Diploma thesis

The Diagnostic, Prognostic, and Predictive Potential of Immune Exhaustion and Senescence Biomarkers in Children and Adolescents with Severe Immune Cytopenias

submitted by

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Graz, 12.02.2021

Affirmation in lieu of an Oath

I hereby formally declare, that I have written the submitted thesis independently and without any illegitimate assistance from third parties.

I confirm that I used no other than the declared sources for the preparation of this academic work. All used sources have been indicated as such and acknowledged by means of complete references in the text.

Graz, 12.02.2021 Julius Christoph Köppen eh.

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Abbreviations

°C	Degree Celsius
ABC	Age associated B cell
AIHA	Autoimmune haemolytic anaemia
ALL	Acute lymphatic leukaemia
ALPS	Autoimmune lymphoproliferative disease
ANA	Antinuclear antibodies
APS	Anti-phospholipid syndrome
CA	Cold agglutinin
CCR	Continuous complete remission
CD	Cluster of differentiation
CHAD	Cold haemagglutinin disease
CHAI	CTLA-4 haploinsufficiency with autoimmune infiltration
CM	Central memory
CMV	Cytomegaly virus
CR	Complete remission
CRF	Case report form
CVID	Common variable immunodeficiency
CyTOF	Cytometry by time of flight
DAT	Direct antiglobulin test
DC	dendritic cell
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EM	Effector memory
Erk	Extracellular-signal regulated kinases
ES	Evans syndrome
FACS	Fluorescence-activated cell sorting
FDA	Food and drug administration
FLT	First line treatment
Fluor.	Fluorochrome
g	Gramm
GE	Gastroenteritis
GOF	Gain of function

GP	Glycoprotein
GVHD	Graft versus host disease
H.p.	Helicobacter pylori
Hb	Haemoglobin
HBV/ HCV	Hepatitis B/ C virus
HC	Healthy control
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRQoL	Health related quality of life
HSCT	Hematopoietic stem cell transplantation
IBLS	ITP Bleeding Scale
IEI	Inborn of immunity
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
ITP	Immune thrombocytopenia
IVIg	Intravenous immunoglobulin
KIR	Killer cell immunoglobulin-like receptors
KLRG	Killer cell lectin-like receptor subfamily G member
L/ dL	Liter/ deciliter
LDH	Lactate dehydrogenase
LOF	Loss of function
LRBA	LPS responsive beige-like anchor protein
mAB	Monoclonal antibody
MAPK	Mitogen-activated-protein kinase
mcL	Microliter
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
mTOR	mammalian target of rapamycin
PBMC	Peripheral blood mononuclear cell
PCH	Paroxysmal cold hemoglobinuria
PI3K	phosphatidylinositol-3-OH kinase

PI3KCD	p110δ catalytic subunit of PI3K
PID	Primary immunodeficiency
PIP3	Phosphatidylinositol 3 phosphate
PLC	Phosphoinositide phospholipase C
PR	Partial remission
RA	Rheumatoid arthritis
RBC	Red blood cell
RCC	Renal cell carcinoma
Rh	Rhesus
RT-PCR	Real-time polymerase chain reaction
SCID	Severe combined immunodeficiency
SIC	Severe immune cytopenia
SLE	Systemic lupus erythematosus
SLT	Second line treatment
STAT	Signal transducers and activators of transcription
Syk	Spleen tyrosine kinase
TCR	T cell receptor
TEMRA	Terminal effector memory T cell CD45RA+
Tfh	T follicle helper cell
Th	T helper cell
TP	Time point
TPO-RA	Thrombopoietin receptor agonist
TPP2	Tripeptidyl peptidase 2
TREC	T cell receptor excision circles
Treg	Regulatory T cell
vW disease	von Willebrand disease

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Zusammenfassung

Einleitung: Schwere Immunzytopenien wie die autoimmunhämolytische Anämie (AIHA), chronische Immunthrombozytopenie (ITP) und das Evans Syndrom (ES) sind häufig mit Immundysregulation assoziiert. Bei einigen angeborenen Immundefekten steht die Immundysregulation in engem Zusammenhang zu frühzeitiger Immunsenescence und einem lymphozytären Exhaustion-Profil. Die Muster dieser Immunprofile können möglicherweise einen Hinweis auf den zugrundeliegenden Pathomechanismus erworbener und angeborener Immundefekte sein.

Patienten/innen und Methoden: Eine longitudinale, prospektive, multizentrische Registerstudie zu schweren Immunzytopenien (sic-reg.org) wurde 2019 initiiert. Das Register schließt pädiatrische und jugendliche Patienten/innen mit Immunzytopenie ein. Aktuell liegt eine frühe Interim-Analyse von Immunphänotyp-Biomarkern der ersten inkludierten Patienten/innen vor. In der ersten Gruppe wurden 12 Patienten/innen (Durchschnittsalter 10 Jahre; Altersverteilung 4-16 Jahre; männlich, n=5) mit ITP, AIHA oder ES eingeschlossen, drei von ihnen bei Rezidiv ihrer Erkrankung als retrospektive Patienten/innen. In der zweiten Gruppe erfassten wir 19 Patienten/innen mit bekanntem angeborenem Immundefekt als Beobachtungspatienten/innen. Bei allen Patienten/innen wurde initial, nach 6 und nach 12 Monaten eine quantitative FACS-Analyse der naiven und Gedächtnis-/T- und B-Subklassen und der altersassoziierten bzw. seneszenten Lymphoyztensubklassen durchgeführt.

Ergebnisse: 10 von 12 Patienten/innen mit Immunzytopenie zeigen ein pathologisches Verlaufsmuster an allen drei Messzeitpunkten in mindestens einer der naiven bzw. Gedächtnis- oder seneszenten Lymphozytensubklassen. Auch acht der 19 Patienten/innen mit angeborenem Immundefekt zeigen mindestens in einer der Lymphozytensubklassen einen pathologischen Verlauf. Daneben präsentierten einige Patienten/innen im Verlauf einzelne auffällige Abweichungen in bestimmten Lymphozytensubklassen.

Schlussfolgerung/Diskussion: Auf Grundlage der Interim-Daten ist die systematische Analyse von Lymphozyten-Differenzierungsprofilen bei Patienten/innen mit schweren Immunzytopenien indiziert, um diagnostische und

prognostische Biomarker-Profile erstellen zu können. Das lymphozytäre Biomarker-Profil kann, in Abhängigkeit von der Grundkrankheit jedoch noch spezifischer gestaltet werden. Durch die geplante Kombination unserer Daten mit der single-cell transcriptome Technik und Analyse epigenetischer Faktoren soll der Pathomechanismus der Immundysregulation weiter aufgeklärt werden.

Abstract

Introduction: Severe immune cytopenias (SIC), such as autoimmune hemolytic anemia (AIHA), immune thrombocytopenia (ITP) and Evans syndrome (ES) are syndromes with a remarkable association to immune dysregulation. Immune dysregulation with consequential occurrence of autoimmunity and immune-mediated cytopenia stands in close relationship to premature immunosenescence and a lymphocyte exhaustion phenotype in many inborn errors of immunity (IEI). The patterns of immune phenotypes might be associated with underlying disease mechanisms, and consequently, with treatment responses of SIC in patients with or without a known IEI.

Methods: This longitudinal, prospective, multicentric study represents an early interim analysis, based on the Sic-reg.org registry including pediatric and adolescent SIC patients. We included 12 study patients (mean age: 10 years, range: 4-16 years; male, n=5) diagnosed with either AIHA, ITP or ES, of which 3 patients were included as retrospective patients after relapse of their cytopenia. In addition, 19 observational patients with known IEI were analysed. Measurement time points were initially, after 6 and after 12 months. At every time point quantitative FACS analyses of naïve and memory T and B cell subsets and age-associated / senescent lymphocyte subsets were performed.

Results: The first observation is that 10 of 12 SIC patients demonstrated a clearly pathological longitudinal pattern of at least one of the naïve or memory or senescent T- or B-lymphocyte subset panels over the first three time points. 8 of 19 observational patients showed at least one pathological longitudinal pattern in a lymphocyte subset. Additionally, certain lymphocyte subsets changed strikingly over time in some patients, allowing a correlation evaluation of these parameters with the clinical courses of the patients.

Discussion: Based on these preliminary results the systematic analysis of lymphocytic differentiation patterns should be further elaborated to serve as prognostic and diagnostic biomarker signatures. First, by analysing our follow up measurements, potential adjustments of the applied lymphocyte marker profile could be adapted to a more specific one. Secondly, by expanding the immune phenotypic characterization into broader differentiation of lymphocyte subsets, and

thirdly, by combining this data with single-cell transcriptome profiling could yield further insights identifying disease-associated and potentially targetable cell types.

Publications

Abstractband der ÖGKJ: Köppen, J; Kindler, O; Schwinger, W; Karastaneva, A; Benesch, M; Seidel, MG Diagnostisches und prognostisch-prädiktives Potenzial von Immune Exhaustion- und Senescence-Biomarker-Profilen bei Kindern und Jugendlichen mit schweren Immunzytopenien
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1. Introduction

1.1. Severe immune-mediated cytopenia (SIC)

Autoimmunity results from failure among the T cell and B cell development. Malfunction can determine on one or several developmental steps. Consequently, autoantigen specific adaptive immune cells evolve as their elimination failed. Severe autoimmune mediated cytopenia are acquired immunologically caused destruction of erythrocytes, platelets, or neutrophiles. Basically, the destruction of hematologic cell lines is classified as primary or secondary. Primary autoimmune cytopenia may occur individually as autoimmune hemolytic anemia, as immune thrombocytopenia, as autoimmune neutropenia, or as bi- or tri-lineage cell destruction called Evans syndrome. Because isolated autoimmune neutropenia in children and adolescents is typically a self-limited benign condition, the term SIC and the present results from an interim analysis of a pilot phase of the prospective registry study (sic-reg) does not include isolated autoimmune neutropenia (see definitions and inclusion criteria below). Secondary immune mediated cytopenia can be caused by heterogenous factors as infections, malignancies, underlying autoimmune diseases and medications. Primary immunodeficiencies or inborn errors of immunity resulting in immune dysregulation are additional causal factors obligatory to evaluate (1, 2). The pathogenesis of autoimmune cytopenia remains unclear. Current hypotheses consist of genetic predisposition, association to certain HLA types and autoreactive lymphocytes due to external factors. Besides, failure or loss of immune self-regulation is seen as crucial factor as well (3). The diagnosis of primary autoimmune cytopenia and potential underlying causes remains challenging. An essential component of the diagnosis consists of the identification or exclusion of secondary causes, because detection of the latter might prompt directed, more efficient treatment of the cytopenia. The main treatment strategy against primary autoimmune cytopenia comprises immune suppressive or immune modulating drugs.

1.1.1. Presumption

As an expression of immune dysregulation SIC are supposed to resemble key characteristics of inborn immune dysregulatory syndromes. With this analysis we will provide additional evidence of lymphocytic premature maturation and

immunosenescence as a shared phenotypical characteristic of SIC and immune dysregulation.

1.1.2. Immune Thrombocytopenia

Introduction

Although ITP has been a well-known hematologic disorder for more than two centuries, crucial aspects regarding etiologic factors or clinical course remain unknown. Especially essential questions related to the pathophysiology and the clinical course of ITP and the according treatment remain unknown. Consequently, treatment therapies must be individually adapted and are performed tentatively. Immune thrombocytopenia is an acquired autoimmune disorder and is characterized by a low platelet count. Thrombocytopenia results from autoimmune mediated platelet destruction as well as from impaired platelet production (4). ITP is a haemolytic disorder presenting in both sexes and affecting all groups of age.

Historical aspects

The history of ITP dates to 1735. In that year Paul Gottlieb Werlhof presented a case report of a 16-year-old girl with cutaneous and mucosal bleeding that occurred after an infectious disease (5). A closer insight of the pathogenesis provided the self-experiment of Harrington–Hollingsworth. It was unclear whether a destruction of platelets or an impaired production represent the underlying cause of ITP mechanism. A self-experiment provided first evidence. After plasma infusion of an ITP patient to the first volunteer Harrington himself, Harrington and James W. Hollingsworth could determine a rapid thrombocytopenia in Harrington. After days he recovered and an indirect proof of platelet destruction was achieved (5). From the 1970s the pathophysiological focussed immunoglobulin mediated platelet destruction, so the direct proof of autoimmune destruction was given. Recently a disbalance in the T lymphocyte subclasses is suggested as additional or underlying autoimmune cause in ITP (5).

Epidemiology

Reports of the prevalence of ITP range from 4.5 to 10.5 cases per 100,000 persons in adults and 4.6 cases per 100,000 in children and adolescents (6). In the 2019 published guidelines for immune thrombocytopenia the American Society of Haematology describes an incidence of 2 to 5 per 100000 people (4).

The clinical course of ITP seems to be age related. The 1-year remission rate in children below 1 year age is 74%. In children from 1 year to 6 years the remission rate is 67% and in adolescents the rate is 62% (7, 8). Data from the International Childhood ITP Study Group demonstrated spontaneous recovery of ITP by the majority of children between 7 to 12 months (8).

Confident epidemiological data to ITP have been provided by the General Practice Research Database (GPRD). Within this database 257 paediatric ITP patients were classified as ITP diagnosed patients (9). The recording duration lasts from 1990 to 2005. The incidence in pediatric patients is 4.2 per 100000. Thereby a dependency on the sex becomes clear. Boys in the age from 2 to 5 years demonstrate a statistically higher incidence of 9.7 compared to girls in the same range of age with an incidence of 4.7 per 100000. The incidence is remarkably higher in younger age groups than in older. Accordingly, the group of adolescents presented an incidence of 2.4 per 100000 with no significant difference between the sexes (9).

Multiple sources suggest that the incidence of ITP shows a seasonal occurrence. The observational study of the Intercontinental Childhood ITP Study Group analysed a cohort of 1104 boys and 909 girls diagnosed with ITP. The cohort demonstrated a peak incidence in spring/early summer and a nadir in autumn (10). In addition, infections are suspected to represent an initialising factor leading to primary acute ITP. A fifth (20,2%) of the pediatric ITP patient cohort presented an infection within 8 weeks prior ITP diagnosis (9). An infection of the upper respiratory track was most common. As the majority of ITP following an infection were diagnosed in winter and the minority were diagnosed in summer these time variation data suggest a correlation to seasonal viruses as initialising factor for ITP occurrence (9).

Pathophysiology

Since the experiment of Harrington–Hollingsworth by self-administering plasma of active ITP patients with the consequence of transient thrombocytopenia the leading hypothesis of platelet destruction was based on autoantibody mediated platelet destruction. The destruction of the platelets was conducted by phagocytic cells of the reticuloendothelial system (11). The current scientific stand reflects a broader spectrum of underlying pathophysiological hypotheses. Certain questions

remain unsolved: At first, in Harrington–Hollingsworth experiment not every blood or plasma sample of ITP patients induced a transient thrombocytopenia. Secondly, platelet autoantibodies can be detected in only 50-75% of patients diagnosed with ITP. At third, John W. Semple et al. demonstrated in 1996 that the pattern of cytokine levels in chronic autoimmune ITP may reflect an early T helper cell (Th cell) activation. The main cytokine found was IL-2. IL-2 secretion was associated in some patients with induction of Th0 or Th1 immune reaction (12). The fourth reason arguing against a mono-mediated pathomechanism in ITP is, that thrombocytes of ITP patients present autoantigens in form of major histocompatibility complexes (MHC) class 1 molecules indicating a multi-mediated immune reaction (11).

Concludingly, this overview suggests that ITP is a heterogeneously mediated autoimmune disease. Different pathophysiological mechanisms might be involved. Four main pathways of pathomechanism in ITP are further focussed.

I) T-cell-mediated destruction

In 2003 Bob Olsson et al. could consolidate the hypothesis of a multifactorial pathogenesis in ITP (11). The group of Olsson performed a DNA microarray for genetic expression in ITP patients. In their study 6 patients with active chronic ITP, 8 patients with ITP in remission and 8 healthy volunteers were included. Active ITP was defined as platelet count $<50 \times 10^9$ cells/l and ITP in remission as platelet count $>150 \times 10^9$ cells/l. Patients with active disease status demonstrated a significantly stronger presence of cytotoxic and Th1 related genes in CD3+ T-cells compared to ITP patients in remission and to the healthy control group. Expression of IFN-gamma, a cytokine characterising the Th1 response, is strikingly increased (11, 13). Based on the predominance of cytotoxic profile in T cells, they investigated the presence of killer cell immunoglobulin-like receptors (KIR) as KIRs are basically supposed to attenuate cytotoxic T cell activity (11).

KIRs contain immunoglobulin similar domains. Depending on the amount (2, 3 and further on) of Ig similar domains the KIR is further designated with -2/ -3/ -etc. The letter D represents domain. In general, KIRs are part of the receptor variability of natural killer cells. That means KIR enable activation or deactivation of natural killer cells. The difference between activating and inhibitory KIR reflects their

cytoplasmatic structure in form of their cytoplasmatic tail. The group of inhibitory KIR is labelled with L for long tail. The main inhibitory KIR are KIR2D-L and KIR3D-L (14). KIRs bind to MHC 1 molecules on potential target cells and prevent their destruction. Based on that inhibitory mechanism KIRs attenuate cytotoxic T cell and natural killer cell reaction (14).

Back to the investigations on T cell mediated platelet destruction, the genetic expression of KIR was noticeably increased in ITP patients in remission. A reduction of genetic expression of KIR was detected in patients with active ITP, demonstrated in Figure 1 b. The expressional presence of KIR was re-evaluated by flowcytometric analysis of CD3+KIR+ cells (CD158a, KIR3DL2; CD158b, KIR2DL3; NKB1, KIR3DL1), which is presented in Figure 1 c. According to the genetic expression of KIR in CD3+ T cells the amount of KIR positive CD3+ T cells in ITP patients in remission is remarkably extended compared to the relative amount in active ITP patients.

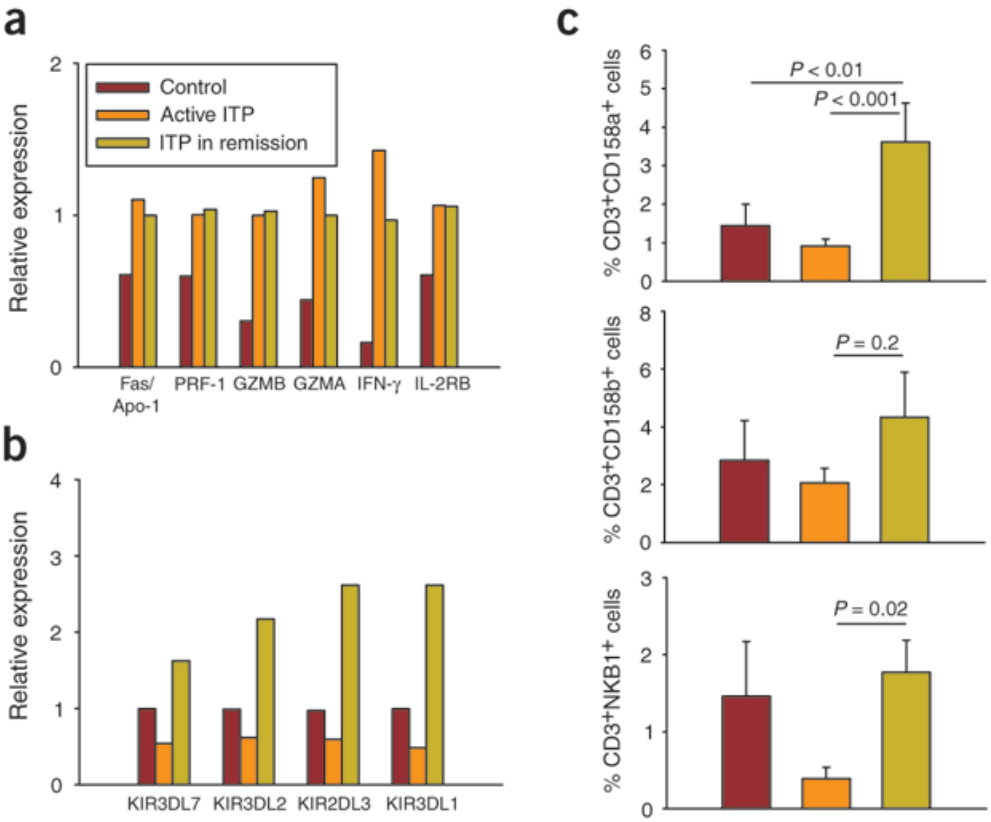

Figure 1 T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. Bob Olsson et al., Nature Medicine, 2003; 9(9):1123-24

Description: PRF-1, perforin 1; GZMB, granzyme B; GZMA, granzyme A; IFN-γ, interferon-γ; IL-2RB, interleukin-2 receptor-β; KIR, killer cell immunoglobulin-like receptor (11)

Increased expression of cytotoxicity associated genes of CD3+ T cells in ITP patients with active disease status (Fig. 1a) combined with increased expression of KIR related genes in CD3+ T cells (Fig. 1b) and increase in KIR positive CD3+ T cells (Fig. 1c) suggest evidence of direct cytotoxic effects of T-cells against platelets (11).

To further proof these findings Olsson et al. performed an in vitro assay. They measured the destruction of radiolabelled autologous platelets by CD14-CD19- mononuclear cells, which includes the population of T cells and natural killer cells. As depicted in Figure 2, platelet destruction was demonstrated in 6 of 8 active ITP patients, whereby no platelet destruction could be found in the control group nor in the patient group ITP in remission (11).

To distinguish between the population of the lytic cells purified CD3+CD8+ T cells and CD3-CD16+CD56+ natural killer cells were used. The destruction of platelets was only demonstratable in CD3+CD8+ cell composite. Whereas in CD3-CD16+CD56+ natural killer cells no lysis of platelets was seen (11).

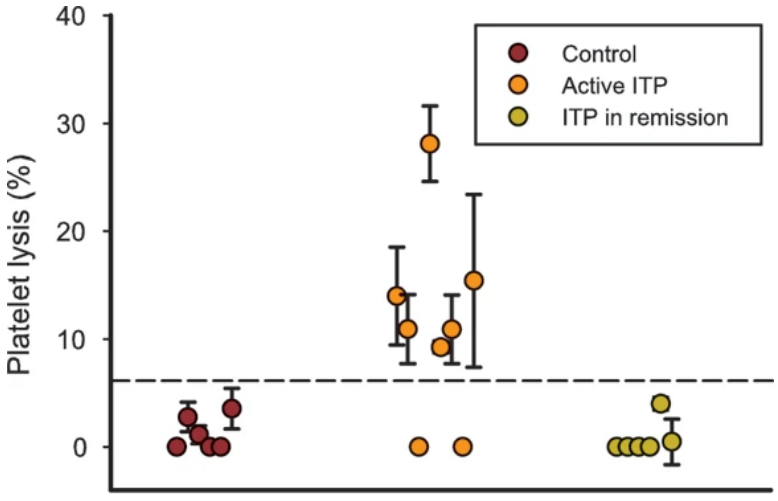

Figure 2 T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. Bob Olsson et al., Nature Medicine, 2003; 9(9):1123-4

Description: detectable cell-mediated cytotoxicity of CD14-CD19- cells against autologous platelets from patients with active ITP (n=8), in remission of ITP (n=6)

and control group (n=6). The dotted line depicts the limit for positive destruction of platelets.

To proof evidence of the suggested immunoregulatory role of Th1/Th2 responses in ITP a Greek research group performed a prospective study of Th1/Th2 gene cytokine expression profiles in 18 children diagnosed with ITP by RT-PCR (15). At initial time point prior any treatment, acute ITP presents with a Th1, Th0/Th1, or 0 (no remarkable genetic cytokine expression) cytokine gene expression (15).

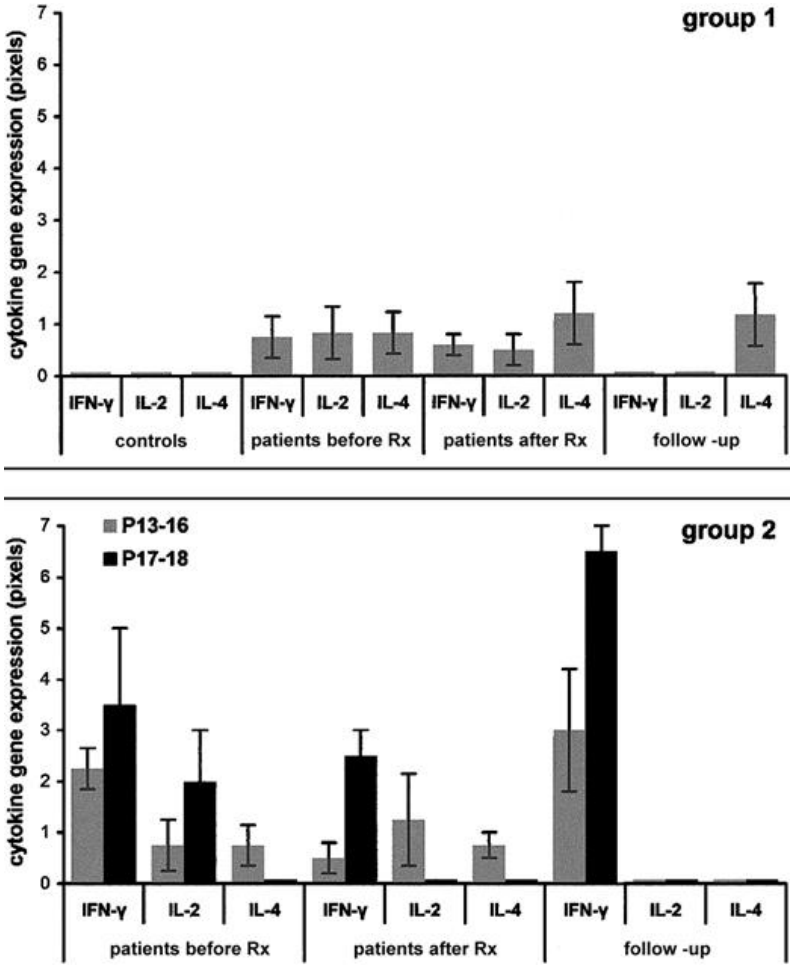

Figure 3 Expression patterns of Th1 and Th2 cytokine genes in childhood idiopathic thrombocytopenic purpura (ITP) at presentation and their modulation by intravenous immunoglobulin G (IVIg) treatment: their role in prognosis. A. Mouzaki et al., Blood, 2002; 100(5): 1774-1779

Description: After isolation of PBMCs of the patient group and control cohort RNA was analysed by RT-PCR. Initial time point equals to patients before treatment with IVIg (before Rx), after Rx equals 24 hours after IVIg therapy.

