Newborn screening for severe combined immunodeficiency: breaking the bubble

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CHAPTER 6

Abnormal results of newborn screening for SCID after azathioprine exposure *in utero*: benefit of *TPMT* genotyping in both mother and child

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TO THE EDITOR

INTRODUCTION

Countries all over the world are progressively implementing newborn screening (NBS) for severe combined immunodeficiency (SCID). NBS for SCID is based on the detection of T-cell receptor excision circles (TRECs), a marker for thymic production of naïve T-cells. However, newborns with a range of conditions associated with T-cell lymphopenia (including other conditions than SCID) are being identified shortly after birth. Due to the global rollout of NBS for SCID, pediatric immunologists are confronted with numerous and various (neonatal) cases of impaired T-cell development. One of the causes of profound T-cell lymphopenia encountered in NBS for SCID is the maternal use of immunosuppressive drugs such as azathioprine during pregnancy [1-6].

Azathioprine is an immunosuppressive cytotoxic drug used for treatment of several autoimmune disorders, including inflammatory bowel disease (IBD). Azathioprine is a prodrug, rapidly metabolized to active 6-thiogunine nucleotides [6-TGN], that are incorporated in the DNA inhibiting purine synthesis and thus cause cell cytotoxicity [7]. Azathioprine is often prescribed during pregnancy to women with IBD to avoid flares and relapse of the disease. Exacerbations of disease activity are associated with an increased risk of pre- and/or dysmaturity. The use of azathioprine during pregnancy is considered relatively safe, however, cases of hematological toxicity and neonatal immunodeficiency have been reported [8].

Azathioprine toxicity (including severe lymphopenia) has been attributed to genetic polymorphisms in the *TPMT* gene, which is responsible for enzymatic catalyzation of azathioprine to the inactive metabolite 6-MMP. Presence of non-functional *TPMT* alleles results in reduced TPMT activity and, thereby, accumulation of active 6-TGN and increased toxicity. TPMT phenotyping and/or genotyping allows individualized azathioprine dosing in patients with either one non-functional *TPMT* allele ('intermediate metabolizers'; prevalence 6 - 11% in Caucasian populations) or two non-functional TPMT alleles ('poor metabolizers'; prevalence 0.3% in Caucasian populations). Guidelines for *TPMT*-informed dosing of azathioprine are available and could prevent toxicity in the newborn [9,10].

Here, we describe four cases of newborns with significant combined T- and B-cell lymphopenia, identified via NBS for SCID, born to mothers using azathioprine. We highlight the case of a girl who was referred to a pediatrician-immunologist with severe T-cell lymphopenia, requiring infection prophylaxis. The T-cell lymphopenia

was caused by *in utero* exposure to high levels of azathioprine/6-TGN due to strongly reduced TPMT enzyme activity. In addition, we report three other cases with significant T-cell lymphopenia after maternal azathioprine use identified by NBS for SCID. All parents provided consent for participation in NBS for SCID and consent for publication.

CASE DESCRIPTION

Maternal history

A 37 year old patient (G2P2) with IBD, was treated with azathioprine 100mg per day throughout pregnancy. No recent 6-TGN levels were known and no *TPMT* genotyping was performed. During pregnancy, a complete blood count (without differential count) showed no anemia, thrombocytopenia or leukopenia. She gave birth to a daughter after an uncomplicated pregnancy (*case A*, see below).

Four weeks after delivery, a differential blood count, 6-TGN/6-MMP levels and TPMT genotyping were performed because of lymphopenia in her newborn daughter. Differential showed lymphopenia (lymphocytes 0.5 x 109/L), high 6-TGN levels (647 pmol/8x108 RBC) with undetectable 6-MMP levels and a heterozygous *3C *TPMT* genotype (intermediate metabolizer).

Patient history (*case A*)

A newborn girl, second child of non-consanguineous parents, was born at 39 4/7 weeks with normal birth weight (3245 grams). She was not breastfed. NBS for SCID was abnormal at day 4 (TRECs 0 copies/punch) and she was referred to the pediatrician-immunologist for further analysis. There were no clinical abnormalities. A differential blood count at day 7 showed no anemia nor neutropenia, but low thrombocytes (52 x 109/L) and severe lymphopenia (0.26 x 109/L). Lymphocyte subset analysis revealed a SCID-like phenotype with (near-)absent T- and B-cells (Figure 1A). Epigenetic immune cell counting (technique for relative leukocyte quantification in dried blood spots [11]) confirmed low relative T- and B-cell counts in the original NBS card. 6-TGN levels were high (618 pmol/8x108 RBC), 6-MMP levels were undetectable. Because of the severe T-lymphopenia, Pneumocystis prophylaxis (trimethoprim-sulphamethoxazole) was started at day 7 and home isolation was advised. Genetic analysis revealed a homozygous *3C *TPMT* genotype (poor metabolizer). The clinical condition of the patient, lymphocyte counts (including TREC analysis) and 6-TGN/6-MMP levels were regularly assessed and normalized during follow-up (Figure 1A-C). Pneumocystis prophylaxis and home isolation were discontinued at week eight (based on CD3+/CD4+ T-cell counts) and the patient was discharged from further follow-up at 18 weeks.

