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# Immune cellular evaluation following newborn screening for severe T and B cell lymphopenia

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ABSTRACT

Newborn screening (NBS) for severe T and/or B cell lymphopenia to identify neonates with severe combined immunodeficiencies (SCID) or agammaglobulinemia rapidly after birth has paved its way into clinical practice. Debate exists on the concept and strategy for rapid verification and stratification of the cellular immune status of positively screened infants. We provide impulses for harmonization of flow cytometric approaches to allow rapid integration in the growing number of immunological laboratories involved in follow-up and subdivision of SCID and non-SCID entities.

## INTRODUCTION

The purpose of neonatal screening programs is the early recognition of treatable genetic diseases that manifest with a high rate of morbidity and mortality. While the implementation of newborn screening tests for metabolic disorders traces back to the mid-1960s, suitable technologies to identify severe inborn errors of immune function have emerged only in recent years.

The estimated incidence of primary immunodeficiency diseases (PID) that would require immediate treatment ranges from 2 to 8 per 100,000 live births, making high demands on the effectiveness and availability of screening tests [3].

In comparison with metabolic diseases, the identification of sensitive and traceable biomarkers poses a challenge due to the genetic diversity of pediatric PID patients.

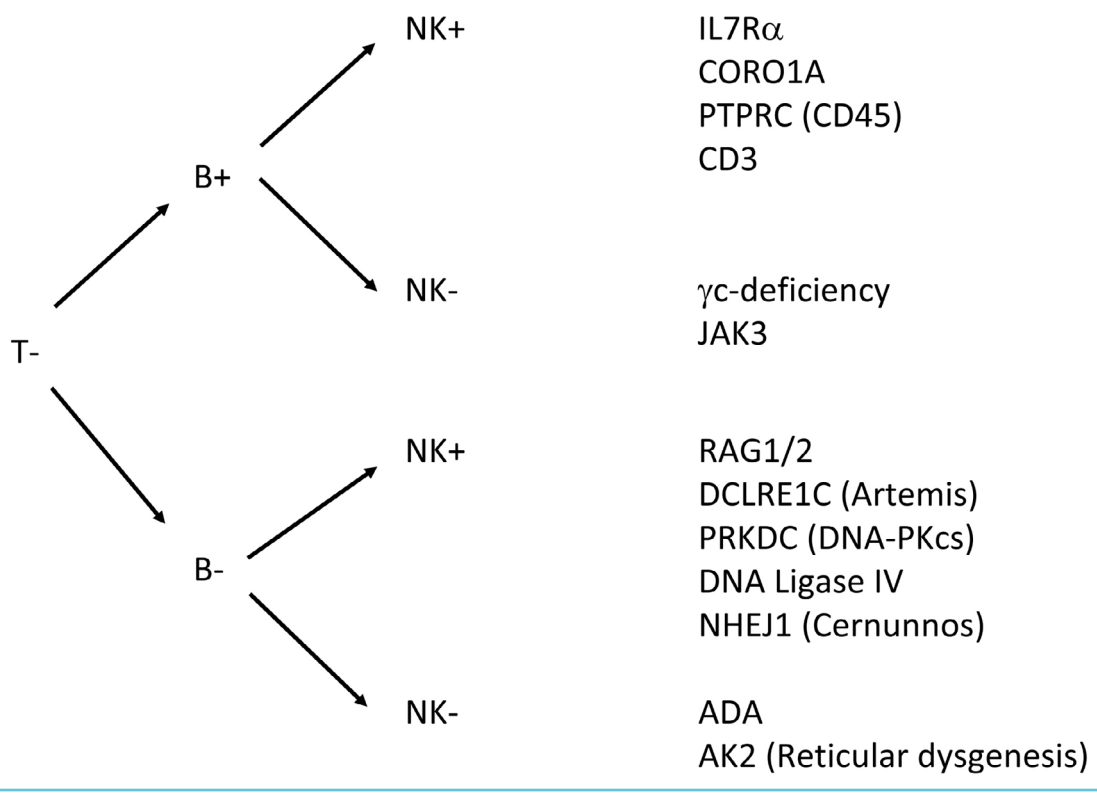
Severe combined immunodeficiency (SCID) is the most severe form of inherited primary immunodeficiency and is a pediatric emergency. Delay in recognizing and detecting SCID can have fatal consequences and also reduces the chances of successful hematopoietic stem cell transplantation (HSCT) [1].

