascioli, Simona, Giorda, Ezio, Rosado, Maria Manuela, Plebani, Alessandro, Auriti, Cinzia, . . . Carsetti, Rita. (2008). CpG Drives Human Transitional B Cells to Terminal Differentiation and Production of Natural Antibodies. *The Journal of Immunology, 180*(2), 800-808. doi:10.4049/jimmunol.180.2.800

Carretero Gomez, J., Vera Tome, A., Arevalo Lorido, J. C., & Munoz Sanz, A. (2003). [Follicular lymphoid hyperplasia, IgA deficiency and coinfecction of Giardia lamblia and Epstein-Barr virus]. *Gastroenterol Hepatol, 26*(6), 397-398.

Casanova, J. L., & Abel, L. (2013). The genetic theory of infectious diseases: a brief history and selected illustrations. *Annu Rev Genomics Hum Genet, 14*, 215-243. doi:10.1146/annurev-genom-091212-153448

Cassidy, J. T., Burt, A., Petty, R., & Sullivan, D. (1969). Selective IgA deficiency in connective tissue diseases. *N Engl J Med, 280*(5), 275.

Cerutti, Andrea. (2008). The regulation of IgA class switching. *Nature Reviews Immunology, 8*, 421. doi:10.1038/nri2322

Chan, A. C., & Behrens, T. W. (2013). Personalizing medicine for autoimmune and inflammatory diseases. *Nat Immunol, 14*(2), 106-109. doi:10.1038/ni.2473

Chaudhuri, J., & Alt, F. W. (2004). Class-switch recombination: interplay of transcription, DNA deamination and DNA repair. *Nat Rev Immunol, 4*(7), 541-552. doi:10.1038/nri1395

Chen, K., Xu, W., Wilson, M., He, B., Miller, N. W., Bengten, E., . . . Cerutti, A. (2009). Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. *Nat Immunol, 10*(8), 889-898. doi:10.1038/ni.1748

Chintalacharuvu, K. R., Chuang, P. D., Dragoman, A., Fernandez, C. Z., Qiu, J., Plaut, A. G., . . . Morrison, S. L. (2003). Cleavage of the human immunoglobulin A1 (IgA1) hinge region by IgA1 proteases requires structures in the Fc region of IgA. *Infect Immun, 71*(5), 2563-2570.

Collin, Matthew, Dickinson, Rachel, & Bigley, Venetia. (2015). Haematopoietic and immune defects associated with GATA2 mutation. *Br J Haematol, 169*(2), 173-187. doi:10.1111/bjh.13317

Cong, Yingzi, Feng, Ting, Fujihashi, Kohtaro, Schoeb, Trenton R., & Elson, Charles O. (2009). A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. *Proc Natl Acad Sci U S A, 106*(46), 19256-19261. doi:10.1073/pnas.0812681106

Crotty, Shane. (2014). T follicular helper cell differentiation, function, and roles in disease. *Immunity, 41*(4), 529-542. doi:10.1016/j.immuni.2014.10.004

Cunningham-Rundles, C. (2001). Physiology of IgA and IgA deficiency. *J Clin Immunol, 21*(5), 303-309.

Cunningham-Rundles, C. (2011). Autoimmunity in primary immune deficiency: taking lessons from our patients. *Clin Exp Immunol, 164 Suppl 2*, 6-11. doi:10.1111/j.1365-2249.2011.04388.x

Cunningham-Rundles, C., & Ponda, P. P. (2005). Molecular defects in T- and B-cell primary immunodeficiency diseases. *Nat Rev Immunol, 5*(11), 880-892. doi:10.1038/nri1713

Cunningham-Rundles, C., Pudifin, D. J., Armstrong, D., & Good, R. A. (1980). Selective IgA deficiency and neoplasia. *Vox Sang, 38*(2), 61-67.

Cuss, Amanda K., Avery, Danielle T., Cannons, Jennifer L., Yu, Li Jun, Nichols, Kim E., Shaw, Peter J., & Tangye, Stuart G. (2006). Expansion of Functionally Immature Transitional B Cells Is Associated with Human-Immunodeficient States Characterized by Impaired Humoral Immunity. *J Immunol, 176*(3), 1506-1516. doi:10.4049/jimmunol.176.3.1506

Davis, Daniel M (2018). *The beautifull cure* (1 ed.). London: Penguin Boogs.

De la Concha, E. G., Subiza, J. L., Fontán, G., Pascual-Salcedo, D., Sequí, J., & Bootello, A. (1982). Disorders of regulatory T cells in patients with selective IgA deficiency and its relationship to associated autoimmune phenomena. *Clin Exp Immunol, 49*(2), 410-418.

Donaldson, G. P., Ladinsky, M. S., Yu, K. B., Sanders, J. G., Yoo, B. B., Chou, W.-C., . . . Mazmanian, S. K. (2018). Gut microbiota utilize immunoglobulin A for mucosal colonization. *Science, 360*(6390), 795-800. doi:10.1126/science.aaq0926

Edwards, E., Razvi, S., & Cunningham-Rundles, C. (2004). IgA deficiency: clinical correlates and responses to pneumococcal vaccine. *Clin Immunol, 111*(1), 93-97. doi:10.1016/j.clim.2003.12.005

Eibel, Hermann. (2015). Early B Cell Development. In Alessandro Plebani & Vassilios Lougaris (Eds.), *Agammaglobulinemia* (pp. 1-17). Cham: Springer International Publishing.

Eren Akarcan, Sanem, Ulusoy Severcan, Ezgi, Edeer Karaca, Neslihan, Isik, Esra, Aksu, Guzide, Migaud, Mélanie, . . . Kutukculer, Necil. (2017). Gain-of-Function Mutations in STAT1: A Recently Defined Cause for Chronic Mucocutaneous Candidiasis Disease Mimicking Combined Immunodeficiencies. *Case reports in immunology, 2017*, 2846928-2846928. doi:10.1155/2017/2846928

Eren, M., Saltik-Temizel, I. N., Yuce, A., Caglar, M., & Kocak, N. (2007). Duodenal appearance of giardiasis in a child with selective immunoglobulin A deficiency. *Pediatr Int, 49*(3), 409-411. doi:10.1111/j.1442-200X.2007.02357.x

Evans, Michael D., Esnault, Stephane, Denlinger, Loren C., & Jarjour, Nizar N. (2018). Sputum cell IL-1 receptor expression level is a marker of airway neutrophilia and airflow obstruction in asthmatic patients. *Journal of Allergy and Clinical Immunology, 142*(2), 415-423. doi:10.1016/j.jaci.2017.09.035

Fadlallah, Jehane, El Kafsi, Hela, Sterlin, Delphine, Juste, Catherine, Parizot, Christophe, Dorgham, Karim, . . . Gorochov, Guy. (2018). Microbial ecology perturbation in human IgA deficiency. *Sci Transl Med, 10*(439). doi:10.1126/scitranslmed.aan1217

Fagarasan, S., & Honjo, T. (2003). Intestinal IgA synthesis: regulation of front-line body defences. *Nat Rev Immunol, 3*(1), 63-72. doi:10.1038/nri982

Fagerberg, L., Hallstrom, B. M., Oksvold, P., Kampf, C., Djureinovic, D., Odeberg, J., . . . Uhlen, M. (2014). Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol Cell Proteomics, 13*(2), 397-406. doi:10.1074/mcp.M113.035600

Fagraeus, A. (1947). Plasma cellular reaction and its relation to the formation of antibodies in vitro. *Nature, 159*(4041), 499.