Table 1. Diagnostic results of case A; Girl, 2nd child of non-consanguineous parents, gestational age 39 4/7 weeks, birth weight 3245 grams. Maternal azathioprine use (1d 100 mg) for inflammatory bowel disease.

	Day 4	Week 1	Week 5	Week 8	Week 18	Normal range
Blood cell counts						
Hemoglobin (Hb) (mmol/L)	-	9.1	7.1	6.3	6.9	6.5 - 8.4
Thrombocytes (x10^9/L)	-	52	337	443	426	150 – 450
Leukocytes (x10^9/L)	-	7.30	3.20	5.10	6.80	6.0 - 17.5
Neutrophils (x10^9/L)	-	5.30	1.50	1.70	2.00	1.5 - 8.5
Flow cytometry						
Lymphocytes (x10^9/L)	-	0.26	0.64	2.31	3.7	4.0 - 13.0
CD3+ cells (x 10^6/L)	-	37	73	866	2575	2300 -7000
CD4+ T-cells (x 10^6/L)	-	31	60	735	2000	1700 -5300
CD4+ naïve T-cells (x 10^6/L)		13	37	606	1800	
CD8+ T-cells (x 10^6/L)	-	4	0	69	434	394 – 1865
CD8+ naïve T-cells (x 10^6/L)		2	0	53	361	
CD19+ B cells (x 10^6/L)	-	0	464	1133	913	600 – 1900
CD56+ NK cells (x 10^6/L)	-	193	52	249	178	200 – 1400
Ig analysis						
IgG (g/L)	-	9.1	6.1	4.1	2.2	2.20 - 11.3
IgA (g/L)	-	-	0.00	0.00	0.08	0.080 - 0.90
IgM (g/L)	-	-	0.02	0.13	0.28	0.070 - 0.65
Epigenetic immune cell counting*						
Relative CD3+ T-cell counts (%)	0.90	1.59	-	15.15	36.45	11.32 - 34.95
Relative B-cell counts (%)	0.05	0.36	-	21.41	18.28	2.28 - 9.36
Relative NK-cell counts (%)	2.75	4.21	-	7.61	3.32	4.18 - 12.21
Screening results and 6-TGN/6-MMP levels						
TREC copies/punch	0 - 0 - 0	0 - 1 - 1	-	10-18-15	139-147-151	>10
B-actin copies/punch (average)	4369	5067	-	3348	3410	>1000
6-TGN pmol/8.10^8 RBC	-	618	126	0	-	Toxic range >450
6-MMP levels pmol/8.10^8 RBC	-	0	0	0	-	
Genetic analysis	*TPMT* genotype: homozygous *3C/*3C. Poor metabolizer.					