Screening for SCID at birth would prevent children from dying before HSCT can be attempted and would increase the success of HSCT. There is strong evidence to show that SCID fulfills the internationally-established criteria for a condition to be screened for at birth [2].

### *Severe combined immunodeficiency – a life-threatening group of disorders*

SCID is a group of life-threatening immune disorders arising from a variety of genetic defects that lead to the absence of lymphocyte development

**Figure 1** T/B/NK-cellular classification of SCID entities



and function [3]. Nearly all patients with SCID have absent T-cells, and are further grouped by the absence or presence of B-cells and NK-cells (Figure 1).

Thus, the absence or severe reduction of functional naïve T and/or B cells at birth would be the preferable biomarker for newborn screening of SCID [4].

The diagnosis of SCID is a pediatric emergency, given that most affected children exhibit extreme susceptibility to bacterial, viral, fungal and opportunistic infections, which are fatal in the first 1-2 years of life without curative treatment.

In most cases, children with SCID appear well at birth and present with recurrent severe infections and failure to thrive at 3-6 months as passively transferred protective maternal immunoglobulins are diminishing.

## DIAGNOSTIC CONCEPT AND STRATEGY

### *Newborn screening algorithm*

Normal T-cell development requires production of precursor T-cells in the bone marrow and subsequent processing of T-cells in the thymus. Although SCID can arise from a variety of genetic defects, there is an abnormality of T-cell development in the thymus in all cases. During normal thymic processing, T cells undergo receptor gene splicing and rearrangement, leading to intracellular accumulation of DNA by-products known as T-cell receptor excision circles (TRECs). When used in NBS assays, TRECs are a surrogate marker of newborns' capability to produce T cells, which is severely hampered in SCID patients [4].

TRECs do not replicate in dividing cells and are diluted out upon cellular division. They are therefore only found in recent thymic emigrant

