Medical Research Archives

ESMED
European Society of Medicine

OPEN ACCESS

Published: January 31, 2023

Citation: Kempis-Calanis LA, Rodríguez-Jorge O, et al., 2023. Unique Characteristics of Neonatal T Cells, Medical Research Archives, [online] 11(1). https://doi.org/10.18103/mra.v11i1.3568

DOI
https://doi.org/10.18103/mra.v11i1.3568

ISSN: 2375-1924

RESEARCH ARTICLE

Unique Characteristics of Neonatal T Cells

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ABSTRACT

Birth causes complex changes in the individual physiology and organ systems and certainly poses a big immune challenge. The sudden encounter with an antigen full world, with the exposure to food antigens, and the colonization of the skin and mucosa with microbiota, require a tolerant immune system. Nevertheless, neonates must also be able to deal with pathogens, which makes their immune system unique. T lymphocytes are responsible for the coordination of the adaptive immune system response, the elimination of infected cells and the type of immune response and memory. It has been shown that neonatal cells have intrinsic differences with adult cells, biased towards an innate response and a tolerant phenotype.

In the perinatal period, the immune system changes from basal signaling and innate like responses, towards stimulus-specific signals, which increase with gestational age. After birth the cells of the immune system continue to change both in composition and function.

In this review, we present the intrinsic differences of neonatal $CD4^+$ and $CD8^+$ T cells, as compared with adult naïve cells. A specific transcriptome profile is present in both $CD4^+$ and $CD8^+$ neonatal T cells, with overexpression of homeobox transcription factors. These cells also present differences in cell signaling and metabolic characteristics, which result in unique functional capabilities. Neonatal $CD4^+$ T cells respond differently from adult cells, with a high production of IL-8, a prevalent Th2 over Th1 profile, and an innate inflammatory response.

The neonatal period is one of the most vulnerable periods of life, with a high morbidity and mortality rate, approaching on average 17 deaths per 1000 live births worldwide. A better understanding of the neonatal immune system will help to ensure a better care of this vulnerable population.

Introduction

Birth causes complex changes leading, among others, to increased oxygen levels, changes in blood circulation, endocrine and metabolic changes [1] and certainly poses a big immune challenge. The sudden encounter with an antigen full world, with the exposure to food antigens, and the colonization of the skin and mucosa with microbiota, require a tolerant immune system. Nevertheless, neonates must also be able to deal with pathogens, which makes their immune system unique [2].

The neonatal period is one of the most vulnerable periods of life, with the highest morbidity and mortality rates, approaching on average 17 deaths per 1000 live births worldwide. Infections account for 24% of deaths and they often leave life-long sequels [3].

The evaluation of the immune system of premature and mature neonates, with single-cell sequencing and the construction of an elastic net model, showed that it changes along gestational age. In the most premature neonates, basal signaling and innate-like responses are dominant, whereas the stimulus-specific signals increase with gestational age [4]. After birth cells continue to change both in composition and in function [5-7] and even in 12 years old children, some differences with the adult immune system still remain [6].

T lymphocytes are responsible for the coordination of adaptive immune system response, the elimination of infected cells and the type of immune memory. It has been shown that neonatal cells have intrinsic differences with adult cells, biased towards an innate response and a tolerant phenotype, but an innate inflammatory response [8-11].

This paper reviews some of the main intrinsic differences of neonatal $CD4^+$ and $CD8^+$ T cells as compared to the naïve adult cells, from the transcriptomic, epigenetic and functional points of view. This will be addressed in five paragraphs, presenting the unique characteristics of $CD4^+$ and $CD8^+$ T cells, the unique and puzzle role of homeobox transcription factors overexpressed by the neonatal cells, as well as their metabolic characteristics. A better knowledge of neonatal T cells could contribute to better address the medical care of the vulnerable population of newborn babies.

Neonatal CD4+ T cells and tolerant phenotype

$CD4^+$ T cells orchestrate the adaptive immune response. For this, T cell have to be activated, proliferate, and differentiate into an effector or memory phenotype. For T cell activation to occur, two types of signals are necessary: (1) the recognition of the MHC-bound antigen through its T cell receptor (TCR), and (2) costimulatory signals through CD28, and/or cytokines produced by APCs. When the T cell receives both types of signals, gene transcription, cytokine secretion, expression of surface molecules, and cell proliferation are promoted [12-14].