Th2 pattern – only IL-4; Th0/Th1 pattern – IL-2/IFN-Gamma; Th1 pattern – IFN-Gamma
First graph: Presentation of 14 healthy paediatric controls and group 1. Group 1 contains patients P1 to P12 with one acute phase of ITP followed by stable remission (grey bars).
Second graph: Presentation of group 2 consisting of P13-P18. Patients P17-P18 demonstrating a chronic course of ITP (black bars) and P13-P16 showing a relapsing course of ITP (grey bars).

According to the varying longitudinal T helper cytokine patterns (the complete description would be T helper cell cytokine gene expression) the authors assumed a disease response classification in ITP patients after acute disease status (15). A longitudinal 0 or Th2 profile might indicate stable remission. Besides, they found evidence for IFN-gamma related differentiation between mild relapsing course of ITP versus the more aggressive form of a chronic ITP as Figure 3 (P13-P18) demonstrates (15). Cytokine expression profiles as response classification in ITP patients provide promising support for a predictive and diagnostical biomarker panel in patients with ITP.

II) Phagocytosis by macrophages

A recent study investigated the efficacy of fostamatinib in adults with chronic ITP. The study was performed as a three phase, multicentre, double-blind, placebo-controlled, randomized trial. 101 patients were administered fostamatinib and 49 patients were treated with placebo for at least a total duration of 24 weeks (16). The primary endpoint was a stable response with platelets >/= 50000/mcL in weeks 14-24 (16).

Spleen tyrosine kinase plays a crucial role in cell activation process during phagocytosis. All Fc receptors signal via spleen tyrosine kinase. The activation further initiates cellular proliferation and differentiation and immune regulation. Fostamatinib is a spleen tyrosine kinase (Syk) inhibitor. Fostamatinib is approved for the therapeutical regime in adults with chronic ITP.

In terms of chronic ITP, the authors included a patient collective of a median ITP duration of 8,5 years. Stable response was more frequent in the cohort treated with fostamatinib indicating Syk inhibition as a crucial factor suppressing ITP pathogenesis (16).

The study provides support for the use of fostamatinib as an effective therapy in ITP. Besides, it presents a certain insight value of ITP pathogenesis. Some patients responded to fostamatinib with disease modulation and platelet increase, some patients did not respond at all. According to the assumption of multiple immune pathways causing ITP the authors suggest a varying predominance of autoimmune reactions. In the case of sufficient response after fostamatinib treatment the authors assume a predominance of autoantibody mediated ITP (16). Oppositely, in non-responsive patients T cell mediated platelet cytotoxicity is the predominant autoimmune reaction (16). Besides the suggested predominance of one immune reaction in ITP, it remains unclear how a predominant immune reaction performs transition into another autoimmune reaction (16).

III) Antibody-dependent cell-mediated cytotoxicity/ complement mediated elimination

There are numerous studies suggesting the presence of autoantibodies directed against glycoprotein IIb/IIIa (GPIIb/IIa) and Ib/IX (GPIb/IX) in patients with ITP. GPIIb/IIIa and GPIb/IX are known as the main targets recognized by autoantibodies (17). The labelling autoantibodies further lead to Fc-mediated phagocytosis by the reticuloendothelial system or by subsequent activation of the complement system to complement mediated phagocytosis or complement induced lysis. A group among Abderrahim Najaoui et al. performed a study with the objective to investigate the expansion of complement activation in patients with ITP (17). Therefore 240 patients with chronic ITP were analysed regarding the presence of bound and free autoantibodies. Secondly, their sera were evaluated on attaching complement. Thirdly, the C1q fixation to immunobead labelled with GPIIb/IIIa in sera containing anti-GPIIb/IIIa antibodies was analysed. In the results the cohort demonstrated that 54% showed detectable platelet bound glycoprotein specific autoantibodies (17). Of these, 11% presented free glycoprotein specific autoantibodies, whereas in 46% presented no detectable autoantibodies (17).

Interestingly, analysing the complement fixation of the sera of each of these subgroups, 30% of the patients without detectable glycoprotein specific autoantibodies showed complement fixation (17). To note, complement fixation occurred in patient without detectable autoantibodies. That leaves the question whether current autoantibody detection is not sufficient for detection below a certain cut-off? Or it might demonstrate a certain immunologic periodicity influencing the presence and quantity of autoantibodies (17).

According to the hypothesis that GP IIa/IIIb and GP Ib/IX are the main targets of autoantibodies in ITP, the results demonstrated in the absence of GP IIa/IIIb and in the absence of GP Ib/IX an inability to fix complement of 67% and 30%, respectively (17). It strongly suggests GP IIa/IIIb and GP Ib/IX as main target for autoantibodies in ITP. In sera of patients containing anti-GP IIb/IIIa autoantibodies 50% presented a C1q fixation to immunobeads coated with GP IIb/IIIa (17).

Two main results summarize autoantibody and complement mediated elimination of platelets as major causal factor in ITP pathophysiology. Firstly, 50% of the 240 patients show ability of complement system activation (17). Thereby, patients presenting detectable autoantibodies are more likely to activate complement system compared to patients without detectable antibodies. Secondly, as 50% of the autoantibody positive sera showed C1q fixation it is most likely that complement fixation activates the classical pathway of the complement cascade (17). Based on these results and assuming complement activation as major causative factor resulting in platelet destruction, inhibitory agent of the classical complement pathway might be a promising therapeutic approach.

According to the pathophysiologic theory of autoantibody opsonized platelets treatment with IVIg in ITP patients is an approved safe and effective treatment. In 2018 Katja M. J. Heitink-Poll´e et al. performed a prospective, multicentre randomized controlled trial on treatment with or without IVIg administration at initial time point of diagnosis (18). The most significant result of this recent study demonstrated that children and adolescents following IVIg treatment presented a more remarkable complete remission rate compared to pediatric cohort with solely observational follow-up (18). Considering clinical complications in both cohorts, severe bleeding occurred in 9% of the observational group compared to only 1% in

the group with initial IVIg single dose (18). Related to the role of autoantibody mediated elimination of platelets in ITP the results of Katja M. J. Heitink-Poll´e et al. suggest that modulation by IVIg of the autoantibody mediated immune reaction is sufficient in preventing severe clinical course (18). The study suggests that presence and reactional status of autoantibodies play a crucial role in the severity and in the pathophysiology of ITP (18). Beside the main comparison between treatment and observational cohort, genetic analysis of the Fc-gamma receptor determining genetic region was performed (18). Fc-gamma receptors consist of a group of heterogenous Fc-gamma receptor subtypes. These receptors are expressed on the surface of various immune cell types to bind on the Fc part of Ig isotypes and promote different immune reactions. The Fc-gamma receptor II (CD32) has different isoforms (Fc-gamma receptor II -a, -b1, -b2, -c) (19). Previously it was described that genetic variation of the Fc-gamma receptor IIc allele – a cell activating Fc receptor – is significantly linked to the appearance of ITP (20). Genetic variation of the Fc-gamma receptor IIc allele induces overactivation of immune cells and promotes immune disbalance with higher susceptibility against autoimmunity (20). According to these findings Katja M. J. Heitink-Poll´e et al. genetically analysed Fc-gamma receptor II and III group in the IVIg and observational cohort (18). As previously described, they detected hetero- and homozygotic genetic alteration (isoleucine to threonine) at position 232 in the Fc-gamma receptor IIb allele (18, 20). All patients with complete response after IVIg administration presented the wild type allele (18). Fc-gamma receptor IIb is a surface receptor primarily inhibiting immune cell activation on its expressing cells (19). Against the background of both previous studies genotyping of Fc-gamma receptor exons might provide an individual profile guiding clinical management of ITP patients (18).

A study group among David E. Schmidt et al. supported the previously described findings. Genetic alterations in the regions encoding for Fc-gamma receptor II and III are associated with higher susceptibility to develop ITP or the transition into chronic ITP (21). It strengthens the evidence of Fc-gamma receptor genotype profiling for individual diagnostic and therapeutic management (21). These findings provide a supportive signal for the investigation of predictive and diagnostic markers in ITP.

IV) MHC I complex associated T-cell cytotoxicity

The majority of autoimmune diseases including ITP demonstrate a Th1 type cytokine profile (11, 12). That includes expansion of IL-2, interferon-gamma and IL-10 in serum of ITP patients (12). Along these findings it is promoted that CD8+ cells interact with Major Histocompatibility Complexes I.

In general, by molecular arrangement of the MHC I complex a certain peptide structure is left. The rest structure of the peptide is free and enables T-cell receptors to interact with. As MHC I complex is found on the surface of platelets it is implicated that CD8+ cells are involved in platelet recognition (22).

The group of Leann M. Hopkins analysed MHC I complexes presented peptides of thrombocytes in ITP patients in comparison to a control group (22). As Table 1 demonstrates, they identified a peptide sequence named GPRGALSLL in four of five ITP patients and in none of the control group (22). However, GPRGALSLL is a sequence of the GPIb receptor. GPIb is of special interest because antiplatelet antibodies often target the GPIb/IX complex (17). The study's limitation is the small cohort of ITP patients, but it supposed platelets presenting GPIb via MHC I complex to the immune system (22). The four ITP patients identified with the GPIb peptide sequence presented the allele HLA-B7 (22). The function and the proportion of HLA-B7 restricted CD8+ cells auto-reactive against GPIb in patients with ITP had to be analysed (22). According to the presence of GPIb associated MHC I complexes on the platelets' surface the question remains whether HLA-B7, GPIb restricted cytotoxic CD8+ cells represent a feature cell population in ITP patients.

Sample	GPIb peptide	HLA type of individual
ITP #1	Yes	A2, AX, B7, B27, Cw2, Cw7
ITP #2	Yes	A3, AX, B7, BX, Cw7, CwX
ITP #3	No	A2, A24, B8, B40, Cw3, Cw7
ITP #4	Yes	A3[a], A24, B7, B40, Cw7, Cw2/15
ITP #5	Yes	A1, A11, B7, B8, Cw7, CwX
Normal #1	No	A2, A29, B57, B60, Cw3, Cw7
Normal #2	No	A2, A30, B60, B63, Cw2, Cw3
Normal #3	No	A3, AX, B14, B8, Cw7, Cw8
Normal #4	No	A11, A29, B7, B51, CwX, CwX
Normal #5	Yes	A1, A3, B7, B8, Cw7, CwX
Normal #6	Yes	A2, AX, B7, BX, Cw7, CwX

Table 1 MHC Class I–Associated Peptides Identified From Normal Platelets and From Individuals With Idiopathic Thrombocytopenic Purpura, 2005; 66: 874-883 Description: ITP patients (n=5) and a control group (n=6). Both groups were HLA typed by molecular methods. The ITP patients identified with GPIb sequence GRPGALSLL present HLA-B7 although previously not selected by HLA typing.

Diagnostics

Evaluation of newly diagnosed ITP in children

The diagnosis of ITP is primarily based on the exclusion of other causes. It is mandatory to evaluate the patient's history, perform a physical examination and analysis of the peripheral blood smear. The physical examination should focus on the spleen size and constitutional signs (fever, weight loss, lymphadenopathy). A direct anti-globulin test (DAT) should be performed to exclude clinical inapparent autoimmunity against red blood cells. The DAT should be performed before treatment is initialized. If the DAT is positive, analysis of haptoglobin, lactate dehydrogenase and reticulocyte count and bilirubin should follow up. Evaluation of ES is substantial as clinical management depends on. According to the International Consensus Report of Investigation and Management of ITP a bone marrow aspiration following unremarkable laboratory check-up is not suggested (23). Bone aspiration is recommended in cases of suspected malignant cells in the blood smear and the combination of splenomegaly with lymphadenopathy (23). In geographic areas with high prevalence, part of the initial work up should be the investigation of a Helicobacter pylori (H.p.) infection by performing an urea breath

test and analysis for H.p. antigen in the patient's stool. In children measurement of Ig levels is recommended to evaluate presence of a primary immunodeficiency (23). FACS and cytogenetic analysis are suggested in patients presenting therapy refractory courses (23).

Evaluation of children with chronic ITP

As in children with recently diagnosed thrombocytopenia a physical examination, analysis of full blood count and the peripheral blood smear should be performed. Bone marrow aspiration and cytogenetical investigation should be reassessed in refractory disease status 3-6 month after initial therapy (23).

Further diagnostical work up can include testing for associated autoimmune diseases. Inclusion of Anti-phospholipid antibodies, anti-cardiolipin antibodies, ANA and quantitative evaluation of Ig (23). Genetic screening can be used in order to rule out primary immunodeficiencies or bone marrow failure syndromes. Exclusion of chronic infections of HBV, HCV, HIV is mandatory and be performed in diagnostic work-up of a first ITP episode as well (23).

Clinical manifestation and symptoms

In the retrospective analysis of 58 children with ITP 164 episodes of severe thrombocytopenia (<20000/mcL) were recorded (24). Almost a fifth (18,9%) remained without any sign of bleeding. Evaluating common symptoms of childhood ITP, this study population showed in 124 episodes cutaneous bleeding as the most common bleeding form (24). Two thirds of the cohort had only cutaneous manifestation and one third was associated with additional bleeding sides. Other common bleeding manifestations feature epistaxis, oral bleeding and gastro-intestinal bleedings (24).

To objectify and quantitatively measure bleeding symptom Lemke K. Page et al. published an ITP Bleeding Scale (IBLS) with eleven grades, demonstrated in Table 2 (25). The prospective study conducted 100 visits of 65 patients with ITP. The majority of the patients were adults with chronic ITP and had a median age of 31,5 years (25). The IBLS includes 11 bleeding sides from grades 0 (none) to 2. To assess the bleeding symptoms the authors incorporated medical history of the patient and physical examination (25). The majority (72%) of grade 1

haemorrhages occurred on the skin; grade 2 bleeding manifested more heterogeneously. 28% appeared on the skin, 46% at oral sides, 13% as gynaecological bleeding as metrorrhagia and 10% as epistaxis (25). Patients with very low platelets count (<30x10^9/l) were evaluated separately (n=38 patient visits of 100 patient visits). Albeit the low count of thrombocytes was poorly associated to bleeding events, strikingly 79% of grade II bleedings appeared in the group of very low platelets count (25).

	All 100 visits			38 visits with platelet count $<30 \times 10^9$/l		
	Bleeding grade			Bleeding grade		
Site	0	1	2	0	1	2
Skin by physical examination (PE)	37	57	6	5	29	4
Oral cavity by PE	83	9	8	25	5	8
Skin by Hx	44	51	5	6	28	4
Oral cavity by Hx	79	11	10	23	8	7
Epistaxis	81	15	4	27	9	2
Vaginal (GYN)	26	5	5	12	1	5
Gastrointestinal (GI)	99	0	1	37	0	1
Urinary (U)	100	0	0	38	0	0
Pulmonary	97	N/A	3	35	N/A	3
Intracranial haemorrhage (ICH)	100	N/A	0	38	N/A	0
Subconjunctival haemorrhage	100	0	N/A	38	0	N/A
Total number of bleeding scores of 0, 1 and 2	846	151	39	284	83	31
% of the total amount of bleeding scores	82%	14%	4%	71%	21%	8%

Table 2 The immune thrombocytopenic purpura (ITP) bleeding score: assessment of bleeding in patients with ITP, L.K. Page et al., Br. J. Hematology, 2007; 138(2): 245-8

Description: Number of visits categorized by bleeding grade for 11 sites.

Treatment

Describing the current therapeutic options of ITP management, I will focus on the guidelines and recommendations related on the therapeutic regime in children and adolescents with ITP.

To sum up, the therapeutic regimes in ITP in adults are similar to these used in paediatric ITP patients. The current data and knowledge based on clinical trials follow more likely investigation in adult ITP patients.

It must be distinguished between acute, persistent/ chronic, and relapsing ITP. Depending on the onset and clinical course different therapeutic targets are defined.

Regarding the question whether patients should rather be treated as outpatient or should be hospitalized the panel of the “American Society of Haematology 2019 guidelines for immune thrombocytopenia” recommend treating children as outpatient featuring following characteristics: patients with newly diagnosed ITP, patients presenting platelet count below $20x10^9$/L and discreet symptoms like mild bleeding or skin manifestation only. 24 to 72 hours after diagnosis patients should undergo follow-up examination (4).

I. Treatment of children with newly diagnosed ITP and/ or mild symptoms

Clinicians must decide between strategy of watchful waiting or treatment initiation. Children with recently diagnosed ITP and discreet symptoms are rather included in the watchful waiting strategy instead of treatment initiation with corticosteroids, IVIg or anti-D Ig administration (4, 23). In case of moderate to severe bleeding, IVIg and anti-D should be administered as single dose. A second dose can be assessed in bleeding or insufficient response (23).

According to the question which type of corticosteroid and treatment duration the American panel recommends prednisone 2-4mg/kg per day for a maximum duration of 5 to 7 days (4, 23). The international consensus guideline suggests a treatment scheme of 3 or 4 doses for 4 days (23).

Similar, the treatment for patients with persistent or chronic ITP initially starts with watchful waiting. Children with reduction in their quality of life or frequent or severe bleeding should be referred to a haematologist and being treated with TPO-RA. Management of insufficient treatment responses includes a change of the TPO-RA drug or a combination with MMF or other immunosuppressant (23).

II. Second-line treatment

According to the Paediatric and Adult Registry on Chronic ITP 38-47% of the children receiving immediate treatment are dependent on second-line treatment after 6-24 moths (26).

Second-line treatment includes splenectomy and the pharmaceutical approach of TPO-RA and rituximab. The American panel suggests the use of TPO-RA rather than rituximab or splenectomy (4).

Recurring bleeding, increased risk of bleeding, fatigue, activity restrictions, and impaired quality of life are common reasons inducing second line treatment. Treatment outcomes (bleeding, platelet count and health related quality of life (HRQoL)) during the management with different second-line treatments were compared (27). 6 months after initial treatment, romiplostim and rituximab showed the most remarkable and stable increase of platelet counts, presented in Table 3 (27). Accordingly, the same drugs demonstrated the most remarkable reduction in bleeding frequency (27).

	n**	Median platelet count, $\times 10^9$/L (range)	Complete response*	Partial Response	No Response
Rituximab					
1 month	42	65 (4-230)	8 (19%)	15 (36%)	19 (45%)
6 months	33	151 (3-412)	17 (52%)	9 (27%)	7 (21%)
12 months	31	156 (4-408)	17 (55%)	8 (26%)	6 (19%)
Oral Immunosuppressants					
1 month	16	60 (1-327)	2 (13%)	3 (19%)	11 (69%)
6 months	8	75 (11-261)	2 (25%)	1 (13%)	5 (63%)
12 months	4	75 (10-216)	1 (25%)	1 (25%)	2 (50%)
Romiplostim					
1 month	29	67 (1-357)	6 (21%)	9 (31%)	14 (48%)
6 months	24	160 (6-598)	17 (71%)	3 (15%)	4 (17%)
12 months	16	147 (29-408)	9 (56%)	4 (25%)	3 (19%)
Eltrombopag					
1 month	20	89 (10-402)	6 (30%)	5 (25%)	9 (45%)
6 months	15	97 (6-301)	4 (27%)	6 (40%)	5 (33%)
12 months	12	106(15-300)	5 (42%)	4 (33%)	3 (25%)

Table 3 Second-line treatments in children with immune thrombocytopenia: Effect on platelet count and patient-centered outcomes, R.F. Grace et al., Am. J. Hematology, 2019; 94(7): 741-50

Description: Platelet response by treatment at time point 1, 6 and 12 months after initial treatment.
Since their last (or initial) study visit patients presented after 1 month, 6 months or 12 months with ≥50% of platelet counts >100 × 10^9/l (complete response), ≥50% of platelet counts >30 × 10^9/l and twice their baseline (partial response) or inapplicable for CR or PR criteria (no response).

The authors stated that clinicians must balance the reasons for treatment. The treatment modalities lead to different outcomes. For children in the need of a rapid response eltrombopag might be most efficacious treatment. Aiming an improvement of HRQoL with sufficient clinical response rituximab or eltrombopag

are suggested (27). In cases of recurrent bleeding the authors demonstrated the most significant efficacy in treatment with romiplostim or rituximab (27).

III. Emergency treatment in children at any stage of their ITP

In life-threatening bleeding conditions, a combinational therapy should be administered. A regimen of a two-/three-folds higher as bolus and continuous infusion of IV high dose steroids (eg. Methyl predniso(lo)ne, 30 mg/kg per day) and IVIg (0.8-1.0 g/kg per day, with or without single-dose IV anti-D (75mg/kg) is indicated. If the bleeding continues despite therapy antifibrinolytics can be administered (23).

IV. Other treatments for ITP in adults and children

Since more than 20 years mycophenolate mofetil (MMF) is commonly used as immunosuppressant drug in prevention of solid organ rejection following transplantation and to treat autoimmune disorders. MMF is transformed to its active metabolite mycophenolic acid, that subsequently inhibits ionise monophosphate dehydrogenase. Ionise monophosphate dehydrogenase is necessary for the purine synthesis in lymphocyte and thereby inhibits the synthesis of DNA (28).

Two studies investigated MMF as treatment option in children with first-line refractory ITP. Maurizio Miano et al. analysed a cohort of 56 children treated with MMF. The cohort consists of primary or secondary (due to ALPS related syndrome) ITP and ES patients (29). 58% of the patients with ITP presented an overall response – complete or partial response – after MMF treatment. In the majority of that subcohort response determined after less than 8 weeks. This rather short duration of response provides an advantage in clinical management and therapeutical procedure (29).

Arun Panigrahi et al. investigated the combinational use of MMF and short duration corticosteroids in paediatric patients with persistent or chronic autoimmune cytopenia (30). All patients (ITP n=6; AIHA n=3) accomplished a complete response of greater than 100 $x10^9$/l platelets or a haemoglobin level of greater than 10g/l during their combination therapy of MMF and short-duration corticosteroids (30). MMF is highlighted as a sufficient second-line treatment. As

co-treatment it provides rapid reduction of corticosteroid treatment and contributes to a long-term remission (30).

Yet, in some patients therapeutic management achieves no sufficient response. For adult patients with chronic ITP fostamatinib, a Syk inhibitor, represents a promising oral treatment option (16). Fostamatinib was proven to reduce bleeding events and severe courses (16). Sufficient stable responses in adult ITP patients indicate, that fostamatinib represents a promising treatment option in therapy-resistent ITP (16).

1.1.3. AIHA

Introduction

Autoimmune hemolytic anemia (AIHA) in childhood and adolescents is a rare immunologic disease that leads to the premature destruction of erythrocytes. The common cause are autoantibodies that target antigens on the surface of red blood cells (31).

AIHA is divided into primary AIHA, without any detected underlying pathology, and secondary AIHA, which is more common. Secondary AIHA is caused by neoplastic pathologies, infections, drug treatment or an underlying immunologic disorder.

Primary AIHA is classified by autoantibody groups that include warm AIHA, cold agglutinin syndrome, paroxysmal cold haemoglobinuria, and mixed-type AIHA.

Historical aspects

A major advance in diagnosis and understanding of AIHA was the development of the antiglobulin test by Coombs et al. 1945. The authors were able to detect autoantibodies that were bound on the erythrocytes' surface. In 1946 Boorman et al. used the direct antiglobulin test (DAT) to determine the association between hemolytic anemia and incomplete antibodies on the erythrocyte's surface (32). The DAT became the diagnostic tool for unambiguous diagnosis of AIHA. After evaluating that some patients with AIHA had a negative DAT result, 1971 Gilliland et al re-activated investigation on the diagnostics in AIHA. The authors demonstrated that DAT-negative patients with AIHA present increased erythrocyte bound IgG levels. The IgG levels were below the detection level of a DAT (32).

Further investigations of the pathophysiology of AIHA followed in the 1970s and 1980s focussing on antibodies and the related complement reaction. Besides, the mechanisms of T cells and the MHC II complex as a crucial antigen structure for antibodies were highlighted (32).

Epidemiology

The nationwide pediatric CEREVANCE study in France, included 265 pediatric patients under the age of 18 (33). The study provided a broad spectrum of epidemiological data for the French population. It demonstrated an incidence of 2-4 per million individuals under the age of 20. Thus, it revised the formerly reported incidences of 0,2 per 1000000 to be 10 to 20fold higher, which can be explained by growing participation of patients and departments (31, 33). AIHA shows an overall incidence of 1-3 cases/100000 and a prevalence of 1 per 100000 people (34, 35).

The mean age of the time point of diagnosis in the CEREVANCE cohort study is 3,8 years and the ratio male/ female is 151/ 114 (33). From 1994 until 2014, the Mayo Clinic studied 35 pediatric patients with AIHA. Their study population showed a median age at diagnosis of 10.0 years with 65% male patients of the whole cohort (36).

Pathophysiology

AIHA is characteristically caused by autoantibodies which bind to antigen structures on the erythrocytes' surface. Autoantibody mediated cytotoxicity results in the premature destruction of erythrocytes. In most cases, autoreactive IgG bind erythrocyte surface antigens. They lead to extracorpuscular destruction and hemolysis in the extra-vascular compartment. In some cases, IgA and IgM can also target erythrocyte surface antigen. Warm IgM occurs in rare cases and is considered an atypical autoantibody. IgA/ IgM and IgM mediated AIHA can lead to severe AIHA courses, why the detection of these atypical autoantibodies is crucial. Since it has been demonstrated that the maximum effectiveness of IgG is around 34-37°C, this autoantibody reaction is classified as a warm autoantibodies. Warm IgG autoantibodies are the most common form and represent 60-70% of the AIHA associated immunoglobulins. The immunoglobulin is directed against the Rh-D antigen. Cold autoantibodies are less common with a frequency of 20-25% in

AIHA. Predominant immunoglobulin is IgM. IgM is activated when patients are exposed to cold temperatures, with maximum activity of 4°C up to temperatures of 27°C. Opsonisation leads to activation of complement cascade and hemolysis in the intravascular compartment (37). Autoantibodies are specific for Anti-I, a subclass of erythrocyte antigens (38). In certain cases, both autoantibody isotypes exist, IgG as well as IgM and both antigen specificities can be detected as anti-Rh and anti-I. These cases are rare with a frequency of less than 5 %. IgG - IgM associated AIHA is known as mixed AIHA (31).

The reasons for autoantibody mediated cytotoxicity are diverse and yet are not solved individually. The loss of immunologic tolerance against erythrocyte related self-antigens can be caused by polyclonal lymphocyte activation. Molecular mimicry is supposed to disturb immune regulation and activation within self and foreign antigens. Defects in the central or peripheral disturbance of immunological tolerance and immunoregulatory deficiency, including changes in the cytokine system, are frequently associated to AIHA (39).