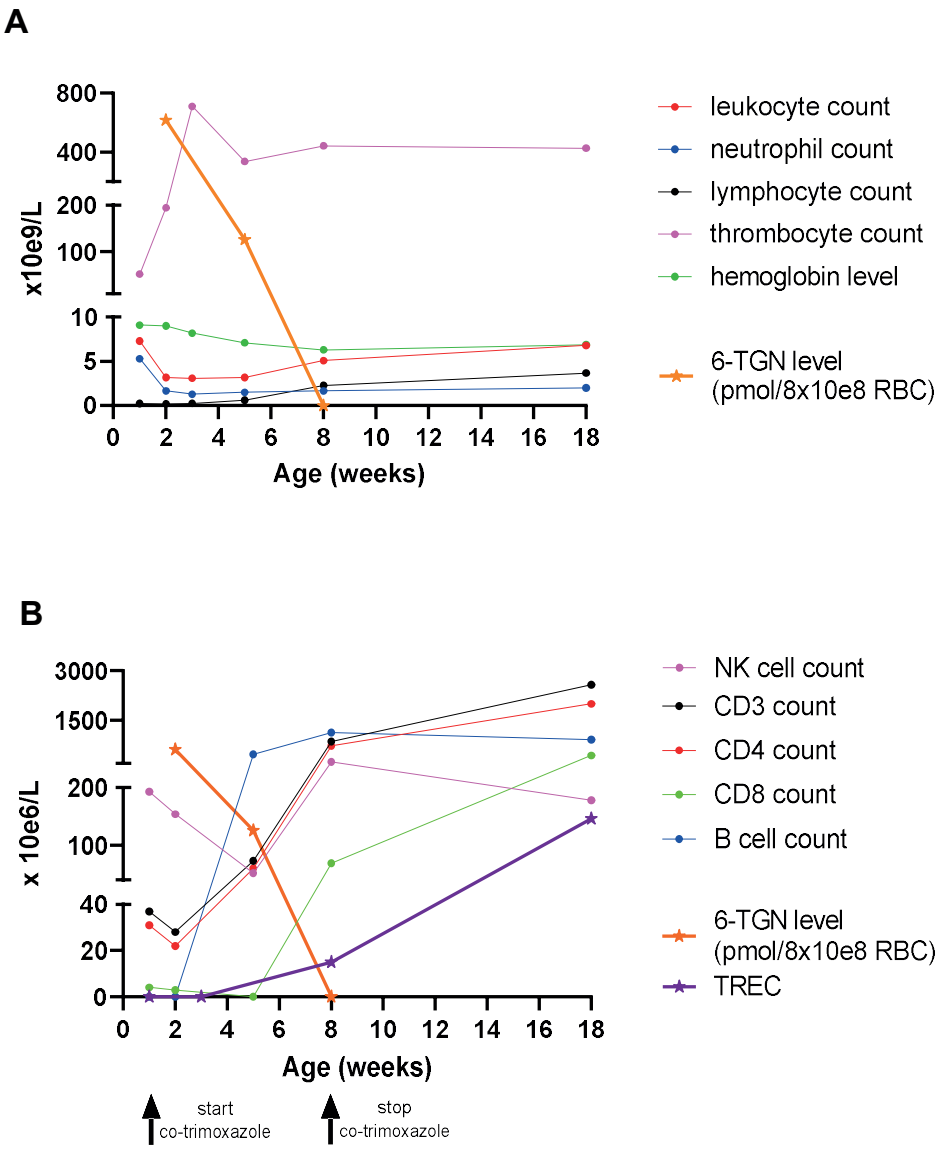

Figure 1. Diagnostic and screening results of case A. **A.** Absolute cell counts, hemoglobin level and 6-TGN levels over time. **B.** Absolute lymphocyte subset cell counts, TRECs and 6-TGN levels over time.

Sister of case A

Because of lymphopenia in *case A*, a retrospective analysis was performed on the original NBS card of her older sister (two years of age). During this pregnancy, mother used azathioprine 50 mg once daily. This healthy sister appeared to have normal TREC levels (26 copies/punch) and normal relative epigenetic CD3+ T-cell counts (18.0%, normal range 11.3 - 35.0%) in her NBS card. Relative epigenetic B-cell counts were very low 0.16% (normal range 2.28 - 9.36). *TPMT* genotyping showed this sister to be an intermediate metabolizer (heterozygous *TPMT* *3C allele).

Additional cases

Three other cases (*cases B-D*) with maternal azathioprine use were identified with NBS for SCID and referred to a pediatrician-immunologist in the same period. TREC counts in these patients varied between 1 and 16 copies/punch. All cases had profound combined T- and B-cell lymphopenia at time of referral and were monitored for up to 18 weeks, until immunological recovery (Figure S1, S2 and S3). Additional *TPMT* genotyping revealed that *case B* was a normal metabolizer (*TPMT* wild type genotype) but exposed to a relatively high dose of azathioprine (200 mg/day). *Case C* was a poor metabolizer (homozygous of *TPMT* *3A allele), exposed to a relatively low dose of azathioprine (75 mg/day). *Case D* was an intermediate metabolizer (heterogeneous for at least one *TPMT* *3 allele) exposed to 100 mg azathioprine/day. Because of low CD3+/CD4+ T-cell counts, *case D* received Pneumocystis prophylaxis until immunological recovery.