Farhadi, E., Nemati, S., Amirzargar, A. A., Hirbod-Mobarakeh, A., Nabavi, M., Soltani, S., . . . Rezaei, N. (2013). AICDA single nucleotide polymorphism in common variable immunodeficiency and selective IgA deficiency. *Allergol Immunopathol (Madr)*. doi:10.1016/j.aller.2013.02.002

Feng, M. L., Zhao, Y. L., Shen, T., Huang, H., Yin, B., Liu, R. Z., . . . Liu, D. Z. (2011). Prevalence of immunoglobulin A deficiency in Chinese blood donors and evaluation of anaphylactic transfusion reaction risk. *Transfus Med, 21*(5), 338-343. doi:10.1111/j.1365-3148.2011.01082.x

Ferreira, A., Garcia Rodriguez, M. C., Lopez-Trascasa, M., Pascual Salcedo, D., & Fontan, G. (1988). Anti-IgA antibodies in selective IgA deficiency and in primary immunodeficient patients treated with gamma-globulin. *Clin Immunol Immunopathol, 47*(2), 199-207.

Ferreira, R. C., Pan-Hammarstrom, Q., Graham, R. R., Fontan, G., Lee, A. T., Ortmann, W., . . . Behrens, T. W. (2012). High-density SNP mapping of the HLA region identifies multiple independent susceptibility loci associated with selective IgA deficiency. *PLoS Genet, 8*(1), e1002476. doi:10.1371/journal.pgen.1002476

Ferreira, R. C., Pan-Hammarstrom, Q., Graham, R. R., Gateva, V., Fontan, G., Lee, A. T., . . . Hammarstrom, L. (2010). Association of IFIH1 and other autoimmunity risk alleles with selective IgA deficiency. *Nat Genet, 42*(9), 777-780. doi:10.1038/ng.644

Fischer, Alain, Notarangelo, Luigi D., Neven, Bénédicte, Cavazzana, Marina, & Puck, Jennifer M. (2015). Severe combined immunodeficiencies and related disorders. *Nature Reviews Disease Primers, 1*, 15061. doi:10.1038/nrdp.2015.61

Fransen, Floris, Zagato, Elena, Mazzini, Elisa, Fosso, Bruno, Manzari, Caterina, El Aidy, Sahar, . . . Rescigno, Maria. (2015). BALB/c and C57BL/6 Mice Differ in Polyreactive IgA Abundance, which Impacts the Generation of Antigen-Specific IgA and Microbiota Diversity. *Immunity, 43*(3), 527-540. doi:https://doi.org/10.1016/j.immuni.2015.08.011

Fujieda, S., Waschek, J. A., Zhang, K., & Saxon, A. (1996). Vasoactive intestinal peptide induces S(alpha)/S(mu) switch circular DNA in human B cells. *J Clin Invest, 98*(7), 1527-1532. doi:10.1172/jci118944

Funakoshi, N., Kanoh, T., Mizumoto, S., & Uchino, H. (1985). Autoantibodies, including antibasement membrane antibody, in patients with selective IgA deficiency. *Tohoku J Exp Med, 145*(1), 49-55.

Goshen, E., Livne, A., Krupp, M., Hammarström, L., Dighiero, G., Slor, H., & Shoenfeld, Y. (1989). Antinuclear and related autoantibodies in sera of healthy subjects with IgA deficiency. *Journal of Autoimmunity, 2*(1), 51-60. doi:https://doi.org/10.1016/0896-8411(89)90107-8

Hammarström, Lennart, Ringdén, Olle, Lönnqvist, Berit, Smith, C. I. Edvard, & Wiebe, Thomas. (1985). TRANSFER OF IgA DEFICIENCY TO A BONE-MARROW-GRAFTED PATIENT WITH APLASTIC ANAEMIA. *The Lancet, 325*(8432), 778-781. doi:http://dx.doi.org/10.1016/S0140-6736(85)91446-1

Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D., & Hayakawa, K. (1991). Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J Exp Med, 173*(5), 1213-1225.

He, B., Xu, W., Santini, P. A., Polydorides, A. D., Chiu, A., Estrella, J., . . . Cerutti, A. (2007). Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. *Immunity, 26*(6), 812-826. doi:10.1016/j.immuni.2007.04.014

Heneghan, M. A., Stevens, F. M., Cryan, E. M., Warner, R. H., & McCarthy, C. F. (1997). Celiac sprue and immunodeficiency states: a 25-year review. *J Clin Gastroenterol, 25*(2), 421-425.

Henriques, A., Silva, I., Ines, L., Souto-Carneiro, M. M., Pais, M. L., Trindade, H., . . . Paiva, A. (2016). CD38, CD81 and BAFFR combined expression by transitional B cells distinguishes active from inactive systemic lupus erythematosus. *Clin Exp Med, 16*(2), 227-232. doi:10.1007/s10238-015-0348-3

Hummelshoj, L., Ryder, L. P., Nielsen, L. K., Nielsen, C. H., & Poulsen, L. K. (2006). Class switch recombination in selective IgA-deficient subjects. *Clin Exp Immunol, 144*(3), 458-466. doi:10.1111/j.1365-2249.2006.03096.x

Hurley, Walter L., & Theil, Peter K. (2011). Perspectives on immunoglobulins in colostrum and milk. *Nutrients, 3*(4), 442-474. doi:10.3390/nu3040442

Ikemiyagi, Masako, Hirai, Toshihito, Ishii, Rumi, Miyairi, Satoshi, Okumi, Masayoshi, & Tanabe, Kazunari. (2017). Transitional B Cells Predominantly Reconstituted After a Desensitization Therapy Using Rituximab Before Kidney Transplantation. *Therapeutic Apheresis and Dialysis, 21*(2), 139-149. doi:10.1111/1744-9987.12508

Indra, Arup K. (2013). Epidermal TSLP: a trigger factor for pathogenesis of atopic dermatitis. *Expert review of proteomics, 10*(4), 309-311. doi:10.1586/14789450.2013.814881

Jacob, C. M., Pastorino, A. C., Fahl, K., Carneiro-Sampaio, M., & Monteiro, R. C. (2008). Autoimmunity in IgA deficiency: revisiting the role of IgA as a silent housekeeper. *J Clin Immunol, 28 Suppl 1*, S56-61. doi:10.1007/s10875-007-9163-2