I. Cytokine dysregulation

For each classification of AIHA, a specific red cell-bound antigen is determined. The underlying loss of tolerance and immune regulation to erythrocyte antigens is unknown. The authors C. Toriani-Terenzi and E. Fagiolo suspected a dysregulation the cytokine - IL-10 and IL-12 – circuit, that promotes the development of AIHA. A skewing towards Th2 phenotype was suggested. Their hypothesis is based on elevated IL-10 levels and limited increase of constitutional high IL-12 levels after mitogen stimulation (40). A predominance of Th2 phenotype correlating to AIHA development could be supported by low basal interferon-gamma levels (40). Thereby interferon-gamma is known to suppress the Th2 profile development (13).

Related to the dysregulated cytokine circuit pattern analysis of Th1 and Th2 cytokine production by unstimulated and mitogen-stimulated whole blood cultures from AIHA patients was performed (41). Reduced production of IFN-Gamma and increased production of IL-4, IL-6, IL-13 after mitogen stimulation expressed an imbalanced Th1/Th2 cytokine profile (41). Besides an imbalance, patients with active disease status presented striking Th1 cytokine reduction. Increased

presence of IL-4 indicates a suppression of Th1 profile as IL-4 inhibits Th1 development (13). Adjunction of Th2 cytokine to whole blood cultures from AIHA patients increased autoantibody occurrence against erythrocytes (41). These findings supposed a predominant Th2 like profile in AIHA patients. Adjunction of TGF-beta to whole blood cultures promoted the development of autoantibodies against erythrocytes (41). Related to a NZB mouse model (New Zealand Black mice are frequently used for autoimmunity models as they spontaneously develop autoimmune disease and clonal B cell tumor), TGF-beta is supposed to insufficiently limit or promote B cell hyper-activity (41-43). That correlates to augmented autoantibody development and disease activity (41).

II. Deficiency of immune regulatory mechanisms

CD4+CD25+ regulatory T cells (Treg) are investigated in several autoimmune mediated diseases. A. Mqadmi et al. demonstrated a controlled induction of AIHA by CD4+CD25+ regulatory T cells (44). To induce AIHA in normal mice the Marshall Clarke and Playfair model was used (44, 45). In this model normal mice repeatedly obtain transfusions of rat RBCs. These mice develop RBC autoantibodies (demonstrated in Figure 4a) and rat specific alloantibodies. The crucial role of CD4+CD25+ regulatory T cells in the development of AIHA was demonstrated strikingly (44). Development of AIHA increased from 30% to 90% after regulatory T cell depletion via anti-CD25-antibody (44). To further confirm the suppressive role of regulatory T cells naïve mice obtained CD4+CD25+ T cells from mice previously transfused with rat RBCs. After transfusion of CD4+CD25+ T cells these mice were immunized by rat RBCs (44). For validation a cohort of naïve mice followed the some transfusion procedure with CD4+CD25- T cells.

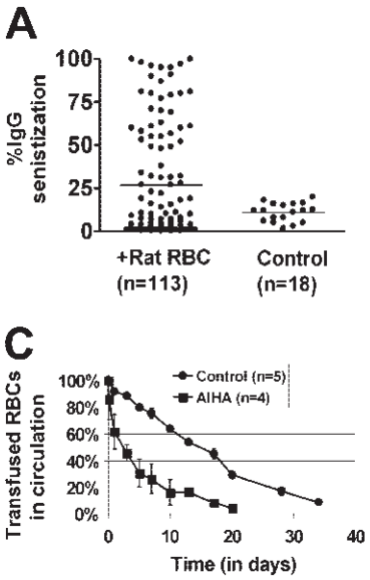

Figure 4 CD4+CD25+ regulatory T cells control induction of autoimmune hemolytic anemia. A. Mqadmi et al., Blood. 2005;105(9):3746-8

Description: For 10 weeks, female normal mice were immunized repeatedly on a weekly basis with rat RBCs.

A) IgG (autoantibodies specific for mouse RBCs) levels, the bar indicates the mean values and n is the number in each group

C) Demonstration of accelerated RBC destruction as a sign of AIHA. RBCs of previously rat RBCs transfused mice (n=4) and RBCs control mice (n=5) are labelled with fluorescent. Fluorescent RBCs are injected in normal, naive mice.

As demonstrated in Figure 5 f the autoantibody production was completely suppressed in mice previously obtained transfusion of CD4+CD25+ (44). CD4+CD25+ regulatory T cells mediate autoantibody specific as anti-rat RBC antibodies developed, demonstrated in Figure 5 g (44). Insufficient regulatory T cell activity supposes to be an essential factor for induction, susceptibility and controlling of autoimmunity.

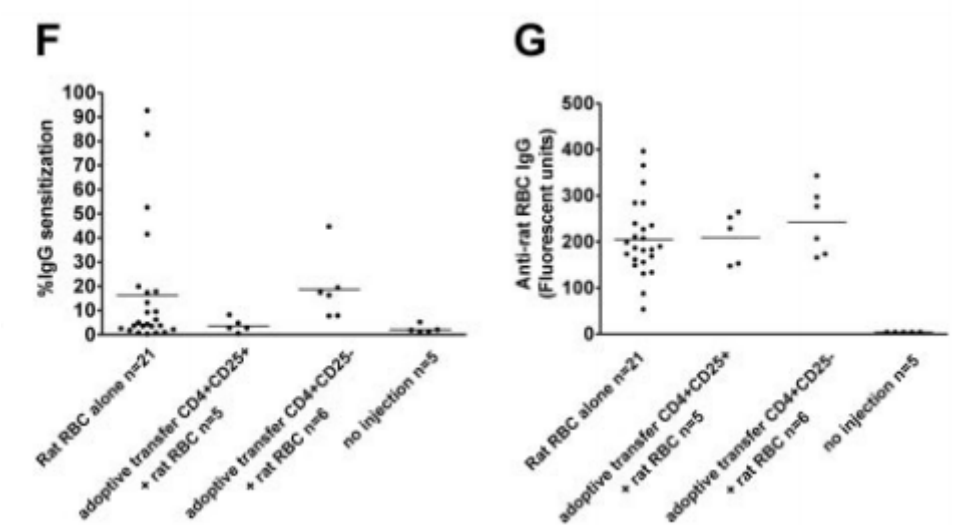

Figure 5 CD4+CD25+ regulatory T cells control induction of autoimmune hemolytic anemia. A. Mqadmi et al., Blood. 2005;105(9):3746-8

Description:
F) CD4+CD25hi or CD4+CD25low cells were transferred into naive female mice, afterwards weekly immunization with rat RBCs for 9 weeks followed. Levels of IgG autoantibodies on mouse RBCs were analysed.
G) Analysis of the same mice cohorts analysing alloantibodies against rat RBCs

Crucial insights of the properties of Treg were provided by analysis of an ex vivo Treg clone population (46). The Treg clones were derived from a patient with active AIHA. Naïve CD4+CD25- Treg are activated (CD4+CD25+ Treg) by self-stimulation via IL-10 secretion (46). The derived clones were Il-10+ CD4+CD25+ Treg (P8(1)). After detection of autoantigen specificity of these Treg clones against Rh peptide 72H-86L, a cell line (P8 line) specific for autoantigen peptide 72H-86L was derived (46). Autoantigen stimulation induced a Treg specific cytokine profile of predominant IL-10 secretion manifesting in the derived clones and in the cell line as demonstrated in Figure 6. In comparison, non-specific stimulation by anti-CD3+CD28+ antibodies resulted in a Th0 cytokine profile (46). IL-10 upregulation in autoantigen induced, IL-10 associated Treg response is supposed to characterise active disease status in AIHA and supports IL-10 as essential factor in immune homeostasis (47).

CD25 is constitutively expressed on Treg during expansion and its expression increases after Treg activation. CD25 is the alpha-chain of the IL-2 receptor. Co-expression of CD25 to the beta- and gamma-chain of the IL-2 receptor results in higher affinity to IL-2 compared to beta- and gamma-chain restricted IL-2 receptors on naïve T cells (13). After specific autoantigen stimulation CD25 expression decreased on the Treg clones and autoantigen specific cell lines (46). Autoantigen specific IL-10 associated Treg activation decreases the Treg activation marker CD25 (46). Expansion of FoxP3, a transcription factor maintaining the function of Treg, in clonal Treg represents a characteristic marker for autoantigen specific Treg activation (46). According to the FoxP3 expansion the focus mainly regards the functional analysis of Treg. Patients with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), are characterised by dysfunctional Treg. They revealed normal levels of CD4+CD25+ FoxP3+ T cells in an anergic status (48). Dysfunctional Treg and CD25 dependent IL-10 absence characterise the CD25 deficiency (49). Both syndromes cause immune dysregulation and autoimmunity (48, 49). The properties of defective (suppressive) Treg highlight functional vs. dysfunctional role of Treg in AIHA.

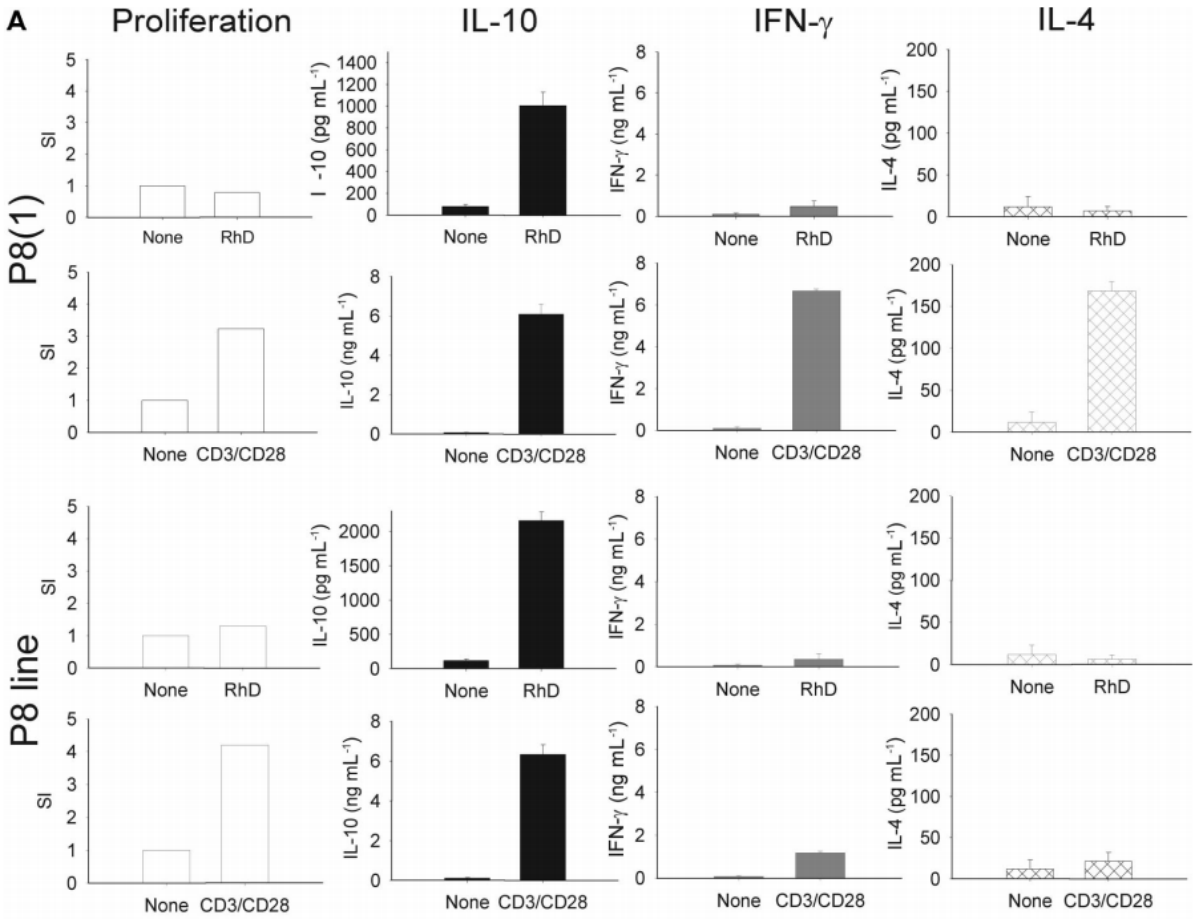

Figure 6 Clonal regulatory T cells specific for a red blood cell autoantigen in human autoimmune hemolytic anemia, F.J. Ward et al., Blood. 2008;111(2):680-7

Description: Comparison of specific autoantigen and unspecific antigen stimulation in Rh peptide specific Treg clones (P8(1)) and Rh peptide specific derived cell lines (P8 line). The autoantigen stimulus induced predominant secretion of IL-10, a regulatory cytokine profile (Tr1 profile). Unremarkable Th1 cytokine IFN-Gamma increase and no Th2 cytokine IL-4 or proliferation were detected.

After stimulation with nonspecific anti-CD3+/CD28+ antibody neither the Treg clones (P8(1)) nor the clonal cells (P8 line) demonstrate a regulatory cytokine profile, concluding that the regulatory cytokine profile (Tr1 profile) might be dependent on the specific autoantigens.

Symptoms and clinical course

Patients with AIHA can presented typical symptoms of anaemia. Most frequent are weakness, dizziness and in severe cases dyspnoea. Marked hemolysis clinically presents as jaundice or rarely as dark coloured urine (33). Besides clinical

symptoms of anemia and hyperbilirubinemia, splenomegaly can indicate a clinical symptom of marked AIHA. Since secondary AIHA occurs in the majority of all cases symptoms beside anemia must be taken into consideration (31, 33, 50). Neoplastic pathologies present with lymphadenopathy, immunologic susceptibility, homeostatic disturbances, B-symptoms primarily unexplainable loss of weight, recurring fever episodes and nightly sweating.

Severe haemolysis is defined as hepatosplenomegaly and hemoglobinuria and can indicate a pronounced form of hematologic disorder regarding structural disorders of hemoglobulin or red blood cells. The extended spectrum of underlying autoimmune conditions could clinically present with endocrine disturbances caused by Graves' disease or type I diabetes. Recurrent infections or protracted infectious courses in childhood can express a primary immunodeficiency (33).

Diagnostics

A few questions are recommended in the approach of patient with suspected AIHA: Is there haemolysis? Is the haemolysis immune-mediated? What type of AIHA is it? AIHA is classified by the thermal properties of the causal autoantibodies and their isotypes.

Initial laboratory testing includes WBC count, red cell morphology, reticulocyte count, indices of haemolysis like haptoglobin, indirect bilirubin and LDH. Pronounced hemolysis can be expressed by hemoglobinemia positive urine analysis. Hemosiderin in the urine can be detected approximately 1 week after intravascular hemolysis has started (31, 50). The DAT indicates the presence of immunoglobulin (Ig)G, IgM, IgA or complement (usually C3d) bound to the red cell membrane. To distinguish between the immunologic subclasses and to classify the primary AIHA the DAT should contain at least monospecific anti-IgG and anti-C3d (50).
If the DAT is negative but AIHA is suspected, a gel column agglutination technique can be performed as a more sensitive method. Subsequently, further serologic test should be performed to evaluate the type of immunoglobulins (50).

Warm AIHA is usually caused by IgG that bind optimally at 37°C. AIHA can be considered as warm AIHA if the DAT reacts positive to IgG, C3 or both.

Cold hemagglutinin disease (CHAD) is usually caused by IgM, which typically binds in vitro at a temperature of 4°C. Usually the DAT is only positive for C3. A quarter of the patients present a positive DAT for IgG. Similar but relevant to distinguish from CHAD is polyclonal cold agglutinin (CA). Laboratory difference consists of the maximum temperature enabling binding to erythrocytes (50). Paroxysmal cold haemoglobinuria (PCH) depends on biphasic autoantibodies. Initially autoantibodies in PCH bind at a low temperature to RBCs surface. With elevating temperature the autoantibodies induce a complement mediated lysis of the RBCs. In PCH the DAT is frequently only positive for C3. After exclusion of CHAD pediatric patients with a DAT positive for C3 (and IgG positive or negative) are recommended undergoing the Donath-Landsteiner Test (50).
Mixed AIHA is characterised by warm IgG and cold IgM autoantibodies. The thermal difference between both isotypes is 30°C. Most frequently the DAT is positive for IgG and C3. Cold associated symptoms as in CHAD are not common in mixed AIHA (50).

AIHA is frequently associated to other pathologies or immunological dysregulations (31, 33, 50). Not least, the diagnostic work-up remains challenging for clinicians. In a French cohort of 265 pediatric patients 141 patients were determined with immune disorder related secondary AIHA; of these, 118 patients presented with associated autoimmune disease and 23 with PID (33). Not only the symptoms of underlying pathology can be masked by marked AIHA symptoms but also the comprehensive diagnostic work-up represent a major challenge (51). Based on the broad spectrum of secondary pathologies table 4 demonstrates a comprehensive diagnostic approach (33).

- Complete blood count (including reticulocytes)
- Blood smear (exclusion of inherited disease and schistocytes)
- Coagulation tests (lupus anticoagulant and antiphospholipid antibodies)
- Extended phenotyped blood grouping
- Bone marrow aspiration, if cytopenia and reticulocytopenia
- Urea, creatinine, AST, ALT, bilirubin, GGT, haptoglobin, LDH
- Urine examination (hemoglobinuria, hematuria, proteinuria)
- Direct and indirect DAT (contact with Blood Center hemobiologist)
- IgG, IgA, IgM dosage (IgG subclasses if > 2 years old) (before initiation of intravenous immunoglobulins)
- Lymphocyte immunophenotyping (before steroids or immunosuppressive treatments): o $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, $CD16^+$, $CD56^+$. If hypogammaglobulinemia, naïve ($CD19^+IgD^+CD27^-$) and memory B ($CD19^+CD27^+$) o Double-negative T cells: $CD3^+$ $CD4^-$ $CD8^-$, TCR α/β^+ o If splenomegaly, hypergammaglobulinemia and elevated double-negative T cells: IL10, circulating FASL, Fas-mediated apoptosis functional tests. If abnormal: further sequencing of *FAS*, *FASL*, *CASP10*.[46]
- Antinuclear antibody (before starting intravenous immunoglobulins). If elevated titer, anti-dsDNA antibodies, other autoantibodies.
- C3, C4, CH50 Microbial serology, genomic or culture identification, and freezing of serum (wide and systematic)
- Chest X-ray and abdominal sonography (spleen size, tumoral syndrome)

Table 4 New insights into childhood autoimmune hemolytic anemia: a French national observational study of 265 children, N. Aladjidi et al., Haematologica. 2011;96(5):655-63

Description: Recommended diagnostical work up on children with newly diagnosed AIHA.

Treatment

Adapted to the response criteria of the 2017 published “Recommendations from the Red Cell Study Group of the Paediatric Haemato-Oncology Italian Association”, treatment responses in AIHA are classified as (31):

I) Complete response (CR): CR is achieved by a haemoglobin (Hb) level greater than or equal to the lowest limit in relation to the group of age. Signs of haemolysis must be excluded. The reticulocyte count and bilirubin concentration should be normal.

II) Partial response (PR): PR is classified as an increase of the Hb of ≥2 g/dL. The Hb concentration does not reach a normal value for the patient's age.

III) No response: No response is characterised by failed increase of Hb and/or dependency on blood transfusions.

I) Emergency therapy

Blood transfusion is recommended if the anemia is life threatening. In children with pronounced hemolysis performing blood transfusion can be less aggressive (31). Besides, a rescue therapy for warm antibody AIHA IVIg or plasma exchange should be considered. Q. A. Hill et al. reported that IVIg in a dose of 0,4–2 g/kg/day for 2–5 days achieved a response rate in one series of 54,4% (50). Thus, plasma exchange is recommended restrictively and preserved for extremely severe cases of hemolysis as evidence is limited (31).

Emergency therapy for life-threatening primary CHAD anemia should consist of an approach with prednisolone or plasma exchange (31).

II) General strategies

Corticosteroids are the established first-line management. As an initial dose for children 2 mg/kg/day are recommended. Response rates of 81-100% are given for children (31, 34, 36, 50). If required, intravenous methylprednisolone can be administered with a dosage of 0.8- 1.6 mg/kg/day (31). In the CEREVANCE study group steroid therapy was initiated as first-line treatment in 92% of patients. In 58% of the cases, a complete remission was attained at the end of the first month (33). Reduction of the corticosteroids remains a major challenge as relapses frequently occur (52).

IVIg can be used in addition to steroid in AIHA. Efficacy of IVIg in pediatric setting was supported and recommended as a dosage for children of 0,4-0,5/kg/day (53). As conclusion IVIg can be administered as an auxiliary treatment option to steroids in more severe cases (31, 53).

Therapy-resistant courses and steroid dependency represent main criteria for induction of second-line treatment. Rituximab is the preferred choice as second-line treatment. Rituximab is a monoclonal anti-CD20-antibody. Originally used in B-cell lymphoma it is widely used in autoimmune diseases as it depletes B lymphocytes by apoptosis inducing CD20 crosslinking. In pediatric AIHA rituximab presents overall response rate greater than 75% (28, 50).

The third line therapy regime is classified as immunosuppressive or -modulatory treatment, respectively. Third line treatments are frequently adjuncts to

corticosteroid therapy and consist of azathioprine, ciclosporin, sirolimus and MMF (50, 52). Although established in the autoimmune treatment single reports in pediatric patients provide limited significance in their efficacy and use (50, 52). In the CEREVANCE cohort more than on third obtained additional treatments to corticosteroids (33). To increase early overall response adjunctive treatment to steroids is recommended; the therapeutical management in pediatric AIHA remains challenging and based on limited controlled studies it is mainly based on personal expertise (33).

1.1.4. Evans Syndrome

Introduction

Evans syndrome (ES) is a rare manifestation of immune dysregulation. ES is defined as simultaneous or sequential occurrence of AIHA and ITP. It is differentiated between primary ES and secondary ES due to autoimmune syndromes or immune disorders. ES can present a relapsing or chronic course. Therefore, patients are usually dependent on prolonged immune suppressive therapy. ES implicates a burden in life quality, augmented health related risk and statistical higher mortality rate. It is essential to further improve diagnostic work up and classification of individual ES courses with the attempt to a sufficient individualized therapy.

Epidemiology

As epidemiologic data of AIHA and ITP are limited, our knowledge about the occurrence of ES is limited. A Danish nationwide population-based cohort study of children diagnosed with ES younger than 13 years was performed between 1981 to 2015 (54). 159 cases of AIHA and 3160 cases of ITP were detected (54). In total 21 children with ES were either subsequently diagnosed (85,7%) or simultaneously diagnosed (14,3%). A prospective French national observational cohort detected 156 children with ES in the period between 1981 to 2014 (55). In 25% the initial cytopenia was AIHA, in 29% ITP and ES in 46% of all cases. The median age of cytopenia onset was 5,4 years, similar to a median onset of 4,7 years in a pediatric cohort with ES associated to PID (ALPS-Fas, CTLA-4, LRBA) (55, 56). The incidence in the Danish pediatric cohort increased from 0,5/1000000 person-years (1981-1990) to 1,2/1000000 person-years (2006-2015); similarly the

prevalence increased from 6,7/1000000 (1990) to 19,3/1000000 (2015) (54). The hazard ratio for mortality in ES compared to general, age-adapted Danish population was 22,3fold increased; confirming that bleeding is the more prominent reason for mortality in ES, the mortality rate for ES is determined 10% (54, 55). The increasing dynamics of epidemiological data might be explained by improved diagnostics and broader awareness of clinicians (54).

Pathophysiology

The most common definition of ES is the manifestation of AIHA and ITP. Several authors use the term Evans syndrome for any combination of two- or trilineage autoimmune cytopenia. Independent whether the autoimmune cytopenia occur simultaneously or subsequently; fact is, the pathophysiologic basis for ES is reflected by a failure of immunity characterised by immune dysregulation and/ or autoimmunity.

Autoimmune cytopenia are strikingly associated to PID: AIHA, ITP and ES are the predominantly occurring autoimmune manifestations in PID (57). Compared to a healthy control population the relative risk yields an 830-fold increase for AIHA, a 60-fold increase in ITP and a 120-fold increase in autoimmune cytopenia (57). Autoimmune cytopenia were associated to all underlying mechanisms of PID particularly T cell deficiencies (57). PID are commonly caused by monogenic defects (39). Failure in particular immune processes or pathways indicate relation between preventing autoimmunity and immune dysregulation and illustrate maintenance of immune tolerance (58).

Evidence for the association between monogenic immune deficiencies and autoimmune cytopenia provided a genetic analysis in paediatric ES (59).

Failure of control of self-reactivity includes several mechanisms: failure in central negative selection, aberrant antigen receptor editing, disturbance of regulatory T cells, disturbed elimination of autoreactive B and T cells (59). 80 non-selected patients with early onset ES underwent genetic testing. 40% presented a known pathogenic mutation in nine known PID genes (59). 25% demonstrated probably pathogenic mutations in 16 different genes all encoding areas relevant for immunologic homeostasis (59). The greater frequency of associated immunologic manifestations, demonstrated in Figure 7, and higher need for second-line therapy

indicate the clinical relevance and the purpose of early individualized management (59).

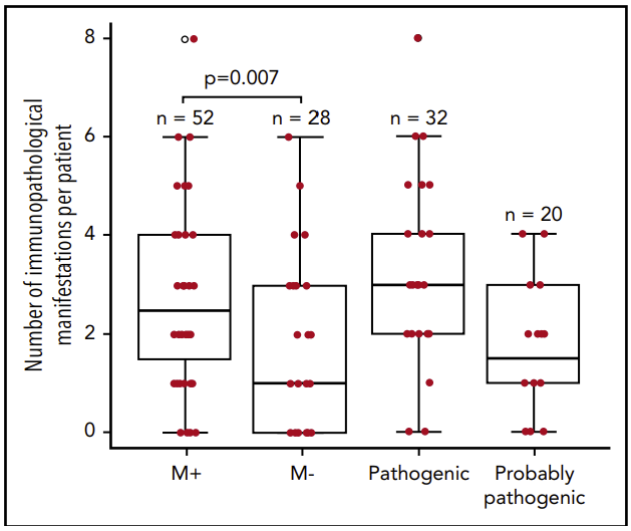

Figure 7 Pediatric Evans syndrome is associated with a high frequency of potentially damaging variants in immune genes, J. Hadjadj et al., Blood. 2019;134(1):9-21

Description: The box plot represents the number of immunologic manifestations per patient. Total of 80 pediatric ES patients (M+ n=52, M- n=28). M+ is subdivided in pathogenic and probably pathogenic mutations. The dots at the top or bottom indicate outlying data. M+ representing the proportion with a positive genetic diagnosis, M- represents the proportion with no genetic abnormality.