DISCUSSION

We describe four cases of newborns with profound combined T- and B-cell lymphopenia, identified via NBS for SCID, born to mothers using azathioprine. *TPMT* genotyping provided valuable additional information during the diagnostic process of these infants with severe lymphopenia, as azathioprine is frequently prescribed during pregnancy and not all newborns from mothers using this drug are affected. Both mothers and newborns with reduced TPMT enzyme activity caused by polymorphisms in the *TPMT* gene had less efficient catalyzation of azathioprine leading to a higher risk of hematopoietic toxicity, including profound lymphopenia (mimicking SCID) in the newborn. Two children with reduced TPMT enzyme activity and severe T-cell lymphopenia even required home isolation and initiation of Pneumocystis prophylaxis. All cases demonstrated complete immunological recovery at 10-18 weeks after birth.

Based on current international guidelines, health care providers prescribing azathioprine to pregnant women usually perform a total leukocyte count without differential, which may leave maternal lymphopenia due to reduced TPMT activity with toxic 6-TGN levels unnoticed [12]. This may result in provision of suboptimal information to (future) parents about the possibility of immunodeficiency and an abnormal result of NBS for SCID in the newborn. Indeed, not all families of our cases were aware of the risk associated with azathioprine use during pregnancy, including the possibility of abnormal NBS results. Parents were given conflicting information during the referral procedure after the abnormal SCID screening result. We earlier reported that referrals in NBS for SCID caused considerable anxiety in parents [13]. In addition, health care providers prescribing azathioprine to the mothers questioned the association between the abnormal NBS result and maternal azathioprine treatment.

With the global rollout of NBS for SCID, there is a strong need to raise awareness on a multidisciplinary scale about maternal azathioprine use and the risk of severe neonatal T-cell lymphopenia with abnormal SCID screening results. More explicit monitoring of maternal lymphocyte counts, 6-TGN/6-MMP levels and *TPMT* genotyping at the start of pregnancy, with adjustment of azathioprine dose without reducing therapeutic efficiency in mothers, may prevent fetal exposure to azathioprine toxicity *in utero*. Moreover, differential blood count analysis in (at-risk) newborns directly after birth may identify these cases prior to NBS for SCID. Maternal and patient history plus laboratory results of both mother and child, will additionally help will help pediatrician-immunologists in the evaluation of these newborns with abnormal SCID screening results. The provision of clear information by the health care providers involved, both during the pregnancy as well as during the referral procedure, is of utmost importance and can severely reduce anxiety for parents [13].

Sharing experiences of cases with profound lymphopenia, identified via NBS, with obstetricians, gastroenterologists, pediatricians and primary health care providers, and a close partnership between physicians on an international level, will help to promote standardization of care for fertile/pregnant women on immunosuppressant medication including azathioprine.

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DECLARATIONS

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Conflicts of interest/Competing interests
The authors declare that they have no conflict of interest.

Availability of data and material
All data generated or analyzed during this study are included in this published article and its supplementary information.

Code availability
Not applicable

Authors' contributions
MB, MvdB and DB designed the study; IP and JS performed flow cytometric and genetic analyses; DB, RB and JvM did the clinical evaluations of the patients; MB, IP, JS, DB analyzed the data; DB coordinated the project; MB and DB wrote the paper; all authors contributed to and approved the final version of the manuscript.

Ethics approval
This study was performed in line with the principles of the Declaration of Helsinki. Approval for the SONNET-study was granted by the Medical Ethics Committee of the Erasmus MC, University Medical Center, Rotterdam (MEC-2017-1146).

Consent to participate
In order to participate in newborn screening for SCID (SONNET-study), parents have to express verbal consent when the heel prick is performed (opt-out consent).

Consent for publication
Parents of cases have provided consent for the publication of the data in this case report.

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SUPPLEMENTARY MATERIAL

Table S1. Diagnostic results of Case B; Boy, 1st child of non-consanguineous parents, gestational age 37.2 weeks, birthweight 3170 grams. Maternal azathioprine use (1d 200 mg) for inflammatory bowel disease. 6-TGN/6-MMP-levels in mother were 526 and 814 pmol/8.10^8 RBC respectively at three months of pregnancy.