**Figure 2** Spectrum of neonatal T cell lymphopenia

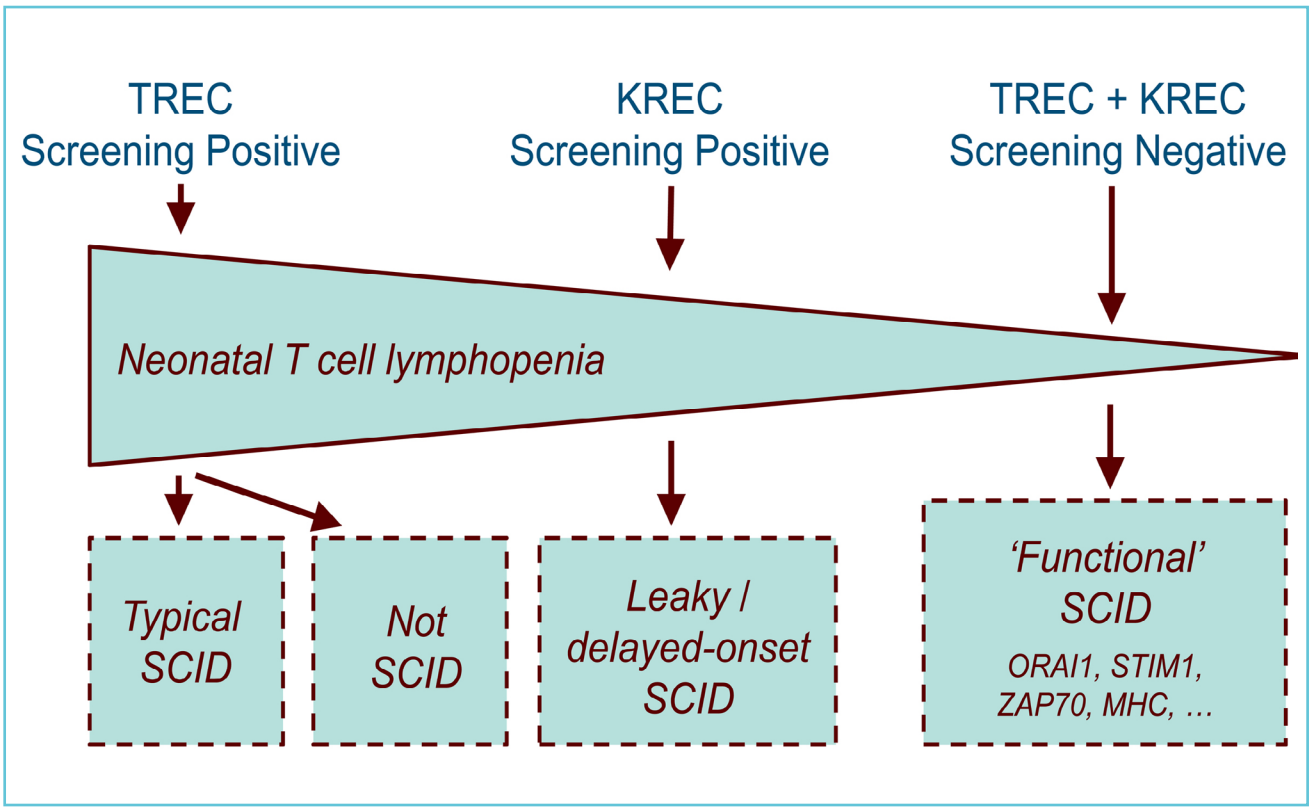