Janssen, Lisanne M. A., Bassett, Paul, Macken, Thomas, van Esch, Jolanda, Pruijt, Hans, Knoops, Arnoud, . . . de Vries, Esther. (2018). Mild Hypogammaglobulinemia Can Be a Serious Condition. *Frontiers in Immunology, 9*(2384). doi:10.3389/fimmu.2018.02384

Janzi, M., Kull, I., Sjoberg, R., Wan, J., Melen, E., Bayat, N., . . . Hammarstrom, L. (2009). Selective IgA deficiency in early life: association to infections and allergic diseases during childhood. *Clin Immunol, 133*(1), 78-85. doi:10.1016/j.clim.2009.05.014

Jatiani, S. S., Baker, S. J., Silverman, L. R., & Reddy, E. P. (2010). Jak/STAT pathways in cytokine signaling and myeloproliferative disorders: approaches for targeted therapies. *Genes Cancer, 1*(10), 979-993. doi:10.1177/1947601910397187

JimÉNez, Adolfo, Alvarez-Doforno, Rita, RodrÍGuez, M. Cruz GarcÍA, Ferreira, Antonio, LÓPez-Trascasa, Margarita, & Fontan, Gumersindo. (1991). Autoantibodies in patients with IgA and IgG2 deficiencies. *APMIS, 99*(1-6), 327-332. doi:10.1111/j.1699-0463.1991.tb05157.x

Johnson, G. L., & Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science, 298*(5600), 1911-1912. doi:10.1126/science.1072682

Jonsson, Stefan, Sveinbjornsson, Gardar, de Lapuente Portilla, Aitzkoa Lopez, Swaminathan, Bhairavi, Plomp, Rosina, Dekkers, Gillian, . . . Stefansson, Kari. (2017). Identification of sequence variants influencing immunoglobulin levels. *Nature Genetics, 49*, 1182. doi:10.1038/ng.3897

https://www.nature.com/articles/ng.3897#supplementary-information

Jorgensen, G. H., Gardulf, A., Sigurdsson, M. I., Arnlaugsson, S., Hammarstrom, L., & Ludviksson, B. R. (2014). Health-related quality of life (HRQL) in immunodeficient adults with selective IgA deficiency compared with age- and gender-matched controls and identification of risk factors for poor HRQL. *Qual Life Res, 23*(2), 645-658. doi:10.1007/s11136-013-0491-9

Jorgensen, G. H., Gardulf, A., Sigurdsson, M. I., Sigurdardottir, S. T., Thorsteinsdottir, I., Gudmundsson, S., . . . Ludviksson, B. R. (2013). Clinical symptoms in adults with selective IgA deficiency: a case-control study. *J Clin Immunol, 33*(4), 742-747. doi:10.1007/s10875-012-9858-x

Jorgensen, G. H., Ornolfsson, A. E., Johannesson, A., Gudmundsson, S., Janzi, M., Wang, N., . . . Ludviksson, B. R. (2011). Association of immunoglobulin A deficiency and elevated thyrotropin-receptor autoantibodies in two Nordic countries. *Hum Immunol, 72*(2), 166-172. doi:10.1016/j.humimm.2010.10.014

Jorgensen, Gudmundur H., Thorsteinsdottir, Ingunn, Gudmundsson, Sveinn, Hammarstrom, Lennart, & Ludviksson, Bjorn R. (2009). Familial aggregation of IgAD and autoimmunity. *J Clin Immunol, 131*(2), 233-239. doi:http://dx.doi.org/10.1016/j.clim.2008.11.013

Jourdan, Michel, Caraux, Anouk, De Vos, John, Fiol, Geneviève, Larroque, Marion, Cognot, Chantal, . . . Klein, Bernard. (2009). An in vitro model of differentiation of memory B cells into plasmablasts and plasma cells including detailed phenotypic and molecular characterization. *Blood, 114*(25), 5173-5181. doi:10.1182/blood-2009-07-235960

Kamae, Chikako, Nakagawa, Noriko, Sato, Hiroki, Honma, Kenichi, Mitsuiki, Noriko, Ohara, Osamu, . . . Nonoyama, Shigeaki. (2013). Common variable immunodeficiency classification by quantifying T-cell receptor and immunoglobulin κ-deleting recombination excision circles. *Journal of Allergy and Clinical Immunology, 131*(5), 1437-1440.e1435. doi:10.1016/j.jaci.2012.10.059

Kau, A. L., Planer, J. D., Liu, J., Rao, S., Yatsunenko, T., Trehan, I., . . . Gordon, J. I. (2015). Functional characterization of IgA-targeted bacterial taxa from undernourished Malawian children that produce diet-dependent enteropathy. *Sci Transl Med, 7*(276), 276ra224. doi:10.1126/scitranslmed.aaa4877

Kawamoto, Shimpei, Maruya, Mikako, Kato, Lucia M, Suda, Wataru, Atarashi, Koji, Doi, Yasuko, . . . Fagarasan, Sidonia. (2014). Foxp3+ T Cells Regulate Immunoglobulin A Selection and Facilitate Diversification of Bacterial Species Responsible for Immune Homeostasis. *Immunity, 41*(1), 152-165. doi:https://doi.org/10.1016/j.immuni.2014.05.016

Kim, Hyun-Jung, Ko, Young Hyeh, Kim, Ji Eun, Lee, Seung-Sook, Lee, Hyekyung, Park, Gyeongsin, . . . Hematopathology Study Group of the Korean Society of, Pathologists. (2017). Epstein-Barr Virus-Associated Lymphoproliferative Disorders: Review and Update on 2016 WHO Classification. *Journal of pathology and translational medicine, 51*(4), 352-358. doi:10.4132/jptm.2017.03.15

Klemola, T., Savilahti, E., Arato, A., Ormala, T., Partanen, J., Eland, C., & Koskimies, S. (1995). Immunohistochemical findings in jejunal specimens from patients with IgA deficiency. *Gut, 37*(4), 519-523.

Koistinen, J. (1976). Familial clustering of selective IgA deficiency. *Vox Sang, 30*(3), 181-190.

Koistinen, J., & Sarna, S. (1975). Immunological Abnormalities in the Sera of IgA-Deficient Blood Donors. *Vox Sanguinis, 29*, 203-213.

Koskinen, S. (1996). Long-term follow-up of health in blood donors with primary selective IgA deficiency. *J Clin Immunol, 16*(3), 165-170.

Koskinen, S., Tolo, H., Hirvonen, M., & Koistinen, J. (1994). Long-term persistence of selective IgA deficiency in healthy adults. *J Clin Immunol, 14*(2), 116-119.

Krutzik, Peter O., Clutter, Matthew R., Trejo, Angelica, & Nolan, Garry P. (2011). Fluorescent Cell Barcoding for Multiplex Flow Cytometry. *Current protocols in cytometry / editorial board, J. Paul Robinson, managing editor ... [et al.], CHAPTER*, Unit-6.31. doi:10.1002/0471142956.cy0631s55

Kunkel, E. J., & Butcher, E. C. (2003). Plasma-cell homing. *Nat Rev Immunol, 3*(10), 822-829. doi:10.1038/nri1203

Lantsoght, E.O.L. (2018). *The A-Z of the PhD Trajectory: A Practical Guide for a Successful Journey*: Springer International Publishing.