An activated $CD4^+$ T cell can differentiate into several effector phenotypes, the best reported being Th1, Th2, Th9, Th17, Th22, and T_{reg}, with distinctive functions. Th1 cells secrete IFN-γ and TNF-α and their main function is to fight intracellular pathogens [15]. Th2 cells secrete interleukin 4 (IL-4), IL-5 and IL-13, and eradicate helmints [16]. Th9 cells secrete IL-9 and are important for tumor immunity [17]. Th17 cells secrete IL-17A and IL-17F and have a protective role against extracellular bacteria and fungi [18]. Th22 cells secrete IL-22 and have a role in skin homeostasis and inflammation [19]. In contrast, T_{reg} cells suppress the immune response of other effector cells by secreting IL-10 and TGF-b [20].

The adaptive immune response early in life is different to that of an adult, which is partly result of the gestational period. During pregnancy, the fetus has a limited exposure to antigens in the uterus and its adaptive immune response is tolerant. The mother's immune system also has to be tolerant to the fetus, which is achieved in part by the T_{regs} that suppress the activation and function of effector cells [21]. If an infection occurs during pregnancy and the fetal immune system is activated, the suppressive activity of T_{regs} could be reduced, the production of pro-inflammatory cytokines like TNF-α and IL1-β augments and labor can be prematurely induced [22-24]. Besides, placental tissues secrete TGF-β, prostaglandin E2 and progesterone, which promote a Th2 differentiation, to avoid a Th1 response that leads to pregnancy termination.

Two possible models have been proposed to explain the differences between neonate and adult T cells responses: (1) fetal and adult T cells arise from different hematopoietic progenitor cells, and neonatal cells have remnants of those fetal cells. (2) There is a continuous progression from the fetal to adult phenotypes, and the newborn cells suffer a progressive change that continues even a couple of months after birth [11].

In general, neonatal $CD4^+$ T cells are considered immunotolerant, with minimal protective functions, which results in a high dependency of the neonates on their innate immune system [25,26]. It has also been reported that peripheral and cord blood from neonates have a predisposition to mount a Th2 response (IL-4, IL-5, IL-10) with the concomitant low production of Th1 cytokines (IFM-gamma, IL-2 and TNF-α) [27]. As a result, neonates are highly susceptible to infections caused by viruses and

intracellular bacteria [28]. In addition, neonatal T cells produce CXCL-8 and express complement receptors (CR), typically considered innate immunity, in comparison to adult T cells [29]. IL-8 secreted by neonatal T cells play a role to recruit and activate neutrophils and γδ T cells during infection and inflammatory lung injury [30,31].

Neonatal CD4+ T cells, like their CD8+ counterparts, present a distinct transcriptomic and epigenetic landscape when compared to adult cells. They present differences in DNA methylation, chromatin landscape and micro-RNA expression. In fact, the long-standing observation that neonatal CD4+ T cells from cord blood present hypermethylation of CpG and non-CpG sites in the promoter of IFN-γ gene [32].

Single-cell and bulk RNA-seq technologies have been used to demonstrate that neonatal CD4+ T cells present a characteristic gene expression profile, which is different from the fetal and adult counterparts. Using a set of 33 differentially expressed genes, Bunis *et. al.* have shown that cord blood naïve CD4+ T cells present an intermediate mean "developmental score" between fetal and adult CD4+ T cells, where the expression of a set of genes is shared with fetal cells and others with adult cells. Of notice, the pathway annotation analysis shows that classical immune functions such as Th1, Th2 and Th17 differentiation is absent in these neonatal CD4+ T cells, which instead expressed genes related to intracellular signaling and cell cycle, and partially retain a T_{reg} signature already present in fetal cells [33,34]. It has also been found that genes from the TCR signaling pathway (CD4, CD3, TCRα, TCRβ, LAT, LCK and ITK) are downregulated in neonatal CD4+ T cells while others are upregulated (JUN, NFKB1), when compared to adult cells. This was associated with a low TCR/CD28-mediated signaling and low expression of activation related genes like CD69, IL-2, IFN-γ, IL-17A, and TNF-α. In this study, the downregulated genes of neonatal CD4+ T cells were linked to inaccessible chromatin, while overexpressed genes in adult cells had a strong association with the open chromatin mark H3K4me3 peaks and accessible chromatin (ATAC-seq) [35]. Another study has also shown differences in the accessibility of chromatin as a feature determining the level of gene expression in neonatal CD4+ T cells and other cord blood cells [36].