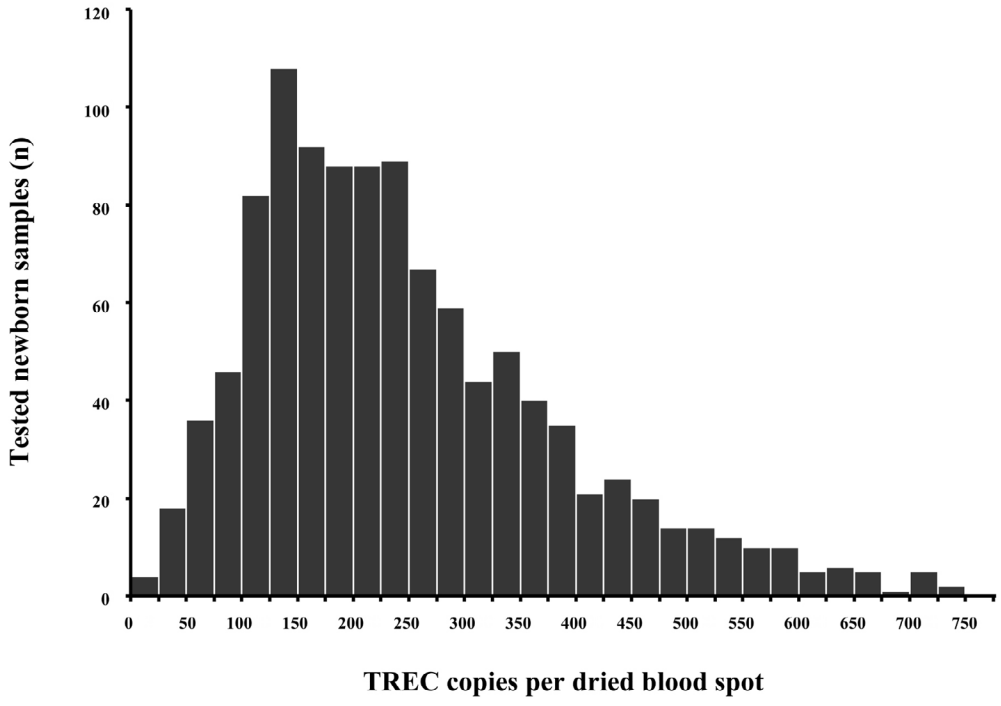
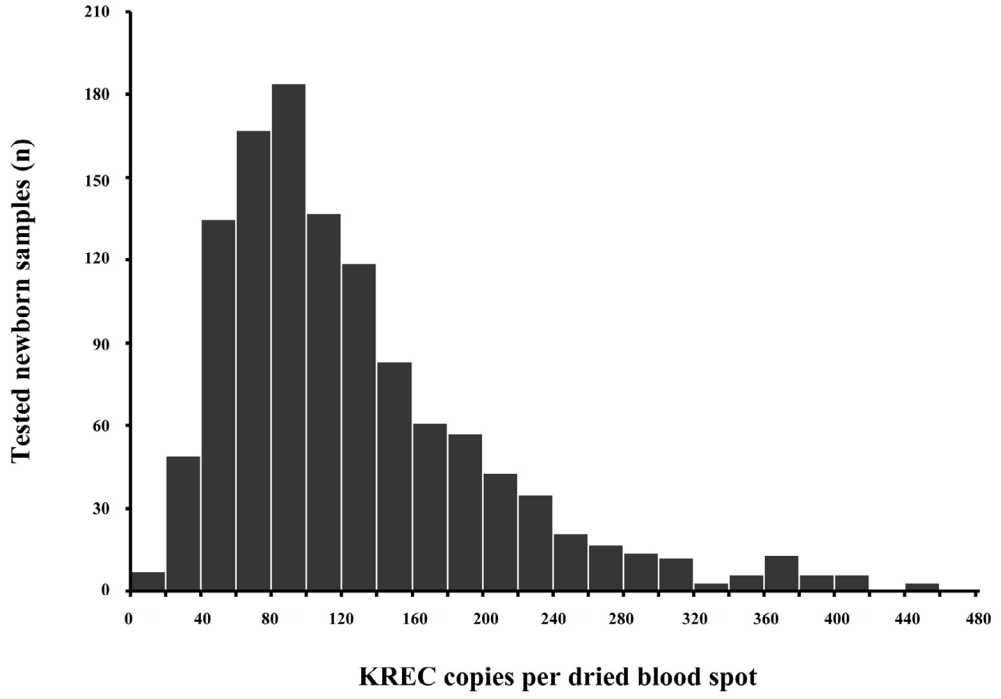