Liblau, R., Fischer, A-M., Shapiro, D. E., Morel, E., & Bach, J-F. (1992). The frequency of selective IgA deficiency in myasthenia gravis. *Neurology, 42*(3), 516-516. doi:10.1212/wnl.42.3.516

Liblau, R. S., & Bach, J. F. (1992). Selective IgA deficiency and autoimmunity. *Int Arch Allergy Immunol, 99*(1), 16-27.

Little, Colleen, Blattner, Collin M., & Young, John, 3rd. (2017). Update: Can breastfeeding and maternal diet prevent atopic dermatitis? *Dermatology practical & conceptual, 7*(3), 63-65. doi:10.5826/dpc.0703a14

Litzman, J., Ševčíková, I., Štikarovská, D., Pikulová, Z., Pazdírková, A., & Lokaj, J. (2000). IgA Deficiency in Czech Healthy Individuals and Selected Patient Groups. *Int Arch Allergy Immunol, 123*(2), 177-180. doi:10.1159/000024438

Litzman, J., Vlková, M., Pikulová, Z., Štikarovská, D., & Lokaj, J. (2007). T and B lymphocyte subpopulations and activation/differentiation markers in patients with selective IgA deficiency. *Clinical & Experimental Immunology, 147*(2), 249-254. doi:10.1111/j.1365-2249.2006.03274.x

Liu, Bi-Sheng, Stoop, Jeroen N., Huizinga, Tom W., & Toes, Rene E. M. (2013). IL-21 Enhances the Activity of the TLR–MyD88–STAT3 Pathway but Not the Classical TLR–MyD88–NF-κB Pathway in Human B Cells To Boost Antibody Production. *The Journal of Immunology, 191*(8), 4086-4094. doi:10.4049/jimmunol.1300765

Liu, Justin K. H. (2014). The history of monoclonal antibody development – Progress, remaining challenges and future innovations. *Annals of Medicine and Surgery, 3*(4), 113-116. doi:https://doi.org/10.1016/j.amsu.2014.09.001

Louis, Ankmalika Gupta, Agrawal, Sudhanshu, & Gupta, Sudhir. (2016). Analysis of subsets of B cells, Breg, CD4Treg and CD8Treg cells in adult patients with primary selective IgM deficiency. *American Journal of Clinical and Experimental Immunology, 5*(1), 21-32.

Ludvigsson, J. F., Neovius, M., & Hammarstrom, L. (2013). IgA deficiency & mortality: a population-based cohort study. *J Clin Immunol, 33*(8), 1317-1324. doi:10.1007/s10875-013-9948-4

Ludvigsson, J. F., Neovius, M., Ye, W., & Hammarstrom, L. (2015). IgA deficiency and risk of cancer: a population-based matched cohort study. *J Clin Immunol, 35*(2), 182-188. doi:10.1007/s10875-014-0124-2

Mackay, F., & Schneider, P. (2009). Cracking the BAFF code. *Nat Rev Immunol, 9*(7), 491-502. doi:10.1038/nri2572

Macpherson, A. J., & Harris, N. L. (2004). Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol, 4*(6), 478-485. doi:10.1038/nri1373

Macpherson, A. J., McCoy, K. D., Johansen, F. E., & Brandtzaeg, P. (2008). The immune geography of IgA induction and function. *Mucosal Immunol, 1*(1), 11-22. doi:10.1038/mi.2007.6

Magri, Giuliana, Comerma, Laura, Pybus, Marc, Sintes, Jordi, Lligé, David, Segura-Garzón, Daniel, . . . Cerutti, Andrea. (2017). Human Secretory IgM Emerges from Plasma Cells Clonally Related to Gut Memory B Cells and Targets Highly Diverse Commensals. *Immunity, 47*(1), 118-134.e118. doi:10.1016/j.immuni.2017.06.013

Malphettes, Marion, Gérard, Laurence, Carmagnat, Maryvonnick, Mouillot, Gaël, Vince, Nicolas, Boutboul, David, . . . Group, Defi Study. (2009). Late-Onset Combined Immune Deficiency: A Subset of Common Variable Immunodeficiency with Severe T Cell Defect. *Clinical Infectious Diseases, 49*(9), 1329-1338. doi:10.1086/606059

Manz, Rudolf A., Thiel, Andreas, & Radbruch, Andreas. (1997). Lifetime of plasma cells in the bone marrow. *Nature, 388*, 133. doi:10.1038/40540

Marasco, E., Farroni, C., Cascioli, S., Marcellini, V., Scarsella, M., Giorda, E., . . . Carsetti, R. (2017). B-cell activation with CD40L or CpG measures the function of B-cell subsets and identifies specific defects in immunodeficient patients. *Eur J Immunol, 47*(1), 131-143. doi:10.1002/eji.201646574

Marconi, M., Plebani, A., Avanzini, M. A., Maccario, R., Pistorio, A., Duse, M., . . . Monafo, V. (1998). IL-10 and IL-4 co-operate to normalize in vitro IgA production in IgA-deficient (IgAD) patients. *Clin Exp Immunol, 112*(3), 528-532.

Mariette, X., Seror, R., Quartuccio, L., Baron, G., Salvin, S., Fabris, M., . . . De Vita, S. (2013). Efficacy and safety of belimumab in primary Sjogren's syndrome: results of the BELISS open-label phase II study. *Ann Rheum Dis*. doi:10.1136/annrheumdis-2013-203991

Martin, Victoria G., Wu, Yu-Chang Bryan, Townsend, Catherine L., Lu, Grace H. C., O'Hare, Joselli Silva, Mozeika, Alexander, . . . Dunn-Walters, Deborah K. (2016). Transitional B Cells in Early Human B Cell Development - Time to Revisit the Paradigm? *Frontiers in Immunology, 7*, 546-546. doi:10.3389/fimmu.2016.00546

Mazanec, M. B., Kaetzel, C. S., Lamm, M. E., Fletcher, D., & Nedrud, J. G. (1992). Intracellular neutralization of virus by immunoglobulin A antibodies. *Proc Natl Acad Sci U S A, 89*(15), 6901-6905.