We have also studied the transcriptomic profile of neonatal CD4+ T cells and found that these cells present an overexpression of metabolism related, innate-like and cell cycle genes, and a low expression of activation-related genes like IFN-γ, and TBX21. We were able to link the overexpression of genes in the neonatal cells with the epigenetic profile of promoters and putative enhancers (Kempis-Calanis et.al. submitted). We have also found that neonatal CD4+ T cells proliferate more than adult naïve CD4+ T cells. Altogether this shows that neonatal CD4+ T cells are able to respond to stimulation, but not in a conventional way, relying more in innate-like responses and a tolerogenic and Th2 bias.

Neonatal CD8+ T cells and innate behavior

The main function of CD8+ T cells is to eliminate infected or nonfunctional cells. To characterize these cells in neonates, our group evaluated the transcriptome of neonatal CD8+ T cells as compared to the adult naïve CD8+ T cells. We observed that the neonatal cells overexpressed genes associated with a neutrophil-like innate immune response, including the genes of antimicrobial peptides (DEFA4, DEFA3 and CTSG), elastase (ELANE) and the transcriptional factor CEBPε involved in development of granulocytes [8]. The neonatal cells were deficient in IFN-γ production and cytotoxic response and produced a higher level of reactive oxygen species, particularly in the mitochondria, which could contribute to the lower cytotoxic function of these cells [37]. We then asked whether this was a transitional immature population or if these neonatal cells were able to mature into a more adult-like phenotype. Indeed, upon activation in the presence of IL-12 signals, these cells downregulated the innate-like genes and expressed the genes associated with T cell receptor (TCR) signaling and the cytotoxic response [9]. This higher activation was related to a more balanced metabolic profile. In agreement with the capability of neonatal CD8+ T cells to mount a strong response under strong challenges, it was reported that in neonatal mice, IFN-γ producing CD8+ T cells were observed after infection with the enteropathogen Y. enterocolitica [38]. The generation of effector cells under acute viral lung infections has been observed in the lungs of 6-week-old children and neonatal mice, with a prevalence of effector rather than memory cell generation [30,39].

Among the specific characteristics of cord blood CD8+ T cells is the high expression levels of the chemokine CXCR3 and the transcription factor Eomes, associated with memory T cells [40]. This Eomes+ innate-like CD8+ T cells were also identified in human fetal thymuses, and young infants up to 3 months old [41]. Remarkably, IL-4 promotes an innate-like program in CD8SP thymocytes, with the expression of CXCR3, CD44 and Eomes but not T-bet.

Another remarkable characteristic of the neonatal cells is the expression of NK cells-like

receptors, among these, the C-type lectin family NKG2 receptors (CD159) and the immunoglobulin (Ig) superfamily killer Ig-like receptors (KIR) expressed in 0.74% and 1.67% neonatal CD8+ T cells respectively [42]. In addition, neonatal CD8+ T cells express the co-inhibitory receptor killer-cell lectin like receptor G 1, which is present in the 20% of neonatal CD8+ T cells [43]. This could also account for the higher activation threshold of the neonatal CD8+ T cells [44].

The puzzling role of Homeobox gene expression in neonatal cells

An intriguing observation regarding the differential gene expression between neonatal and adult naive T cells is their transcription factor signature expression. Many transcription factors encoding genes expressed at higher level in neonatal T cells are generally expressed in hematopoietic and immature T cell precursors and found overexpressed in T-Acute Lymphocytic Leukemia (T-ALL). These genes include LMO2, HHEX, MEIS1, HOXA9, HOXA10 and BCL11A in CD4+ T cells (Kempis-Calanis, submitted) and BCL11A, MYCN, HOXA3, and MEOX1 in CD8+ T cells [8,9]. Remarkably, these genes are expressed in normal hematopoietic or early T cell precursors in the thymus (i.e. CD34+ thymocytes) but are then switched off during T cell differentiation and maturation [45,46]. Moreover their ectopic or sustained expression in the T cell lineage is associated with a differentiation blockage and hyperproliferation [45]. For instance, the HOXA family of genes play a key role in controlling cell identity and differentiation of hematopoietic stem cells (HSC) and T cell progenitors [47-49]. The progressive down-modulation of HOXA transcripts has been reported during thymic cell maturation [46,50], while the sustained expression of HOXA genes during T cell differentiation has been shown to impair T cell maturation and to induce oncogenic transformation [47,50,51]. Similarly, LMO2, MEOX1, MEIS1 are major T-ALL oncogenes and are normally expressed in HSCs and contribute to the stemness of the HSC cells [45]. Whether the expression of these transcriptional regulators might impose an "immature" epigenetic state contributing to the proliferative and more tolerant immune response phenotype of the neonatal T cells will require further investigation.