**Figure 3** Representative distribution of TREC and KREC copy numbers in neonatal dried blood spot samples




(A)



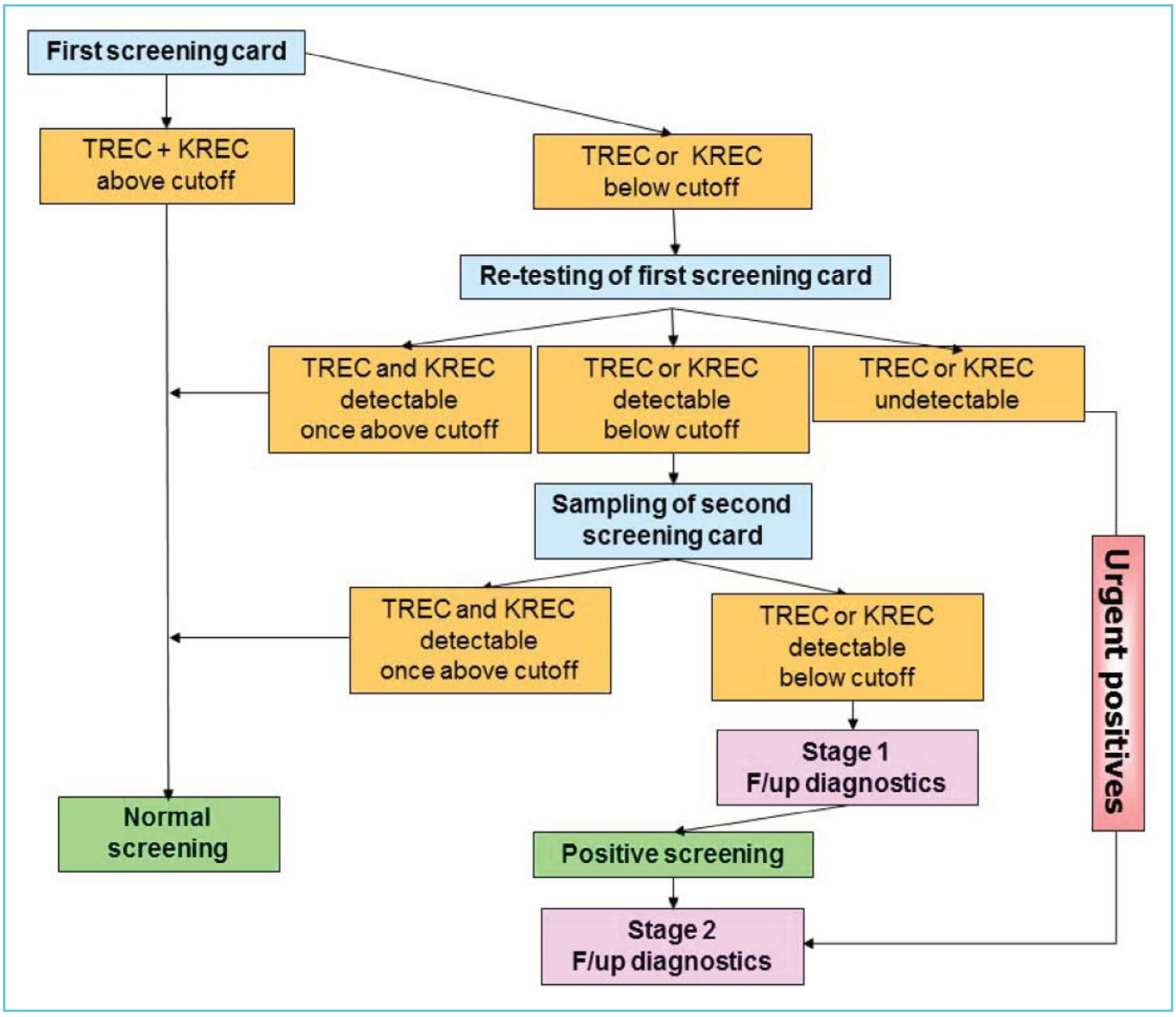

(B)

naïve T-cells. This aspect is important, as in certain conditions such as engraftment of maternal T-cells or expansion of a few oligoclonal T-cells in Omenn syndrome, a substantial amount of T-cells can be found in an infant with SCID. As these T-cells have undergone multiple rounds of cell division, TRECs are diluted and the TREC value is low despite high numbers of T-cells in peripheral blood.

As some leaky, variant, or delayed-onset forms of SCID will not be detected at birth based on a single TREC assay, the addition of other screening markers such as kappa-deleting recombination excision circles (KREC), which detect defects of B-cell development, has been proposed and might be considered helpful (Figure 2) [4].

Screening for severe T-cell lymphopenia by TRECs is not standardized and employs different methods, leading to marked differences in cut-offs for the number of newly formed T-cells in the ongoing screening programs in various countries. This, in turn, has resulted in significant

**Figure 4** Proposed flow-chart for the follow-up of positive newborn screening results for TRECs and KRECs



difference in the number of patient recalls and diagnostic procedures, including flow cytometry and other cellular testing stages. Typical testing results in a large cohort of healthy newborn using a commercially available screening kit for TRECs and KRECs are depicted in Figure 3 [5].