MD, Carsten Speckmann, PhD, Annette Uhlmann, MSc, Sam Doerken, PhD, Martin Wolkewitz, Pohl, Annette, & MD, Stephan Ehl. (2015). A prospective outcome study of patients with profound combined immunodeficiency (P-CID). *LymphoSign Journal, 2*(2), 91-106. doi:10.14785/lpsn-2015-0002

Medvedovic, J., Ebert, A., Tagoh, H., & Busslinger, M. (2011). Pax5: a master regulator of B cell development and leukemogenesis. *Adv Immunol, 111*, 179-206. doi:10.1016/b978-0-12-385991-4.00005-2

Meesilpavikkai, Kornvalee, Dik, Willem A., Schrijver, Benjamin, Nagtzaam, Nicole M. A., Posthumus-van Sluijs, Sandra J., van Hagen, P. Martin, & Dalm, Virgil A. S. H. (2018). Baricitinib treatment in a patient with a gain-of-function mutation in signal transducer and activator of transcription 1 (STAT1). *Journal of Allergy and Clinical Immunology, 142*(1), 328-330.e322. doi:10.1016/j.jaci.2018.02.045

Mei, H. E., Yoshida, T., Sime, W., Hiepe, F., Thiele, K., Manz, R. A., . . . Dorner, T. (2009). Blood-borne human plasma cells in steady state are derived from mucosal immune responses. *Blood, 113*(11), 2461-2469. doi:10.1182/blood-2008-04-153544

Mei, Henrik E., Frölich, Daniela, Giesecke, Claudia, Loddenkemper, Christoph, Reiter, Karin, Schmidt, Stefanie, . . . Dörner, Thomas. (2010). Steady-state generation of mucosal IgA plasmablasts is not abrogated by B-cell depletion therapy with rituximab. *Blood, 116*(24), 5181-5190. doi:10.1182/blood-2010-01-266536

Mei, Henrik E., Yoshida, Taketoshi, Muehlinghaus, Gwendolin, Hiepe, Falk, Dörner, Thomas, Radbruch, Andreas, & Hoyer, Bimba F. (2007). Phenotypic Analysis of B-Cells and Plasma Cells. In Andrew P. Cope (Ed.), *Arthritis Research: Methods and Protocols Volume 2* (pp. 3-18). Totowa, NJ: Humana Press.

Mei, Henrik E., Yoshida, Taketoshi, Sime, Wondossen, Hiepe, Falk, Thiele, Kathi, Manz, Rudolf A., . . . Dörner, Thomas. (2009). Blood-borne human plasma cells in steady state are derived from mucosal immune responses. *Blood, 113*(11), 2461-2469. doi:10.1182/blood-2008-04-153544

Mellemkjaer, L., Hammarstrom, L., Andersen, V., Yuen, J., Heilmann, C., Barington, T., . . . Olsen, J. H. (2002). Cancer risk among patients with IgA deficiency or common variable immunodeficiency and their relatives: a combined Danish and Swedish study. *Clin Exp Immunol, 130*(3), 495-500.

Mendelson, Avital, & Frenette, Paul S. (2014). Hematopoietic stem cell niche maintenance during homeostasis and regeneration. *Nature medicine, 20*, 833. doi:10.1038/nm.3647

Mestas, Javier, & Hughes, Christopher C. W. (2004). Of Mice and Not Men: Differences between Mouse and Human Immunology. *The Journal of Immunology, 172*(5), 2731-2738. doi:10.4049/jimmunol.172.5.2731

Milner, Joshua D., & Holland, Steven M. (2013). The cup runneth over: lessons from the ever-expanding pool of primary immunodeficiency diseases. *Nat Rev Immunol, 13*(9), 635-648. doi:10.1038/nri3493

Min, Hong-Ki, Kim, Sung-Min, Park, Jin-Sil, Byun, Jae-Kyeong, Lee, Jennifer, Kwok, Seung-Ki, . . . Park, Sung-Hwan. (2016). Fn14-Fc suppresses germinal center formation and pathogenic B cells in a lupus mouse model via inhibition of the TWEAK/Fn14 Pathway. *Journal of Translational Medicine, 14*(1), 98. doi:10.1186/s12967-016-0846-4

Minguet, S., Klasener, K., Schaffer, A. M., Fiala, G. J., Osteso-Ibanez, T., Raute, K., . . . Del Pozo, M. A. (2017). Caveolin-1-dependent nanoscale organization of the BCR regulates B cell tolerance. *Nat Immunol, 18*(10), 1150-1159. doi:10.1038/ni.3813

Mohammadi, J., Ramanujam, R., Jarefors, S., Rezaei, N., Aghamohammadi, A., Gregersen, P. K., & Hammarstrom, L. (2010). IgA deficiency and the MHC: assessment of relative risk and microheterogeneity within the HLA A1 B8, DR3 (8.1) haplotype. *J Clin Immunol, 30*(1), 138-143. doi:10.1007/s10875-009-9336-2

Montag, D. T., & Lotze, M. T. (2006). Rapid flow cytometric measurement of cytokine-induced phosphorylation pathways [CIPP] in human peripheral blood leukocytes. *Clin Immunol, 121*(2), 215-226. doi:10.1016/j.clim.2006.06.013

Morgan, Mary E., van Bilsen, Jolanda H. M., Bakker, Aleida M., Heemskerk, Bianca, Schilham, Marco W., Hartgers, Franca C., . . . Toes, René E. M. (2005). Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Human Immunology, 66*(1), 13-20. doi:http://dx.doi.org/10.1016/j.humimm.2004.05.016

Moschese, Viviana, Chini, Loredana, Graziani, Simona, Sgrulletti, Mayla, Gallo, Vera, Di Matteo, Gigliola, . . . Specchia, Fernando. (2018). Follow-up and outcome of symptomatic partial or absolute IgA deficiency in children. *Eur J Pediatr*. doi:10.1007/s00431-018-3248-1

Mukherjee, S., & Bhattacharya, P. (2011). Severe anaphylactic reaction in IgA deficient patient following transfusion of whole blood. *Asian journal of transfusion science, 5*(2), 177-177. doi:10.4103/0973-6247.83248

Muramatsu, M., Nagaoka, H., Shinkura, R., Begum, N. A., & Honjo, T. (2007). Discovery of activation-induced cytidine deaminase, the engraver of antibody memory. *Adv Immunol, 94*, 1-36. doi:10.1016/s0065-2776(06)94001-2

Narita, I., Kondo, D., Goto, S., Saito, N., Watanabe, Y., Yamazaki, H., . . . Gejyo, F. (2001). Association of gene polymorphism of polymeric immunoglobulin receptor and IgA nephropathy. *Intern Med, 40*(9), 867-872.

Nechvatalova, J., Pavlik, T., Litzman, J., & Vlkova, M. (2017). Terminally differentiated memory T cells are increased in patients with common variable immunodeficiency and selective IgA deficiency. *Cent Eur J Immunol, 42*(3), 244-251. doi:10.5114/ceji.2017.70966

Nechvatalova, J., Pikulova, Z., Stikarovska, D., Pesak, S., Vlkova, M., & Litzman, J. (2012). B-lymphocyte subpopulations in patients with selective IgA deficiency. *J Clin Immunol, 32*(3), 441-448. doi:10.1007/s10875-012-9655-6

Ochtrop, Manuella L. Gomes, Goldacker, Sigune, May, Annette M., Rizzi, Marta, Draeger, Ruth, Hauschke, Dieter, . . . Peter, Hans Hartmut. (2011). T and B lymphocyte abnormalities in bone marrow biopsies of common variable immunodeficiency. *Blood, 118*(2), 309-318. doi:10.1182/blood-2010-11-321695

Odegard, V. H., & Schatz, D. G. (2006). Targeting of somatic hypermutation. *Nat Rev Immunol, 6*(8), 573-583. doi:10.1038/nri1896

Olerup, O., Smith, C. I., & Hammarstrom, L. (1990). Different amino acids at position 57 of the HLA-DQ beta chain associated with susceptibility and resistance to IgA deficiency. *Nature, 347*(6290), 289-290. doi:10.1038/347289a0

Olin, Axel, Henckel, Ewa, Chen, Yang, Lakshmikanth, Tadepally, Pou, Christian, Mikes, Jaromir, . . . Brodin, Petter. (2018). Stereotypic Immune System Development in Newborn Children. *Cell, 174*(5), 1277-1292.e1214. doi:https://doi.org/10.1016/j.cell.2018.06.045

Ozsoylu, S., Karabent, A., & Irken, G. (1988). Selective IgA deficiency in childhood ITP. *Eur J Haematol, 41*(1), 95.