Correlating to the clinical relevance the probably pathologic genetic variations might induce dysfunctional regulation of T cell proliferation by Treg or increased effector response due to GOF and LOF mutations (59)

As described, ES derives due to autoimmune reactions or immune dysregulation. PID, variations in immunologic genes and IEI associated with deficient immune regulation are classified and characterised based on the related immune deficient pathway (39, 51, 58). Failure of intracellular metabolic depletion due to TPP2-deficiency induces premature immunosenescence and autoimmune mediated ES (60). CVID phenotype (panhypogammaglobulinemia) patients and CVID patients manifest with immune dysregulatory lymphoproliferation and ES (58, 61, 62). Evans syndrome can result in the context of impaired apoptotic process of lymphocytes. Pediatric patients with ALPS characteristic αβ-positive double-

negative (CD4−CD8−) T lymphocytes and patients with disease defining defective Fas-mediated lymphocyte apoptosis presented Evans syndrome. Immune dysregulation manifesting by lymphoproliferation and insufficiently restricted lymphoproliferation due to deficient apoptosis might display causal factors for ES (61, 63). Patients with CTLA-4 defect and LRBA defect typically present childhood ES. By suppression of activation CTLA-4 (CD152) balances T cell proliferation. LRBA is supposed to function as intracellular guiding protein. Defect in both manifests with immune dysregulation (lymphoproliferation), multiorgan autoimmunity and early onset ES (39, 56).

Clinical Manifestations and Symptoms

The symptoms vary between clinical manifestation of AIHA and ITP. The following symptoms may be among patients presenting simultaneously two-line cytopenia like ES or subsequently cytopenia: AIHA leads to pallor, fatigue, dyspnoea, and often fever. The presence of hepato-splenomegaly, jaundice, and hematuria or hemoglobinuria may also be detected but indicate a marked anemia. Thrombocytopenia characteristically presents with petechiae, bruising and muco-cutaneous bleeding (52). To characterise the clinical course the follow-ups of 156 paediatric patients were studied (55). Complete remission (CR) was defined as platelet count >100 g/L and Hb ≥11 g/dL with reticulocytes counts of ≤120 g/L, and ongoing or discontinued treatment for less than 12 months. Continuous complete remission (CCR) was defined as complete remission without any treatment for more than 12 months. After 5 years 74% of the patients achieved CR or CCR (55). The challenging clinical predictability is represented by 74% of the patients relapsing after a median duration of 8 months after initial diagnosis (55). The severity of ES is characterised by ITP as the high mortality in ES is mainly depending on ITP related consequences. Demonstrating the discrepancy of relapse free survival in both cytopenia Figure 8 displays the clinical severity and challenge of ITP in ES (55).

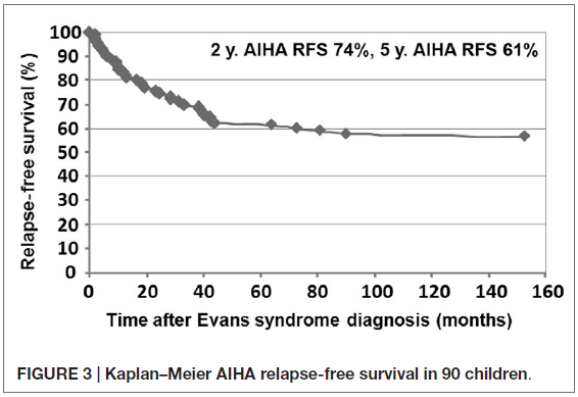

FIGURE 3 | Kaplan–Meier AIHA relapse-free survival in 90 children.

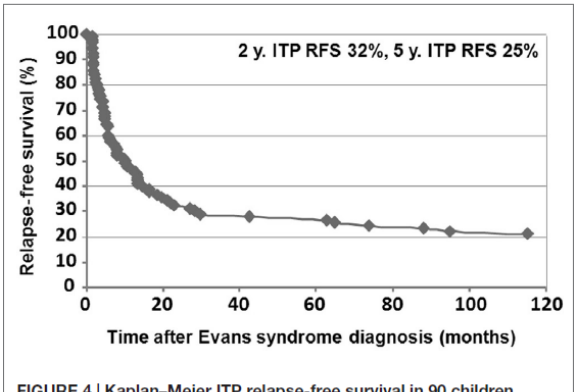

FIGURE 4 | Kaplan–Meier ITP relapse-free survival in 90 children.

Figure 8 Evans Syndrome in Children: Long-Term Outcome in a Prospective French National Observational Cohort. N. Aladjidi et al., Front Pediatr. 2015; 3:79

Description: Relapse free survival analysis of the first 90 patients represented by a Kaplan-Meier curve for AIHA and ITP.

Diagnostics

For the diagnostical process in determination of ES an intensively examination of the patient's anamneses must be performed. Certain risk factors must be contemplated such as infections, malignancies, underlying autoimmune disorders. This extensive examination of the patient's status and history is crucial in order to determine a primary or a secondary ES. Finally, the treatment of the patient is particularly depending on primary or secondary ES (64).

Laboratory diagnosis should follow diagnostical approaches similar to AIHA and ITP. To evaluate an AIHA the patient must fulfil a Hb level of <11g/L, a positive DAT test and one haemolysis criteria. Reticulocytosis of >120g/L, indirect bilirubin >17mcmol/L and / or haptoglobin <10mg/dl (64).

Laboratory testing should include complete blood count including reticulocytes. Further, a blood smear, measurement of total and indirect bilirubin and LDH.

To classify ES as a secondary ES diagnostical methods should include diagnostic work-up regarding autoimmune diseases, PID or infections:

I. Exclusion of autoimmune disorders like SLE or anti-phospholipid syndrome (APS). For SLE detection of anti-dsDNA-antibodies, anti-nuclear-antibodies and anti-Smith-antibodies must be analysed. For determination of APS anti-beta2-glycoprotein I antibodies and anti-cardiolipin-antibodies must be evaluated. Rheumatoid arthritis is diagnosed by analysis of rheumatoid factor and anti-cyclic-citrullinated peptide-antibodies.
II. In order to determine an immunodeficiency as cause for secondary ES frequently associated immunodeficiency disorders should be excluded. ALPS with a chronic lymphadenopathy and characteristic double negative T cells. Exclusion of CVID, characteristically presenting with isotype deficiency, is performed by determination of immunoglobulin levels focussing IgA and IgG. CTLA4 and LRBA defects may have to be determined by genetic sequencing. Furthermore, the complement factors C3, C4 and activity of the complement system by measurement of CH50 should be analysed. (52)
III. Common viral infections should be ruled out as well frequently associated infection with CMV, EBV, HIV. The patient should be screened for HBV and HCV or an infection with helicobacter pylori (52). Furthermore, examination should focus on detection of malignancies as lymphoma or leukaemia (64). Also bone marrow related functional or structural disorders should be evaluated (65).

Treatment

Treatment in patients with ES is classified into first-line and second-line treatment. A relapsing or chronic course of ES indicates that the therapeutic approach probably does not affect the autoimmune mediated pathway or the underlying immunologic dysregulation.

The main goal in therapy is defined as continuous complete remission.

I. First line treatment

As first-line therapeutic approach predniso(lo)ne 1-2mg/kg/day is administered. It is recommended that patients undergo a re-evaluation after three weeks. If a complete remission is achieved tapering off should be initialised over a duration of six months. Besides, patients presenting with a partial response should obtain two more consecutive weeks of predniso(lo)ne (64). To anticipate relapsing courses, it is recommended to treat full dose of predniso(lo)ne for three to four weeks (52).

IVIg represents the second first-line treatment. Usually 0,4g/kg/day for four to five days are administered. In cases of predominant AIHA it is common to raise the dosage to 0,5g/kg/day (52, 64).

II. Second line treatment

In two cohort studies rituximab demonstrated sufficient results. Of five patients diagnosed with ES and treated with rituximab three patients responded with a remarkable increase in their platelet counts within two months after starting with rituximab. In another cohort initial response occurred after a mean duration of 12 months in 86% of the patients (64).

MMF is an immunosuppressant drug inhibiting the inosine monophosphate dehydrogenase which is crucial for lymphocyte proliferation. MMF is commonly used in treatment in immune cytopenia. In a cohort study it has been shown that 13 of 16 patients achieved a complete response (29). Usually the dosage is 600mg/m^2 twice a day. After three months the first re-evaluation should be performed.

Ciclosporin, a drug used in multiple T cell mediated immune disorders, can be administered at 6mg/kg/day. Continuous monitoring of the ciclosporin blood level should be performed due the severe adverse side effects like nephrotoxicity and malignancy (64).

Similar to AIHA or ITP splenectomy is categorized as second- or third-line treatment option. It is related to severe surgical outcomes and adverse effects as post-surgical infection and infectious susceptibility. Of note, it is contraindicated to perform splenectomy in children younger than six years due to increased risk of

septicaemic course in terms of streptococcus pneumoniae, Neisseria meningitidis and haemophilus influenzae infections (66).

Therapeutic management in ES remains challenging. A previous study reported second line treatment in 69% of the patients and re-modification in second-line treatment in 47% (55). The frequent association of ES to immune deficient processes contributes to the complexity of diagnostics and the frequent insufficient therapeutic approach (55, 56).

1.2. Premature Maturation and senescence in lymphocytes (Immunosenescence)

Immunosenescence is strikingly linked to autoimmunity. Immunosenescence characteristically presents in T cells and B cells with premature maturation or antigen-experienced subpopulation skewing, respectively.

The dysfunctional mechanism of T cells can be categorized. Based on their different phenotypic markers and their activated signalling pathway dysfunctional T cells are classified in anergic, exhausted and senescent (67). Anergic T cells are the result of incomplete co-stimulation by CD28 during TCR ligation or co-inhibitory signaling of high affinity CTLA-4 (CD152). Chronical antigen over-stimulation and overexpression of inhibitory factors, as PD-1, CTLA-4, CD160, Tim-3 and LAG-3, lead to the condition of immune exhaustion. Characteristic phenotypical markers of senescence are CD57, CD160 and killer cell lectin-like receptor subfamily G member 1 (KLRG-1) and absence of CD28. On one hand, there is physiological, cellular life span related immunosenescence occurring by age. Telomer shortening (DNA damage or aberrant structure in telomers) induce telomer senescence signals. A phosphatidylinositol 3-kinase-like protein kinases conducts telomer senescence signals and induces upregulation of p21 resulting in G1 cell arrest (67, 68). On the other hand, there is premature immunosenescence occurring in an acute reaction to an infectious or autoimmune mediated stimulus. Premature immunosenescence is independent from ageing, telomere independent and potentially reversible. The unaffected telomeres might maintain by upregulated telomerase activity. The p38-MAPK is reported to be upregulated by Map kinase kinases 3 and 6. The p38-MAPK is classified as one of four subgroups of MAPK and implements cellular response among differentiation including

senescence, development, and cell death. Upregulation of p38-MAPK is associated with premature senescence (67, 69).

The main hallmarks shared by age-dependent immunosenescence and premature immunosenescence are increased proportion of terminally differentiated effector memory T cells, reduced proportion of naïve cells, dysregulated apoptosis of lymphocytes and impaired lymphocyte proliferation with skewing of the subpopulations (70).

J. Fessler et al. determined these distinct features of immunosenescence in a prospective, cross-sectional study of ankylosing spondylitis patients (SpA) and rheumatoid arthritis (RA) patients compared to healthy controls (71). Firstly, the TREC levels in SpA and RA patients were decreased. TRECs occur in terms of T cell receptor rearrangement and are composed of excised unused DNA fragments. Their detection in the blood indicates a T cell receptor rearrangement process. J. Fessler et al. determined a reduced thymic output in patients with aSpA and RA as TREC levels in naive T-cells were reduced (71).

Premature immunosenescence is characterised by CD57 expression on the surface of CD4+ and CD8+ T cells. CD57 is a beta-1,3-glucuronyltransferase composed of the carbohydrate epitope human natural killer (HNK)-1 (72). CD4+ and CD8+ T cells expressing CD57 represent a group of cells characterised as antigen specific, senescent and performed numerous cell cycles (73). Premature immunosenescence is also characterised by a skewed lymphocyte subpopulation of the naïve, central memory, effector memory and terminal differentiated T cells. Contrary to that, immune exhaustion describes a state in which chronic infection and constant antigen stimulation lead to lymphocytic adaptation in terms of progressive loss of effector functions. As well as an associated acquirement of regulatory and inhibitory surface receptors and disturbances in the cytokine profile (74).

Inborn errors of immunity, in detail monogenic PID, characterised by immune deregulation (-deficiency, -proliferation and susceptibility) and autoimmunity feature reminiscent lymphocytic immunosenescence. They develop by multiple pathways: PIDs associated with an underlying monogenic disorder firstly, mimic constant signalling, secondly, present disturbance in lymphocytic regulation, thirdly, present compromised lymphocytic apoptosis (74). Exemplary for monogenic mutations associated with a PID and immunological phenotypic

alterations is the cohort study including 39 patients with PIK3CD GOF mutation (74). E. S. J. Edwards et al. presented CD8+ T cells skewed towards effector memory population and increased expression of CD57 (74). Overall, the CD8+ T cells demonstrated an accelerated differentiation and concomitant insufficient cytotoxicity. Interestingly, B cell ligands for regulation of CD8+ T cell and natural killer cells (NK cell) cytotoxicity were upregulated. Ligands of 2B4 (CD48) and CD27 (CD70) were upregulated in PIK3CD GOF mutation affected B cells (74). After CD40 stimulation also ligands for PD-1, PD-L1 and PD-L2 were significantly upregulated (74). PD-L1, constitutively expressed, and PD-L2 balance T cell activation and differentiation to effector cells (75).To augment the effect of further and more detailed understanding of the consequences of monogenetic mutations leading to PID Edwards et al. hypothesize an immunomodulatory therapeutic approach among the accessibility of the CD48-2B4 and PD-1-PD-L1/PD-L2 axes (74). Alterations in the T cell maturation were presented by J. Bier et al. as well by investigating 37 patients in a mean age of 20 years with GOF mutation in PIK3CD (76). The authors found parallels to PIDs like CVID, STAT3 LOF and STAT1 GOF. Firstly, the authors found an increase in T follicular helper cells (Tfh) in PIK3CD GOF mutation patients. As a consequence from that the authors suggests that the Tfh cell expansion is due to dysregulation of other cell compartments particularly B cells. This results in a disturbed support of Tfh cells during a T cell dependent humoral immune response. Secondly, the production of Th2 cytokines by CD4+ T cells in PIK3CD GOF mutation patients was increased with a skewing towards IL-4, IL-5 and IL-13 production (76). Investigating 51 patients with SpA (mean age = 40,7 years) and 51 patients with RA (mean age = 52,3 years) J. Fessler et al. reported that both patient groups presented a reduced proportion of CD4+CD45RA+ and CD8+CD45RA+ T cells in comparison to 51 healthy controls (71). Premature senescence is no phenomenon particularly associated to autoimmune mediated cytopenia. Premature senescence is detectable in a broader spectrum of autoimmune diseases.

The association between premature senescence and skewed maturation within the lymphocytic populations was also remarkably demonstrated by R. Ruiz-Garcia et al. in four patients with GATA binding protein 2 deficiency due to a mutation in the GATA2 gene (77). GATA binding protein 2 deficiency is associated to cytopenia. In the study of four patients (P1 12 years, P2 26 years, P3 11 years and

P4 32 years), one patient (P4) presenting cytopenia during the period of analysis, skewed maturation profiles of lymphocytes were detected. The senescent populations of CD4+ and CD8+ T cells were increased (77).
Interestingly, the phenomena premature immunosenescence is an immunologic condition presenting in the context of hematopoietic stem cell transplantation as well. Ga Hye Lee et al. investigated T cell sets of parental donors and pediatric recipients of haploidentical hematopoietic stem cell transplantation (78). They conducted flowcytometry based phenotypic and functional analyses and analysis of telomere length of the T cells. Of note, in patients the population of senescent CD28- or CD57+ T cells was significantly expanded. Furthermore, both, CD4+CD28- T cells and CD4+CD28+ T cells showed reduced cytokine production. As patients' T cells preserved telomere length the authors confirmed characteristics of premature immunosenescence in T cells (78).
H. Suen et al. made the observation that the T-cell clones of patients with multiple myeloma demonstrate features of premature senescent T cells: T cells presented phenotypically as KLRG-1+, CD57+, CD160+, and CD28− (67). Further, this study displayed, that these clonal T cells have identical telomere length to non-clonal T cells of age-matched controls. As a consequence, this suggests that their senescence is telomere independent and potentially reversible and assumably reflects premature immunosenescence (67).

1.3. Synopsis

What is unknown?

Several single- or multicenter trials aiming specific, clinical epidemiological approaches or interventional studies in cITP, AIHA, or ES. Furthermore, cytopenia-specific basic scientific research or translational studies have been conducted (60, 77, 79, 80).
Severe immune cytopenias (SIC) are frequently associated with immune dysregulation. Immune dysregulations that are directly associated to monogenetic mutations are classified as primary immunodeficiency (39, 60, 63, 77, 80). In both conditions immunologic dysregulation expresses as premature immunosenescence and maturational shifting of lymphocytes (60, 77, 79, 80). Investigation on the predictive and diagnostic potential of characteristic lymphocyte profiles must expand. Assembling of a characteristic immune profile is

substantial as early autoimmunity frequently indicates underlying immune deficient processes (81).

Working hypothesis

Therefore, analysis of maturational and premature immunosenescent lymphocyte panels might be a suggestive tool in diagnosis of severe immune cytopenia and indicative criteria to further immunological investigation on PID. Subsequently, following more specific analysis of lymphocyte alterations in cytopenia will yield broader and concrete knowledge of causative association between lymphocytes' status in autoimmune cytopenia and primary immunodeficiencies. Related to the previous metaphor of B. Grimbacher et al. of two sides of the same coin: one side describes clinical manifestations of autoimmunity, in this case severe immune cytopenia. The other side of the coin represents single gene defects resulting in PIDs (39).

Figure 9 aims to demonstrate the complex linkage between PIDs and SICs. The interface of PIDs and SICs contains abnormalities representing reminiscent hallmarks shared by PIDs and SICs: Premature immunosenescence and maturational shifts in lymphocytes as well as alterations in activation and prolific status of lymphocytes, coined as immunometabolism. Alterations in the cytokine secretion are expressions of the immune dysregulation. (Mono)Genetic defects result in PIDs and present significant correlation to SIC.

Major differences in microbiome composition, as reported characteristic in both conditions, mirror the common area of immune dysregulation, in which the linkage between PIDs and autoimmunity reveals.

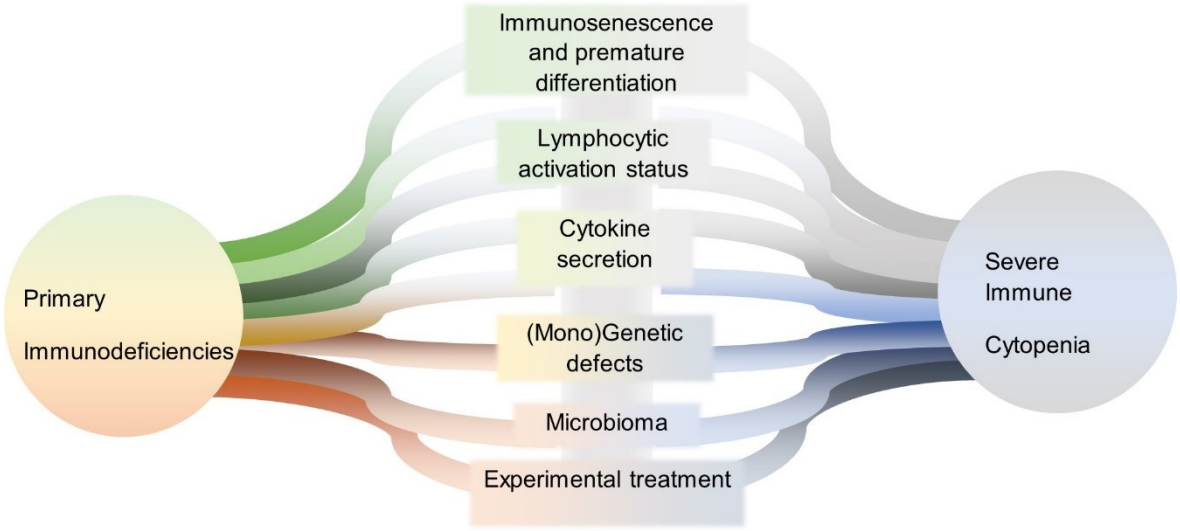

Figure 9

Description: PIDs and SICs as expression of immune dysregulation. Common phenomena and characteristics as linkage between PIDs and SICs. Many more phenomena might be undetected yet.

Study design

We investigated a general immunologic panel and lymphocytic differential panel including maturation and premature senescence markers in the study group, the retrospective group, and the observational group. Our analyses focused on pathological disturbances in subpopulations of lymphocytes and maturation shifts within lymphocytes. Furthermore, investigations of longitudinal patterns in maturation or premature senescence along the three time points were performed. Further research and merging of unpublished results of additional immunologic and genetic investigation will generate more specific insights of the immunologic pathophysiology in autoimmune cytopenia and associated PIDs.

Benefits of the research

Examination of premature immunosenescence and maturational shifts in lymphocytes will yield further understanding of the linking pathway between PIDs and SIC. By characterization of the lymphocytic phenotype we explore the lymphocytic environment regarding activation, proliferation and differentiation, regulation, and suppression.

Translational analyses of SIC patients will contribute to an assembled diagnostic approach among the interface of autoimmune cytopenia and PID. The future

diagnostic work up of autoimmune cytopenia will embrace diagnosis of PID as well. Of note, based on detailed understanding of immunologic disturbances the diagnostic approach in SIC will become more specified and therefore accelerate the diagnostic work up and restrict the disease related activity status.
As patients with autoimmune cytopenia present variable and unpredictable clinical courses including relapses more detailed determination of lymphocytic environment in SIC provides predictive value in the clinical course of affected patients. In addition, evaluation of lymphocyte profiles reveals lymphocytic properties that might expose targets for individualized therapies.
With the constant increase of insights regarding checkpoint components in lymphocytic differentiation, activation, proliferation we gain the possibility to target treatment. Simultaneously, it establishes the possibility to predict and balance the most effective therapeutic approach for any affected individual.

2. Materials and Methods

2.1. Immunologic Laboratory Analyses

Next to routine chemistry laboratory analyses, particular immunologic phenotype analysis was performed in Graz, Austria. That included flow cytometry with a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, Calif) in combination with a panel of mABs from Beckman Coulter, Immunotec SAS, Marseille, France. Pre-sorting of the patients' PBMC were performed consistently and according to CD4+CD3+ and CD8+CD3+ standardized labelling and sorting. The mABs including fluorochromes used in B and T cell pre-sorting and final gating are presented in Table 5.

B-Ly	AB + Fluor.	T-Ly	AB + Fluor.
	CD21 FITC		CD57 FITC
	CD11c PE		CD197 PE
	CD19 ECD		CD8 APC
	CD45 KrO		CD4 APC AF700
			CD45RA PB
			CD45 KrO

Table 5. Description: B cell and T cell sorted mABs and fluorochromes.

For sorting the final cell subpopulations of CD4+ and CD8+ T cells, we labelled CD4+ and CD8+ T cells with mABs anti-CD197 (CCR7) and anti-CD45RA. Maturation gating determines Naïve (CD197+CD45RA+), central memory (CD197+CD45RA-), effector memory (CD197-CD45RA-), terminally differentiated effector memory (CD197-CD45RA+) cells as presented Figure 10.

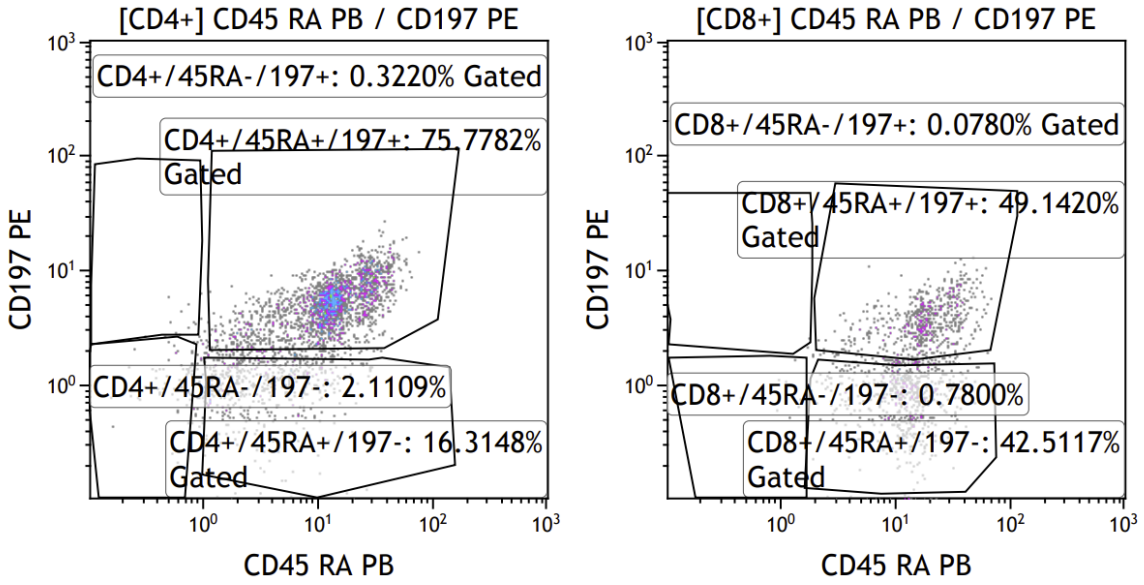

Figure 10

Description: Exemplary demonstration, on the left demonstration of CD4+CD3+ maturational gating and on the left the maturational gating for CD8+CD3+ cells.

As senescent T cells characteristically acquire CD57 (and lost CD28 expression) we labelled senescent CD4+CD3+ and CD8+CD3+ T cells by anti-CD57+. Gating for CD57+ CD4+CD3+ and CD8+CD3+ T cells is represented in Figure 11.