To ensure adequate follow-up of infants with likely SCID identified by TREC screening and to limit the number of false-positive results at the same time, algorithms have been designed by screening centers together with clinical immunologists [5]. In most cases this will include the following (Figure 4): All infants undergo screening by the TREC (and KREC) assay. If normal, no further intervention is recommended. In infants with TREC levels below the cut-off, the first screening card will be retested for TREC as well as DNA amplification by quantifying beta-actin levels. If the beta-actin level is normal and TRECs are still below the cut-off the primary care provider is contacted for two scenarios: 1) an emergency scenario - if TRECs are undetectable (~1 in 20.000 cases), the infant needs urgent confirmatory testing by flow cytometry and treatment by a clinical immunologist; 2) an intermediate scenario – if TRECs are detectable, yet below the established local cut-off value, re-testing of a second screening card is performed: Tracking of newborns is thus initiated in case of repeated abnormal test results for TREC and/or KREC copy numbers after examination of at least three independent dried blood punches of the first dried blood spot submitted.

### *Parent and patient interaction*

As first action within the tracking procedure, the obstetric unit and the parents of the newborn should be contacted to obtain additional information on the status of the child (Stage 1). While the parents of the newborn should be informed without delay after the examination of the second separate dried blood card, even if the findings are normal, a detailed explanation of the significance of the test results, if they are abnormal again, will be provided only at a specialized immunodeficiency center. The parents will be directed to such a center in the area (Stage 2), and the center will be informed about the patient to be expected.

Subsequently, a specialized treatment center should be selected that is close to home: in the case of suspected severe naive T- and/or B-lymphopenia, intensive hygiene measures and possibly early, strict isolation to prevent opportunistic infections are necessary steps to be taken. In order to perform HSCT or gene therapy, a transfer to a specialized transplant center may be necessary.

### *Flow cytometric analyses in Stage 1 and Stage 2*

Screening for neonatal T and/or B cell lymphopenia reveals not only patients with SCID or agammaglobulinemia, but also genetic, metabolic and other medical conditions associated with low TREC and/or KREC copies in the dried blood spot card. Whereas the most common reasons for low TREC/KREC copies refer to neonates with preterm birth, 22q11 microdeletion syndrome and Trisomy 21, radiosensitivity disorders such as Ataxia telangiectasia (ATM) or Nijmegen breakage syndrome, chylothorax or spina bifida, there is also a fraction of newborns found resulting from side-effects of maternal medication during pregnancy (i.e. azathioprine, methotrexate or Rituximab treatment) [5-7].

Thus, both in testing Stages 1 and 2 there is an imminent need for verification of the cellular immune status of positively screened infants and stratification of SCID, leaky-SCID and non-SCID patients.

In order to decrease patient harm and parental concern, all testing stages will have to be instructed and supervised by a pediatric immunologist, with 24/7 availability. Minimal diagnostic