Cell signaling in neonatal cells

T cells are activated upon recognition of the peptide-MHC complex by the TCR, and costimulatory molecules presented by dendritic cells or secreted by these or other neighboring cells in the lymph nodes. The costimulatory molecules CD80 and CD86 whose ligand is CD28 in the surface of T cell is the best studied costimulatory pair, but other receptors, cytokines and parts of the antigens traveling in the lymph can also provide important activation signals.

In a very general way, signals through the TCR start by the phosphorylation of immunoreceptor signaling motifs (ITAMs) present in the intracellular domains of the invariable molecules named CD3 and ζ, which are associated with the antigen recognition pair (α/β or γ/δ chains). This phosphorylation is due to the enzymatic activity of Lck, which is associated with the intracellular domains of co-receptor molecules CD4 and CD8. Early signaling proceed with the recruitment of ZAP 70 kinase to the phosphorylated ITAMS, which in turn phosphorylates and activates Lat, a transmembrane protein that is responsible for the recruitment and activation of several intermediary proteins that end up activating three main families of transcription factors: AP-1, NFκB and NFAT, through the activation of MAP kinase cascades, PKCθ and Calcium waves, respectively [52].

It has been reported that TCR early signaling could be impaired in neonatal CD4+ T cells, due to differences in chromatin accessibility and a lower expression of important signaling molecules, such as Lat and Lck [28,35]. Other authors found, however, that calcium flux signals and MAP (Mitogen Activating Protein) kinase activation cascades were even higher in neonatal CD4+ T cells, probably due to the higher expression of miR-181a. Despite these higher activation of the MAP kinase Erk, AP-1 transcription factor activation was impaired, compromising neonatal CD4+ T cell activation [53]. The high calcium waves present in neonatal cells could potentially lead to an imbalance in transcription factors' activation. The transcription factor NFAT, activated by calcium waves, is implicated in both, activation and anergy. This is because NFAT binds to DNA motifs often associated with AP-1 in dual motifs sites. When AP-1 is diminished, however, as reported for neonatal cells, NFAT can form homodimers and bind to DNA motifs associated with T cell exhaustion and anergy [54,55]. A single-cell sequencing study of cord blood mononuclear cells, showed a higher expression of the inhibitor of NFκB, IκBα, in the neonatal cells, which could potentially further contribute to an unbalance in the main transcription factors' activation in neonatal cells [36]. We have also found differences in the expression of signaling molecules in the neonatal cells, both in CD8+ and in CD4+ T cells (Kempis-Calanis et. al. submitted and [8].

Among the genes that both neonatal CD4+ and CD8+ T cells overexpress are genes associated with cell cycle. This overexpression is related with

a higher rate of homeostatic proliferation in both cell types and a higher clonal expansion in response to stimulation only in the CD4+ T cell population (Kempis-Calanis et. al. submitted and [8].

The cytokine IL-12 is also important to induce T cell activation and differentiation, particularly into the Th1 phenotype and cytotoxic functions, which are important for the elimination of intracellular pathogens to which neonatal cells are particularly susceptible. In neonatal cells, IL-12 is not expressed, increasing slowly its levels during childhood [6]. We showed that neonatal CD8+ T cells respond to activation in the presence of IL-12 with an adult-like expression profile [9].

Downstream of the TCR signaling cascade, TCR signals are blocked by the overexpression of the phosphatase DUSP-9 and the ubiquitin ligase Cblb (Rodriguez-Jorge unpublished results), further inhibiting the TCR signaling cascade.