Pabst, Oliver. (2012). New concepts in the generation and functions of IgA. *Nat Rev Immunol, 12*(12), 821-832.

Palm, N. W., de Zoete, M. R., Cullen, T. W., Barry, N. A., Stefanowski, J., Hao, L., . . . Flavell, R. A. (2014). Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell, 158*(5), 1000-1010. doi:10.1016/j.cell.2014.08.006

Pan, X., Ji, Z., & Xue, J. (2016). Percentage of Peripheral CD19+CD24hiCD38hi Regulatory B Cells in Neonatal Sepsis Patients and Its Functional Implication. *Med Sci Monit, 22*, 2374-2378.

Pape, K. A., Catron, D. M., Itano, A. A., & Jenkins, M. K. (2007). The humoral immune response is initiated in lymph nodes by B cells that acquire soluble antigen directly in the follicles. *Immunity, 26*(4), 491-502. doi:10.1016/j.immuni.2007.02.011

Perri, Valentina, Gianchecchi, Elena, Scarpa, Riccardo, Valenzise, Mariella, Rosado, Maria Manuela, Giorda, Ezio, . . . Fierabracci, Alessandra. Altered B cell homeostasis and Toll-like receptor 9-driven response in patients affected by autoimmune polyglandular syndrome Type 1: Altered B cell phenotype and dysregulation of the B cell function in APECED patients. *Immunobiology*. doi:http://dx.doi.org/10.1016/j.imbio.2016.09.001

Polosukhin, V. V., Richmond, B. W., Du, R. H., Cates, J. M., Wu, P., Nian, H., . . . Blackwell, T. S. (2017). Secretory IgA Deficiency in Individual Small Airways Is Associated with Persistent Inflammation and Remodeling. *Am J Respir Crit Care Med, 195*(8), 1010-1021. doi:10.1164/rccm.201604-0759OC

Pott, Marian-Christopher, Frede, Natalie, Wanders, Jennifer, Hammarström, Lennart, Glocker, Erik-Oliver, Glocker, Cristina, . . . Grimbacher, Bodo. (2017). Autoantibodies against BAFF, APRIL or IL21 - an alternative pathogenesis for antibody-deficiencies? *BMC immunology, 18*(1), 34-34. doi:10.1186/s12865-017-0217-9

Quach, T. D., Rodriguez-Zhurbenko, N., Hopkins, T. J., Guo, X., Hernandez, A. M., Li, W., & Rothstein, T. L. (2016). Distinctions among Circulating Antibody-Secreting Cell Populations, Including B-1 Cells, in Human Adult Peripheral Blood. *J Immunol, 196*(3), 1060-1069. doi:10.4049/jimmunol.1501843

Radbruch, A., Muehlinghaus, G., Luger, E. O., Inamine, A., Smith, K. G., Dorner, T., & Hiepe, F. (2006). Competence and competition: the challenge of becoming a long-lived plasma cell. *Nat Rev Immunol, 6*(10), 741-750. doi:10.1038/nri1886

Rakhmanov, M., Keller, B., Gutenberger, S., Foerster, C., Hoenig, M., Driessen, G., . . . Warnatz, K. (2009). Circulating CD21low B cells in common variable immunodeficiency resemble tissue homing, innate-like B cells. *Proc Natl Acad Sci U S A, 106*(32), 13451-13456. doi:10.1073/pnas.0901984106

Rockey, J. H., Hanson, L. A., Heremans, J. F., & Kunkel, H. G. (1964). BETA-2A AGLOBULINEMIA IN TWO HEALTHY MEN. *J Lab Clin Med, 63*, 205-212.

Rolf M. Zinkernagel, Martin F. Bachmann, Thomas M. Kündig, Stephan Oehen, Hanspeter Pirchet, and, & Hengartner, Hans. (1996). ON IMMUNOLOGICAL MEMORY. *Annual Review of Immunology, 14*(1), 333-367. doi:10.1146/annurev.immunol.14.1.333

Rosser, E. C., & Mauri, C. (2015). Regulatory B cells: origin, phenotype, and function. *Immunity, 42*(4), 607-612. doi:10.1016/j.immuni.2015.04.005

Savilahti, E., Klemola, T., Carlsson, B., Mellander, L., Stenvik, M., & Hovi, T. (1988). Inadequacy of mucosal IgM antibodies in selective IgA deficiency: excretion of attenuated polio viruses is prolonged. *J Clin Immunol, 8*(2), 89-94.

Savilahti, E., Pelkonen, P., & Visakorpi, J. K. (1971). IgA deficiency in children. A clinical study with special reference to intestinal findings. *Arch Dis Child, 46*(249), 665-670.

Schmidt, R. E., Grimbacher, B., & Witte, T. (2017). Autoimmunity and primary immunodeficiency: two sides of the same coin? *Nat Rev Rheumatol, 14*(1), 7-18. doi:10.1038/nrrheum.2017.198

Scott, L. J., Bryant, A., Webster, A. D., & Farrant, J. (1994). Failure in IgA secretion by surface IgA-positive B cells in common variable immunodeficiency (CVID). *Clin Exp Immunol, 95*(1), 10-13.

Sellars, Maclean, Kastner, Philippe, & Chan, Susan. (2011). Ikaros in B cell development and function. *World journal of biological chemistry, 2*(6), 132-139. doi:10.4331/wjbc.v2.i6.132

Shkalim, V., Monselize, Y., Segal, N., Zan-Bar, I., Hoffer, V., & Garty, B. Z. (2010). Selective IgA deficiency in children in Israel. *J Clin Immunol, 30*(5), 761-765. doi:10.1007/s10875-010-9438-x

Shrivastava, S., TrehanPati, N., Kottilil, S., & Sarin, S. K. (2013). Decline in immature transitional B cells after hepatitis B vaccination in hepatitis B positive newborns. *Pediatr Infect Dis J, 32*(7), 792-794. doi:10.1097/INF.0b013e31828df344

Shulzhenko, N., Dong, X., Vyshenska, D., Greer, R. L., Gurung, M., Vasquez-Perez, S., . . . Morgun, A. (2018). CVID enteropathy is characterized by exceeding low mucosal IgA levels and interferon-driven inflammation possibly related to the presence of a pathobiont. *Clin Immunol, 197*, 139-153. doi:10.1016/j.clim.2018.09.008

Simell, B., Kilpi, T., & Kayhty, H. (2006). Subclass distribution of natural salivary IgA antibodies against pneumococcal capsular polysaccharide of type 14 and pneumococcal surface adhesin A (PsaA) in children. *Clin Exp Immunol, 143*(3), 543-549. doi:10.1111/j.1365-2249.2006.03009.x

Simon, Q., Pers, J. O., Cornec, D., Le Pottier, L., Mageed, R. A., & Hillion, S. (2016). In-depth characterization of CD24(high)CD38(high) transitional human B cells reveals different regulatory profiles. *J Allergy Clin Immunol, 137*(5), 1577-1584.e1510. doi:10.1016/j.jaci.2015.09.014

Smith, Graeme C.M., & Jackson, Stephen P. (1999). The DNA-dependent protein kinase. *Genes & Development, 13*(8), 916-934.