	Day 4	Week 1	Week 10	Normal range
Blood cell counts				
Hemoglobin (Hb) (mmol/L)	-	9.2	6.0	6.5 - 8.4
Thrombocytes (x10^9/L)	-	354	536	150 - 450
Leukocytes (x10^9/L)	-	4.30	4.51	6.0 - 17.5
Neutrophils (x10^9/L)	-	2.38	1.15	1.5 - 8.5
Flow cytometry				
Lymphocytes (x10^9/L)	-	0.76	2.25	4.0 - 13.0
CD3+ cells (x 10^6/L)	-	660	1314	2300 - 7000
CD4+ T-cells (x 10^6/L)	-	404	990	1700 - 5300
CD4+ naïve T-cells (x 10^6/L)	-	411	883	
CD8+ T-cells (x 10^6/L)	-	106	272	394 - 1865
CD8+ naïve T-cells (x 10^6/L)	-	91	255	
CD19+ B cells (x 10^6/L)	-	0	718	600 - 1900
CD56+ NK cells (x 10^6/L)	-	68	126	200 - 1400
Immunoglobulin (Ig) analysis				
IgG (g/L)	-	5.8**	2.8	2.20 - 11.3
IgA (g/L)	-	0	0.06	0.080 - 0.90
IgM (g/L)	-	0.01	0.2	0.070 - 0.65
Epigenetic immune cell counting*				
Relative CD3+ T-cell counts (%)	7.05	14.40	-	11.32 - 34.95
Relative B-cell counts (%)	0.15	0.39	-	2.28 - 9.36
Relative NK-cell counts (%)	1.47	2.25	-	4.18 - 12.21
Screening results and 6-TGN levels				
TREC copies/punch	4 - 10 - 16	23 - 18 - 15	-	>10
B-actin copies/punch (average)	3589	1078	-	>1000
6-TGN pmol/8.10^8 RBC	-	< 50	-	Toxic range >450
Genetic analysis	TPMT genotype: 'wild type', normal metabolizer (no variants in the TPMT*2, *3A, *3B or *3C allele).			

d, Day; 6-TGN, 6-thioguanine nucleotides; TRECs, T-cell receptor excision circles. *Relative cell counts in percentages (%) of total leukocytes. ** Week 3 measurements.