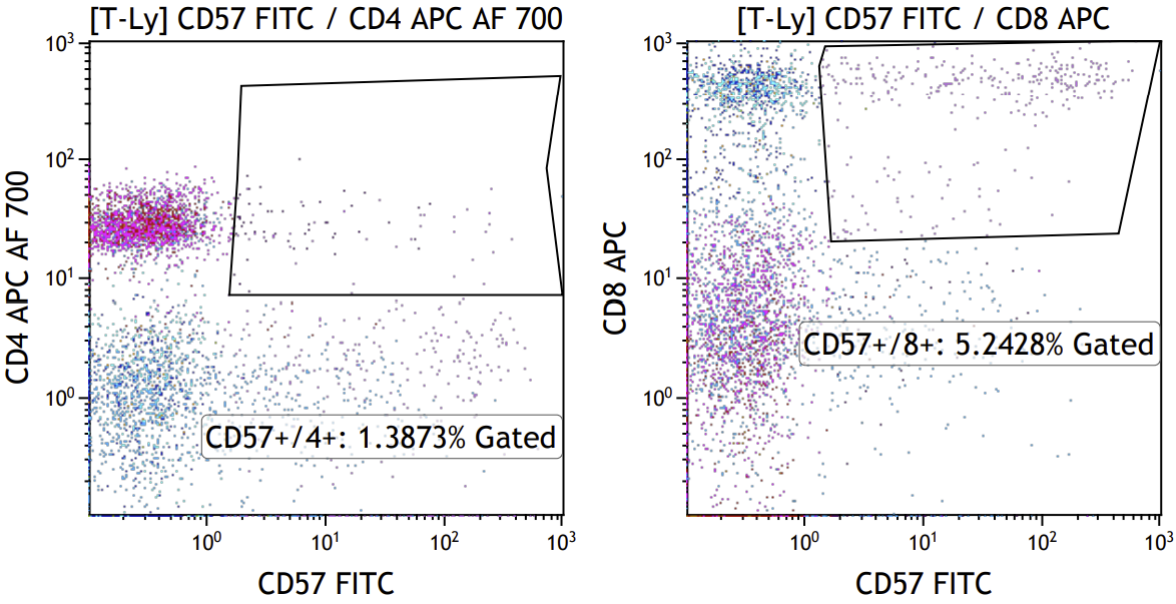

Figure 11

Description: Exemplary demonstration, gating for senescent T cells, on the left CD4+CD57+ and on the right CD8+CD57+ T cells.

Pre-sorting of the patients' PBMC were performed consistently and according to B memory cells standardized labelling and sorting protocol. Sorting of B memory cell differentiation was performed according to subpopulations Naïve (CD27-IgD+), non-class switched (CD27+IgD+) and class switched (CD27+IgD-).

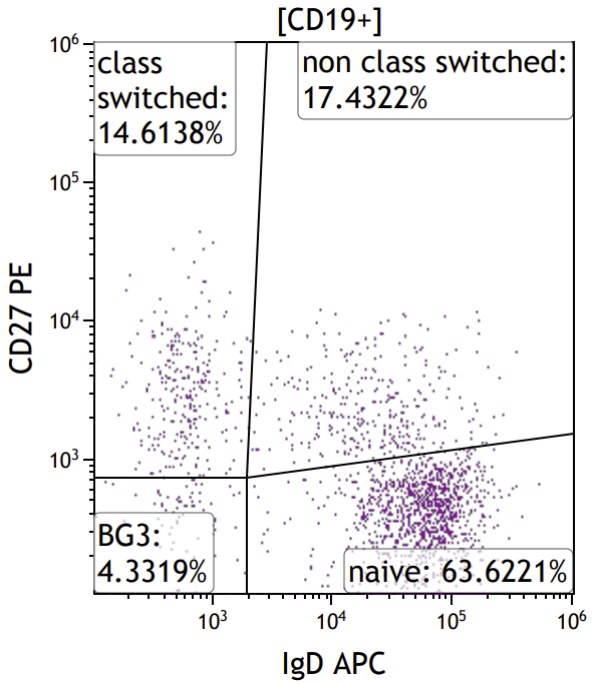

Figure 12

Description: Exemplary demonstration, gated for differentiation of B memory cells.

Sorting of age associated cell B cells (CD21lowCD11c+) was performed after standardized pre-sorting of CD19+ B cells; age associated B cells gating is demonstrated in Figure 13.

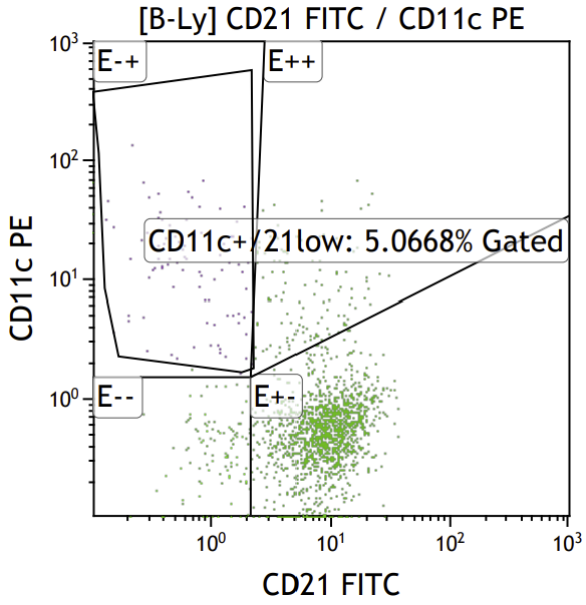

Figure 13

Description: Exemplary demonstration, gated CD21lowCD11c+ ABC

We appreciate the valuable cooperation with B. Egner, A. Raicht and Prof. W. Schwinger performing and evaluating the FACS analyses of the included study patients as well as the exemplarily represented FACS analyses.

2.2. Patients and data acquisition

This interim analysis is based on the sic-reg.org registry study (pilot phase) guided by the research unit of the pediatric department of hematology and oncology in Graz, Austria.

At the time point of data conclusion all patients were listed as current patients treated and sequentially examined at the department of pediatric hematology and oncology.

Inclusion criteria for the sic-reg.org registry study are defined for each SIC condition. In general, patients had to be between 6 months and 25 years.

Persistent or chronic ITP was diagnosed according to international definitions, including clinical findings as wet or dry purpura or hematoma at time point of consultation or regarding patient's history, and laboratory tests as described in the introduction. Inclusion criteria were defined as a duration of ITP longer than six months.

Diagnosis of AIHA was also diagnosed on basis of clinical and laboratory findings. Inclusion criteria consist of a positive DAT. In addition, laboratory criteria include hemolysis signs like elevated LDH, elevated indirect hyperbilirubinemia, signs of anaemia as reduced Hb and intensified erythropoiesis by elevated reticulocytes in the peripheral blood. In case the DAT is negative but AIHA is intensively suspected, repetition of DAT in terms of an enhanced DAT is recommended. Clinical signs displayed pallor, weakness, icteric sclerae.
Evans syndrome is defined by inclusion criteria of two-lineage cytopenia.
Exclusion criteria were determined as either active or previous malignancy or hematopoietic stem cell transplantation.
Exclusion criteria consist of exclusion of hematological-oncological causes and others. These include exclusion of neoplasm, congenital thrombocytopenia, bone marrow failure and exclusion of drugs and toxins. Anemia due to other causes as enzymopathies (e.g. G6PD deficiency, pyruvate kinase deficiency), hemoglobinopathies (e.g. sickle cell disease, thalassemia), membranopathies (e.g. hereditary spherocytosis, elliptocytosis), developmental disturbances (e.g. iron deficiency, vitamin B12/ folate deficiency) must be excluded.

9 study patients were prospectively included from the first manifestation of their cytopenia. Patients were further treated and clinically evaluated at the pediatric department of hematology and oncology in Graz, Austria. Furthermore, 3 retrospective patients were included. The diagnosis of their cytopenia was in the past or the patients demonstrated a relapse of their previously diagnosed cytopenia.
In addition, 19 observational patients with known primary immunodeficiency or bone marrow failure or hematological or rheumatic disease.
At initial time point all study patients underwent blood samples and stool samples. We performed a Case Report Form (CRF) obtaining general patients' information, medical history, evaluation of the suspected cytopenia and treatment and sequential follow ups.
The CRF was composed of subheadings. Interrogation of the most relevant facts regarding the patients' histories and clinical statuses including infections. An ITP Bleeding Score Assessment (IBLS) and an AIHA/ ES Dynamics Assessment was evaluated for every study patient. IBLS and AIHA/ ES Assessment were evaluated

at further follow up examinations as well. Besides, comorbidity, drug therapy other than cytopenia related and exclusion of hematological-oncological causes were proven. Laboratory testing included general laboratory testing and quantitative and qualitative general immunologic testing.
Additionally, infectious testing was performed including serology analyses before IVIg administration or at least 6 months after IVIg administration regarding herpes group viruses, hepatitis C, adenovirus, and parvovirus B19.
Examination and sampling were conducted initially as time point 1, after 6 months as time point 2, after 1 year as time point 3. At the time point finalizing this diploma thesis follow up examinations after 2 years (time point 4) and 3 years (time point 5) were performed on an increased number of patients.
At every time point FACS analyses of lymphocytes and lymphocytic subpopulation was performed. Further, at every time point stool sample should be taken by the patient. Besides, the CRF follow up forms should be evaluated with the patient.

Comparative extended routine immunologic laboratory data were obtained by previous immunological reports of premature senescence and exhaustion and lymphocytic maturation alterations [Stepensky et al., Blood 2015; 125(5):753-61; Rensing-Ehl et al., Blood. 2014;124(6):851-860; Lucas et al., Nat Immun 2014; 15(1):88-97].

2.3. Data Presentation

Due to the small patient number, a descriptive data analysis was performed. Figures were designed by using Prism 9.0 (GraphPad software, La Jolla, CA, USA). Tables were designed by using Microsoft Excel (Microsoft Excel software, Redmond, Washington).

2.4. Ethics Statement

The sic-reg.org study was performed in compliance with current guidelines for good clinical practice and the Declaration of Helsinki and conducted under an ethics approval (30-155 ex 17/18) granted by the Medical University Graz (IRB00002556).

3. Results

3.1. Study population

Study Patients	DOB	Age (years)	Gender	Main diagnosis	Age of manifestation	Initial Symptoms	Initial treatment	Additional treatment
K01	23.07.2009	11	f	Evans	8	n.a.	Prednisolone	none
G03	28.07.2005	15	f	Evans/ITP	13	asymptomatic	IVIg	Prednisolone + Eltrombopag + MMF
G04	11.01.2004	16	m	Evans	13	h/o petechia	IVIg	recurring IVIg + Eltrombopag + MMF
G02	01.05.2012	8	f	ITP	6	h/o petechia	Prednisolone	none
G01	23.07.2013	7	f	ITP	3	petechia ubiquitair	IVIg	recurring IVIg
G05	11.03.2015	5	m	AIHA	3	pallor, weakness	Prednisolone	none
G06	05.03.2012	8	f	AIHA	7	pallor, icteric sclerae, fatigue	Prednisolone	none
G10	14.02.2011	9	m	ITP	5	hematoma	IVIg	Prednisolone
G12	22.02.2014	6	f	ITP	5	hematoma	IVIg	Prednisolone
Retrospective Patients								
G09	30.04.2009	11	m	ES/ AIHA	7	icterus	IVIg	IVIg, prednisolone, methylprednisolone, MMF, dexamethasone
G08	03.03.2016	4	m	ITP	1	hematoma, petechia	IVIg	Prednisolone, MMF
G07	19.10.2004	16	f	Evans	10	icterus, pallor	Prednisolone	MMF

Study Patients	Primary cause/ trigger	Side diagnosis	Relapse	Infections	IBLS	Initial AIHA / ES Dynamics Assessment	Occurence of ITP and AIHA			
							Simult.	Sequent.	AIHA first	ITP first
K01	none	none	none	none	n.a.	n.a.				
G03	none	2011 bicytopenia (platelets & leucocytes)	none	febrile infection	3	3	G03			
G04	viral GE (1W before onset)	h/o vW disease	multiple relapses (thrombocytopenia)	none	3	3		G04		G04
G02	viral URT infection (1W before onset)	selective IgA deficiency	none	none	0	0				
G01	none	deficiency of B-cells, NK-cells and monocytes	multiple relapses	febrile infection	5	5				
G05	viral URT infection	none	none	none	0	1				
G06	none	unclear: hypo-haptoglobinopathy	none	none	0	1				
G10	none	none	multiple relapses	none	2	2				
G12	EBV infection	partial albinism	none	febrile infection	4	4				
Retrospective Patients										
G09	febrile infection	NC-deficiency	multiple relapses (thrombocytopenia)	febrile infection	1	1		G09	G09	
G08	n.a.	none	n.a.	none	6	0				
G07	n.a.	Gibert's syndrome	multiple relapses	recurring fever attacks	0	1		G07	G07	

Table 6. Study population of 9 Study patients and 3 retrospective patients.

DOB – date of birth, h/o – history of, GE – gastroenteritis, MMF – Mycophenolate mofetil, Sequent. – sequentially, simul. – simultaneously, URT – upper respiratory tract, n.a. – not applicable

We included 12 patients with severe immune cytopenia. Of these, 3 patients were included after a relapse of their autoimmune cytopenia as retrospective patients. The mean age is 10 years (median age 8,5 years) and the range from 4 to 16 years. The sex distribution is 7 female and 5 male patients. The mean age of onset is 7 years (median age of onset 6,5 years) with 5 patients presenting an associated infectious trigger prior cytopenia onset. 5 patients were diagnosed with ES, 2 patients with AIHA and 5 patients with ITP. Of the 5 patients presenting an ES, 3 patients showed a sequential manifestation with 2 patients presenting AIHA first and 1 patient demonstrated ITP as first cytopenia. One ES patient demonstrated simultaneous symptoms of anemia and thrombocytopenia. In one ES patient the course of manifestation is unclear. 10 patients demonstrated initial symptoms or symptoms recent to the time point of diagnosis. 8 patients obtained additional or second-line treatment whereby 4 of them received multiple treatment regimes. 5 patients demonstrated multiple relapses, 2 of whom received extended treatment regimen. 7 of 12 patients were diagnosed with an additional hematologic or immunologic manifestation prior to or at the time point of cytopenia diagnosis; one patient demonstrated partial albinism without detection of any associated immune deficiency or related syndrome.

We included 19 observational patients with either known inborn error of immunity, hematologic or oncologic neoplasm, bone marrow deficiency, inborn error of metabolism or autoimmune disease. The mean age is 13,8 years (median age 14 years) and the age range 4 to 29 years. The sex ratio is 6 female and 13 male patients.

3.2. Results – Single time points

CD8+CD3+ cells - time point 1

Almost one third of the patients showed a remarkable decrease in their naïve proportion of CD8+ T cells. Instead, the proportion of CM is in the normal range. As previously described the proportion of EM cells is remarkably increased. In addition, the proportion of TEMRA in some of these patients is augmented.

Besides, we detected four patients with remarkably increased TEMRA proportion. These four patients present with Naïve and CM cells in the normal range. The EM proportion is decreased. G03, GB05, GB06 and GB15 demonstrate a contrary distribution. These four patients present marked increase in naïve proportion and subsequent decrease in their EM population. CM and TEMRA proportion are unaltered.

Thereby, all these three observational patients are heterogenous in terms of clinical analysis. G03 is a 14-year-old girl diagnosed with ES. GB05 is a female adolescent patient diagnosed with renal cell carcinoma (RCC). On the other hand, GB06, an adult male patient, is registered and analysed after continuous remission of ALL. GB15 is a male pediatric patient diagnosed with combined immunodeficiency.

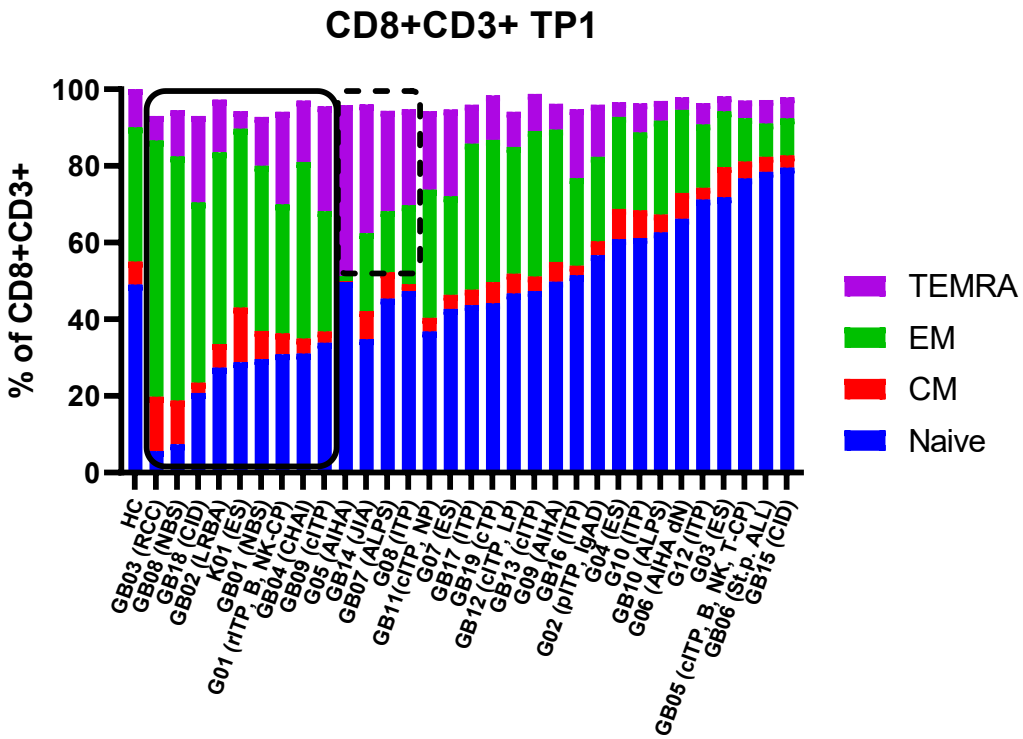

Figure 14 CD8+CD3+ T cells time point 1

Senescent CD8 - time point 1

We did not detect remarkable abnormalities in the CD3+CD8+CD57+ population of CD3+CD8+ T cells compared to the healthy control. Solely GB14 showed values outside the healthy control range. GB14 is a five-year-old boy with juvenile idiopathic arthritis.

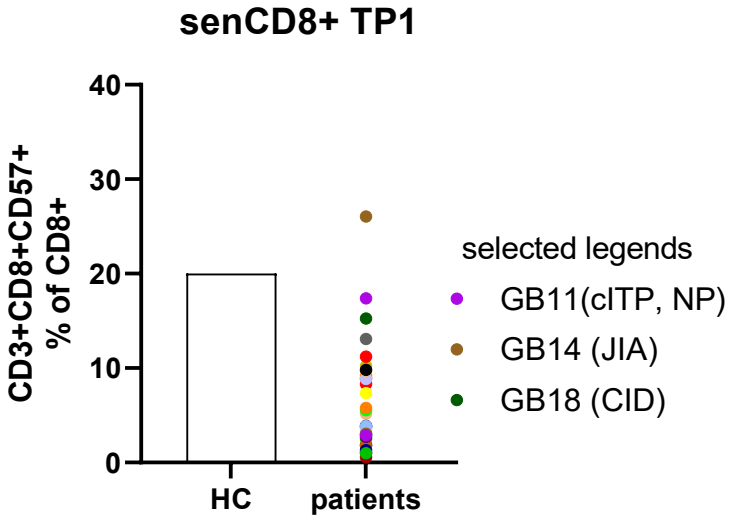

Figure 15 senCD8+ T cells time point 1

CD8+CD3+ cells - time point 2

Five patients demonstrate a predominance in the EM (and TEMRA) proportion. Within this group we detected a decrease in the Naïve proportion.

G02 presents remarkable decreased naive as well as central memory cell proportions. Effector memory cell counts demonstrate an average ratio. On the other hand, the TEMRA proportion is remarkably increased. Of note, at time point 1 G02 showed a course of CD8+CD3+ cell distribution without any distributional abnormalities.

As mentioned in the analysis of time point 1 G03 and GB15 demonstrate a repetitive pattern of CD8+CD3+ dispersion with increased Naïve and decreased EM proportion.

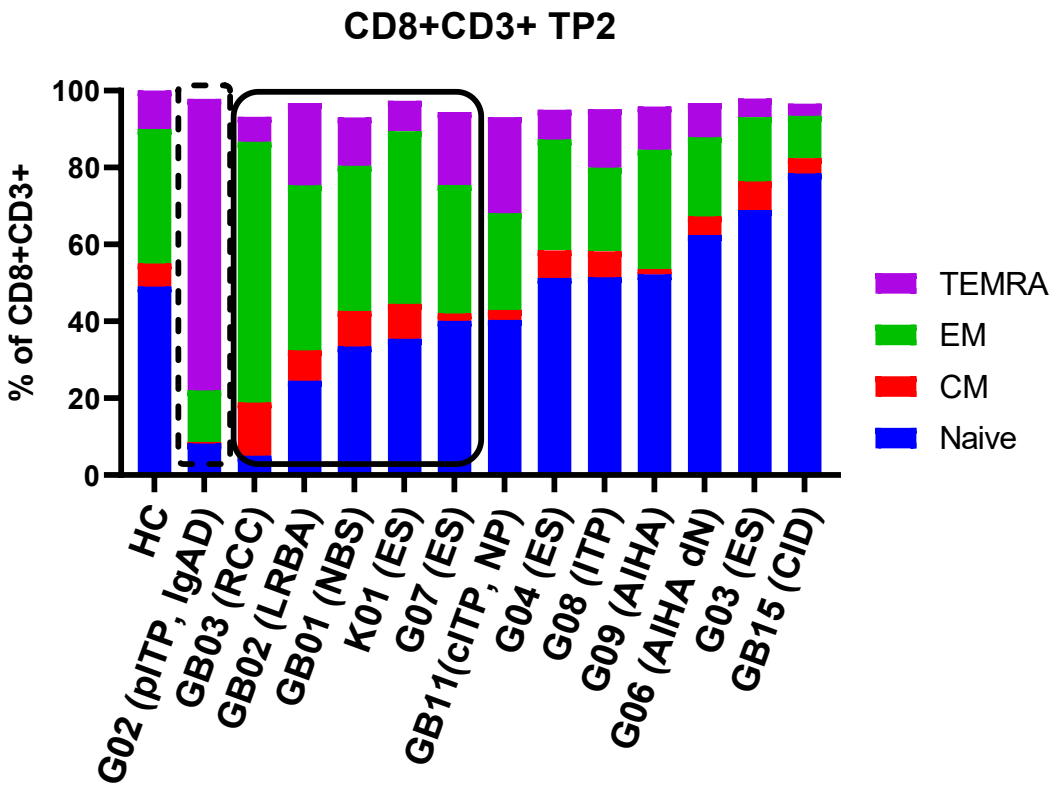

Figure 16 CD8+CD3+ T cells time point 2

Senescent CD8+ - time point 2

Within the senescent compartment of CD8+ cells GB03 demonstrates a slightly elevated value. GB11 presents elevation outside the range, whereby the previous value in time point 1 was elevated but in the range.

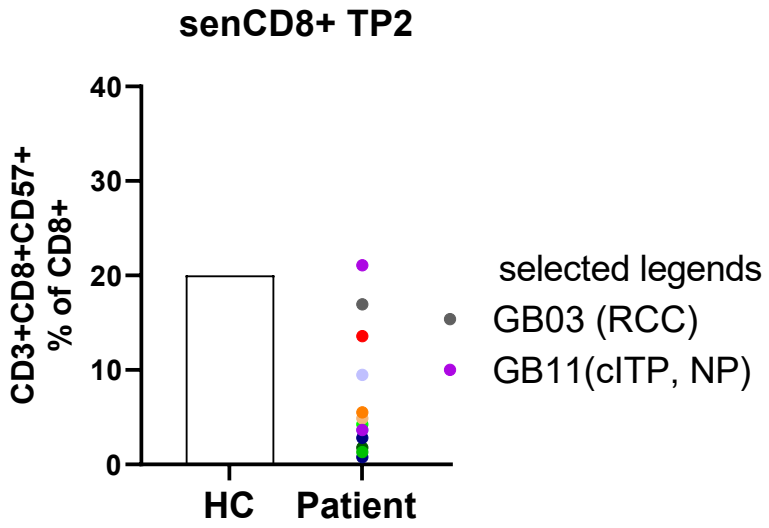

Figure 17 senCD8+ T cells time point 2

CD8+CD3+ cells - time point 3

In time point 3 we detect six patients who showed a predominance of their EM compartment. According to the previous time points these six patients present with reduced Naïve cell proportion.

At time point 3 the distributional pattern of GB03 remains. Naïve cells are remarkably decreased. The proportion of EM cells shows marked predominance related to reduced CM compartment.

As previously described in time point 3 patients G03 and GB05 present a similar pattern of differentiation. An increased proportion of Naïve cells and accordingly reduced proportion of effector memory cells.

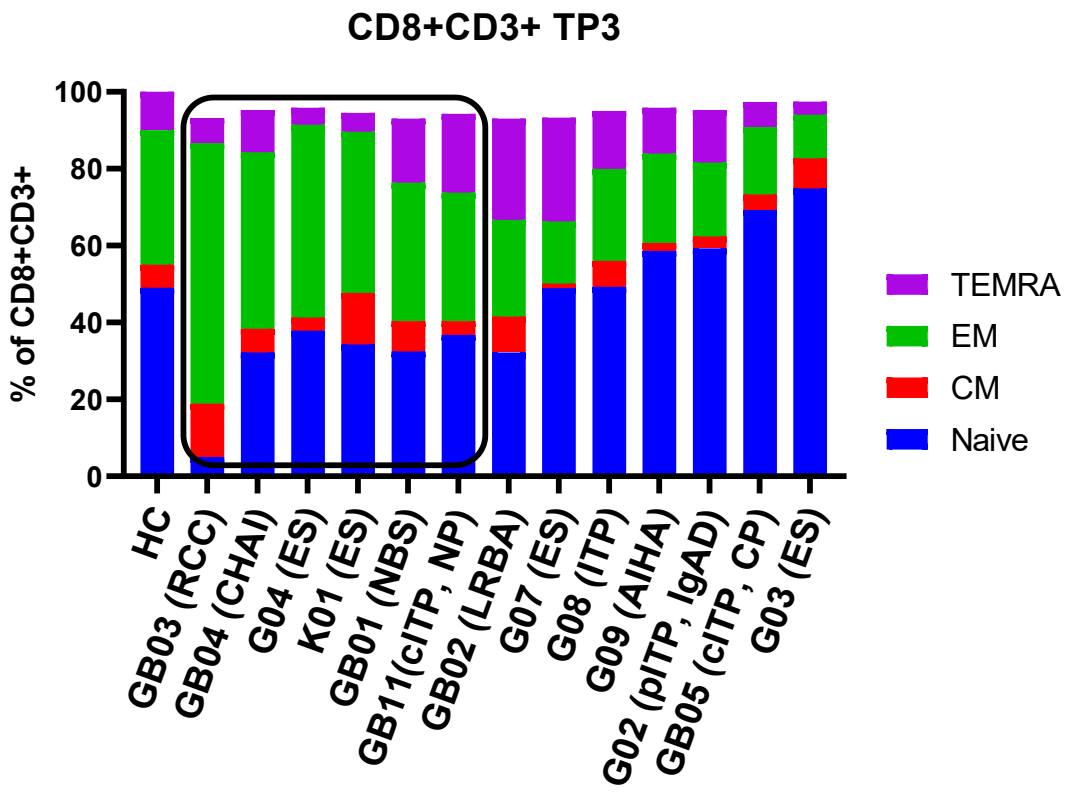

Figure 18 CD8+CD3+ T cells time point 3

Senescent CD8+ - time point 3

Similar to time point 2 in time point 3 only GB02, GB03 and GB11 present with high, but in range values of senescent CD8+ T cells in comparison to healthy controls.