Soheili, H., Abolhassani, H., Arandi, N., Khazaei, H. A., Shahinpour, S., Hirbod-Mobarakeh, A., . . . Aghamohammadi, A. (2013). Evaluation of natural regulatory T cells in subjects with selective IgA deficiency: from senior idea to novel opportunities. *Int Arch Allergy Immunol, 160*(2), 208-214. doi:10.1159/000339867

Soler-Palacin, P., Cobos-Carrascosa, E., Martin-Nalda, A., Caracseghi, F., Hernandez, M., & Figueras-Nadal, C. (2016). [Is familial screening useful in selective immunoglobulin A deficiency?]. *An Pediatr (Barc), 84*(2), 70-78. doi:10.1016/j.anpedi.2015.04.017

Spencer, J., & Sollid, L. M. (2016). The human intestinal B-cell response. *Mucosal Immunol, 9*(5), 1113-1124. doi:10.1038/mi.2016.59

Suchanek, O., Sadler, R., Bateman, E. A., Patel, S. Y., & Ferry, B. L. (2012). Immunophenotyping of putative human B1 B cells in healthy controls and common variable immunodeficiency (CVID) patients. *Clin Exp Immunol, 170*(3), 333-341. doi:10.1111/j.1365-2249.2012.04656.x

Svachova, V., Sekerkova, A., Hruba, P., Tycova, I., Rodova, M., Cecrdlova, E., . . . Viklicky, O. (2016). Dynamic changes of B-cell compartments in kidney transplantation: lack of transitional B cells is associated with allograft rejection. *Transpl Int, 29*(5), 540-548. doi:10.1111/tri.12751

Szabo, K., Papp, G., Szanto, A., Tarr, T., & Zeher, M. (2016). A comprehensive investigation on the distribution of circulating follicular T helper cells and B cell subsets in primary Sjogren's syndrome and systemic lupus erythematosus. *Clin Exp Immunol, 183*(1), 76-89. doi:10.1111/cei.12703

Tebbe, B., Wilde, B., Ye, Z., Wang, J., Wang, X., Jian, F., . . . Hoerning, A. (2016). Renal Transplant Recipients Treated with Calcineurin-Inhibitors Lack Circulating Immature Transitional CD19+CD24hiCD38hi Regulatory B-Lymphocytes. *PLoS One, 11*(4), e0153170. doi:10.1371/journal.pone.0153170

Torow, Natalia, & Hornef, Mathias W. (2017). The Neonatal Window of Opportunity: Setting the Stage for Life-Long Host-Microbial Interaction and Immune Homeostasis. *The Journal of Immunology, 198*(2), 557-563. doi:10.4049/jimmunol.1601253

Toussaint, Marie, Jackson, David J., Swieboda, Dawid, Guedán, Anabel, Tsourouktsoglou, Theodora-Dorita, Ching, Yee Man, . . . Johnston, Sebastian L. (2017). Host DNA released by NETosis promotes rhinovirus-induced type-2 allergic asthma exacerbation. *Nature medicine, 23*(6), 681-691. doi:10.1038/nm.4332

Tsubata, Takeshi. (2017). B-cell tolerance and autoimmunity. *F1000Research, 6*, 391-391. doi:10.12688/f1000research.10583.1

Underhill, D. M., Gordon, S., Imhof, B. A., Nunez, G., & Bousso, P. (2016). Elie Metchnikoff (1845-1916): celebrating 100 years of cellular immunology and beyond. *Nat Rev Immunol, 16*(10), 651-656. doi:10.1038/nri.2016.89

van Asperen, P. P., Gleeson, M., Kemp, A. S., Cripps, A. W., Geraghty, S. B., Mellis, C. M., & Clancy, R. L. (1985). The relationship between atopy and salivary IgA deficiency in infancy. *Clin Exp Immunol, 62*(3), 753-757.

van de Kerkhof, P. C., & Steijlen, P. M. (1995). IgA deficiency and psoriasis: relevance of IgA in the pathogenesis of psoriasis. *Dermatology, 191*(1), 46-48. doi:10.1159/000246487

van der Burg, M., van Zelm, M. C., Driessen, G. J., & van Dongen, J. J. (2012). New frontiers of primary antibody deficiencies. *Cell Mol Life Sci, 69*(1), 59-73. doi:10.1007/s00018-011-0836-x

van der Vlugt, Luciën E. P. M., Haeberlein, Simone, de Graaf, Wouter, Martha, Tyrill E. D., & Smits, Hermelijn H. (2014). Toll-Like Receptor Ligation for the Induction of Regulatory B Cells. In Gaetano Vitale & Francesca Mion (Eds.), *Regulatory B Cells: Methods and Protocols* (pp. 127-141). New York, NY: Springer New York.

van Egmond, Marjolein, Damen, Cora A., van Spriel, Annemiek B., Vidarsson, Gestur, van Garderen, Evert, & van de Winkel, Jan G. J. (2001). IgA and the IgA Fc receptor. *Trends in Immunology, 22*(4), 205-211. doi:https://doi.org/10.1016/S1471-4906(01)01873-7

van Schouwenburg, Pauline A., IJspeert, Hanna, Pico-Knijnenburg, Ingrid, Dalm, Virgil A. S. H., van Hagen, P. Martin, van Zessen, David, . . . van der Burg, Mirjam. (2018). Identification of CVID Patients With Defects in Immune Repertoire Formation or Specification. *Frontiers in Immunology, 9*(2545). doi:10.3389/fimmu.2018.02545

Viladomiu, M., Kivolowitz, C., Abdulhamid, A., Dogan, B., Victorio, D., Castellanos, J. G., . . . Longman, R. S. (2017). IgA-coated E. coli enriched in Crohn's disease spondyloarthritis promote TH17-dependent inflammation. *Sci Transl Med, 9*(376). doi:10.1126/scitranslmed.aaf9655

Villarino, A. V., Kanno, Y., Ferdinand, J. R., & O'Shea, J. J. (2015). Mechanisms of Jak/STAT signaling in immunity and disease. *J Immunol, 194*(1), 21-27. doi:10.4049/jimmunol.1401867