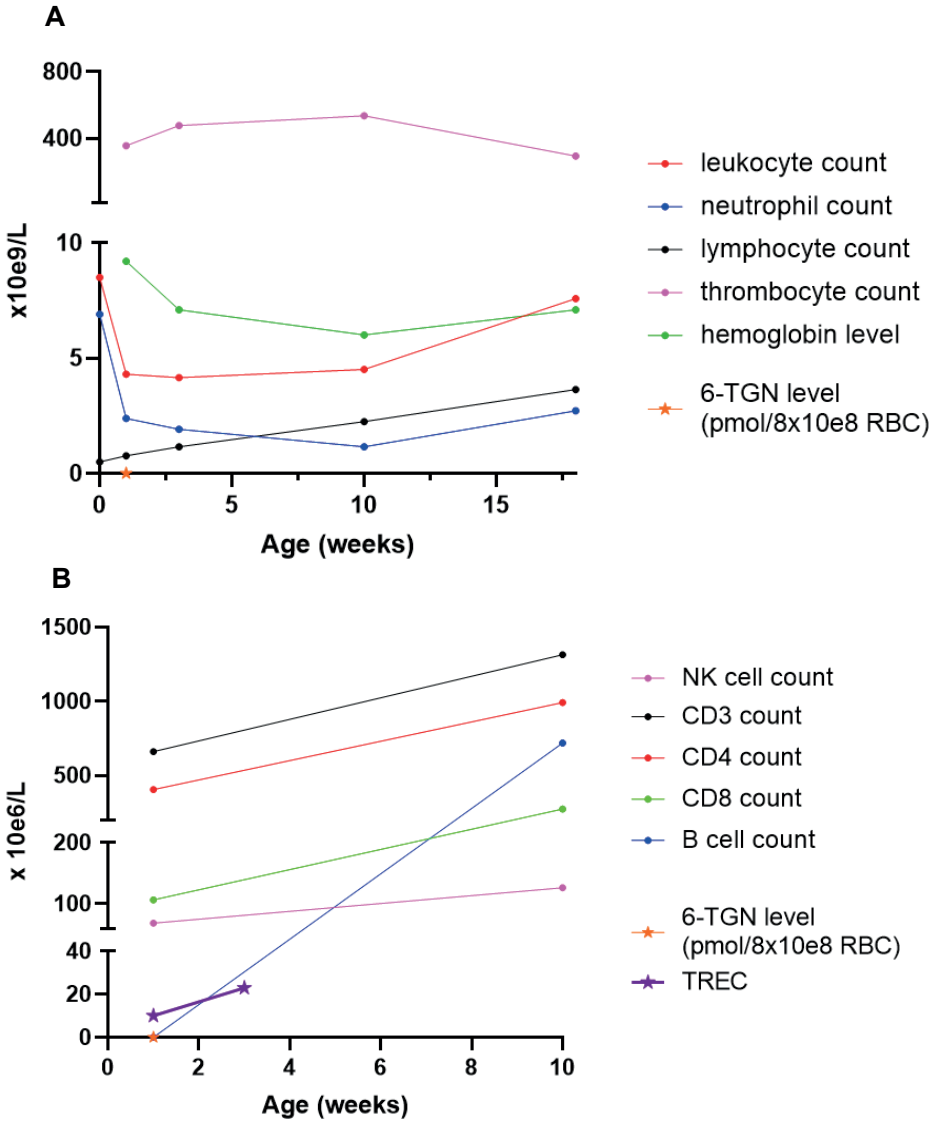

Figure S1. Diagnostic and screening results of case B. **A.** Absolute cell counts, hemoglobin level and 6-TGN levels over time. **B.** Absolute lymphocyte subset cell counts, TRECs and 6-TGN levels over time.

Table S2. Diagnostic results of Case C; Boy, 4th child of non-consanguineous parents, gestational age 40.1 weeks, birthweight 3032 grams. Maternal azathioprine use (1d 75 mg) for inflammatory bowel disease.

	Day 4	Week 1	Week 10	Normal range
Blood cell counts				
Hemoglobin (Hb) (mmol/L)	-	10.2	6.2	6.5 - 8.4
Thrombocytes (x10^9/L)	-	258	402	150 - 450
Leukocytes (x10^9/L)	-	4.30	5.80	6.0 - 17.5
Neutrophils (x10^9/L)	-	1.86	1.49	1.5 - 8.5
Flow cytometry				
Lymphocytes (x10^9/L)	-	0.60	3.43	4.0 - 13.0
CD3+ cells (x 10^6/L)	-	720	1406	2300 - 7000
CD4+ T-cells (x 10^6/L)	-	580	1101	1700 - 5300
CD4+ naïve T-cells (x 10^6/L)	-	-	909	
CD8+ T-cells (x 10^6/L)	-	80	216	394 - 1865
CD8+ naïve T-cells (x 10^6/L)	-	-	188	
CD19+ B cells (x 10^6/L)	-	0	1845	600 - 1900
CD56+ NK cells (x 10^6/L)	-	10	96	200 - 1400
Immunoglobulin (Ig) analysis				
IgG (g/L)	-	10.9	-	2.20 - 11.3
IgA (g/L)	-	0	-	0.080 - 0.90
IgM (g/L)	-	0.01	-	0.070 - 0.65
Epigenetic immune cell counting*				
Relative CD3+ T-cell counts (%)	4.61	13.18	-	11.32 - 34.95
Relative B-cell counts (%)	0.24	0.56	-	2.28 - 9.36
Relative NK-cell counts (%)	1.56	3.30	-	4.18 - 12.21
Screening results				
TREC copies/punch	7 - 3 - 6	11 - 18 - 16	-	>10
B-actin copies/punch (average)	4137	7240	-	>1000
Genetic analysis	TPMT genotype: homozygous *3A/*3A. Poor metabolizer.			

d, Day; 6-TGN, 6-thioguanine nucleotides; TRECs, T-cell receptor excision circles. Relative cell counts in percentages (%) of total leukocytes.