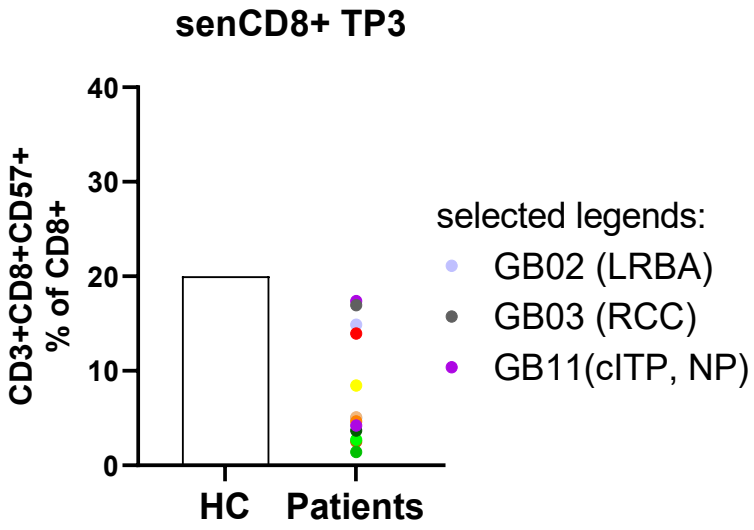

Figure 19 senCD8+ T cells time point 3

CD4+CD3+ - time point 1

More than one third of all patients presents with remarkably decreased Naïve cell proportions and a predominant EM compartment. The TEMRA proportion presents no noticeable alterations. Three patients of this group are patients of the study or retrospective group. The other ten patients are patients of the observational group.

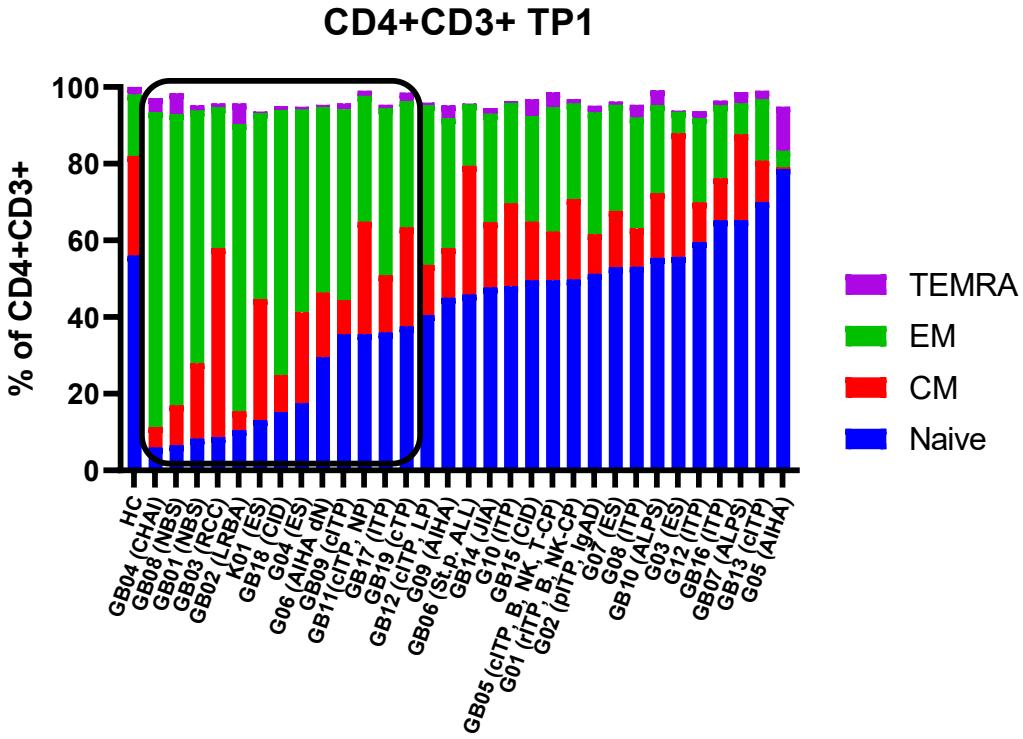

Figure 20 CD4+CD3+ T cells time point 1

Senescent CD4 – time point 1

GB02, GB07 and GB11 demonstrate elevation of senCD4+ cells. GB02 shows a proportion of senCD4+ T cells 2fold greater than the high in range value of the healthy control. G04 and GB14 present high normal values.

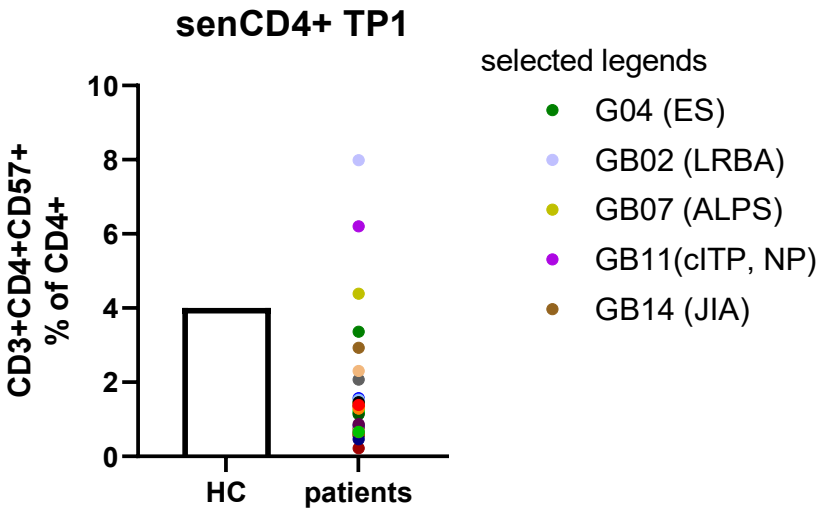

Figure 21 senCD4+ T cells time point 1

CD4+CD3+ - time point 2

Similar to the CD8+CD3+ T cell distribution in time point 2 G02 presents tremendous abnormalities in the differential distribution of CD4+CD3+ T cells. We detected six patients with reduced Naïve proportion and predominant EM compartment. There is no related alteration in the TEMRA compartment.

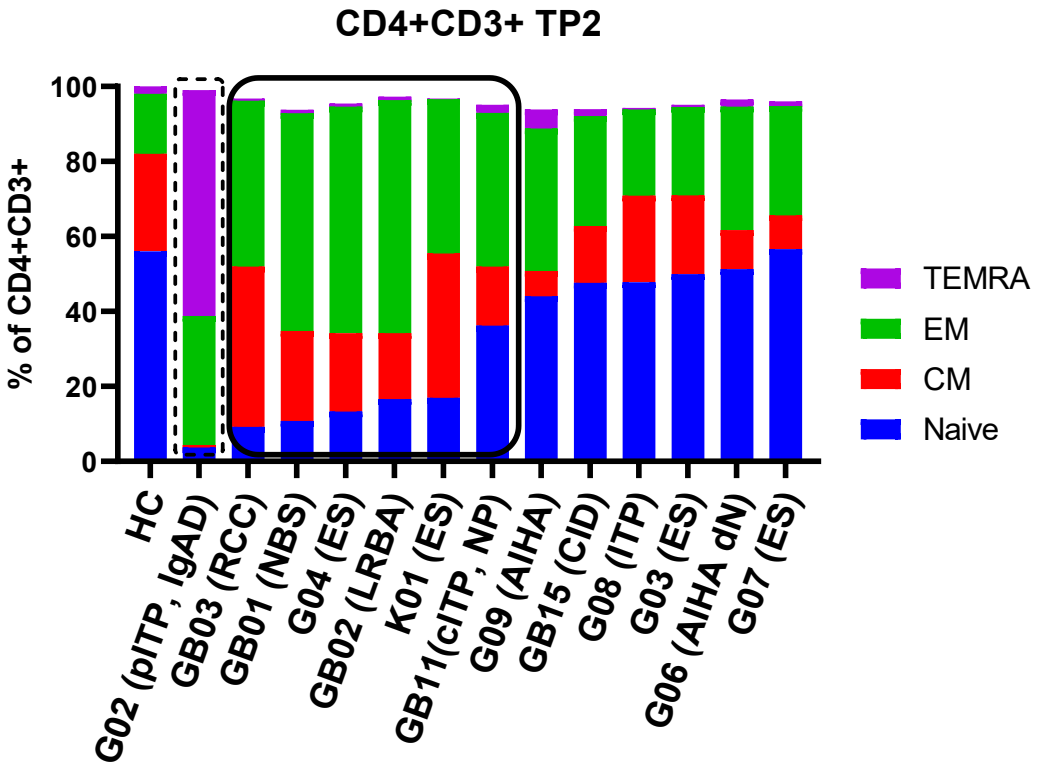

Figure 22 CD4+CD3+ T cells time point 2

Senescent CD4 – time point 2

In time point 2 three patients present elevated senCD4+ cell counts. GB02 demonstrates according to the previous time point an unaltered elevation of senescent CD4+ T cells. G04 and GB11 are marginally increased compared to the healthy control.

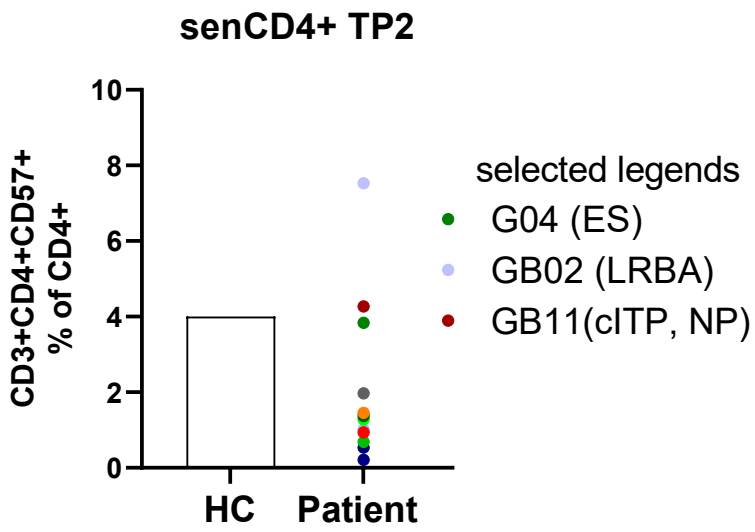

Figure 23 senCD4+ T cells time point 2

CD4+CD3+ - time point 3

At time point 3 we detected five patients presenting reduced Naïve proportion and predominant elevation of EM compartment. Like in the previous time points the TEMRA proportion was unaltered.

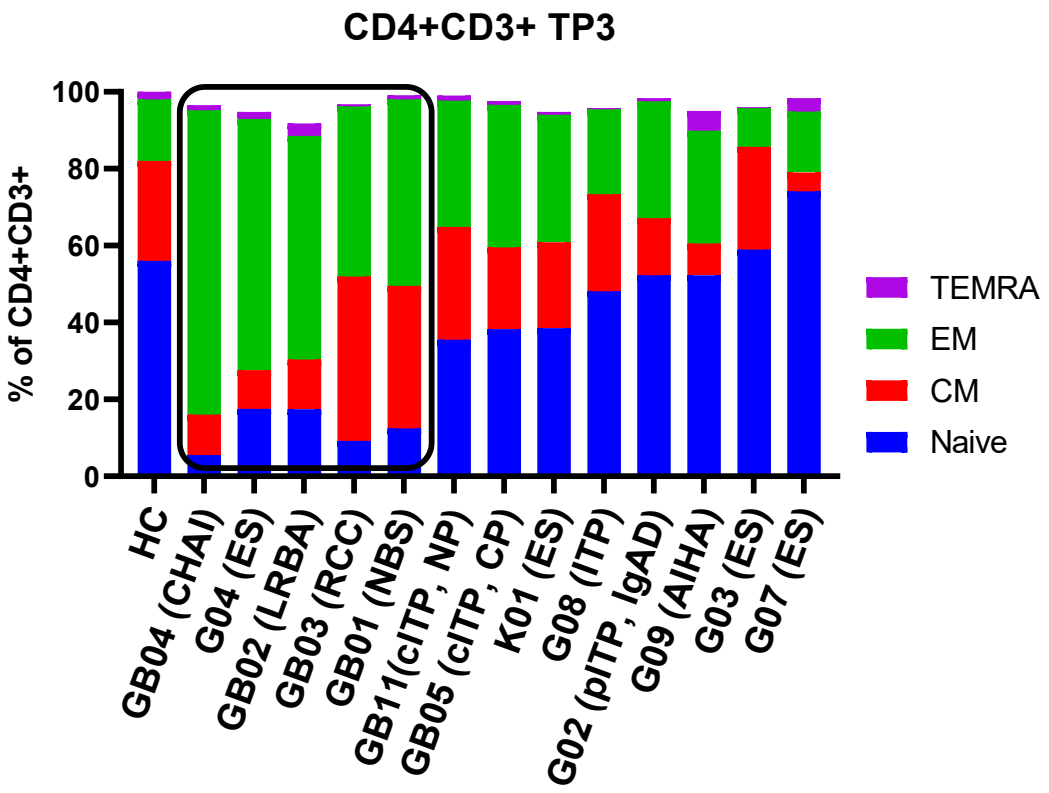

Figure 24 CD4+CD3+ T cells time point 3

G07 presented unremarkable differential distribution in the previous time points. G07 is a female adolescent patient of the retrospective patient group. The initial diagnosis is AIHA. Related to multiple relapses, she developed an ES. The measurement of time point 3 is simultaneously to the patient's transition from relapse to remission.

Senescent CD4 – time point 3

At time point 3 all subjects are in range except G04 and GB02. G04 shows an almost 2fold increase in senescent CD4+ proportion. GB02 presents a marked increase in senescent population even compared to the previous time points.

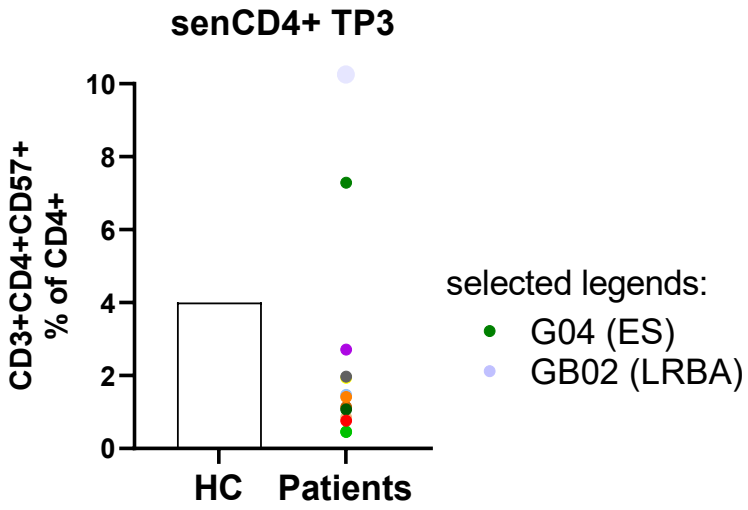

Figure 25 senCD4+ T cells time point 3

B memory cells - time point 1

We detected six patients with remarkably reduced Naïve cell proportions. The reduction in Naïve cells is related to four patients with marked class-switched population (K01, GB14, G06, GB16) and two patients with pronounced non-class-switched proportion increase (GB01, GB08; both diagnosed with NBS).
Of note, five patients present predominant Naïve cell compartment. Thereby, non-class-switched and class-switched compartments are tremendously reduced.

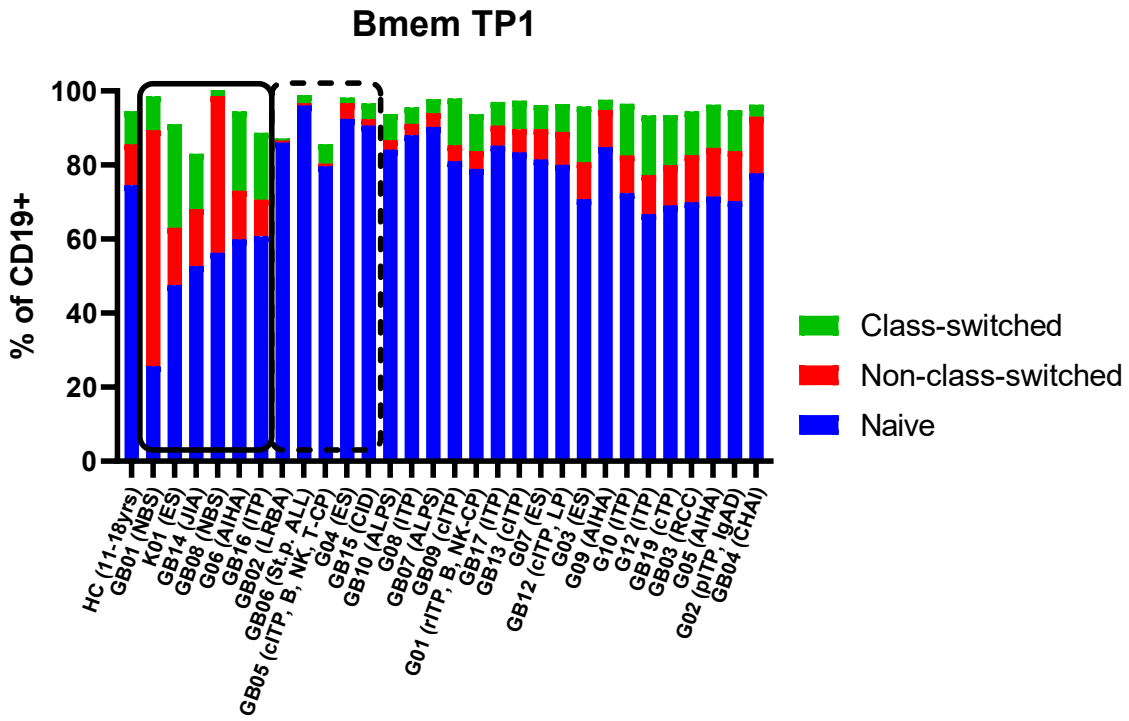

Figure 26 B memory cells time point 1

Age associated B cells - time point 1

The most remarkable increase present patients GB14, GB01 and GB08. These three patients show a 10fold increase compared to the healthy control.

In general, we detected an elevation within the majority of all patients. The majority of patients presents elevated propportions in the range 3% to 10% of CD19+ cells. G09 and GB04 demonstrate values greater 15%

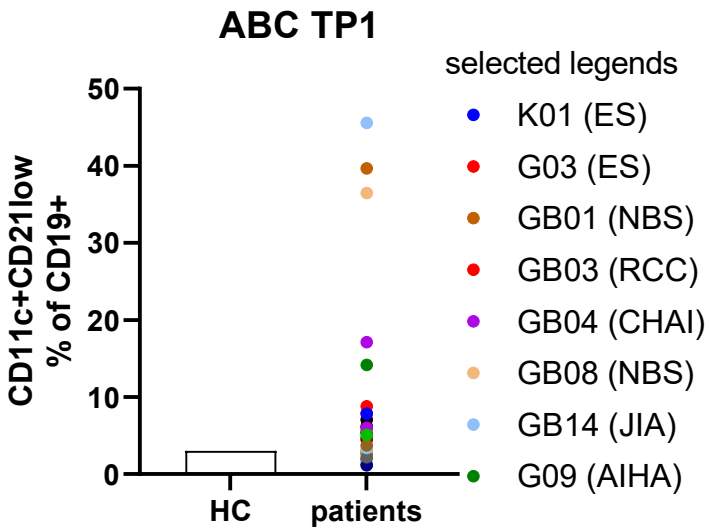

Figure 27 Age associated B cells time point 1

B memory cells - time point 2

At time point 2 we determined three patients with reduced Naïve cell proportions. GB01 shows a clear predominance of non-class-switched cells, whereby K01 and G06 demonstrate a balanced predominance of non-class-switched and class-switched cells present.

Three patients present abnormal distributions in terms of a predominant Naïve cell compartment. The compartments of non-class-switched and class-switched cells tend to undetectable.

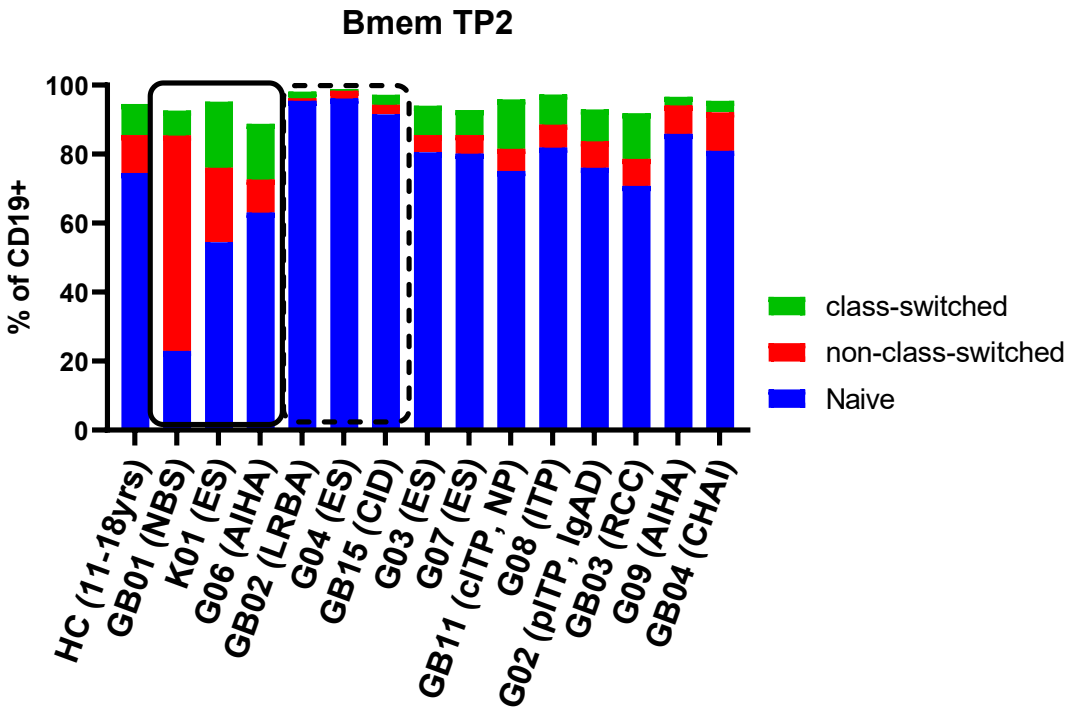

Figure 28 B memory cells time point 2

Age associated B cells - time point 2

It is clearly displayed that the majority of the patients presents with increased ABC proportions compared to the healthy controls. The majority presents with a population of CD19+ cells larger than 3% and less than 10% of CD19+ cells. GB01 shows, similar to time point 1 an increased proportion. At time point 2 GB01 demonstrates a reduced proportion of CD19+ compared to time point 1.

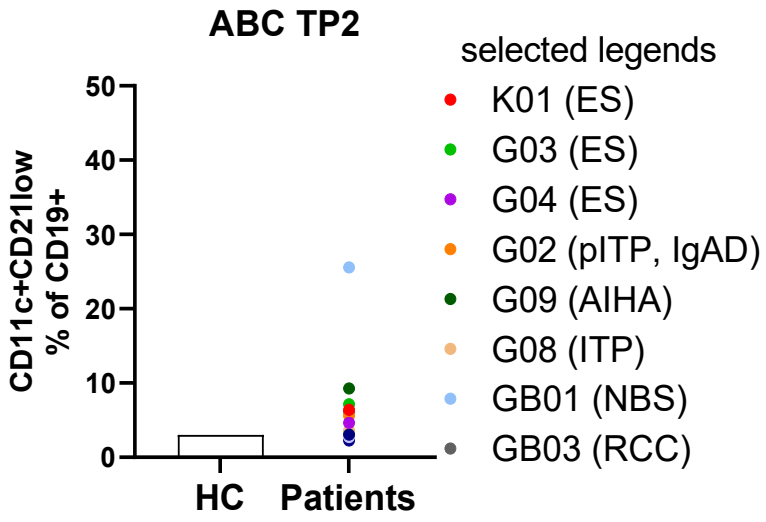

Figure 29 Age associated B cells time point 2

B memory cells - time point 3

At time point 3 two patients present with reduced Naïve cell compartment. GB01 demonstrates a tremendous elevation of non-class-switched cells with related reduction of the Naïve proportion.
Besides, we detected three patients with remarkably increased Naïve cell compartment and accordingly to the previous time points reduction of non-class-switched and class-switched cells towards barely detectable proportions.

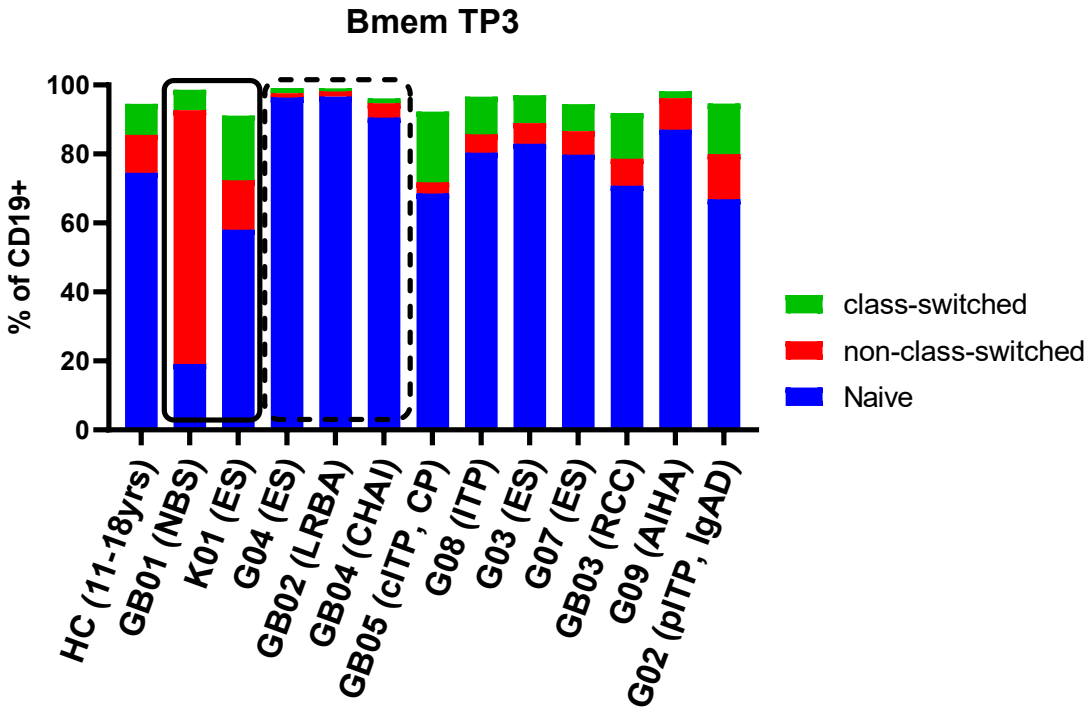

Figure 30 B memory cells time point 3

Age associated B cells - time point 3

Repetitively, the majority of the patients show elevated proportions compared to the healthy control in a similar range as at time point 1 and 2. The majority presents proportions in the range between greater than 3% and less than 10% of CD19+ cells. As in the previous time points GB01 presents a remarkably increased population, but tendentially lower according to the previous time points. GB04 shows the strongly elevated proportion that is comparable to the patient's time point 1 proportion.