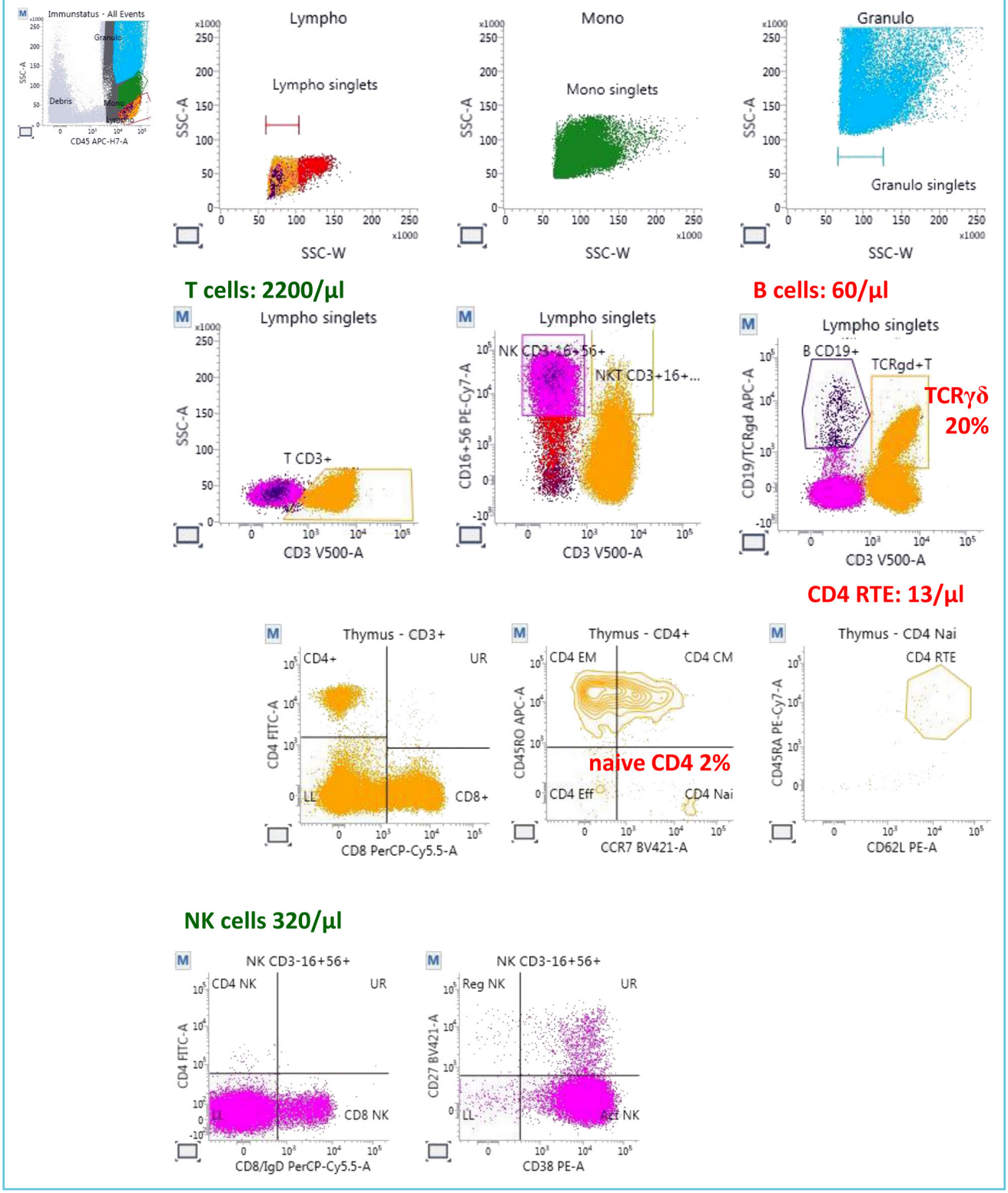
**Table 1** Diagnostic recommendation for Stage 1 and Stage 2 centers following positive NBS with TRECs and/or KRECs

| | |
|---|---|
| **Stage 1** | |
| - 24/7 availability of a paediatric immunologist | |
| - Immediate medical examination and counseling | |
| - Differential blood count | |
| - IgM, IgG, IgA, IgE serum levels | |
| - HIV testing mother and child | |
| - Flow cytometric analysis based on neonatal reference values for | |
| | T cells (CD3/CD4/CD8) |
| | T cell naivety (CD45RA and CD45RO) |
| | B cells (CD19) |
| | NK cells (CD3/CD16/CD56) |
| **Stage 2** | |
| - All of the above | |
| - Additional flow cytometric analyses for | |
| | T cell naivety (CCR7) |
| | Recent thymic emigrants (CD4/CD31/CD45RA) |
| | αβ and γδ T cells (CD3/αβTCR/γδTCR) |
| - In case of presence of >100 T cells/µl | |
| | Exclusion of maternal T cells |
| | Lymphocyte proliferation studies (PHA and/or anti-CD3/CD28) |
| | Radiosensitivity testing of lymphocytes |
| | Analysis of the TCR Vbeta repertoire (Omenn-Syndrome) |
| | ADA and PNP enzyme activity levels |
| | Whole Exome or Genome Sequencing |