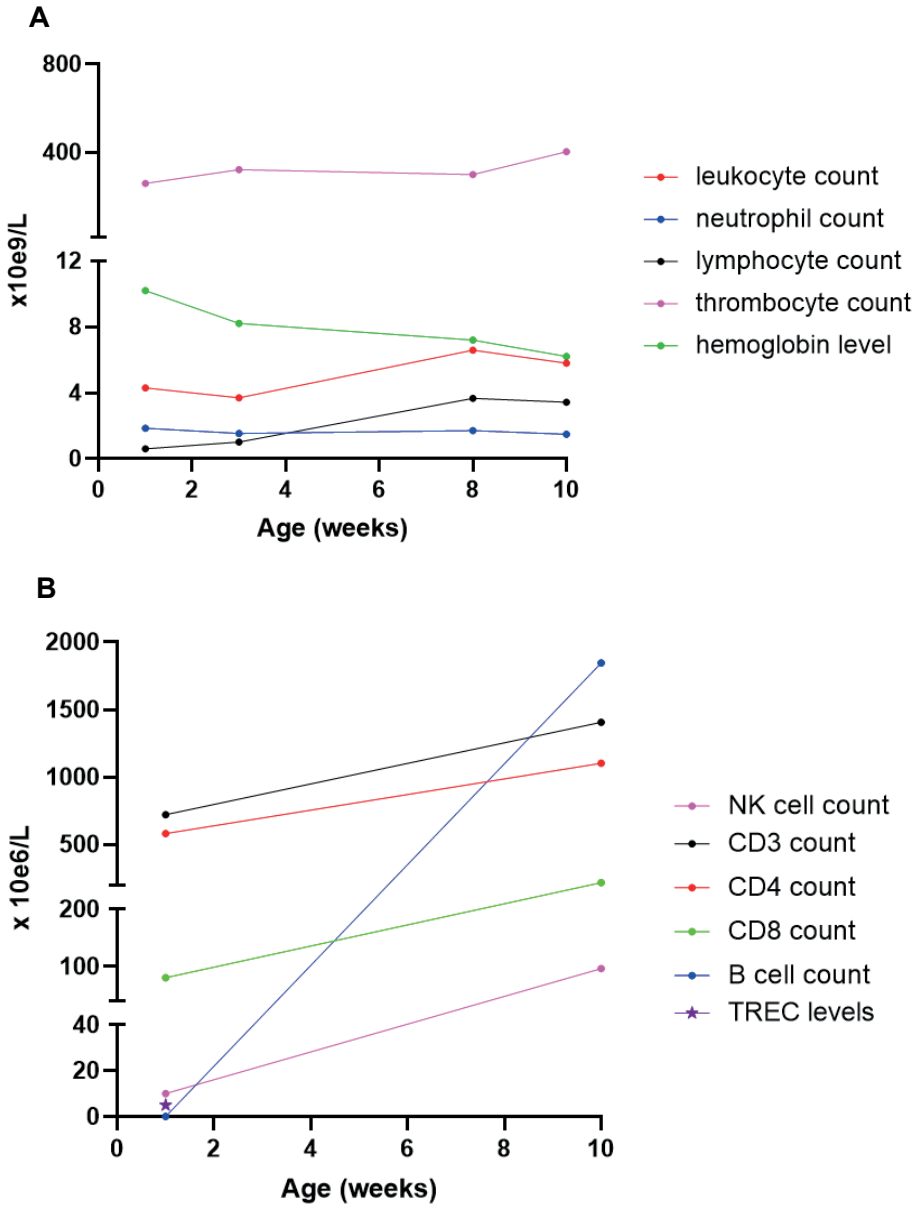

Figure S2. Diagnostic and screening results of case C. **A.** Absolute cell counts and hemoglobin level over time. **B.** Absolute lymphocyte subset cell counts and TREC levels over time.

Table S3. Diagnostic results of Case D; Boy, 1st child of non-consanguineous parents, gestational age 40.4 weeks, birthweight 3840 grams. Maternal azathioprine use (1d 100 mg) for inflammatory bowel disease.

	Day 4	Week 1	Week 6	Week 12	Week 26	Normal range
Blood cell counts						
Hemoglobin (Hb) (mmol/L)	-	10.6	7.3	6.4	7.0	6.5 - 8.4
Thrombocytes (x10^9/L)	-	301	549	378	570	150 - 450
Leukocytes (x10^9/L)	-	7.0	5.1	6.6	11.9	6.0 - 17.5
Neutrophils (x10^9/L)	-	4.07	1.88	2.49	2.74	1.5 - 8.5
Flow cytometry						
Lymphocytes (x10^9/L)	-	0.70	1.98	3.15	8.33	4.0 - 13.0
CD3+ cells (x 10^6/L)	-	383	872	1961	4491	2300 - 7000
CD4+ T-cells (x 10^6/L)	-	209	667	1538	3262	1700 - 5300
CD4+ naïve T-cells	-	133	489	1307	2734	
CD8+ T-cells (x 10^6/L)	-	101	170	310	1165	394 - 1865
CD8+ naive T-cells	-	88	120	217	860	
CD19+ B cells (x 10^6/L)	-	1	625	965	2146	600 - 1900
CD56+ NK cells (x 10^6/L)	-	68	392	134	370	200 - 1400
Immunoglobulin (Ig) analysis						
IgG (g/L)	-	7.3	-	2.37	3.58	2.20 - 11.3
IgA (g/L)	-	<0.04	-	0.05	0.21	0.080 - 0.90
IgM (g/L)	-	<0.04	-	1.1	0.50	0.070 - 0.65
Epigenetic immune cell counting*						
Relative CD3+ T-cell counts (%)	8.11	12.64	16.24	-	28.99	11.32 - 34.95
Relative B-cell counts (%)	0.11	0.09	12.9	-	18.0	2.28 - 9.36
Relative NK-cell counts (%)	1.30	3.47	4.72	-	2.96	4.18 - 12.21
Screening results						
TREC copies/punch	1 - 3 - 5	6 - 4 - 9	14-16-23	-	66	>10
B-actin copies/punch (average)	1882	2886	1648	-	1376	>1000
Genetic analysis	TPMT genotype: heterogenous for at least one *3 allele. Based on allele frequency most probable *1/*3A genotype. Intermediate metabolizer.					

d, Day; 6-TGN, 6-thioguanine nucleotides; TRECs, T-cell receptor excision circles. Relative cell counts in percentages (%) of total leukocytes.