Vincent, Fabien B., Morand, Eric F., Schneider, Pascal, & Mackay, Fabienne. (2014). The BAFF/APRIL system in SLE pathogenesis. *Nat Rev Rheumatol, advance online publication*. doi:10.1038/nrrheum.2014.33

Vo Ngoc, D. T., Krist, L., van Overveld, F. J., & Rijkers, G. T. (2017). The long and winding road to IgA deficiency: causes and consequences. *Expert Rev Clin Immunol, 13*(4), 371-382. doi:10.1080/1744666x.2017.1248410

Wang, N., & Hammarstrom, L. (2012). IgA deficiency: what is new? *Curr Opin Allergy Clin Immunol, 12*(6), 602-608. doi:10.1097/ACI.0b013e3283594219

Wang, N., Shen, N., Vyse, T. J., Anand, V., Gunnarson, I., Sturfelt, G., . . . Hammarstrom, L. (2011). Selective IgA deficiency in autoimmune diseases. *Mol Med, 17*(11-12), 1383-1396. doi:10.2119/molmed.2011.00195

Wang, Ning, Shen, Nan, Vyse, Timothy J., Anand, Vidya, Gunnarson, Iva, Sturfelt, Gunnar, . . . Hammarström, Lennart. (2011). Selective IgA Deficiency in Autoimmune Diseases. *Molecular Medicine, 17*(11-12), 1383-1396. doi:10.2119/molmed.2011.00195

Wang, Y. Y., Zhang, L., Zhao, P. W., Ma, L., Li, C., Zou, H. B., & Jiang, Y. F. (2014). Functional implications of regulatory B cells in human IgA nephropathy. *Scand J Immunol, 79*(1), 51-60. doi:10.1111/sji.12128

Wardemann, H., Yurasov, S., Schaefer, A., Young, J. W., Meffre, E., & Nussenzweig, M. C. (2003). Predominant autoantibody production by early human B cell precursors. *Science, 301*(5638), 1374-1377. doi:10.1126/science.1086907

Watanabe, Kakeru, Sugai, Manabu, Nambu, Yukiko, Osato, Motomi, Hayashi, Tatsunari, Kawaguchi, Miho, . . . Shimizu, Akira. (2010). Requirement for Runx Proteins in IgA Class Switching Acting Downstream of TGF-β1 and Retinoic Acid Signaling. *The Journal of Immunology, 184*(6), 2785-2792. doi:10.4049/jimmunol.0901823

Weaver, Lawrence T., Arthur, Helen M. L., Bunn, James E. G., & Thomas, Julian E. (1998). Human milk IgA concentrations during the first year of lactation. *Arch Dis Child, 78*(3), 235.

Wehr, C., Kivioja, T., Schmitt, C., Ferry, B., Witte, T., Eren, E., . . . Warnatz, K. (2008). The EUROclass trial: defining subgroups in common variable immunodeficiency. *Blood, 111*(1), 77-85. doi:10.1182/blood-2007-06-091744

Weller, S., Braun, M. C., Tan, B. K., Rosenwald, A., Cordier, C., Conley, M. E., . . . Weill, J. C. (2004). Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood, 104*(12), 3647-3654. doi:10.1182/blood-2004-01-0346

Wenzel, S. (2012). Severe asthma: from characteristics to phenotypes to endotypes. *Clinical & Experimental Allergy, 42*(5), 650-658. doi:doi:10.1111/j.1365-2222.2011.03929.x

Weterings, E., & van Gent, D. C. (2004). The mechanism of non-homologous end-joining: a synopsis of synapsis. *DNA Repair (Amst), 3*(11), 1425-1435. doi:10.1016/j.dnarep.2004.06.003

Whiteman, H. J., & Farrell, P. J. (2006). RUNX expression and function in human B cells. *Crit Rev Eukaryot Gene Expr, 16*(1), 31-44.

Williams, A., Reljic, R., Naylor, I., Clark, S. O., Falero-Diaz, G., Singh, M., . . . Ivanyi, J. (2004). Passive protection with immunoglobulin A antibodies against tuberculous early infection of the lungs. *Immunology, 111*(3), 328-333.

Yazdani, R., Latif, A., Tabassomi, F., Abolhassani, H., Azizi, G., Rezaei, N., & Aghamohammadi, A. (2015). Clinical phenotype classification for selective immunoglobulin A deficiency. *Expert Rev Clin Immunol, 11*(11), 1245-1254. doi:10.1586/1744666x.2015.1081565

Zan, H., Cerutti, A., Dramitinos, P., Schaffer, A., & Casali, P. (1998). CD40 engagement triggers switching to IgA1 and IgA2 in human B cells through induction of endogenous TGF-beta: evidence for TGF-beta but not IL-10-dependent direct S mu-->S alpha and sequential S mu-->S gamma, S gamma-->S alpha DNA recombination. *J Immunol, 161*(10), 5217-5225.

Zeidler, H. (2013). Niki de Saint Phalle's lifelong dialogue between art and diseases: psychological trauma of sexual abuse, transient selective IgA deficiency, occupational exposure to toxic plastic material, chronic lung disease, rheumatoid arthritis. *Joint Bone Spine, 80*(3), 332-337. doi:10.1016/j.jbspin.2012.09.026

Zielen, S., Bauscher, P., Hofmann, D., & Meuer, S. C. (1993). Interleukin 10 and immune restoration in common variable immunodeficiency. *Lancet, 342*(8873), 750-751.

Zotos, D., Coquet, J. M., Zhang, Y., Light, A., D'Costa, K., Kallies, A., . . . Tarlinton, D. M. (2010). IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med, 207*(2), 365-378. doi:10.1084/jem.20091777

Original publications

I. Andri Leo Lemarquis, Helga Kristin Einarsdottir, Rakel Natalie Kristinsdottir, Ingileif Jónsdóttir and Bjorn Runar Ludviksson, “Transitional B cells and TLR9 responses are defective in selective IgA deficiency” Frontiers in Immunology. 2018 Apr 27;9:909. doi: 10.3389/fimmu.2018.00909. eCollection 2018.

II. Andri Leo Lemarquis, Fannar Teodor Palsson, Helga Kristin Einarsdottir and Bjorn Runar Ludviksson, “Mapping of signalling pathways linked to IgA production reveals a defective IL-21 driven STAT3 activation in selective IgA deficiency”, Frontiers in Immunology. 2019 Mar 18;10:403. doi: 10.3389/fimmu.2019.00403. eCollection 2019.

III. Andri Leo Lemarquis, Ida Karnsund, Fannar Teodor Palsson, Anna G Vidarsdottir, Helga Kristin Einarsdottir, Gudmundur Jorgensen, Olov Ekwall, Ingileif Jonsdottir and Bjorn Runar Ludviksson. “Selective IgA deficient individuals have a serological and transcriptomic germinal centre associated immune dysregulation associated with autoantibody production”, Manuscript.

Paper I

Paper II

Paper III