Altogether neonatal TCR signaling is inhibited at several points, from early signaling to downstream transcription factors' unbalanced activation and the overexpression of inhibitory molecules. Other signals should be used to reach a more balanced transcription factor activation, when neonatal T cell activation is required for vaccine formulations and during infections. We and others have shown that one such pathway to reach AP-1 and NFκB activation is through TLR engagement, particularly TLR5 [56-58].

Nutrition and metabolism during neonatal T cell responses

As we have previously stated, neonatal T cell activation is limited and biased towards an innate-like response. We and others have published transcriptomic analyses showing that neonatal CD4+ and CD8+ T cells have a basal glycolytic program (Kempis-Calanis, et.al. submitted and [8,9,37]. However, the metabolism of these cells has not been well characterized and metabolic reprogramming upon activation has not been evaluated.

A big amount of literature has shown an important cross-regulation of metabolism and the immune system, called immunometabolism. This cross-regulation is at the core of many inflammatory, chronic, and infectious diseases, and T cell function has been strongly linked to the metabolic activity of the cell.

Human T cells switch from their quiescent oxidative metabolic program to a highly anabolic one, characterized by elevated glycolysis, pentose phosphate, and glutaminolysis pathways, during an immune response, a process known as metabolic reprogramming or metabolic rewiring. Metabolic reprogramming is necessary to acquire the energy and biosynthetic precursors needed for proliferation (cell division), differentiation, gene expression, and effector functions, upon T cell activation and has been widely studied in adult cells from human and animal models, especially in the tumor microenvironment [59-61].

Nutrient uptake and nutrient availability have been linked to the activity of specific pathways in cells, to determine if specific metabolic pathways can alter the activation and differentiation of T cells. Indeed, specific metabolic pathways have a role in T cell differentiation and function, such as the need for aerobic glycolysis for pro-inflammatory Th1/Th17responses and fatty acid oxidation for T_{reg} activation [62-64]. Glucose metabolism is essential for a proper generation of effector T cells and the expression of pro-inflammatory cytokines such as IFN-γ (Buck et al., 2015).

In our previous work, we have shown that in order to reach adult-like expression of activation-related genes like T-bet, IFN-γ, GZMB expression, as well as glycolysis-related and OXPHOS related genes, neonatal CD8+ T cells need IL-12 along with TCR/CD28 stimulus, which has been show to induce important metabolic changes to promote T cell proliferation and function [9]. Based on these studies, we have proposed that the metabolic status and high production of ROS impair the activation and function of neonatal CD8+ T cells. We have also found that neonatal CD4+ T cells overexpress metabolism-related genes (Kempis-Calanis, submitted), potentially contributing to their activation profile and higher proliferation capacity.

Altogether, studies in humans along with several studies in mice point towards highly proliferative (homeostatic) neonatal naïve CD4+ and CD8+ T cells, which is accompanied by a highly glycolytic program already present in basal conditions [8,65] in agreement with the bias towards a rapid response with low memory generation of the neonatal cells [11,44]. In this regard, the metabolic program could negatively influence the activation response of these cells, since it is well established that in other to achieve full activation and generation of effectors and memory compartments, T cells have to engage an aerobic glycolysis program (effectors) which some cells abandon for an OXPHOS one (memory cells)[62,66-68]. If the metabolic profile of neonatal T cells controls T cell activation, the metabolic modulation of metabolism with specific nutrients could possibly restore T cell activation, differentiation, and function in neonates.

Concluding remarks

The specific response of neonatal CD4+ and CD8+ T cells is associated with a particular transcriptome and epigenetic profile, which obeys

the birth transition but leaves newborn babies vulnerable to infections, causing a high morbidity and mortality rates. A graphical abstract of our findings is presented in Figure 1. The immune-tolerant window of newborns is important for their healthy development; however, several avenues could be imagined to improve neonatal T cell response when needed. One of them is the use of alternative ways to balance transcription factors' activation, in unblocked pathways in the neonatal cells. One such way is the TLR signaling pathway, for instance by the use of the TLR5 agonist, flagellin, to induce AP-1 and NFκB transcription factors and a protective Th1 response in vaccine formulations [56,58]. The other is through nutrient use to improve the metabolic state of the cells.