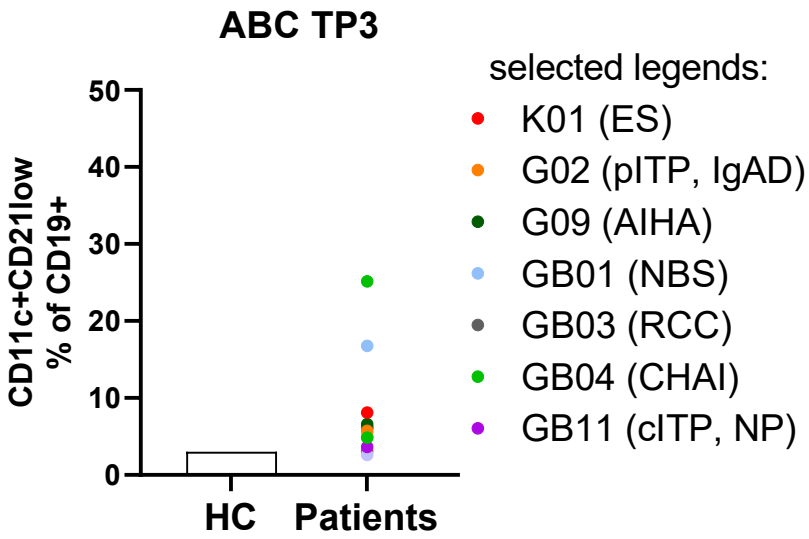

Figure 31 Age associated B cells time point 3

3.3. CD4/CD8 ratio

We calculated the CD4/CD8 ratio of the patients of the study and retrospective group. According to the collective data of the CD4+CD3+ and CD8+CD3+ T cells and B cells, at final data ending point of this interim analysis not every patient provided data for the follow up time points. At time point 1 we analysed the ratio of 12 patients, at time point 2 of 6 patients and at time point 3 of 8 patients.

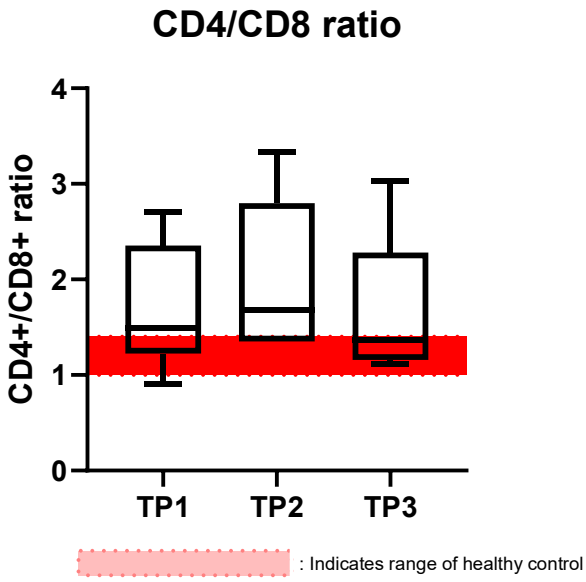

Figure 32 CD4/CD8 ratio of the study and retrospective group for the time points 1-3

Generally, the CD4/CD8 ratio in the study group and retrospective group appears abnormal. In all three time points the median is greater or equal to the upper edge of the healthy control group range. That indicates 50% the patients having a greater CD4/CD8 ratio than 1,40. In time points 1 and 2 the median is outlying of the range, being greater than 1,40. In time point 3 the median equals the level of the upper range.

In time point 2 the lower interquartile range is outside the normal range being greater than 1,40 with a minimum equally to the lower interquartile. That means all patients' CD4/CD8 ratios are greater than 1,40.

3.4. Results – Longitudinal patterns

Similarly to other investigations of single time point graphs we investigated the longitudinal course of patients regarding determinable patterns. These patterns describe a subset distribution that is constant at all three time points. It is to note, that in the context of the interim analysis the term pattern is equivalent to profile or signature.

Our attempt was to investigate every longitudinal representation of a subset and examine this course on a recurrent pattern.

CD4+CD3+ pattern I

The patients G04 and K01 represent a remarkable pattern in the CD4+CD3+ subsets. G04 is a male adolescent of 15 years. His clinical course is illustrated in Figure 34. The initial diagnosis of ITP was determined in 02/2017 whereby he did not present any symptoms in terms of purpura previous to the first diagnosis. Similarly, one week before the first diagnosis the patient suffered from a viral gastroenteritis. A relevant side diagnosis at that time point was a von Willebrand disease type I (vW disease type I). The vW disease type I was first diagnosed 05/2009. Right after the diagnosis of ITP he was treated twice with IVIg and showed a very sufficient response of increasing thrombocytes. In 02/2018 after a routine check-up, he was diagnosed with Evans syndrome. Following that two times repeated IVIg were administered. Subsequently, although treatment regimen was extended by continuous administration of eltrombopag, the patient showed

multiple relapses of ITP and ES while the IVIg administration was repeated. Due to these multiple relapses 09/2019 eltrombopag was changed to MMF.

K01 is a patient diagnosed with ES. He was successfully treated with mycophenolate mofetil (MMF) from the start, while undergoing continuous remission during this treatment.

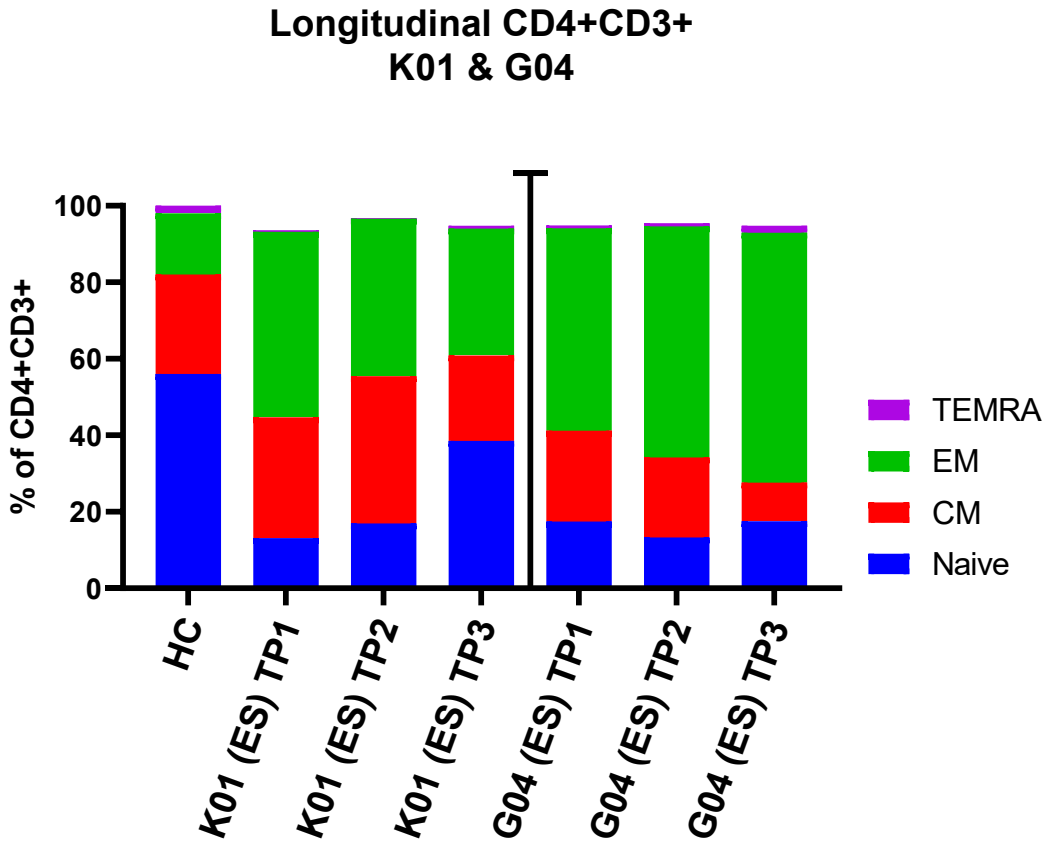

Figure 33 Longitudinal CD4+CD3+ T cells of K01 and G04

The healthy donor (HC) graph demonstrates the relation of CD4+CD3+ maturity stages. At the time point (TP) 1 K01 and G04 demonstrate a similar pattern. The naïve proportion shows a remarkable decrease whereby the EM population is increased. The pattern of G04 remains consistent regarding TP2 and TP3. Over time the proportion of EM decreased in TP2 and TP3 whereby firstly CM proportion increased (TP2) and subsequently naïve cells proportions adapted. It may represent a tendency to physiological subset adaption.

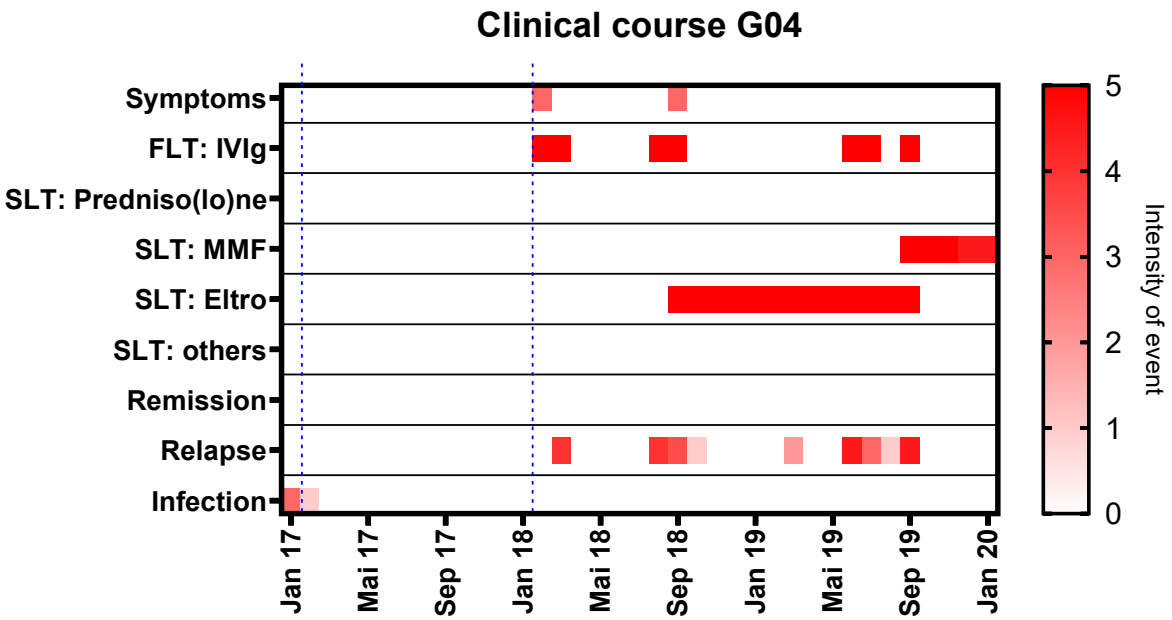

Figure 34 Clinical course of G04

Description*:* visualisation of the clicial course of patient G04; Modified heat-map displaying by colour intensity the level or extent of symptoms, treatment, clinical course and infections, respectively; FLT: first line treatment, SLT: second line treatment; MMF: Mycophenolat mofetil; Eltro: TPO-RA. Blue dotted line(s) visualize(s) the time point of diagnosis/es.

All clinical visualisations depicted consistently beginn at the date January 2017 and end at final data closing January 2020

With the visualization of the clinical course of G04 the focus is mainly on the recurrent relapses. For instance, TP2 and TP3 are close to relapses. TP2 is instantly close to the first relapse on 08/2018 while TP3 is close to the suspected relapse of AIHA (discrete hemolysis with solely increased reticulocytes) on 03/2019. Contrary to an active immune reaction in G04 resulting in two relapses the pattern of CD4+CD3+ T cells remained constantly.

In contrast to the pattern of G04 stands the course of patient K01. K01 is a female patient, 10 years old and diagnosed with ES. From the time point of first diagnose of ES she was successfully treated with MMF to a continuous state of remission. Over time and during the remission the patient's CD4+CD3+ subset pattern changes to a distribution which might reflects adaption to physiological subset

dispersion. That represents eventually a correlation between successful treatment and normalisation of CD4+CD3+ T cell subsets.

CD4+CD3+ pattern II

GB01 is a male patient 8 years old and diagnosed with Nijmegen breakage syndrome. Similarly, GB02 is a male patient as well, yet adolescent and diagnosed with LRBA deficiency. Patient GB03 is an adolescent female and was diagnosed with RCC. Further, GB04 is an adult male patient and diagnosed with CHAI.

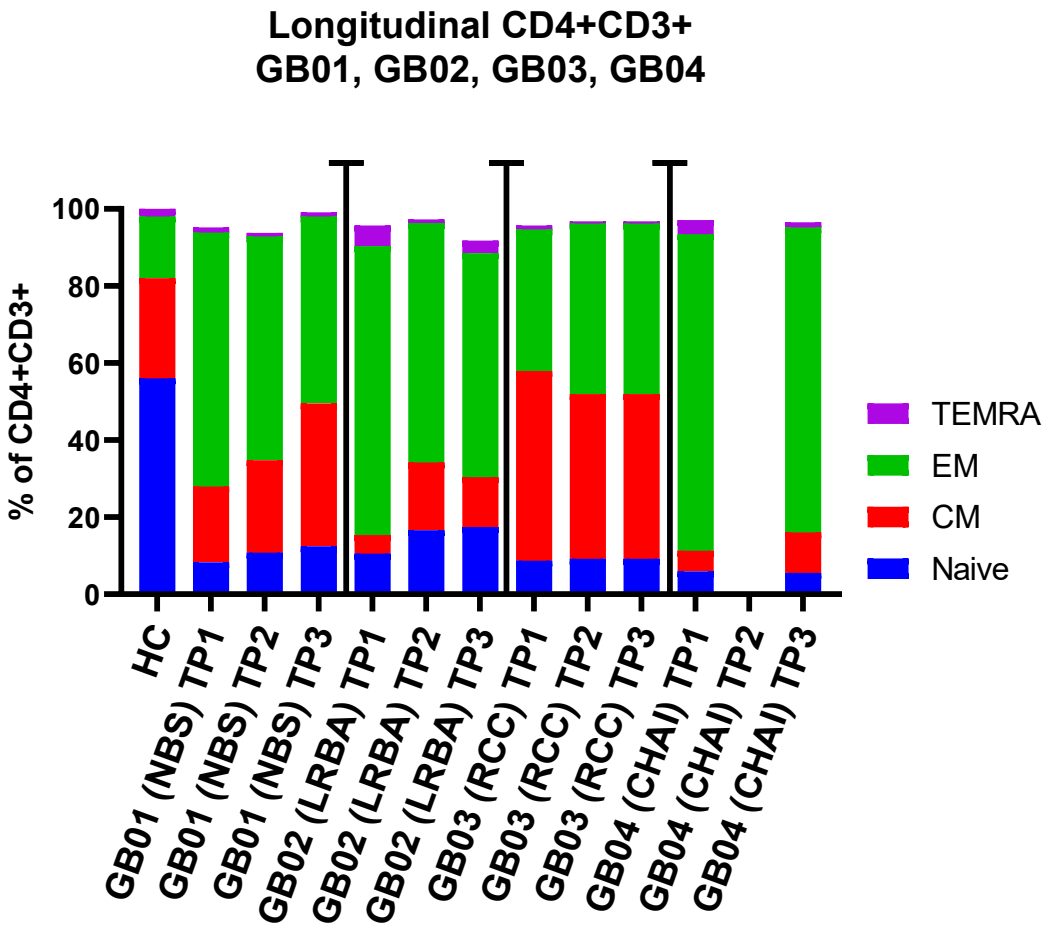

Figure 35 Longitudinal CD4+CD3+ T cells of GB01-04

On three time points, the CD4+CD3+ distribution of these four supervised patients resemble the distributional pattern of K01 and G04. In general, the naïve cell population is decreased. Besides, the distribution of maturational stages of CD4+CD3+ T cells distinguishes in three ways, which is demonstrated by arrows in Figure 36. Over time GB03 illustrates decreased naïve cell proportion and a constant increased and an equal relation of CM to EM cells. In contrast, GB01

demonstrates an increasing proportion of CM cells by relatively reducing the predominant proportion of EM cells. In addition, the pattern of GB02 and GB04 reflect a continuous overbalance of the EM population. Laboratory analysis of GB04 at time point 2 was not performed.

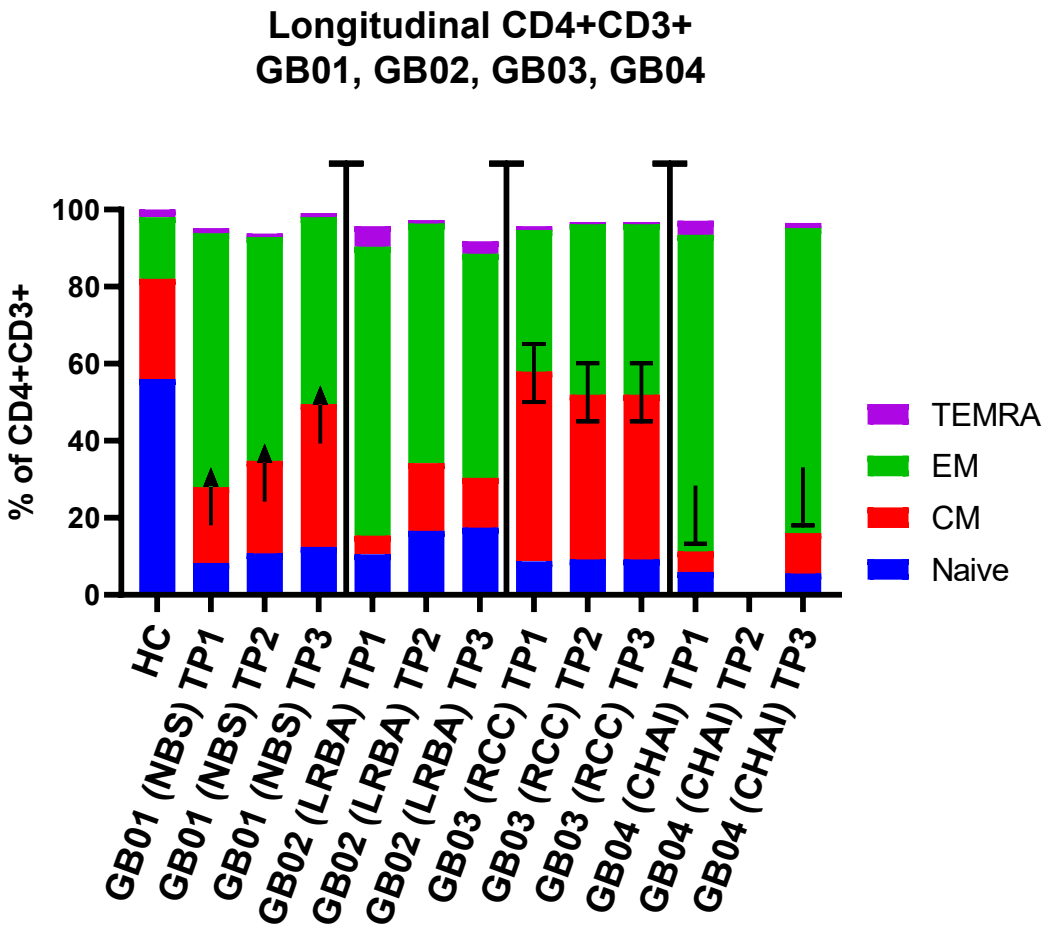

Figure 36 Longitudinal CD4+CD3+ T cells pattern

CD8+CD3+ pattern

A longitudinal pattern can be specified in the CD8+CD3+ set throughout the course of patient G03, GB05 and GB15. Due to the absence of the patients, the TP2 measurement of GB05 and the TP3 measurement of GB15 are not applicable.

Patient G03 is a female adolescent. Her clinical course is illustrated in Figure 37. The initial diagnosis confirms as ES by a positive Coombs test. Since 2011 a two-line cytopenia in form of thrombocytopenia and neutropenia was known. At the time of diagnosis, single IVIg administration was initiated. Subsequently, the

administration of prednisolone was induced continuing for four months due to recurrent thrombocytopenia. Eltrombopag was started as a supportive treatment regime. At the same time, MMF replaced prednisolone as an immunosuppressive therapy. After discontinuing the patient's therapy an ITP relapse occurred, consequently followed by the reinstalling of the MMF therapy. During the month of initial diagnosis, patient G03 had an infection prior to the occurrence of ES. In 02/2020, a second self-limiting infection occurred, whereby the infection did not cause intensification of cytopenia.

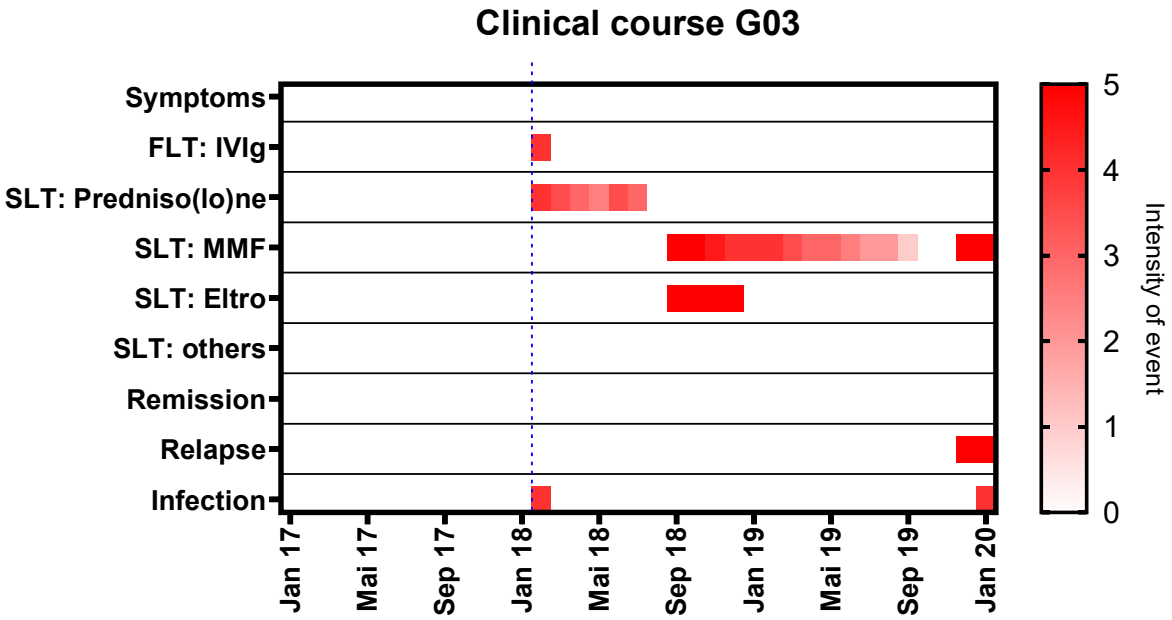

Figure 37 Clinical course of G03

Description*:* visualisation of the clicial course of patient G03; Modified heat-map displaying by colour intensity the level or extent of symptoms, treatment, clinical course and infections, respectively; FLT: first line treatment, SLT: second line treatment; MMF: Mycophenolat mofetil; Eltro: TPO-RA. Blue dotted line(s) visualize(s) the time point of diagnosis/es.

All three time points of the patients G03, GB05 and GB15 illustrate a distinctive pattern in the CD8+CD3+ subset. In general, the pattern demonstrates a clearly visible 50% increase in the naive population. Yet, it should be noted that for patient G03, the CD8+CD3+ pattern remains stable, even though the measurement at

time 2 was carried out before the start of MMF therapy, while the measurement of time point 3 was performed during the process of gradual MMF reduction shortly before treatment termination.

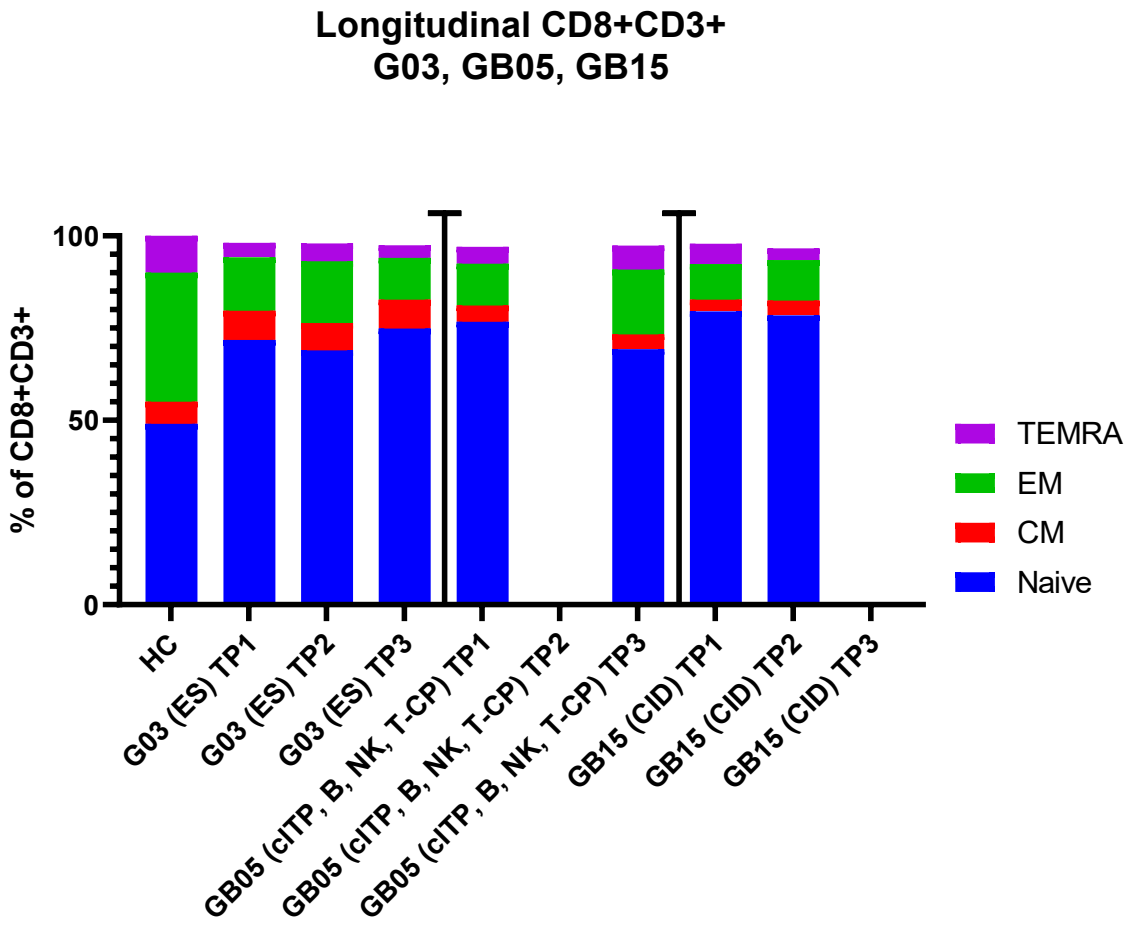

Figure 38 Longitudinal CD8+CD3+ T cells of G03, GB05, GB15

CD8+CD3+ singe patient pattern

At time point 2, patient G02 displays a noticeable pattern. Throughout, patient G02 demonstrates CD8+CD3+ distribution within the range compared to HC at time point 1 and time point 3. The proportions of naive, CM and EM cells are remarkably reduced with a simultaneous increase of the TEMRA proportion.