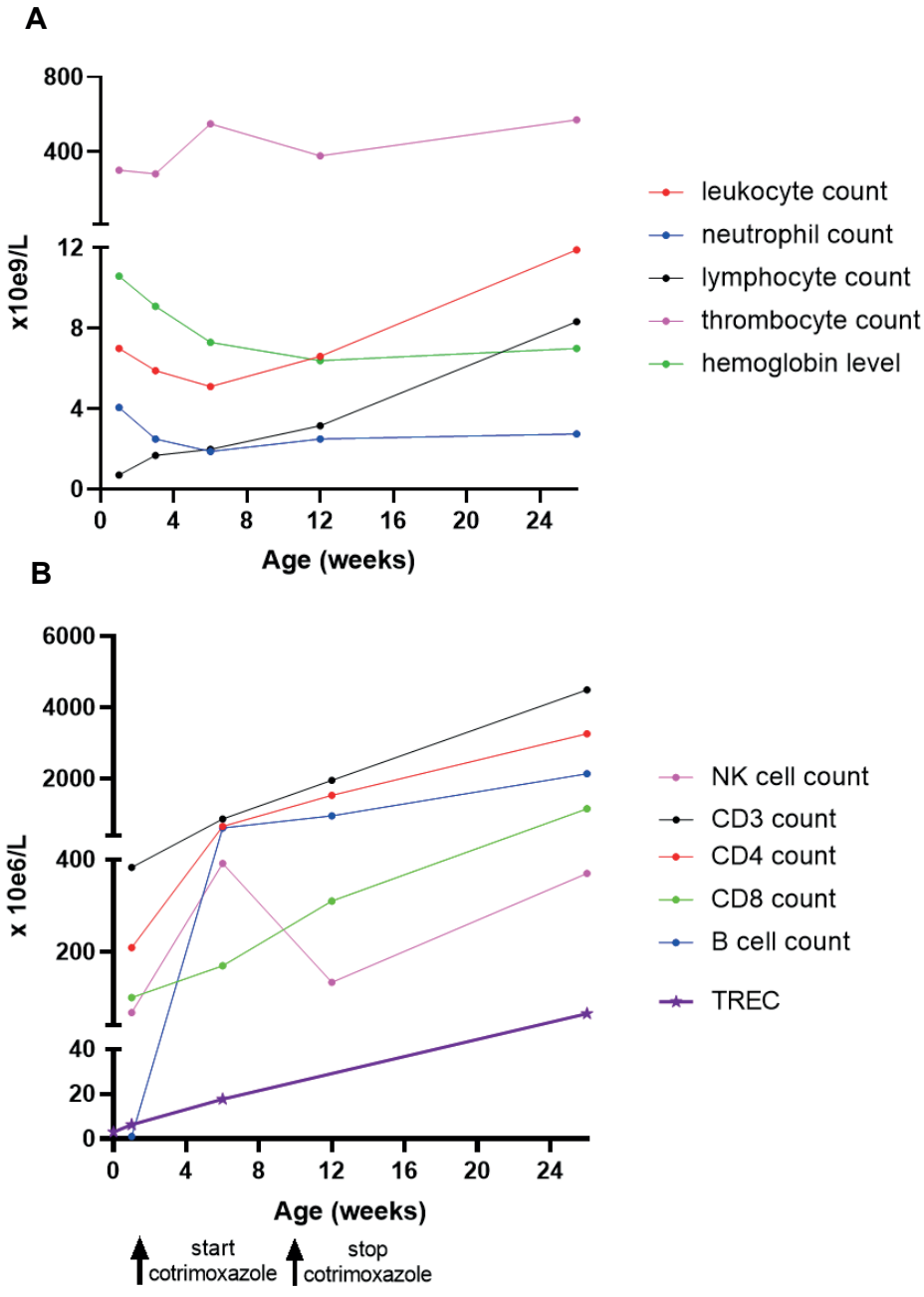

Figure S3. Diagnostic and screening results of case D. **A.** Absolute cell counts and hemoglobin level over time. **B.** Absolute lymphocyte subset cell counts and TREC levels over time.