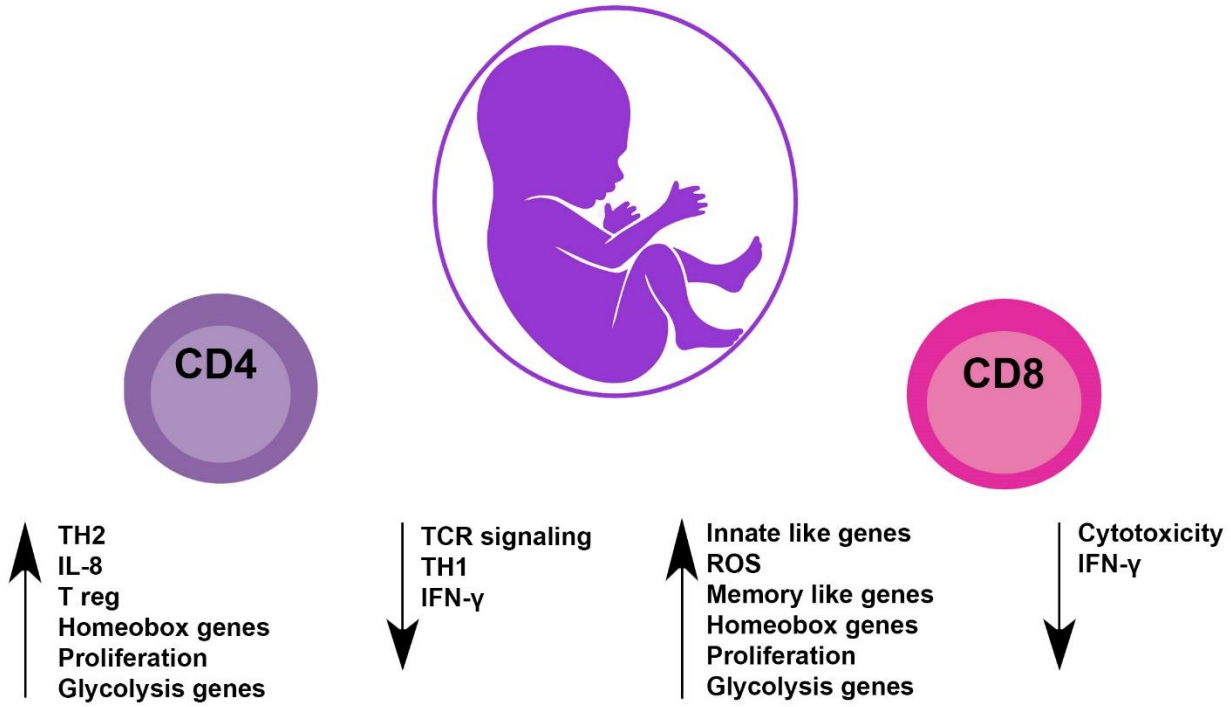

Figure 1: Graphical abstract of the characteristics of neonatal $CD4^+$ and $CD8^+$ T cells

It was reported that hypercholesterolemic mothers give birth to neonates with a stronger pro-Th2 gene expression profile and a propensity to allergy up to 3 years old [69]. This highlights the importance of proper maternal and newborn nutrition, and diet composition, for the immune development of neonates and infants [70].

A proper maternal diet, supplementation with immunonutrients and bioactive compounds, and the regulation of specific metabolic pathways, could be interesting therapeutic strategies to target immune cell maturation, metabolic fitness, and the immune response of neonates and adults (68-70). In the case of neonatal T cells, this could lead to improved cellular-mediated immune responses and health.

Conflicts of interest Statement

"The authors have no conflicts of interest to declare."

Funding Statement

Work in the MAS and OR-J laboratory was supported by CONACYT grants FC 1690 and CF 2019/1727995. Work in the laboratory of SS was supported by recurrent funding from INSERM and Aix- Marseille University, as well as by funding from INCA (PLBIO018-031 INCA_12619). This work was also supported by ECOS/ANUIES/SEP/CONACYT grant M17S02.

Acknowledgments

We thank the blood donnors, mothers, babies and staff of Hospital José G. Parres, Hospital Temixco, Centro Estatal de la Transfusión Sanguínea and Servicios de Salud Morelos for granting the access to neonatal blood and leukocyte concentrates. We also thank the Transcriptomics and Genomics Marseille-Luminy (TGML) platform for sequencing RNA-seq samples. TGML is a member of the France Genomique consortium (ANR-10-INBS-0009).

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