**Table 2** **Diagnostic consensus of the Primary Immune Deficiency Treatment Consortium**

| |
|---|
| **Typical SCID** |
| - Absence or very low number of T cells (CD3 T cells <300/µL) and no or very low T-cell function (<10% of lower limit of normal) as measured by response to PHA |
| - Or T cells of maternal origin present |
| **Leaky SCID** |
| - Reduced number of CD3 T cells (CD3 T cells <1000/µL) |
| - Absence of maternal engraftment |
| - <30% of lower limit of normal T-cell function (as measured by response to PHA) |
| **Omenn syndrome** |
| - Generalized skin rash |
| - Absence of maternal engraftment |
| - Detectable CD3 T cells, ≥300/µL |
| - Absent or low (≤30% of normal) T-cell proliferation to antigens to which the patient had been exposed |
| - Or Hepatomegaly, Splenomegaly, Lymphadenopathy |
| - Or increased IgE level, increased absolute eosinophil count |
| **Reticular dysgenesis** |
| - Absence or very low number of T cells (CD3 T cells <300/µL) |
| - No or very low (<10% of lower limit of normal) T-cell function (as measured by response to PHA) |
| - Severe neutropenia (absolute neutrophil count <200/µL) |
| - Sensorineural deafness and/or absence of granulopoiesis at bone marrow examination and/or a deleterious AK2 mutation |

**Figure 5** **Immunophenotype of a leaky-SCID (RAG2 mutation) identified by TREC/KREC newborn screening**



testing recommendations for Stage 1 and Stage 2 centers is depicted in Table 1 [8].

There exists a minimal diagnostic consensus, yet of fluid nature and certainly subject to future discussions, for the classification of neonates with typical SCID, leaky SCID, Omenn syndrome, reticular dysgenesis, and idiopathic T cell lymphopenia [8]. These diagnostic criteria are reproduced in Table 2 and should be applied in the cellular and immune-functional testing strategies despite of ongoing genetic analyses.

The aim is to disclose those patients from a HSCT-track that will not benefit from this therapy or might even yield harm during the transplantation conditioning phase, such as seen in individuals with chromosome repair disorders (i.e. ATM). In this context, flow cytometric analyses subsequent to positive NBS with TRECs and/or KRECs will have to rely on best established-practice, or better harmonized and widely-available diagnostic products, and reference values validated for the cell staining/lysis protocol that is applied [Table 1] [9]. As an example, the PID working party of the EuroFlow consortium provided a Primary Immunodeficiency Orientation Tube (PIDOT) with polychromatic flow cytometric markers for classification or T-, B- and NK-cells, thereby fulfilling requirements of Stage 1 testing after NBS [10]. To further endorse harmonization across different labs, such panels will be available in lyophilized antibody format to allow pre-production, storage and uniform staining approaches. Similarly to surface-marker phenotypic staining, there also are protocols available for T/B/NK cell functional analyses of the DNA damage repair capability (γH2AX assay), as well as cellular proliferation upon phytohemagglutinin (PHA) or anti-CD3/28 treatment [11, 12]. Exemplarily, Figure 5 shows testing results of a Stage 2 flow cytometric analysis from a newborn identified with leaky-SCID due to a RAG2-mutation upon positive NBS with TRECs and KRECs.

## CONCLUSIONS AND PERSPECTIVE

As newborn screening for severe primary immunodeficiency diseases (PID) - characterized by T and/or B cell lymphopenia - is becoming clinical routine practice in a growing number of countries, there still is debate about the definition of PID and a lack of harmonized approaches to the immune cellular phenotype and functional testing during diagnostic follow-up. In the future, more specific large scale and age-selected studies in the field of neonatal care and primary immunodeficiency diseases are required to provide reliable cornerstones to line clinical decisions upon and provide earliest-possible and safeguarded care for PID patients.

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