Patient G02 is 8 years old and female. Her clinical course is demonstrated in Figure 39. In the beginning of 2018, the patient was diagnosed with ITP. As a side diagnosis a selective IgA deficiency was determined. Consequently, it limited the therapeutically procedure with the exclusion of IVIg. The effect of the initial therapy with prednisolone was sufficient; a continuous remission of ITP was induced and

corticosteroids were tapered off. In total the steroid therapy lasted for four months. The measurement of time point 2 occurred four months after the termination of steroid therapy and the measurement of time point 3 was conducted in stable remission. It should be noted that at time point 2, patient G02 showed the highest platelet count after discontinuing steroid therapy. Furthermore, patient G02 showed an increase in platelets at time point 2, which gradually decreased to a stabilized range between 70,000 and 100,000 per mcL during remission.

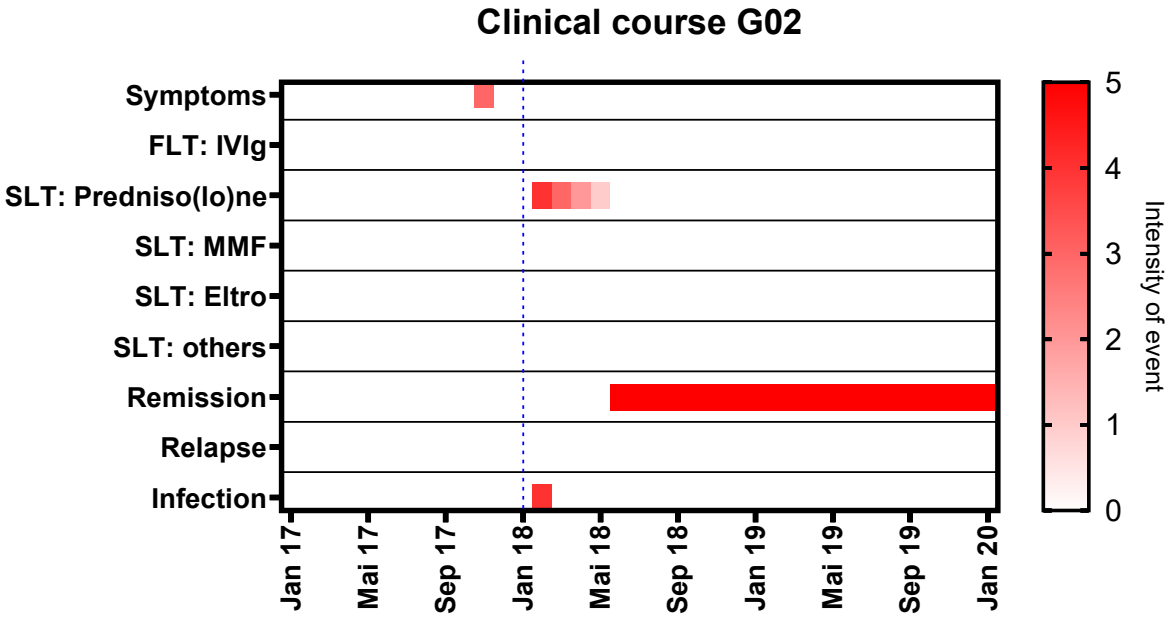

Figure 39 Clinical course G02

Description*:* visualisation of the clicial course of patient G02; Modified heat-map displaying by colour intensity the level or extent of symptoms, treatment, clinical course and infections, respectively; FLT: first line treatment, SLT: second line treatment; MMF: Mycophenolat mofetil; Eltro: TPO-RA. Blue dotted line(s) visualize(s) the time point of diagnosis/es.

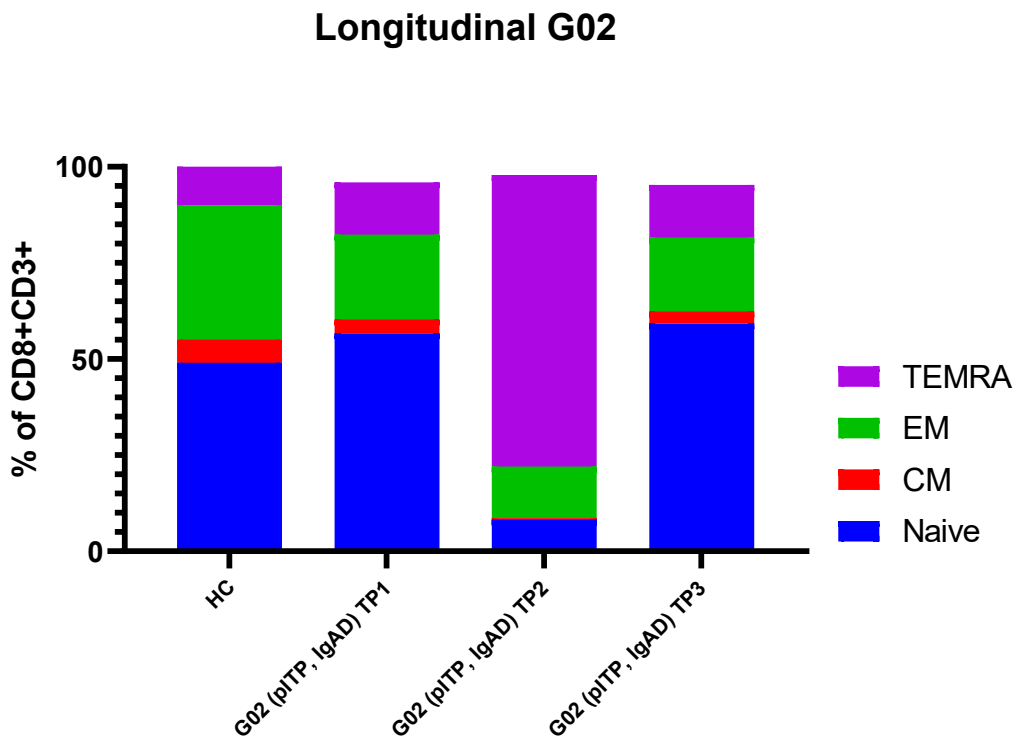

Figure 40 Longitudinal CD8+CD3+ T cells of G02

B memory cells pattern

When investigating the longitudinal pattern of the patients´ B memory cells it becomes evident that G04, GB02 and GB15 present a similar pattern of B memory cell dispersion. It should be noted that time point 3 follow up of GB15 is outside data ending.

The pattern clearly presents an increase of naïve B-cells whereby the relative amount of non-class-switched B-cells and class-switched B-cells decreases and partly converges to 0%. In sum, the patient's relative amount of naïve B cells has increased, and the relative proportion of memory B cells has decreased in comparison to HC. It is to note, that despite the relapsing course of G04 the distributional pattern of his B cells remained stable.

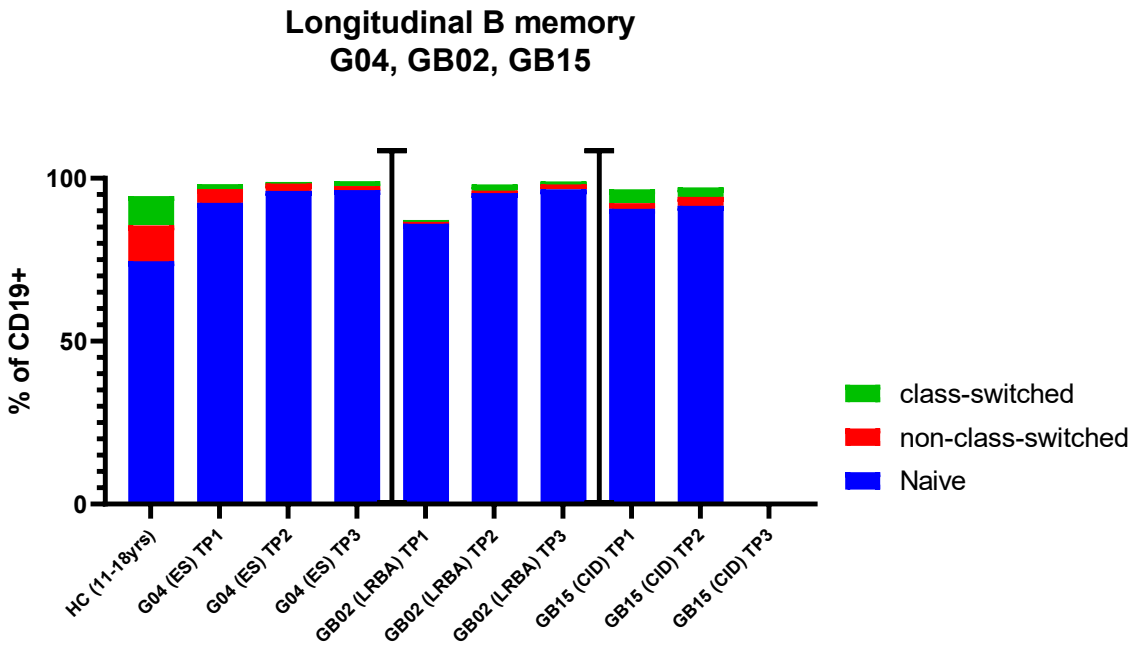

Figure 41 Longitudinal B memory cells of G04, GB02, GB15

B memory cell single patient pattern

Over the three time points two more patients presented a notable distribution of their B cell population. K01 as well as GB01 demonstrate a pathological distribution of naïve B cells and memory B cells. The population of Naïve B cells is decreased in K01. GB01 shows a even more marked decrease of Naïve cells. In K01 the relation of B cell maturation stages is modified in such a way that non-class-switched proportion remains within the normal range. Hereby, the relative part of class-switched cells increases. GB01 demonstrates a continuous and distinct decrease of Naïve cell proportion as the non-class-switched proportion strikingly increased.

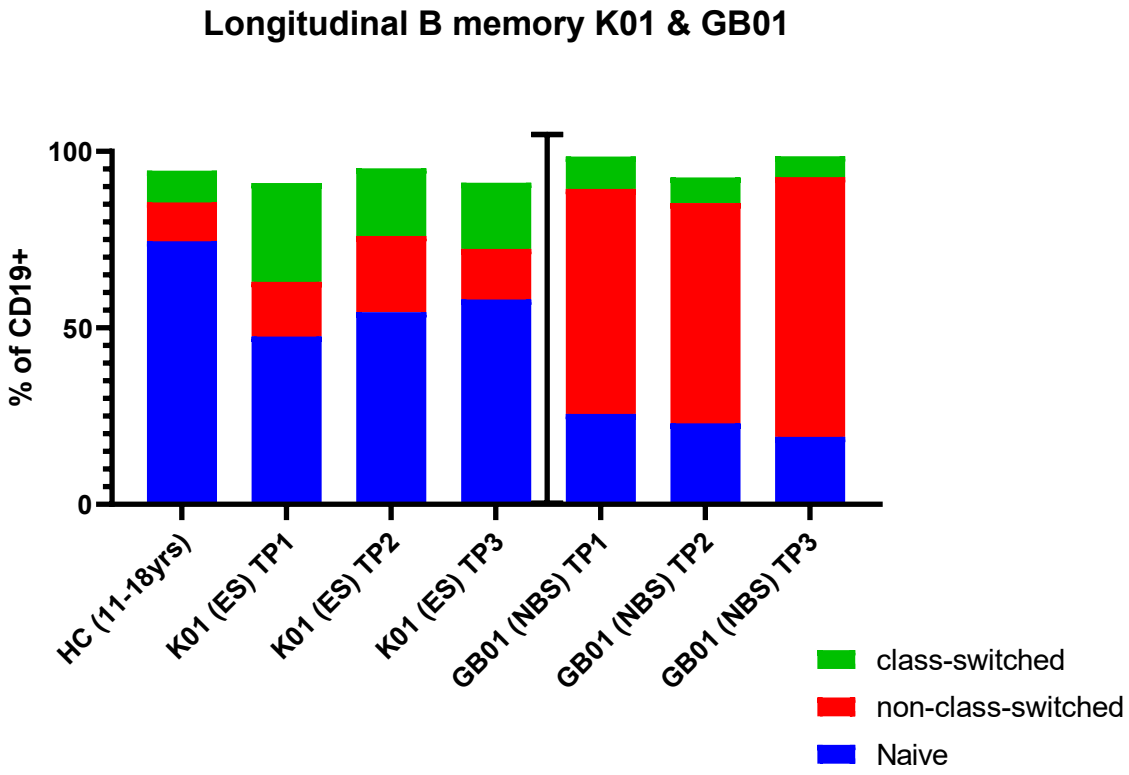

Figure 42 Longitudinal B memory cells of K01 and GB01

4. Discussion

This early interim analysis of the sic-reg.org pilot study provides comprehensive results of maturational and senescent subpopulations of B and T cells of patients affected by SIC. On all time points we detected tremendous abnormalities in B and T cell subsets in the majority of the patients. In several patients we determined a maturational shift in T and B cells. We could repeatedly demonstrate premature maturation and senescence in lymphocytes. Our data represent a reminiscent pattern primarily according to previously described PID presenting with SIC, as to autoimmune conditions and hematologic malignancy.

Our repeatedly determined patterns of premature maturation and immunosenescence in T and B cells suggest a diagnostic potential. Evaluation and detection of primary SICs remains a challenge for clinicians. Even after diagnosis of SIC clinical courses remain unpredictable and individual risk assessment is restricted. Not least, the patients‘ main burden consists of repeated treatment modifications. The demonstration of premature maturation and immunosenescence markers as detected in this pilot phase will prompt additional experimental immune phenotypic investigations in more patients that might allow a specification of the diagnostic approach in the condition of severe immune mediated cytopenia in the future.

Premature maturation in CD4+CD3+ and CD8+CD3+ T cells

At all three time points we detected that a majority of the patients presented with memory-marker formation in CD4+CD3+ and CD8+CD3+ T cells. We detected patients with constant longitudinal memory-marker formation. Our representation of premature maturation is related to previous described PIDs presenting with SIC. Numerous PIDs show noticeable memory-marker formation in CD4+CD3+ and CD8+CD3+ T cells (60, 76, 77, 79, 80). Premature maturation was detectable in malignancy setting as well (67). Of note, common autoimmune manifestations demonstrate premature lymphocytic maturation (71).

Premature senescence in B cells

Analysing the ABC distribution, we detected premature senescence in B cells in the majority of patients. Premature senescence is reminiscent to previously reported ABC elevation PIDs with autoimmune cytopenia (60, 82).
Disturbance in the B cell compartment provides a link to the pathogenesis of autoantibody formation in SIC. Further investigation of the B cell compartment is necessary as in paediatric ITP patients a decline in CD19+CD24hiCD38hi B regulatory cells and increase of CD19+CD27+ B memory cells was detected (83). In addition to B cell differentiation further research will also include immunoglobulin levels and isotype distribution in patients presenting with SIC.

Contrary picture in B memory cells

We could demonstrate a phenotype in B memory cells with premature maturation. That corresponds to a previously reported study in patients with primary ITP with upregulated CD27+ memory cells (84). Based on that it is propagated that an increased CD27+ memory formation represents a maker for disease activity (84). In contrast to that, CVID patients previously demonstrated profound decline of class-switched B cells and predominance of Naïve CD27- cells (85, 86). Independent from the underlying mutation ALPS patients present reduced CD27+ memory B cells. It remains unclear whether the disturbed CD95 pathway is solely responsible for B cell differentiation abnormalities (82). A similar phenotype of reduced memory CD27+ formation was detected in patients with CGD and hyper-IgE syndrome (87, 88). Reaffirming, evaluation of CD27+ memory B cells provides diagnostic potential in hyper-IgE syndrome (88). Besides diagnostical implementation of memory B cells, it remains unclear whether the underlying mutation in these PIDs results in structural and/ or functional alterations in the germinal centre reaction (82, 87).

Absent premature senescence in CD4+CD3+ and CD8+CD3+ T cells

Increased premature senescence phenotype in CD4+CD3+ and CD8+CD3+ T cells is reported by numerous immune deficient or autoimmune conditions. Numerous PIDs, autoimmune conditions like RA, hematologic malignancies present increase of CD57 expression on CD4+CD3+ and CD8+CD3+ T cells, they present an immunosenescence phenotype (60, 72, 77, 79, 89). Despite no

significant phenotype of premature immunosenescence in CD4+CD3+ and CD8+CD3+ T cells, by ongoing accumulation of data we might support present evaluations of premature senescence in T cells. Immunosenescence shows increasingly evidence as a marker for immune dysregulation and is suggested as a marker for an immune-risk type (72). Targeting cell regulatory signalling indicates a promising therapeutic approach in modulating immune response (90). Cumulative insights of the intracellular signalling and transcription dependent cell regulation indicate the group of mitogen-activated protein kinase (MAPK). Blocking of p38-MAPK and Erk-MAPK (both MAP-kinases activating B- and T cell relevant transcription factors) are suggestive for reversing immunosenescence, as MAPK signalling supposes to be substantial for induction of immunosenescence (67, 75, 90). It is confirmed but the condition remains unclear to which Treg cells induce immunosenescence (90). As a substantial factor for peripheral tolerance and preventing autoimmunity, a diminished or dysfunctional Treg cell population is highly suggestive as a main cause leading to SIC (39, 48, 49). Ongoing investigations in the present study are focusing on various subsets of Treg cells.

Continuously increased CD4+/CD8+ ratio

We detected an increased CD4+/CD8+ ratio at all time points. Consequently, we confirmed a previous detection of increased CD4+/CD8+ ratio in primary ITP patients. With elevation in the CD4+/CD8+ ratio we supported the hypothesis of immune dysregulation in the context of ITP (91). Corresponding to the assumption of immune dysregulation, PI3K deficient patients present an increased CD4+/CD8+ ratio (80).

Immune dysregulation as feature of inborn errors of immunity (IEI) may manifest with autoimmune cytopenia. IEI integrates the spectrum of primary immunodeficiencies, autoimmunity, autoinflammatory manifestation and malignancy predisposition (92, 93). More than 400 monogenic IEI are known. They are caused by monogenic germline mutations leading to GOF or LOF of the encoded protein. Immune dysregulated processes unify the majority of IEI and SIC. Premature maturation and immunosenescence observed in SIC patients may thus suggest that underlying immune dysregulation exists as in IEI. Lymphocytic metabolism, particularly T cell metabolism, represents an inherent origin of

immune dysregulation. Dysregulated T cell metabolism is categorized as a main pathophysiologic pathway: Numerous IEI result by alterations within the cell cycle in terms of proliferation, differentiation, and apoptosis (39). The differentiation of function and activation or senescence status is precisely and substantially regulated by the T cell metabolic status. In a complex way the differentiational signature is linked to the metabolic signature of T cells. The strong association of lymphocytic metabolism and differentiation and activation status in lymphocyte subsets was previously reported, concretely, differentiation and expansion of CD4+CD3+ and CD8+CD3+ effector memory subsets. Contrary, the intrinsic factor of lymphocyte metabolic signature is the mTOR-Akt pathway. Numerous of categorized PIDs are associated to the mTOR-Akt pathway. Based on the linkage between lymphocytic metabolic signature and differentiation status a categorisation of metabolic states in lymphocytes correlating to associated pathological conditions was developed and is presented as Figure 43 (94). Supportive for the hypothesis of alterations in the mTOR-Akt pathway are PIDs associated with the assumed hypometabolism or hypermetabolism in lymphocytes. The GOF mutation in the catalytic subunit p110Delta is an example of a hypermetabolic PID. Hyperactivation of mTOR-Akt leads to immune mediated cytopenia and premature maturation and senescent shift in B cells and T cells (80). Haploinsufficiency in CTLA-4 or mutations in the ligand-binding or dimerization domain of CTLA-4 may represent another example of a hypermetabolic PID. CTLA-4, an inhibitory member of the CD28 family, binds to the B7 ligands (CD80 and CD86) with greater affinity and affects greater avidity than CD28; thus, inhibiting T cell stimulation via CD28. Aberrant genetic status of CTLA-4 removes the suppressing, competing function of CTLA-4 against CD28. Normally CD28 activates the PI-3 kinase which activates PIP3. PIP3 activates T cell transcription program, secretion, and proliferation. Without inhibition by CD28 consequently, lymphocytic metabolism via mTOR-Akt would be increased (75). Rapamycin, a drug blocking the mTORC1 Raptor regulated pathway, is used as an immunomodulatory/ -suppressive drug among others in CTLA-4 deficient patients (95, 96). The mTORC1 pathway is mainly responsible for proliferation in lymphocytes and inhibition promotes apoptosis and induces immunomodulatory modifications in T lymphocyte subsets (75, 97).

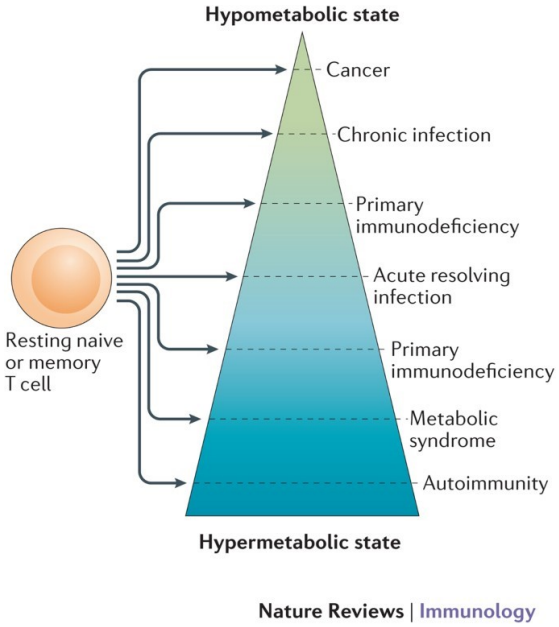

Figure 43 The spectrum of T cell metabolism in health and disease, Glenn R. Bantug et al, Nat. Rev. Immunol., 2018; 18(1):19-34
Description: Visualization of an unbalanced metabolic reprogramming. The authors assume different metabolic state depending on the T cell status. Naïve and memory cells are support by catabolism of glucose and fatty acids. Effector T cells developing due to acute inflammation increase both anabolic and catabolic pathways. PIDs are displayed on both sides of the scale, autoimmunity in the setting of hypermetabolic state.

Besides the approach to investigate the lymphocytic metabolism to gain insight of lymphocytic proliferation and activation another field of interest are abnormalities in telomer length. Although premature immunosenescence is, by definition, independent from telomer length we investigated the telomer length in numerous of our patients. These data, however, were not subject of this diploma thesis and await further analysis (data not shown).

Currently, the intestinal microbiome receives a lot of attention. The human immune system is in constant exposure to a vast number of non-pathogenic germs on its epithelial surfaces such as the gut; its importance to shape immune tolerance is being increasingly appreciated. Microbiome metabolites impact the host immune system via receptors and/or metabolic integration. Metabolites promote differentiation in B cells, the generation of effector T cells and induce regulatory T

cells (98). Besides that, the microbiome is partly responsible for thymic development of self-tolerance of T cells. In addition, it was recently reported that the host's microbiome regulates production of type I interferons (IFN-alpha and IFN-beta) by plasmacytoid dendritic cells (pDC) (99). The type I interferon receptor (IFNAR) is responsible for metabolic equilibrium in pDC before pathogen contact (99). Thereby, pDC interact with conventional dendritic cells by balancing levels of proinflammatory IL-12 levels (100).

Together, these approaches illustrate that for further investigation the number and detail of lymphocyte subpopulations to be analysed must be increased. Furthermore, it is recommendable to investigate the cytokine profile. The additional data provided by microbiota examination and analyses of telomer length should be combined with more itemized perspective on the lymphocytic subsets. For more detailed scaling of lymphocytic status, it is planned to conduct investigation on lymphocyte subsets with cytometry by time-of-flight (CyTOF) (101). CyTOF analysis will provide better understanding of phenotypic organisation of lymphocytes and the interrelation between the lymphocytic subsets depending on their proliferation and activation status.

Further accumulation of data beyond the present interim study will provide our hypotheses with evidence or corrective approaches. At the time point of reviewing this diploma thesis we meanwhile included 30 study patients and 20 observational patients with a follow-up of 3 years. Within this context, continuation of the proposed studies shall provide essential and substantial broadening of initial FACS analyses of untreated patients. Cumulative data from this ongoing project will allow validation and modification of our deducted hypotheses and the development of novel approaches for diagnostical implementation.

5. Conclusion and outlook

In summary, we detected a reminiscent phenotypical pattern of effector memory skewing in CD8+CD3+ and CD4+CD3+ T cells, strongly indicating premature maturation. By examination of B cells, we could also detect elevated age associated B cell proportions. This promising reminiscence is based on resembling phenotypes of lymphocytes in several immune dysregulated conditions like primarily PIDs, autoimmune conditions and malignancy. Based on and configured by numerous preliminary studies we support premature maturation formation and immunosenescence in lymphocytes as translational approach for a predictive and diagnostic biomarker profile in SIC.

By accumulation of more patient data and more detailed analytic panels as adjunction to preliminary data we aim to gather reliable and valid evidence and modification of deducted hypotheses. Prospective insights of individual study populations and assigning data to clinical courses and treatment will support validation of premature maturation and immunosenescence as biomarkers for risk evaluation and therapy stratification in SIC and IEI.

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